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Mechanisms of Cellular Adaptive Sensitivity Changes: Applications to Opioid Tolerance and Dependence*

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I. Introduction

EXCITABLE cells of many types demonstrate the ability to adapt their sensitivity to neurotransmitters and pharmacological agonists (Fleming et al., 1973; Fleming, 1976; Fleming and Westfall, 1988). There is general consensus that tolerance to and dependence upon opioids represent cellular adaptations. However, the mechanisms underlying these phenomena and the relationships between them are poorly understood. Moreover, the literature on opioid tolerance and dependence contains frequent inconsistencies and contradictions. A theme developed by this review is that the term tolerance currently is being applied in the literature to two or more different phenomena. Tolerance may be the manifestation of several different mechanisms of cellular adaptation. Through a review of the pertinent literature, we will show that the cellular adaptive process involved in a given determination of "tolerance" probably depends on the particular cells being studied and/or the procedure used to experimentally induce tolerance. Furthermore, some of these adaptive processes may logically be associated with dependence and some may not. Much can be gained by putting the experimental evidence regarding the characteristics and mechanisms of tolerance and dependence into the perspective of the wider literature of cellular adaptation.

Tolerance and dependence in the intact organism can involve large portions of the neuraxis, with changes in several transmitters and complex interactions of neuronal circuits. Although, at times, it will be necessary to refer to such factors, their detailed analysis is beyond the scope of this review. Rather, the emphasis is on adaptations at the cellular level.

To avoid confusion it is necessary to establish definitions of some of the terms to be used in this review. *Supersensitivity* is a descriptive term used as defined by Fleming et al. (1973). It represents a shift of the concentration-response curve for an agonist to the left relative to its control position. Although this shift is sometimes accompanied by an increase in the slope and maximum response, supersensitivity commonly occurs without a change in slope or maximum. *Subsensitivity* is a related term used to describe the condition in which the concentration-response curve is shifted to the right (Fleming et al., 1973) and/or demonstrates a decrease in slope or maximum response.

The term *adaptive super-* or *subsensitivity* is used to describe a change in the position of a concentration-response curve in those instances in which the sensitivity change is the consequence of an altered relationship between stimulus and response at the cellular level, in contrast to an altered disposition of the agonist (Fleming and Westfall, 1988). *Postjunctional, disuse* and *nondeviation supersensitivity* are all synonymous with adaptive supersensitivity but are less appropriate terms within the context of current knowledge. For discussion of the evolution of terminology in adaptive supersensitivity, see references by Fleming (1975) and Fleming and Westfall (1988).

Desensitization and *tachyphylaxis* are terms, often used

interchangeably, that designate a rapidly occurring, for example, within minutes, loss of responsiveness (Harden, 1983). This loss of responsiveness involves a change in the stimulus-response relationship within the cell and may also be considered an adaptive change (Harden, 1983). Just as the onset is rapid, so generally is the recovery (Harden, 1983). For the purpose of this review, we shall use the term desensitization within such a framework and distinguish it from adaptive subsensitivity and supersensitivity which develop more gradually (Fleming et al., 1973; Fleming, 1976).

Homologous desensitization is a loss of responsiveness that is specific for agonists acting via a single type of receptor, whereas *heterologous* desensitization signifies a loss of responsiveness involving multiple receptor systems (Su et al., 1976a,b). As will be discussed later, such distinctions are of great importance in identifying the level of cellular function that is responsible for the desensitization.

Tolerance represents the loss of responsiveness to a drug as a consequence of prior exposure to the drug and has been applied particularly to drugs of abuse. In the past, it has been customary to distinguish between rapidly developing subsensitivity, by calling it tachyphylaxis, and slowly developing subsensitivity, by calling it tolerance (Goodman and Gilman, 1965). In the more recent literature on the opioids it has become common to use the term tolerance for both rapidly and slowly developing subsensitivity. This has tended to obscure the possibility that the cellular mechanisms underlying the two phenomena may be quite different. This review will attempt to keep that distinction in the forefront.

The term *dependence* refers to the state in which the drug is required for the maintenance of normal physiological function. If the drug is removed from its receptor, characteristic disturbances, collectively referred to as the *abstinence syndrome* or *withdrawal response,* are precipitated. These disturbances are generally qualitatively opposite to the effect of the drug that induced the dependence. Thus, because the acute opioid effects are usually depressant, morphine dependence has been referred to as a state of "latent hyperexcitability" (Seevers and Deneau, 1963), masked by an ongoing action of the drug and revealed after withdrawal of the drug. The *withdrawal response* may be a complex behavioral effect (Clouet and Iwatsubo, 1975) or a response in a single cell (Johnson and Duggan, 1981a).

Full appreciation of the changes in the concentration-response curve for an agonist which accompany the development of tolerance of a tissue to the agonist requires some understanding of modern receptor theory. The following brief discussion of some of the concepts of receptor theory are based upon the contributions of Stephenson (1956), Furchgott (1955; 1966), Furchgott and Bursztyn (1967), and Nickerson (1956), as detailed in the excellent review by Ruffolo (1982).

The position and shape of a concentration-response curve can be affected by dispositional factors such as neuronal uptake or rapid metabolism of the agonist (Fleming et al., 1973; Trendelenburg, 1986). If such factors are absent or controlled, then the horizontal position, slope, and maximum of a concentration-response curve are determined by three factors. These are (*a*) the *affinity* of the agonist for its receptor, (*b*) the density of those receptors in the tissue, and (*c*) *intrinsic efficacy.* The term intrinsic efficacy represents the amount of response generated per unit of receptor occupation. The concept recognizes the fact that different agonists that combine with the same receptor do not necessarily result in equal coupling of the receptor to subsequent cellular events leading to response. It was Stephenson (1956) who first introduced the term *efficacy.* Subsequently, Furchgott (1966) introduced the term *intrinsic efficacy. Efficacy* is equal to intrinsic efficacy multiplied by the concentration of available receptors in the tissue. Thus, the value of *intrinsic efficacy* is that it separates the coupling of receptor occupation to response from the concentration of receptors. It follows, therefore, that a change in the sensitivity of a tissue to an agonist, such as the development of tolerance, may involve alterations in one or more of the following: (*a*) disposition of the drug, (*b*) the affinity of the receptor for the drug, (*c*) the density of receptors in the tissue, or (*d*) the intrinsic efficacy of the drug-receptor combination in the tissue.

A corollary of the concept of efficacy is the concept of *receptor reserve* or *spare receptors.* It was presumed by Stephenson (1956) that an agonist with high efficacy could produce a maximum response in a tissue without occupying all of the available receptors. The first strong evidence that this assumption was correct came from the work of Nickerson (1956) who demonstrated that there were concentrations of an irreversible antagonist (N-1-naphthylmethyl-N-ethyl-β-chloroethylamine), which shifted the concentration-response curve for the contractile effects of histamine in the ileum to the right *without* a decrease in maximum. Higher concentrations of the antagonist depressed the maximum. Because the antagonist bound irreversibly to the receptors, the effect of the lower concentrations of the antagonist could only be explained if it were assumed that the agonist could achieve maximum response with less than the full complement of receptors in the tissue available (i.e., "free" or not bound to the antagonist).

In recent years, the concept of receptor reserve has been readily accepted because of the large number of agonists that have EC_{50} values considerably lower than their respective dissociation constants. The EC_{50}‡ is the

‡Abbreviations used: EC_{50}, 50% effective concentration; ED_{50}, 50% effective dose; IC_{50}, 50% inhibitory concentration; 5-HT, 5-hydroxytryptamine; GTP, guanosine 5′-triphosphate; GTPase, guanosine 5′-triphosphatase; GDP, guanosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; ADP, adenosine 5′-diphosphate; PG, prostaglandin. DAMGO, (D-Ala^2,MePhe^4,Gly^5-ol)-enkephalin; DRG, dorsal root ganglion.

concentration producing half-maximum response and the dissociation constant is equal to the concentration that achieves occupancy of half the receptors. Hence, a ratio of EC_{50} to dissociation constant of less than 1.0 indicates the existence of spare receptors. Indeed, in some tissues for some agonists there are orders of magnitude of receptor reserve. For examples and calculations, see the review by Ruffolo (1982).

On the other hand, if the efficacy of an agonist in a given tissue is quite low, it may not be able to produce a maximum response equal to that produced by an agonist of higher efficacy even when occupying all available receptors. Such a drug is a *partial agonist.* Note that, because efficacy equals the concentration of receptors times the intrinsic efficacy of the drug, a given drug with modest intrinsic efficacy can be a partial agonist in a tissue with relatively low receptor density and a full agonist in another tissue with a higher density of the same receptors.

The equations developed for modern receptor theory allow one to predict the changes in a concentration-response curve that would follow from a change in the density of receptors (Fleming and Westfall, 1988). This is important because there has been a tendency in the literature to explain large changes in sensitivity on the basis of relatively small changes in receptor density without considering the quantitative relationship. For example, the calculations of Fleming and Westfall (1988) indicate that a 50% increase in receptor density could only account for a 1.5-fold increase in sensitivity to the agonist. Thus, if the sensitivity is increased 5- to 10-fold, one must consider the probability that other cellular changes in addition to changes in receptors contribute to the altered sensitivity. The calculations further demonstrate that, if there is no receptor reserve, the primary effect of a modest increase in receptor concentration would be an increase in maximum response.

The literature on opioids and on opioid tolerance and dependence is enormous. This review is oriented toward cellular mechanisms. There are many excellent reviews that the reader may wish to consult to expand this topic. Some published since 1980 are Chapman and Way (1980), Collier (1980), Herz and Schulz (1982), Nicoll (1982), Duggan and North (1983), Martin (1983), Woolverton and Schuster (1983), Goldstein and James (1984), Zieglgänsberger (1984), Koob and Bloom (1988), and McFadzean (1988).

II. Characterization of Adaptive Super- and Subsensitivity

The literature on this subject is extensive and has been reviewed several times in the last 15 years. The purpose of this section is to give adequate background information on the subject to enable the reader to understand its potential application to the topic of opioid tolerance and dependence. With this goal in mind, reference to major review articles combined with selected primary references will be used.

Historically, the recognition of adaptive supersensitivity begins with the phenomenon of denervation supersensitivity, first observed in the middle 19th century. Early experiments with denervation supersensitivity have been reviewed by Cannon and Rosenblueth (1949) and Fleming et al. (1973). Although Cannon and Rosenblueth (1949) speculated with remarkable insight regarding the cellular basis of denervation supersensitivity, real progress in identifying the cellular mechanisms of denervation supersensitivity did not begin until the 1950s and 1960s. Two breakthroughs occurred during that period. Thesleff (1960) presented electrophysiological evidence that supersensitivity of denervated skeletal muscle was associated with a spread of receptors beyond their usual location in the end plate, without apparent change in the density of receptors within the end plate itself. In later years the spread of receptors and the increase in the total number of receptors per muscle cell were confirmed with radiolabeled ligands (Colquhoun, 1974).

The second major discovery of that period was made by Trendelenburg (1963) who established that noradrenergic denervation produced two distinct types of supersensitivity in a given tissue. One of these was highly specific for norepinephrine and a few closely related amines and was fully developed within about 24 h. This supersensitivity, which was due to loss of neuronal uptake of amines consequent to the degeneration of the nerve terminals, came to be known as *presynaptic* or *prejunctional* supersensitivity (Trendelenburg, 1966) and subsequently as *deviation* supersensitivity (Fleming, 1975). The second component of denervation supersensitivity was not limited to norepinephrine and related amines and developed much more gradually (Trendelenburg, 1963). There has been a long evolution of terms for this phenomenon. It has been called *postsynaptic* or *postjunctional* supersensitivity (Trendelenburg, 1966), *disuse* supersensitivity (Sharpless, 1975), *nondeviation* supersensitivity (Fleming, 1975), and, most recently, *adaptive* supersensitivity (Fleming and Westfall, 1988).

Deviation supersensitivity can be induced with drugs that inhibit membrane transport and/or metabolism of neurotransmitters (Fleming, 1976; Trendelenburg, 1986) and occurs with cholinergic (Westfall et al., 1974; Bird and Aghajanian, 1975; McConnell and Simpson, 1976) and dopaminergic (DiRenzo et al., 1988), as well as noradrenergic, denervation. Deviation supersensitivity is not presently known to be related to tolerance and dependence at the cellular level and is not a subject of this review.

Experimentally, adaptive supersensitivity has generally been induced by chronic interference in the neurotransmission to effector cells (skeletal muscle, smooth muscle, cardiac muscle, exocrine glands, other neurons, etc.). Briefly, the chronic procedures used include sur-

gical interruption of nerve pathways, chemical destruction of neurons, depletion of transmitter, pharmacological inhibition of transmitter release, blockade of postjunctional receptors for the transmitter, and chronic changes in sensory stimuli, such as light, in instances in which neurotransmission is reflexly controlled by external stimuli (Fleming, 1976; Fleming and Westfall, 1988). These procedures have in common the chronic interruption of a neurotransmission process to effector cells.

Adaptive supersensitivity always involves a change in sensitivity that is compensatory in nature. That is, if one chronically interrupts a stimulatory pathway, the result is supersensitivity to stimulatory substances. If one chronically interrupts an inhibitory pathway, such as the nigrostriatal dopaminergic pathway, supersensitivity develops to inhibitory substances. Numerous examples are given in reviews (Fleming et al., 1973; Fleming, 1976; Fleming and Westfall, 1988). This is the basis for the choice of the term adaptive. One may think of adaptive sensitivity changes as a means by which cells compensate for marked and prolonged changes in the net stimulation they receive.

Chronic enhancement of stimulatory neuroeffector transmission produces subsensitivity of effector systems (Fleming et al., 1973; Fleming, 1976). Unfortunately, adaptive super- and subsensitivity have generally been studied in separate systems. Nevertheless, there are examples of the same group of effector cells developing supersensitivity after chronic reduction in stimulatory neurotransmission and subsensitivity after chronic enhancement of the same neuroeffector transmission (Emmelin, 1964; Bito et al., 1971; Overstreet et al., 1973; Russell and Overstreet, 1987). The examples are numerous enough to suggest that both the supersensitivity and the subsensitivity are mutual expressions of cellular homeostatic mechanisms that compensate for chronic changes in the net stimulus the cells receive (Fleming et al., 1973; Fleming and Westfall, 1988). A good example is the supersensitivity of the iris sphincter muscle in animals kept in constant dark and subsensitivity of the same muscle in animals kept in constant light.

A characteristic of adaptive sensitivity changes in contrast to deviation supersensitivity is the delay in the appearance of the former. The alteration in neurotransmission that is used to induce the adaptive sensitivity change must be maintained for several days to several weeks. The time required is *independent* of the procedure used to induce the sensitivity change (for example, surgical denervation versus transmitter depletion). The delay is a characteristic of the effector cells being investigated. For example, adaptive supersensitivity develops fully in the cat heart within 3 days, whereas 14 to 28 days are required for the full development of supersensitivity of the nictitating membrane of the cat (see review by Fleming et al., 1973).

An aspect of adaptive sensitivity change which has not been adequately understood or appreciated by the scientific community is the degree of specificity or nonspecificity of the change in sensitivity. It is often assumed, for example, that chronic depression of a specific neurotransmission process (e.g., noradrenergic) causes an increase in sensitivity only to that transmitter (e.g., norepinephrine) and other agonists acting upon the same receptor system. Such is often not the case; the sensitivity may also increase to a divergent group of agonists with no pharmacological relationship to the neurotransmitter. This issue is of critical importance as a clue to the cellular mechanisms responsible for the changes in sensitivity. The specificity of sensitivity is best put into perspective by discussing it in relationship to each of several effector systems.

A. Skeletal Muscle

The magnitude of the increase in sensitivity of denervated skeletal muscle to cholinoceptor agonists is enormous. For example, denervation of the rat hemidiaphragm increases the sensitivity of the muscle to carbachol by a factor of 50 (McConnell and Simpson, 1976). The dramatic nature of that change has often obscured the fact that sensitivity is modestly altered to a number of substances that do *not* act upon nicotinic cholinoceptors. These include potassium ion, 5-hydroxytryptamine (5-HT), caffeine, histamine, and bradykinin (Fleming et al., 1973).

B. Smooth Muscle

This tissue offers a marked contrast to skeletal muscle. If one is careful to eliminate any component of deviation supersensitivity, with all smooth muscles studied, adaptive supersensitivity is nonspecific. The sensitivities to a number of unrelated agonists are increased to a similar degree, and when differences do occur they are less than one order of magnitude (Fleming et al., 1973; Fleming, 1976; Fleming and Westfall, 1988).

A few examples will help one to appreciate this characteristic. Preganglionic denervation of the cat's nictitating membrane induces supersensitivity to the contractile effects not only of the normal transmitter, *l*-norepinephrine (16-fold), but also of methoxamine (9-fold), acetylcholine (8-fold), and barium (7-fold) (Fleming et al., 1973). There is a difference of only 2-fold between the smallest change (barium) and the largest (norepinephrine). Note, also, that the change in sensitivity for methoxamine (an α-adrenoceptor agonist like norepinephrine) is nearly identical with the changes for acetylcholine and barium. Chronic treatment of rabbits with reserpine, which depletes the noradrenergic transmitter, induced supersensitivity of aortic strips to phenylephrine (3-fold), acetylcholine (4-fold), and potassium (2-fold) (Taylor and Green, 1971) and supersensitivity of the saphenous artery to norepinephrine (2.4-fold),

methoxamine (2.8-fold), histamine (2.3-fold), and potassium (1.4-fold) (Abel et al., 1981). Cholinergic denervation of the rat iris sphincter (Banro et al., 1987) produced supersensitivity to acetylcholine (28-fold in one series, 10-fold in another). It also produced a 50% loss of cholinesterase and, therefore, the change in acetylcholine sensitivity could include a component of deviation supersensitivity. The increase in sensitivity to bethanechol, a nonester and not hydrolyzed by cholinesterase, was 7-fold and to 5-HT, 4-fold.

C. Cardiac Muscle

Chronic depletion of norepinephrine in the hearts of dogs (Westfall and Fleming, 1968;) and rabbits (Tenner and Carrier, 1978) produces modest supersensitivity to β-adrenoceptor agonists and to calcium. Similar depletion produces supersensitivity of guinea pig atria to isoproterenol but not histamine, calcium, theophylline, or pilocarpine (Torphy et al., 1982). Recently, Tenner et al. (1988) demonstrated adaptive supersensitivity of guinea pig ventricular tissue to impromidine, a relatively selective agonist for histamine H_2-receptors. It is likely that previous attempts to demonstrate supersensitivity to histamine failed because of its nonselective effects on both H_1- and H_2-receptors. Guinea pig atria do develop adaptive supersensitivity to agents, such as forskolin and analogs of guanosine 5′-triphosphate (GTP), which activate the adenylate cyclase system at points *beyond* the β-adrenoceptor and the histamine H_2-receptor (Hawthorn et al., 1987). In general, although cardiac adaptive supersensitivity resulting from interruption of adrenergic neurotransmission is not strictly limited to agonists acting on adrenoceptors, the supersensitivity is much more specific than that found in smooth muscle (Fleming, 1984; Fleming and Westfall, 1988).

D. Exocrine Glands

Both the adrenergic and the cholinergic innervations of salivary glands are stimulatory. Chronic interruption of *either* innervation leads to supersensitivity to *both* adrenoceptor and cholinoceptor agonists (Emmelin, 1961; 1965). Adaptive supersensitivity also occurs in sweat glands (Reas and Trendelenburg, 1967), but its specificity has not been tested.

E. Neurons

There are a great many reports of supersensitivity in the central nervous system (CNS) with a number of examples referenced in reviews by Fleming (1976) and Fleming and Westfall (1988). In many instances, there has not been a clear separation of adaptive supersensitivity from deviation supersensitivity, and only rarely have the specificity/nonspecificity been determined (Fleming and Westfall, 1988). In some instances, experiments interpreted to show that supersensitivity in a CNS response is specific have not been properly designed (Fleming and Westfall, 1988). The reliable evidence regarding specificity or nonspecificity of adaptive supersensitivity in the CNS is still too sparse to generalize.

Later in this review, evidence will be discussed regarding a hypothesis that slowly developing tolerance to, and dependence on, morphine in the guinea pig ileum is an expression of adaptive sensitivity changes. Within the present context it is important to note that both tolerance and dependence involve nonspecific changes in sensitivity of the neurons in ileum to a number of unrelated agonists (section VI).

F. Factors Regulating Cellular Sensitivity

There have been intensive attempts to identify the factor or factors that regulate cellular sensitivity. Some appreciation of the difficulty of the problem can be gained from the following quotation from Fleming and Westfall (1988).

> It is evident that some aspect of the neuroeffector relationship has a suppressing effect on the sensitivity of the effector cells. Removal of that factor, whatever it may be, allows sensitivity to increase. For nearly as long as the effect of denervation on sensitivity has been recognized, there has been speculation about, and more recently, intense investigation of, the factor or factors which control sensitivity. The factor could be the level of activity of the effector cells themselves, in which case the role of the nerve is only secondary as the physiologic modulator of that cellular activity. If the controlling factor is the target cell activity, one must define which specific cellular activity is critical. For example, in a muscle cell, is the frequency of contraction, the frequency of action potentials, the frequency of subthreshold junction potentials, some aspect of transmembrane ion movement or the intracellular concentration of calcium ions (to name a few possibilities) the ultimate factor signaling the cell to adjust its sensitivity? On the other hand, sensitivity may be regulated by a specific trophic substance released from the nerve. Such a trophic factor could be the transmitter itself or a separate substance. If there is a trophic factor other than the transmitter it might be released in intimate association with the transmitter or independently. Gathering clear evidence for or against these various possibilities has been exceedingly difficult.

Work on this question has been thoroughly reviewed (Drachman, 1974; Fleming, 1976; Fleming and Westfall, 1988), and only the conclusions will be given here. There is evidence of a factor, other than a transmitter, transported along nerves, the loss of which leads to the development of supersensitivity in the muscle. The evidence comes from experiments in which drugs, such as colchicine, which block axonal transport without abolishing neurotransmission, induce supersensitivity. Although the drugs used do cause some changes in neurotransmission, the effect on transmission is moderate. Thus, the existence of a trophic substance other than the transmitter must be seriously considered.

On the other hand, there are numerous studies that directly link the neurotransmitter and/or activity of the effector cell itself as the factors regulating sensitivity of

the effector cell. Any procedure that markedly reduces neurotransmission (see above) will induce supersensitivity. It is difficult to understand how all of these procedures (depletion of transmitter, inhibition of release of transmitter, specific antagonists of postsynaptic receptors, etc) could interfere with the release or action of some trophic substance, other than the transmitter, as well.

Particularly intriguing are a large number of studies implicating changes in some activity of the effector cells themselves as the trigger for sensitivity changes. Low levels of electrical stimulation, for example, which do not induce contractions, will prevent or reverse denervation supersensitivity in skeletal muscles. This suggests that sensitivity changes in response to modest alterations in transmembrane flux or intracellular content of an ion. In smooth muscle and glands, supersensitivity induced by the interruption of a stimulatory innervation can be abolished or prevented by chronic administration of a stimulatory drug, *other than the transmitter*. Again, this suggests that activity of the effector cell may be the regulatory factor.

As will be discussed in a later section, opioids inhibit some neurons by increasing the outward movement of K^+ and hyperpolarizing the cell membrane. If sensitivity is regulated by the cell's own activity, such as ion fluxes or concentrations, chronic hyperpolarization by an opioid might induce adaptive supersensitivity to stimulatory agonists and subsensitivity to inhibitory agonists including itself. As discussed in section VI, this is precisely what happens in the guinea pig ileum.

III. Cellular Mechanisms of Sensitivity Changes

A. Desensitization

Rapid loss of response to opioids occurs in some cellular systems. Particularly, as studied in cell cultures, this loss of response appears to be a desensitization reaction. There seems to be little question that desensitization processes are initiated by agonists interacting with their receptors. For comparative purpose, this section will briefly review what is known concerning the phenomenon of desensitization in two non-opioid receptor systems that have been extensively studied. The nicotinic cholinoceptor and the β-adrenoceptor have been chosen for this purpose. These two receptor systems represent different arrangements of coupling of receptor to response and corresponding distinct differences in the desensitization process. Furthermore, the mechanisms of desensitization have been well defined in both systems.

1. Nicotinic receptor. There have been excellent reviews of the biophysics and molecular structure and function of the nicotinic receptor in recent years, including those by Adams (1981), Changeux (1981), Conti-Tronconi and Raftery (1982), Heidmann et al. (1983), Popot and Changeux (1984), and McCarthy et al. (1986). The analysis presented here is heavily dependent upon those reviews.

The early work on the electrophysiology of the nicotinic receptor and the Na^+-conducting channel which it regulates was done on the neuromuscular junction of lower vertebrates, particularly frogs, and of mammals. The subsequent discovery that the electric organs (electroplaques or electroplax) of certain genera of fish, notably *Electrophorus* and *Torpedo,* possessed remarkably high concentrations of nicotinic receptors provided a source for isolation and chemical identification of the receptor. Electrophysiological and molecular studies on both the muscle end plate and the electroplaque have clearly established that the receptor-ion channel systems are virtually identical functionally in those two systems (Changeux, 1981), although the molecular weights of the subunits vary somewhat among different tissues (McCarthy et al., 1986).

The nicotinic receptor is composed of four differing subunits, ranging from 40,000 to 65,000 daltons, with a stoichiometric relationship of 2:1:1:1 (Popot and Changeux, 1984; McCarthy et al., 1986). The subunits appear to be arranged as $\alpha_2\beta\delta\Delta$-helices which penetrate the membrane completely and surround a "central pit" which is almost certainly the ion channel (McCarthy et al., 1986). The binding sites for acetylcholine reside on the α-subunits, and the binding of two agonist molecules per receptor is required to open the channel (Changeux, 1981; McCarthy et al., 1986). Opening of the channel involves a conformational change in which side chains are presumed to move away from the center of the channel (Changeux, 1981; McCarthy et al., 1986). From the above, it is clear that the nicotinic receptor and the Na^+ channel represent a molecular unit without the need for intermediary coupling proteins.

Desensitization of the nicotinic receptor of the muscle end plate was described by Katz and Thesleff in 1957. It was concluded by those workers that the receptor must exist in two states with differing affinities for agonists. In 1980, Neubig and Cohen proposed a four-state model which has continued to fit the available data.

The four states are currently designated as follows: (*a*) R, a "resting" state with low affinity for the agonist which is susceptible to being activated upon occupation by the agonist, (*b*) A, the "active" conformation with the channel open, (*c*) D, a "desensitized" conformation with the channel closed and affinity for agonists high, and (*d*) I, an "intermediate," high-affinity state with the channel closed (Changeux, 1981; Heidmann et al., 1983). It has been possible to correlate the time course of the opening and closing of the channel with the rate of change of affinity states of the receptor (Heidmann et al., 1983). The affinities of differing conformations of the nicotinic receptor-rich membranes from *Torpedo marmorata* have been measured as approximately 50 μM for the R state, 1 μM for the I conformation, and 3 nM for the D conformation (Heidmann et al., 1983). These are presumably comparable to the three different affinities demonstrated

for acetylcholine before and after dispersion of receptor-rich membranes with sodium cholate, K_D values of 1 μM, 0.1 μM, and 30 nM (see Changeux, 1981). It should be noted that the K_D of the low-affinity state is well within the range of concentrations calculated to be achieved in the synaptic area by nerve activity (Changeux, 1981).

As the concentration of agonist is increased, there is a linear acceleration of the desensitization process, occurring within seconds at high concentration (Changeux, 1981). The rate of onset of desensitization is influenced by membrane potential, temperature, divalent cations, local anesthetics, barbiturates, and long-chain alcohols (Changeux, 1981). Recovery from desensitization is rapid after removal of the agonist, requiring only a few seconds (Changeux, 1981). Based upon electrophysiological data, the transformation from the R (resting) to the hypothetical A (open channel) conformation must be very rapid, i.e., within a millisecond. Such a change is too fast for biochemical measurement. Simultaneous with the rapid conversion from the R to the A state, conversion to either the I and/or D state, depending upon the agonist concentration, is initiated. The conversion to the I conformation has a time course of 10 to 100 ms, whereas the conversion to the D form, either directly or via the I form, is of the order of seconds. After the receptor is in the I or D configuration, the channel is closed and a state of desensitization exists until the agonist is removed. For more detailed discussion of the kinetics, see the papers by Changeux (1981) and Heidmann et al. (1983).

It is notable that with the system at rest, that is, in the total absence of agonist, 80% of the receptor population is in the R (low affinity) form and 20% in the D (high affinity, "desensitized") form (Changeux, 1981). Because the D form exists in the absence of agonist, the desensitizing effect of the agonist involves a shift of a preexisting equilibrium away from the R conformation in favor of the D conformation (Changeux, 1981).

Huginer and Greengard (1983) reported that the nicotinic receptor can be phosphorylated by a cyclic adenosine 5′-monophosphate (AMP)-dependent protein kinase. This phosphorylation of the receptor increased the rate of desensitization by agonists, but the functional significance of such a phosphorylation is not known (Huginer,1987b).

2. β-Adrenoceptor. In contrast to the nicotinic receptor, in which the receptor and the sodium channel constitute a single molecular entity, the β-adrenoceptor is a single, independent molecule. Its activation by an agonist triggers coupling reactions among a series of closely associated, but independent, proteins in the cell membrane leading ultimately to formation of the intracellular messenger, cyclic AMP. The cyclic AMP activates a cyclic AMP-dependent protein kinase. The various responses to β-adrenotropic agonists depend upon the location and function of specific proteins that are phosphorylated by the protein kinase. It is highly likely that among the proteins that become phosphorylated are ones associated with some ion channels (Huginer, 1987a).

To understand the desensitization process in the β-adrenoceptor-adenylate cyclase system, one needs to know some detail of the activation process. The analysis which follows is based upon several recent reviews (Harden, 1983; Gilman, 1984; Schramm and Selinger, 1984; Levitzki, 1986). Whereas the dissection of the molecular structure and function of the nicotinic receptor was aided by the remarkably large concentrations of the receptor in muscle end plates and, even more, in electroplaques, β-receptors do not occur in such densities and often occur in complex tissues in which only one of several cell types possesses significant concentrations of the receptors. The latter problem has been resolved by heavy reliance upon data from red blood cells, such as the turkey erythrocyte, and cultured cell lines from certain tumors. Three proteins are involved in the initial steps of cellular activation via β-adrenoceptors. The first is the receptor itself (R) which is a single unit of 60,000 daltons. The second is one of a series of guanine nucleotide (GTP)-binding proteins (G proteins), G_s: a heterotrimer with subunits α (45,000 daltons), β (35,000 daltons), and γ (10,000 daltons). The third protein is the catalytic unit, adenylate cyclase.

At rest, the receptor is in a relatively low-affinity state for the agonist, and guanosine 5′-diphosphate (GDP) is bound to G_s. The combination of the agonist with the receptor leads to a further association of the agonist-receptor complex with G_s. When the agonist-receptor complex and G_s combine, the receptor undergoes a conformational change in which its affinity for the agonist increases, "locking" the receptor into the complex. G_s undergoes a conformational change which allows GTP to displace GDP. The combination of GTP with the agonist-receptor-G_s complex leads to a dissociation of G_s and the receptor, a decrease in affinity of the receptor for the agonist, and a likely dissociation of the agonist from the receptor. The $G_s \cdot$GTP complex interacts with the catalytic unit that is activated, cyclic AMP is generated, and GTP is hydrolyzed to GDP.

There are several recent reviews in which desensitization of β-adrenoceptor-mediated responses are discussed. The present analysis draws heavily upon those by Harden (1983), Sibley and Lefkowitz (1985), Lefkowitz and Caron (1986), and Levitzki (1986). Several processes of desensitization have been identified with differing time courses ranging from minutes to hours. It should be emphasized that all of these processes are considerably slower than desensitization in nicotinic receptors (seconds) and very much faster than the slowly developing adaptive super- and subsensitivity (days to weeks) to be described in a subsequent section of this review. Three of the four processes of desensitization of β-adrenoceptor-mediated responses are homologous and the fourth is heterologous. The four processes are (*a*) uncoupling of

the receptor from its interaction with G_s (homologous), (*b*) sequestration of the receptor away from the outer surface of the membrane (homologous), (*c*) a decrease in measurable β-adrenoceptors per cell, often referred to as "down-regulation" (homologous), and (*d*) loss of activation of adenylate cyclase by the β-adrenoceptor and, *simultaneously,* by other receptors normally capable of its activation (heterologous).

Although a great deal is known about these processes, it is still difficult to put together a totally clear and complete picture. A number of factors are responsible for some confusion. It is still unclear to what degree the various processes of homologous desensitization are interdependent. Yet, the term is used at times as if homologous desensitization were a single process. Furthermore, not all cell types demonstrate all of the above processes. For example, turkey erythrocytes demonstrate heterologous, but not homologous, desensitization (Levitzki, 1986).

Sequestration of receptors can be distinguished from loss of receptors (down-regulation) by several procedures (Harden, 1983). In whole-cell preparations, binding studies with hydrophilic ligands cannot distinguish between the two processes because the ligands detect only receptors on the cell surface. However, with a lipophilic ligand which can measure receptors *inside* as well as on the surface, additional binding sites for *sequestered* receptors can be detected. In homogenized preparations, if receptors have been sequestered, they can be detected by ligand binding in vesicles of lower density than the usual membrane particles. No such binding occurs in the low-density vesicles when the receptors have been truly "lost" by down-regulation.

In the continued presence of agonist, the uncoupling of the β-adrenoceptors from the G protein-adenylate cyclase system and the sequestration ("internalization") of the receptor occur within a few minutes and are accompanied by a *decrease* in affinity of the receptor for the agonist (Harden, 1983). This combined process of uncoupling and sequestration occurs with a half-time of approximately 10 min (Harden, 1983; Staelin and Hertel, 1983) in S49 lymphoma cells. In contrast, in the same cells, the actual loss of receptors occurs with a half-time of approximately 4 h. A similar separation apparently occurs in astrocytoma cells (Su et al., 1979; 1980; Harden, 1983). During prolonged exposure, the decline in adenylate cyclase stimulation in homogenates or membrane preparations occurs more rapidly than the decline in ligand binding during the first few hours. However, after 6 to 12 h the decreases in the two measures become equal, and at 12 h, ligand binding is only 20% of control. This is presumably because uncoupling of the receptors and/or their sequestration rapidly reduce the response while actual receptor loss takes several hours to catch up. Recovery from the rapidly developing phases is also rapid. At a time when the response of the enzyme has decreased by 80% but only 35% of the receptors have been lost, removal of the agonist restores the response to within 35% of control with a half-time of about 7 min (Su et al., 1979; 1980; Harden, 1983).

The development of both homologous and heterologous desensitization has been demonstrated in a number of cells and tissues. Heterologous desensitization has a slower time course and is less marked in degree (Harden, 1983). For example, exposure of rat myometrium to either isoproterenol or prostaglandin (PG) E_2 leads to rapid desensitization selectively to the agonist used and a more slowly developing loss of response to the other agonist. Although heterologous desensitization tends to be slower than the uncoupling-sequestration processes of homologous desensitization, it is, nevertheless, fairly rapidly developing in some cell lines (Harden, 1983). Examples are $T_{1/2}$ values of 2 to 3 min (WI-38 fibroblasts), 30 min (astrocytoma cells), and 1 h (C6–2B rat glioma cells). Cells may become desensitized, not only to several agonists (including β-adrenotropic substances) that activate adenylate cyclase via different receptors, but also to guanine nucleotides, sodium fluoride, and forskolin, agents that activate the transduction process at points beyond the receptor (Lefkowitz and Caron, 1986). It is likely that more than one mechanism exists for heterologous desensitization among various types of cells (Harden, 1983).

During heterologous desensitization, both the β-adrenoceptor and the catalytic subunit of the enzyme are phosphorylated (Lefkowitz and Caron, 1986). A cyclic AMP-dependent kinase seems to be involved in the phosphorylation and the phosphorylation is enhanced if the receptor is occupied by the agonist. Lefkowitz and Caron (1986) proposed that other protein kinases, not dependent upon cyclic AMP, may also be able to contribute to this phosphorylation of the receptors and the adenylate cyclase, thus expanding the potential heterologous nature of the desensitization.

Phosphorylation appears to be involved in homologous desensitization also, although it is *not* mediated by a cyclic AMP-dependent kinase (Lefkowitz and Caron, 1986). Indeed, there appears to be no dependence of any of the steps of homologous desensitization upon cyclic AMP. Lefkowitz and Caron (1986) presented a model of proposed steps involved in homologous desensitization. The first step is depicted as uncoupling and phosphorylation of the receptor. In the second step, the receptor, still functionally normal, is sequestered. The location of the sequestered receptors may be either at the inside surface of the cell membrane or within cytoplasmic vesicles. Lefkowitz and Caron emphasized that it is still uncertain whether uncoupling and sequestration are truly separate events. However, a recent report (Cheung et al., 1989) provided evidence that the region (domain) of the β-adrenoceptor that is essential for coupling to G_s is also essential for the sequestration to take place. In

the continued presence of the agonist, sequestered receptors gradually are lost and must be restored by intracellular processing of the original receptors or resynthesized (Harden, 1983; Lefkowitz and Caron, 1986).

B. Chronic Adaptive Subsensitivity and Supersensitivity

There are abundant examples in the literature of slowly developing increases or decreases in sensitivity (Fleming et al., 1973; Fleming, 1976; 1984; Fleming and Westfall, 1988). As previously discussed, these changes are routinely compensatory, i.e., they are in a direction that compensates for chronic decreases or increases in net stimulus received by the cells. Multiple mechanisms are involved. Some of the mechanisms, for example, changes in receptor density, are similar to those associated with desensitization. On the other hand, some mechanisms, such as changes in membrane potential and the density of Na^+/K^+ pump sites, are clearly different.

The one characteristic that appears to clearly separate desensitization from slowly developing adaptive sensitivity changes is their relative time courses. Most desensitization processes occur within seconds or minutes. The exceptions are down-regulation of receptors and heterologous desensitization. However, even these occur with half-times of a few hours and are virtually at their maximum within 12 to 24 h. On the other hand, slowly developing sensitivity changes generally do not appear for several days, in some instances require weeks to reach their maximum, and disappear equally slowly when the cause is removed (Fleming et al., 1973; Fleming, 1976; 1984; Fleming and Westfall, 1988). The one apparent exception to the slow time course has been the pineal gland. However, it is likely that the rapid changes in activation of adenylate cyclase via β-adrenoceptors observed in the pineal gland represents classic desensitization and recovery (Harden, 1983). There are three possible explanations for the dramatic differences in time course between desensitization and slowly developing adaptive sensitivity changes. (*a*). *The two phenomena are essentially the same but have very different time courses in different types of cells.* This possibility is raised because the two phenomena have routinely been investigated by separate groups of investigators who select distinctly different target cells. For example, desensitization research has heavily depended upon specialized tumor cell lines and blood cells. In contrast, adaptive sensitivity changes have been studied almost exclusively in muscle, nerve, and gland cells within intact organs or whole animals. Both selections have been appropriate in terms of the questions being asked and the procedures being applied. However, although we are unaware of any study of both phenomena in the same tissue, rapid desensitization has been observed in intact tissues and animals (Harden, 1983). (*b*). *The two phenomena are essentially the same, but whole tissue-whole animal approaches often do not allow adequately rapid changes in agonist concentration necessary for rapid changes in desensitization.* In other words, perhaps, slowly developing subsensitivity, for example, is merely desensitization with an artificially expanded time scale. This explanation is inadequate because slowly developing sensitivity changes have been documented to occur several days beyond the time that maximal changes in transmitter levels have been reached (Fleming et al., 1973). In desensitization, the subsensitivity and peak increases in transmitter levels should coincide closely in time. (*c*). *Desensitization and slowly developing sensitivity changes may represent distinctly different cellular phenomena.* As will be discussed subsequently in this review, some instances of slowly developing adaptive sensitivity changes involve alterations in cellular function that have never been associated with desensitization. There are very good reasons for considering at least some examples of adaptive sensitivity changes as phenomena totally independent of classical desensitization. There are equally excellent reasons to believe that rapid desensitization and slowly developing tolerance to opioids are examples of distinctly separate phenomena.

1. Subsensitivity. Slowly developing, adaptive subsensitivity ("tolerance") has been demonstrated in a number of tissues, induced by procedures that cause a chronic enhancement of neurotransmission. The most thoroughly investigated relationship of this kind has been with inhibition of cholinesterase. Following chronic inhibition of cholinesterase, especially with irreversible inhibitors, a subsensitivity to the physiological manifestation of the inhibitor and to cholinoceptor agonists develops. Generally, the subsensitivity is of the order of 2- to 4-fold and occurs in organs such as ileum, heart, salivary glands, iris, and brain (Fleming et al., 1973).

Routinely, there is a delay of several days after the maximal inhibition of cholinesterase in the full development of the subsensitivity and a corresponding delay in the recovery of normal sensitivity following recovery to normal levels of cholinesterase (Fleming et al., 1973). The important question of the specificity of the subsensitivity has not been thoroughly addressed. However, there is some evidence that the subsensitivity is nonspecific in cat salivary glands but specific in rat ileum (Fleming et al., 1973).

Investigation of cellular mechanisms of subsensitivity has concentrated primarily on changes in ligand binding to cholinoceptors. Numerous investigations have demonstrated a decrease in maximum binding to muscarinic receptor sites and a few to nicotinic sites (Russell and Overstreet, 1987). Usually, but not always, the change in maximum binding occurs without significant changes in affinity. The magnitude of the decrease in binding ranges, in general, from about 25 to 80 (see, for example, Gazit et al., 1979; Schiller, 1979; Uchida et al., 1979; Ehlert et al., 1980; Costa et al., 1981; McKinnney and Coyle, 1982; Costa and Murphy, 1983; Yamada et al., 1983).

Two factors make it difficult to assess the importance of the receptor changes to the subsensitivity. (*a*) The changes in binding are often modest in degree and investigators have made little attempt to relate the magnitudes of receptor change and sensitivity change. This problem will be discussed further (section III, B, 2). (*b*) Few studies have attempted to investigate, simultaneously, the *rate of change* in sensitivity and ligand binding.

An approach to the second problem was presented by Ehlert et al. (1980). Rats were treated with an initial dose of diisopropylfluorophosphate, 2 mg/kg, followed by maintenance doses of 1 mg/kg every other day. Twenty-four hours after the last dose, acetylcholinesterase activity in the brain was reduced by 90%. Subsensitivity (tolerance) to the pharmacological effects of diisopropylfluorophosphate was measurable on days 3 to 5 and maximal on days 7 to 9. The density of muscarinic binding sites ([^{3}H]quinuclidynal benzilate) in the brain was decreased 41% with a $t_{1/2}$ of approximately 1.6 days. This more rapid loss of binding sites relative to sensitivity leads one to question whether the loss of receptors can account for the subsensitivity. However, because the end points used to evaluate tolerance were functions of peripheral cholinergic activity (diarrhea, tremor, salivation, lacrimation), brain receptor binding is not the most appropriate comparison. The loss of peripheral muscarinic receptors may follow a slower time course but was not investigated.

The decrease in binding of cholinoceptor ligands has been measured most often in rats. The absence of significant changes in receptors in studies with guinea pigs raises the question of species differences in the potential role of receptor down-regulation in subsensitivity induced by inhibitors of cholinesterase (Russell and Overstreet, 1987). However, differences in doses of the inhibitors and the degree of enzyme inhibition induced across species lines again make definite judgments difficult (Russell and Overstreet, 1987). There is some evidence that the subsensitivity induced by chronic inhibition of cholinesterase is the consequence of increased activity of the target cells rather than the increased levels of acetylcholine per se (Uchida et al., 1979). If that should prove to be correct, it represents another argument against simple down-regulation of receptors as an explanation of the phenomenon.

2. Supersensitivity. Extensive discussion of the identification and characteristics of slowly developing supersensitivity (henceforth called, simply, supersensitivity) has been presented in section II. This is a complex phenomenon that evolves from multiple cellular changes. Two or more cellular changes can collectively contribute to supersensitivity in a given type of cell and the functional change or changes that is/are of primary importance vary with the type of cell and, possibly, with the species (Fleming, 1976; 1984; Fleming and Westfall, 1988).

Alterations in four cellular characteristics have been proposed to underlie adaptive supersensitivity in various effector tissues. These are (*a*) increases in the density of specific receptors, (*b*) changes in the activity of the Na^+/K^+ pump and related changes in membrane potential, (*c*) changes in adenylate cyclase or the G protein coupling system, and (*d*) changes related to calcium. These have been extensively reviewed previously (Fleming, 1976; Fleming, 1984; Fleming and Westfall, 1988) and only a brief synopsis of the current state of knowledge of them will be given here.

An increased density of receptors in the cell membrane was first proposed as a cause of supersensitivity in denervated skeletal muscle. The hypothesis was based upon experiments in which acetylcholine was applied locally by microelectrophoresis at discrete locations along the surface of single skeletal muscle fibers (Thesleff, 1960). In control cells, only the end plate region responded to low concentrations of acetylcholine. However, after denervation, there was a gradual spread of sensitivity to acetylcholine outward from the end plate until the entire membrane was as sensitive to the transmitter as was the end plate. Subsequently, binding of radioactive ligands highly selective for nicotinic cholinoceptors confirmed the concept of "spread of receptors" (Colquhoun et al., 1974; McConnell and Simpson, 1976). The magnitude of the increases in receptor density in denervated skeletal muscle is great. For example, McConnell and Simpson (1976) reported a 20-fold increase in binding sites for α-bungarotoxin in denervated rat diaphragm muscle.

The role of increases in the concentration of receptors in adaptive supersensitivity is equivocal in most tissues other than skeletal muscle. Increases in binding sites for ligands selective for various receptors are rarely increased more than 50% in supersensitive tissues relative to control tissues (Fleming, 1976; Fleming and Westfall, 1988). Calculations based upon receptor theory have determined that such modest changes in the number of receptors cannot account for the magnitude of changes in sensitivity that occur (see paper by Fleming and Westfall, 1988, for an extensive discussion of this point).

In numerous smooth muscles, adaptive supersensitivity occurs without any measurable changes in receptors (Fleming and Westfall, 1988). This is consistent with the nonspecificity of adaptive supersensitivity in smooth muscle (Fleming et al., 1973; Fleming and Westfall, 1988). A number of investigators have reported increased ligand binding to receptors for neurotransmitters and modulators associated with supersensitivity in neurons (Fleming, 1976). However, invariably, the magnitude of the change in receptors is too small to fully account for the magnitude of supersensitivity (Fleming and Westfall, 1988).

The above can be summarized as follows. An increase in numbers of receptors is the largest factor contributing to supersensitivity in skeletal muscle. In contrast, this

cellular change generally contributes *nothing* to supersensitivity in smooth muscle and is probably only one of two or more mechanisms that collectively underlie supersensitivity in neurons.

Denervation of skeletal muscle leads to a partial depolarization of the cell membrane of up to 15 mV. This change *precedes* in time the spread of receptors (Albuquerque et al., 1971). This depolarization explains the modest supersensitivity of denervated skeletal muscle to K^+ and, when considered in combination with the spread of receptors, can quantitatively account for the magnitude of supersensitivity to nonhydrolyzable nicotinic agonists (Fleming and Westfall, 1988). A partial depolarization contributes to adaptive supersensitivity in the guinea pig vas deferens (see discussion and references in the paper by Fleming and Westfall, 1988) and the rabbit saphenous artery (Abel et al., 1981).

The depolarization in the guinea pig vas deferens and the rabbit saphenous artery have been correlated with changes in the Na^+/K^+ pump. In both preparations, the Na^+/K^+ pump is electrogenic, exchanging Na^+ inside for K^+ outside at a ratio of greater than 1:1 (Thomas, 1972; Fleming, 1980). In both preparations, supersensitivity, depolarization, and a decline in electrogenic pumping occur with similar time courses (see references and discussion in paper by Fleming and Westfall, 1988). Extensive investigation of the guinea pig vas deferens further demonstrated that a number of procedures that induce supersensitivity led to simultaneous decreases in the activity of Na^+, K^+ adenosine 5′-triphoshatase (ATPase) (Gerthoffer et al., 1979) and in the density of Na^+/K^+ pump sites (Wong et al., 1981). Collectively, the evidence strongly indicates that decreases in the activity of the Na^+/K^+ pump constitute the primary mechanism for adaptive supersensitivity in the guinea pig vas deferens and an important mechanism in the rabbit saphenous artery (Fleming and Westfall, 1988).

Changes in the Na^+/K^+ pump have also been suggested to contribute to supersensitivity of denervated skeletal muscle, but the evidence is controversial (see discussion in the paper by Fleming and Westfall, 1988).

Supersensitivity of the generation of cyclic AMP in response to agonists has been reported in a number of tissues. In many instances the evidence provided does not allow one to determine whether the enhanced response is secondary to changes in receptors, changes in substrate [adenosine 5′-triphosphate (ATP)], or other factors (Fleming and Westfall, 1988). There is growing support for an altered coupling of receptors to adenylate cyclase or an actual increase in adenylate cyclase with adaptive supersensitivity in cardiac tissue (Chiu, 1978; Torphy et al., 1982; Hawthorn et al., 1987).

A number of investigators have implicated altered interactions of cells with calcium in supersensitive skeletal muscle, smooth muscle, cardiac muscle, and neurons [see paper by Fleming and Westfall (1988) for references and discussions]. At the present time, the molecular bases of the altered calcium dynamics are not known, although some studies implicate calmodulin.

Initially, the array of cellular changes that do or may contribute to adaptive supersensitivity appears somewhat bewildering. However, it appears that there may be a few specific proteins (receptors, Na^+, K^+ ATPase, G proteins, adenylate cyclase, calmodulin) in a given cell, quantitative changes in which can alter the cell's sensitivity to transmitters, hormones, and drugs. The quantity of each protein presumably represents the genetic expression/repression of that protein. Which of these proteins are most susceptible to altered expression or repression may determine the cellular basis for the development of adaptive supersensitivity in that type of cell.

IV. Measurement of Opioid Tolerance and Dependence

In section I, *tolerance* was defined as the loss in responsiveness to a drug, and *dependence* was defined according to the abnormalities that appeared after the drug was withdrawn. Several criteria may be used to demonstrate these phenomena in animals treated chronically with morphine. For dependence, these are (*a*) the appearance of physiological abnormalities when morphine is removed from its receptors, either by discontinuing its administration or by using a specific antagonist such as naloxone; and (*b*) supersensitivity to the excitant effects of external stimuli after withdrawal. For tolerance, the criteria are (*a*) a decrease in the response to the same dose of the drug or (*b*) an increase in the dose of the drug required to elicit a given response. Usually, comparisons are made of the effects of morphine in pretreated and in untreated animals; the latter are sometimes referred to as opioid "naive."

Although at first sight these criteria appear uncomplicated, the phenomena of tolerance and dependence are actually not easy concepts. Consequently, their identification and measurement have often proved to be difficult tasks. Some of the problems and pitfalls that may be encountered, and the precautions that are necessary in assessing tolerance and dependence, are considered in this section.

A. Dependence

Typically, to induce dependence, animals are pretreated by a schedule of doses of morphine that are steadily increased, for example, from 1 to 100 mg/kg, over a period of several days. In principle, the strategy required to establish whether the animals are dependent is straightforward. The experimenter need only interrupt the actions of morphine and determine whether abnormalities ensue as a consequence. Historically, concepts of dependence were based on abnormalities observed when administration of the opioid was discontinued. More recently, the use of naloxone has often been the

preferred method of intervention because the antagonist abruptly reduces the concentration of agonist at its receptors. It, therefore, rapidly reveals the full magnitude of any abnormalities, which may be masked by an ongoing action of the agonist. However, either method of removing morphine from its receptors should reveal abnormalities if the animals are dependent.

The effect produced by naloxone is compared with that evoked in naive animals. For the purpose of the present discussion, it is assumed (as is often observed experimentally) that naloxone is without effect in naive animals. A response to naloxone in the pretreated animals is then often interpreted as a withdrawal sign indicating that the animals are morphine dependent. What is frequently overlooked, however, is that an effect of naloxone may be expected in morphine-pretreated animals, even if they were not dependent. Because animals are pretreated with large doses of morphine, the resulting high concentrations in the circulation must be presumed to produce a receptor-mediated agonist action that is subject to antagonism by naloxone. There are, therefore, at least two explanations why naloxone affects physiological functions in pretreated animals. The first is that, by antagonizing an ongoing action of morphine, naloxone may restore the function to normal. The second is that, by antagonizing an ongoing agonist action of morphine, naloxone reveals an abnormality in the function. It is the second of these possibilities that provides evidence for dependence. Thus, an effect of naloxone is not sufficient in itself to establish that the pretreated animals are dependent.

In some experiments, the crucial distinction between a simple antagonism of an agonist action and the precipitation of a withdrawal response is quite easy. A well-known example is observed in mice that have been treated chronically with morphine. When naloxone is administered, the mice exhibit an extraordinary behavior, vigorous jumping (Way et al., 1969), which is not seen in normal, untreated animals and which is only evoked by naloxone in mice that have been pretreated with an opioid. Thus, the so-called "withdrawal jumping" is a clear indication of morphine dependence.

It is seldom, however, that naloxone elicits a response that is so easily identified as an abnormal behavior. In the majority of cases, naloxone simply alters the level of activity of a physiological function, such as blood pressure, respiration rate, a spinal reflex, the firing of a neuron, or the activity of an enzyme. To be able to conclude that a change in one of these parameters (for example, blood pressure) indicates dependence, it is necessary to compare the blood pressures of pretreated and control animals after administration of naloxone. If the blood pressures are similar, the effect of naloxone in the pretreated animals indicates merely that the blood pressure has been restored to normal. If the blood pressures of the two groups of animals are different, the effect of naloxone may be taken as a withdrawal response indicative of dependence. Again, an effect of naloxone does not, per se, distinguish between these two possibilities.

A major difficulty may be encountered if the parameter under study is not readily quantifiable. For example, in electrophysiological experiments, in which recordings are made from single neurons, the frequency of action potential discharge exhibits very large variations among different neurons, so that a meaningful estimate of an average firing frequency is not possible. If naloxone increases the firing frequency of a particular neuron in a morphine-pretreated animal, it is difficult to distinguish whether this represents a mere antagonism of an agonist effect of morphine or a withdrawal response indicative of dependence. The normal firing frequency of the neuron is not known and the firing observed after naloxone cannot be compared with an average frequency of a normal population of neurons. An exception to this difficulty is found in the locus coeruleus, in which neurons discharge at a rather regular frequency that is relatively constant among different neurons.

In the majority of studies on single neurons, however, alternate means must be sought to determine whether the excitation of neurons is abnormal. For example, although the basal firing rates of different neurons may vary enormously, the changes in firing produced by drugs or by natural stimuli may be more constant and quantifiable. In the experiments of Satoh et al. (1976b), cortical neurons in morphine-pretreated rats were supersensitive to the increase in firing rates produced by glutamate and acetylcholine. Thus, dependence was demonstrated by criterion (*b*), by the supersensitivity to excitant agonists.

B. Tolerance

The strategy required to measure tolerance, like that for dependence, also appears straightforward at first sight, based on the criteria described above. It should only be necessary to compare responses to a particular dose of morphine in pretreated and in control animals. In fact, however, there may be serious uncertainties about the dose of morphine present and about the magnitude of the response produced in the pretreated animals.

In a typical experiment, the effects of a test dose of morphine on a chosen physiological parameter are compared in pretreated and control animals. If the magnitude of the effect of morphine is reduced in the pretreated animals, these are considered to be tolerant to morphine. This conclusion is valid only if there is no morphine in the circulation at the time the test dose was administered. Criterion (*a*) above would then be satisfied; there is a reduced response to the same dose of the drug. However, the conclusion is tenuous and, perhaps, invalid, if morphine is already present in the circulation when the test dose is administered. Under these conditions, some of the opioid receptors will already be occupied by morphine.

The magnitude of the effect produced as a consequence should be known because it may markedly influence the response produced by subsequent administration of the test dose. More than 100 years ago, Witkowski (1877) pointed out that the apparent tolerance to the cardiovascular effects of morphine was most readily explained on the basis that the morphine already present in the circulation was sufficient to produce a maximal response. Because of the intense schedule of pretreatment doses that are used in many experiments, it does seem possible that many of the available receptor sites could be occupied when test doses are administered. If so, morphine would be expected to be less effective than in control animals. A diminished effect of a test dose of morphine, therefore, does not necessarily indicate tolerance but may merely indicate a paucity of available receptor sites. Even if the system has an excess of receptors (spare receptors) for the opioid agonist, there is a similar problem. The agonist present from the pretreatment may only be occupying a small portion of the available receptors but producing a near maximum effect. An added amount of the agonist might occupy additional receptors but cause little effect.

What needs to be quantified in experiments in which large concentrations of morphine are already present in the circulation is not the effect of a test dose but rather the physiological parameter that is the target for the agonist action. If the parameter (for example, blood pressure) is partially restored to normal in spite of the combined effect of the test dose and the morphine already present in the circulation, this indicates tolerance to morphine.

Uncertainties about the dose and the response are not encountered if tolerance is examined after all morphine has been removed from its receptors. Appropriate measurement of tolerance under these conditions is well illustrated by, for example, the experiments of Goldstein and Schulz (1973) in the isolated guinea pig ileum in vitro. Ileal preparations from untreated guinea pigs and from those treated chronically with morphine were washed extensively to eliminate all morphine from the tissue. The inhibitory effects of morphine on ileal contractions were then examined. Tissues from morphine-pretreated guinea pigs were tolerant to morphine, as indicated by the shift to the right in the concentration-response curve. This approach satisfied criterion (*b*) above for the assessment of tolerance; an increase in the dose of morphine was needed to elicit a given response (a 50% reduction in the magnitude of contractions of the ileum).

Tolerance decays after morphine dissociates from its receptors, however, so that experiments of this type may not measure the full extent of tolerance. Recognition of this possibility has influenced the strategy used to investigate tolerance in vitro. Tissues from pretreated animals are often removed and immediately placed in a solution containing morphine. This serves to maintain continuous occupancy of the receptors in vitro and, hence, to sustain tolerance induced in vivo. Tissues are dissected and equilibrated in the presence of the maintenance concentration, and tolerance is investigated subsequently by measuring the effects produced by additional concentrations of morphine.

It should be noted, however, that opioid maintenance introduces difficulties in measuring tolerance. For example, morphine may be added from the beginning of an experiment in vitro, at a concentration of 200 nM. It is then necessary to numerically add this concentration to each of those amounts that are used subsequently to establish concentration-response relationships. Some, but not all, studies have taken the maintenance concentration into account. A more difficult problem is that no study has taken into account the *response* that is produced by the maintenance concentration. Because no measurements are made before addition of the maintenance concentration, the magnitude of the *response* produced is never known. Therefore, a precise concentration-response curve cannot be obtained and the degree of tolerance measured is also imprecise.

Thus, opioid maintenance in vitro may facilitate detection of phenomena of tolerance that may decay rapidly after withdrawal. At the same time, however, it may impose limitations on measuring the phenomenon. Serious inaccuracies could result if tolerance is measured in the presence of a large maintenance concentration.

V. Cellular Mechanisms of Tolerance and Dependence

Several hypotheses concerning the possible mechanisms of tolerance and/or dependence to opioids at the cellular level have been advanced. These include qualitative or quantitative changes in receptors, alterations in the coupling of receptors to adenylate cyclase or changes in the cyclase itself, and a partial depolarization of neurons.

A. Receptors and the Adenylate Cyclase System

In 1974 Collier and Roy determined that, in rat brain homogenates, morphine, heroin, and methadone inhibited the formation of cyclic AMP induced by PGE_1 and PGE_2. The inhibition was concentration dependent, occurred with concentrations obtainable in vivo, and was antagonized by naloxone. They suggested that inhibition of cyclic AMP formation was the basis of the analgesic action of opioids and that tolerance and dependence could be mediated by a "compensating hypertrophy of a part of the inhibited PGE-cyclic AMP mechanism." In support of this hypothesis, it has been demonstrated (Collier and Francis, 1975; Ho et al., 1975) that the withdrawal response in rats was enhanced by dibutyryl cyclic AMP and inhibitors of phosphodiesterase, while being reduced by a stimulant of phosphodiesterase. Collier further refined these hypotheses in subsequent reviews (Roy and Collier, 1975; Collier, 1977; 1980).

In support of the concept that the adenylate cyclase system plays a role in dependence, Collier and colleagues discovered a phenomenon which they termed a "quasi-morphine abstinence syndrome" (Collier, 1974; Collier et al., 1974; 1975; Francis et al., 1975). In essence, Collier and colleagues found that substances that elevated cyclic AMP in the brain, such as 5-HT and phosphodiesterase inhibitors, acutely induced a behavioral response that was indistinguishable from a naloxone-induced abstinence syndrome.

Naloxone has been reported to increase plasma cyclic AMP in morphine-dependent mice (Muraki et al., 1981). In contrast, Nakaki et al. (1981) reported that morphine, itself, increased plasma cyclic AMP in rats and that this effect diminished with continued exposure to morphine.

The investigations of cyclic AMP related to tolerance and dependence listed above generated considerable interest. It is important to recognize, however, that in all of those experiments one cannot determine whether the cyclic AMP changes were occurring in the same cells as morphine was acting upon. Thus, they could reflect activity in neurons secondary to alterations in other neurons directly affected by morphine. Further impetus for the adenylate cyclase hypothesis was to come from a number of laboratories working with a pure tumor cell line.

1. Pure cell lines. Nirenberg's Laboratory (Klee and Nirenberg, 1974; Sharma et al., 1975a; 1977) investigated the action of opioids in membranes of a hybrid strain of neuroblastoma × glioma cells (NG108–15). The membranes contained both opioid receptors and adenylate cyclase. Opioids inhibited adenylate cyclase in a concentration-dependent, naloxone-sensitive manner. Inhibitory potency correlated well with binding affinity of the agonists. Goldstein et al. (1977) reported that endorphin in a concentrate from porcine pituitary also inhibited cyclic AMP formation. As might be expected, the inhibitory effect of opioids on cyclic AMP formation in NG108–15 cells is associated with inhibition of the sequential cascade of biochemical events including the activation of cyclic AMP-dependent protein kinase and/or ornithine decarboxylase (Bachrach et al., 1979). Subsequently, Sharma et al. (1975b) and Lampert et al. (1976) established that chronic administration (12 h or more) of morphine or Met-enkephalin induced a compensatory increase in adenylate cyclase activity and levels of cyclic AMP. After removal of the opioid, the ability of adenosine or PGE_1 to increase cyclic AMP levels was enhanced. The effects of morphine were stereoselective and antagonized by naloxone. All aspects of the dual regulation of adenylate cyclase activity by PGE_1 and opioids can be demonstrated in homogenates of NG108–15 cells as well as in intact cells (Klee and Streaty, 1981).

Traber et al. (1975) demonstrated that prolonged incubation of NG108–15 cells with each of a variety of inhibitory agents (morphine, methadone, norepinephrine, carbachol) enhanced the formation of cyclic AMP induced by PGE_1. Furthermore, they provided evidence that the enhanced response was dependent upon protein synthesis. The augmenting effect was lost within 3 h after removal of the agonists. Subsequent investigation in Nirenberg's laboratory (Wilkening and Nirenberg, 1980) indicated that the enhanced activity of adenylate cyclase induced by prolonged exposure to opioids may be the consequence of a change in the proportion of low- and high-activity forms of the enzyme rather than to an increase in the total amount of the enzyme and appears to involve membrane phospholipids (Griffin et al., 1986). The formation or mobilization of an activating factor could be involved (Wilkening and Nirenberg, 1980; Loh et al., 1988). The rebound increase of cyclic AMP after opioid withdrawal is accompanied by a similar rebound in cyclic AMP-dependent protein kinase and ornithine decarboxylase (Benalal and Bachrach, 1985).

A rebound increase in cyclic AMP formation induced by PGE_1 when naloxone was administered to NG108–15 cells pretreated with opioids has been noted by some laboratories (Law et al., 1983b; Griffin et al., 1983) but not found by others (Wüster et al., 1983). When it has been observed, the effect was less than reported originally by Sharma et al. (1975b). The reason for the variability of this response was discovered by Mussacchio and Greenspan (Greenspan and Musacchio, 1984; Musacchio and Greenspan, 1986). The rebound response in cyclic AMP appears to be a function of the rate of dissociation of the opioid agonists in which the cells are incubated. Thus, incubation for 12 to 48 h with (D-Ala,^{2} D-Leu^5)-enkephalin (DADLE) or morphine (rapidly dissociating agonists) led to a rebound effect when naloxone was added. In contrast, if etorphine, a more slowly dissociating agonist, was the incubating agent, naloxone did not induce rebound unless, prior to the addition of PGE_1 and naloxone, the etorphine was replaced with DADLE.

In 1982, Law et al. determined that loss of the inhibitory effect of opioids in NG108–15 cells was a multistep process. Incubation with etorphine for less than 3 h induced a decline in the ability of the opioid to inhibit PGE_1-induced cyclic AMP formation and a 19% increase in adenylate cyclase activity. However, there was little change in the specific binding of [^{3}H]diprenorphine. Longer exposure, i.e., 24 h or more, led to a down-regulation of opioid receptors, as indicated by a 60% decrease in maximum specific binding. Further refinement of the process came from experiments with [^{3}H] DADLE (Law et al., 1984). Chronic treatment with the agonist resulted in internalization of the ligand-receptor complex, involving transfer of the complex from the membrane-enriched fraction to the lysosome-enriched fraction. Subsequently, the receptors were degraded in the lysosomes.

The opioid receptors of NG108–15 cells are a single, homogeneous population of the δ type (Law et al., 1983a).

Simantov et al. (1982) found that agonists with high affinity for δ-receptors in NG108–15 cells induced down-regulation of those receptors, whereas morphine, which has low affinity for δ-receptors, did not induce down-regulation. Subsequent work in Loh's laboratory (Law et al., 1983b) further emphasized the separation of changes in adenylate cyclase and down-regulation of opioid receptors. Extensive quantitative experiments established that only full agonists can produce down-regulation (24 to 72 h exposure), whereas either full or partial agonists could induce the rapid desensitization. Furthermore, for any given agonist, desensitization was a function of receptor occupation, and down-regulation was a function of physiologic effect. In other words, an agonist with high efficacy (and, therefore a large proportion of spare receptors) could induce down-regulation in concentrations that were too small to induce desensitization. In spite of the quantitative relationship between receptor occupancy and desensitization, some agonistic effect is also essential because antagonists do not induce desensitization.

Adenylate cyclase is activated and inactivated by two separate GTP-binding proteins (Griffin et al., 1985) generally called G_s and G_i in current terminology (formerly termed N_s and N_i by some authors). Both proteins are activated by GTP and by its stable analog, Gpp(NH)p. In homogenates of NG108–15 cells, the inhibition of cyclic AMP formation via either opioid receptors or α_2-adrenoceptors involves a GTP-dependent process (Wilkening et al., 1980). G_i is inhibited by adenosine 5′-diphosphate (ADP)-ribosylation via the action of pertussis toxin. The use of pertussis toxin and Gpp(NH)p made it possible to establish that opioids inhibit adenylate cyclase in NG108–15 cells via an inhibitory GTP-binding protein (G_i) (Burns et al., 1983; Costa et al., 1983; Kurose et al., 1983).

Wüster and Costa (1984) used pertussis toxin to analyze the mechanism of DADLE's actions in NG108–15 cells. DADLE produced a concentration-dependent inhibition of PGE_1-induced stimulation of cyclic AMP formation. Pretreatment with pertussis toxin antagonized the inhibitory effect of DADLE, also in a concentration-dependent manner. The effect of the toxin was primarily to reduce the maximum inhibitory effect of DADLE with little change in the median effective concentration. The authors also demonstrated that exposure of the cells to DADLE for 24 h induced similar changes in the concentration-response curves for DADLE's acute inhibitory effect on cyclic AMP generation. Based upon striking similarities of the effects of chronic exposure to DADLE and those of pertussis toxin, Wüster and Costa (1984) suggested that desensitization in NG108–15 cells was the result of a loss of the coupling capacity of G_i.

However, the similarities between ADP-ribosylation and desensitization found by Wüster and Costa (1984) may be fortuitous due to methodological problems. In their desensitization experiments, "care was taken to maintain during the acute testing period the drug concentration which was employed for the chronic treatment, in order not to disturb the newly established equilibrium." Thus, the acute effects of PGE_2 and of opioids, for example DADLE, were determined by adding them in the presence of 1 to 100 nM DADLE. Under those conditions, it is difficult to interpret concentration-response data for two reasons: (*a*) One does not know how much stimulation by PGE_1 would have occurred in the *absence* of the incubating concentration of opioid. One cannot assume no inhibition by the incubating opioid simply because there is desensitization. Thus, there is no control level of cyclic AMP stimulation; (*b*) the concentration-response curves are plotted in terms of the acute concentrations added. For example, adding 1 nM DADLE in the presence of 10 nM makes 11 nM not 1 nM, as plotted by the authors. As a result of these problems, it is not possible to determine whether or not desensitization changed the median effective concentrations of the opioids.

Just as described for β-adrenoceptors (section III, A, 2), internalization of receptors for DADLE occurs in a neuroblastoma cell line, N4TG1 (Blanchard et al., 1982; 1983). Law et al. (1985a) found that chronic exposure to DADLE (10 nM) induces internalization of opioid receptors in NG108–15 cells. By pretreating the cells with pertussis toxin (100 ng/ml) for 3 h, they established that the internalization of the receptors does *not* require G_i. The internalization was accompanied by uncoupling of the receptor from G_i. It seems likely, therefore, that the desensitization observed by Wüster and Costa (1984) was due to uncoupling of the receptor from G_i, internalization of the receptor and, possibly, to down-regulation of the receptor.

The guanosine 5′-triphosphatase (GTPase) activity of NG108–15 membranes has also been found to decrease with prolonged exposure to opioids (Vachon et al., 1985; 1987a,b). Analogous to the situation with G_s and the β-adrenoceptor, the GTPase activity associated with opioid receptors in NG108–15 cells is a function of G_i. The desensitization is a two-step process with half-lives of 7 and 120 min (Vachon et al., 1987b). The rapid component probably corresponds to uncoupling of the receptor from G_i and the slow component to receptor down-regulation. Partial agonists were unable to desensitize the GTPase activity (Vachon et al., 1987).

The knowledge gained from NG108–15 cells has been summarized by Loh et al. (1988). There are many similarities between desensitization to opioids in these cells and desensitization in the β-adrenoceptor system coupled to adenylate cyclase. An early event following the combination of an opioid with its receptor is an alteration in the affinity of the receptor for the agonist (Law et al., 1985b; Costa et al., 1985). Desensitization is a multistep process beginning with uncoupling of the receptor from

its G protein. Subsequently, the receptor is internalized and eventually degraded, resulting in down-regulation. All of these steps result in homologous desensitization.

In addition there is another, apparently independent, event that results from chronic exposure to and removal of an opioid, an increase in the intrinsic activity of adenylate cyclase. Such a change is heterologous, because it affects actions of a number of unrelated agonists which lead to either stimulation or inhibition of the enzyme. For example, this response manifests cross-dependence between opioids and clonidine (Lee et al., 1988).

Tolerance to opioids has been observed in other isolated tumor cell lines. When neuroblastoma cells SH-SY5Y have differentiated into neurons, they demonstrate opioid-induced inhibition of PGE_1- or forskolin-induced stimulation of adenylate cyclase activity (Yu and Sadee, 1988). These cells possess μ- and δ-receptors in a ratio of approximately 5 to 1. Prolonged exposure to morphine produces a tolerance that reaches a maximum of 4-fold within 12 h.

Puttfarcken et al. (1988) investigated tolerance in 7315c pituitary tumor cell membranes. Inhibition of adenylate cyclase in these cells is mediated solely by μ-receptors. Tolerance to the inhibitory effects of μ-agonists involves cellular mechanisms very similar to those described in NG108–15 cells and with the β-adrenoceptor. Puttfarcken et al. studied membranes from 7315c cells after 5, 24, and 72 h of incubation with morphine (100 nM). At 5 h, there was evidence of uncoupling of the receptor from the G protein. At 24 h, there was a 20% and at 72 h a 60% decrease in binding sites for a μ-selective antagonist. Thus, an early desensitization is followed by a slower down-regulation of receptors.

The homologous processes of desensitization and down-regulation would produce tolerance of the cells to opioids. However, such a change would not lead subsequently to a withdrawal response indicative of dependence. On the other hand, the heterologous increased activity of adenylate cyclase could be expected to be associated with both supersensitivity to stimulatory agonists (analogous to dependence) and subsensitivity to inhibitory agonists, including opioids (analogous to tolerance).

One must be cautious in applying these conclusions to the broader concepts of tolerance and dependence in intact animals. The application of very high concentrations of opioids to these cells under uniform conditions for many hours is not generally achieved in vivo, nor has a link been established between cellular mechanisms in NG108–15 cells and in normal neurons.

2. Neurons. The role of adenylate cyclase in analgesia and other effects of opioids in the CNS are not clearly defined (see discussion and references in the paper by Loh et al., 1988). In the striatum, only actions via δ-receptors are mediated by adenylate cyclase, receptors that may not mediate analgesia. Nevertheless, the possibility remains open that other opioid receptors in other areas of the brain are coupled to adenylate cyclase as an intermediate in analgesia.

Much more attention has been given to the possibility of tolerance being associated with down-regulation or changes in affinity of opioid receptors. Unfortunately, the picture is not clear in spite of an extensive literature. Early investigators using ligand binding generally found either no changes or changes that did not fit the development of tolerance temporally or in magnitude. However, those early investigators generally examined whole brain and the investigations preceded recognition of multiple types of opioid receptor (Loh et al., 1988).

Early investigations of the possibility that qualitative changes in opioid receptors could be associated with tolerance and/or dependence were carried out by Takemori and associates (Takemori et al., 1973; Tulunay and Takemori, 1974a,b; Kitano and Takemori, 1977). These investigators calculated affinities of naloxone versus morphine and other agonists or partial agonists as analgesics using in vivo pA_2 determinations (Takemori et al., 1973; Tulunay and Takemori, 1974a,b).

The pA_2 calculation (−log concentration of antagonist causing a 2-fold shift in the agonist concentration-response curve) was developed by Schild (1947) whose mathematical derivations indicated that, when a number of conditions were met in in vitro experiments, pA_2 is equal to $-\log K_B$, where K_B is the dissociation constant of the antagonist for the receptor (Schild, 1957; Arunlakshana and Schild, 1959). A number of investigators have used *"apparent"* pA_2 determinations in vivo to derive an apparent dissociation constant. An actual K_B calculation requires that one be able to estimate the concentration of the antagonist in the biophase of the receptors under equilibrium conditions. Generally, that is not feasible in vivo. Nevertheless, under proper conditions, the true K_B may be assumed to be a function of the in vivo pA_2 (Cox and Weinstock, 1964; Green and Fleming, 1967; Takemori et al., 1969).

Cox and Weinstock (1964) were the first to apply in vivo pA_2 estimations to opioid drugs. Takemori's group developed defined conditions for determining in vivo pA_2 values for naloxone in antagonizing morphine's analgesic effect in the phenylbenzoquinone test in mice (Takemori et al., 1969; 1972; Smits and Takemori, 1970). Subsequently, they used these procedures to determine that pretreatment of mice with morphine, 2 mg/kg, 2 h before the experiment, induced an apparent 2-fold increase in the affinity of the analgesic receptors for naloxone (Takemori et al., 1973). This effect on the affinity for naloxone lasted for more than 24 h. Similar changes were found in experiments in which analgesia was determined by the tail-flick test (Takemori et al., 1973). Another study in the same laboratory, but not using pA_2 determinations, found similar increases in the effectiveness of

other opioid antagonists resulting from pretreatment with morphine (Tulunay and Takemori, 1974a).

It is interesting that, under conditions in which the in vivo pA_2 value for naloxone was changed by pretreatment with morphine, there was no induction of tolerance to morphine (Takemori et al., 1973). Nevertheless, Tulunay and Takemori (1974a) suggested that ". . . this increased efficacy of narcotic antagonist due to narcotic pretreatment might be a sensitive indicator of the initiation and development of tolerance to narcotic analgesics."

Work with striatal slices from mice further strengthened the conclusion that prior exposure of mice to morphine led to an increased affinity of opioid receptors to naloxone (Kitano and Takemori, 1977). Naloxone was shown to produce concentration-dependent release of ^3H from striatal slices incubated with [^3H]morphine. In slices taken from animals that had received a morphine pellet, implanted 72 h before the experiment, ^3H was released more rapidly and by lower concentrations of naloxone than it was from control preparations. The maximum amount of releasable ^3H was not changed. Kitano and Takemori (1977) concluded that the affinity of the receptors for morphine was decreased and the affinity for naloxone increased in the morphine-pretreated animals. Whether or not the striatal cells involved in the binding and release of [^3H]morphine exhibited tolerance to morphine was not determined.

In 1974, Tulunay and Takemori (1974b) produced tolerance to the analgesic effects of morphine in mice by several different regimens of morphine treatment. They found that the 50% effective dose (ED_{50}) of morphine in the presence of a set dose of naloxone, 160 μg/kg, increased with the development of tolerance to morphine. They also reported an increase in the in vivo pA_2 value for naloxone in mice, each of which had received an implanted morphine pellet 72 h before the experiment. It was concluded that naloxone's increased effectiveness "appears to be a sensitive indicator of the development of tolerance."However, that interpretation depends upon the manner in which one analyzes the data. In a situation in which the sensitivity to an agonist varies from group to group, the ED_{50} of the agonist in the presence of a set dose of an antagonist may not be the best indicator of differences in effectiveness of the antagonist between groups. Rather, the ratio of ED_{50} values for the agonist in the presence and absence of a dose of the antagonist within each group is a better indicator. If one applies such an analysis to the data in figure 1 of the paper by Tulunay and Takemori (1974b), one finds only small differences in the ratios of ED_{50} values and no correlation with the degree of tolerance. The increase in pA_2 value is apparently based upon the data in table 1 (Tulunay and Takemori, 1974b). If one constructs Schild plots from the data in that table, one finds that the curves in tolerant and control animals deviate markedly from parallel, and therefore, the pA_2 values are not comparable.

Several laboratories have used in vitro pA_2 determinations of naloxone versus morphine or normorphine in the ileum from guinea pigs as an indication of qualitative changes in opioid receptors associated with opioid tolerance. Tolerance was induced by standard procedures involving subcutaneous implantation of pellets. Ward and Takemori (1976a) reported a decrease in pA_2 indicative of a 7-fold decrease in affinity of the opioid receptors for naloxone. Subsequent work by Tallarida et al. (1982), in which pA_2 values were precisely constrained to slopes of -1 in Schild plots, confirmed that finding, with a decrease in the pA_2 of 0.6 log units (4-fold decrease in affinity) in pieces of ileum from guinea pigs in which morphine pellets had been implanted. In contrast, Huidobro-Toro et al. (1981) reported an *increase* in the pA_2 of naloxone in pieces of ileum exhibiting tolerance. However, the results from Huidobro-Toro et al. were based on Schild plots with slopes that deviated markedly from -1, probably because preparations were tested in the presence of normorphine, 100 nM, above and beyond the amount used to calculate the pA_2. The experiments of Ward and Takemori (1976a) and Tallarida et al. (1982) were not encumbered by that complication, yielded slopes very close to unity, and, therefore, accurately reflected a decrease in receptor affinity for naloxone in the opioid tolerant ileum.

Porreca and Burks (1983) used the irreversible antagonist, β-chlornaltrexamine to estimate the dissociation constant (K_A) of the opioid receptors for the agonist, normorphine, in the isolated guinea pig ileum. The standard morphine pellet implantation procedure was used to induce tolerance. They reported that the K_A for normorphine was not different between control and opioid-tolerant preparations, indicating no change in affinity for the agonist. They also calculated that the concentration of normorphine that inhibits the nerve-mediated cholinergic contractions by 50% (IC_{50}) was only one-sixth of the K_A in naive preparations. In contrast, the ratio of IC_{50} to K_A in tolerant preparations was approximately unity. Porreca and Burks (1983) suggested that tolerance is probably related to changes in the chain of events that couple the receptor to the final response, a conclusion supported by similar experiments by Chavkin and Goldstein (1984). The decrease in sensitivity to a number of inhibitory substances (Taylor et al., 1988), the increase in sensitivity to excitatory substances (Johnson et al., 1978), and the lack of change in binding of [^3H]etorphine (Cox and Padhya, 1977) in the morphine-tolerant ileum also lead to the conclusion that tolerance is the result of a cellular adaptation beyond the level of the receptor. Without a change in affinity for opioid agonists, it is difficult to relate changes in affinity for antagonists to the development of tolerance in the ileum.

Early binding studies with radioactive ligands suggested that chronic exposure to morphine by means of

pellet implantation in rats induced both tolerance of the animals to morphine and a decrease in the affinity of specific binding sites in brain stem slices for [^{3}H]morphine and [^{3}H]etorphine (Davis et al., 1975; 1979). The effect on binding was gradually reversible when the pellets were removed. Subsequently, Oishi et al. (1983) identified two binding sites in the mouse brain for [^{3}H]naloxone. Implantation of a morphine pellet for 3 days resulted in a selective 50% decrease in affinity of the low-affinity site for naloxone. There were no changes in maximum binding.

In contrast, Sivam et al. (1982) reported that both acute and chronic treatment of mice with morphine increased the number of both high- and low-affinity sites in the brain without a change in affinity. Other laboratories have presented evidence of decreases in maximum binding associated with chronic exposure to opioids without changes in affinity. These include a 25% decrease in [^{3}H]naloxone binding in intact brain cells from rats in which morphine pellets were implanted (Rogers and El-Fakahany, 1986) and a 30% decrease in striatal binding of [D-serine^2][tyrosyl-3,5-^{3}H]-enkephalin-[5-L-leucine, 6-L-threonine] following chronic administration of enkephalin by osmotic minipump (Steece et al., 1986).

In a particularly extensive investigation, Tao et al. (1987) administered etorphine by osmotic minipump to rats for up to 7 days. They measured tolerance, withdrawal, and ligand binding in a number of brain sites. Tolerance, as measured by the tail-flick test, to etorphine was greater than 4-fold and withdrawal responses were elicited by naloxone. Decreases of specific binding of [^{3}H]diprenorphine to membranes from cortex, midbrain, and striatum of 35 to 65% occurred within 7 days of treatment. By using [^{3}H]DADLE and displacing a portion of it with morphiceptin, Tao et al. (1987) were able to assess μ- and δ-sites separately in the membranes of the same brain regions. The data indicated a decrease of 40 to 60% in μ-sites in the three regions. Binding for δ-sites was unchanged in striatum but decreased 25% in cortex and 40% in midbrain by 7 days of etorphine treatment. The decreased binding was due to a decrease in maximum binding, not an increase in K_D. Tao et al. concluded that, although chronic opioid exposure did induce down-regulation of opioid receptors, there was no direct correlation between the receptor change and the magnitude of tolerance.

A number of laboratories have reported no changes in opioid receptor binding associated with chronic exposure to opioids. These include Höllt et al. (1975) (mouse brain), Rubini et al. (1982) (mouse vas deferens), Perry et al. (1982) (rat brain without cerebellum), Matsui and Yamamoto (1984) (guinea pig stria terminalis), and Geary and Wooten (1985) (quantitative autoradiography in several regions of rat brain).

Several recent studies have consistently found that chronic exposure of animals (Moudy et al., 1985; Tempel et al., 1985) or isolated fetal mouse spinal cord-ganglion explants (Tempel et al., 1986) to opioid antagonists induces increases ("up-regulation") of opioid-binding sites. There seems little doubt that opioid receptors can undergo adaptive up- and down-regulation. Nevertheless, the inconsistency of reported changes in opioid ligand binding induced by chronic exposure to opioids and the relatively small changes in binding that have been observed lead to the conclusion that tolerance and dependence in opioid systems are not readily explained by changes in density or affinity of receptors.

B. Membrane Potential

The hypothesis that adaptive changes in membrane potential could provide the basis for tolerance and dependence presents a different approach to the problem that has received relatively little attention until recently. The concept has arisen particularly from the characteristics of tolerance and dependence in the longitudinal muscle-myenteric plexus of the guinea pig ileum (section VI, A). Implantation of morphine pellets in guinea pigs results in tolerance of the isolated longitudinal muscle-myenteric plexus that is paralleled by a nonspecific subsensitivity to unrelated inhibitory substances and supersensitivity to stimulatory substances.

Nonspecific sensitivity changes are a prime characteristic of the adaptive changes that occur with chronic suppression of neurotransmission to a number of excitable cells, especially smooth muscle (section II). Nonspecific sensitivity changes in some cell types have been closely linked to alterations in resting membrane potential (Fleming et al., 1973; Fleming and Westfall, 1988, and section III, B). The nonspecificity of the sensitivity changes in the morphine-tolerant myenteric plexus led Johnson et al. to propose in 1978 that the myenteric neurons were undergoing an adaptive partial depolarization. Such a depolarization, by placing resting potential closer to the threshold for firing action potentials, could produce supersensitivity to a variety of excitatory agents and subsensitivity to a variety of inhibitory agents.

The ability of opioids to inhibit contractions of the longitudinal muscle of the guinea pig ileum to electrical stimulation may be a function, at least in part, of a hyperpolarization of the S neurons of the myenteric plexus induced by opioids (section VI). Although opioids hyperpolarize the ganglion cells acutely, cells that are and have been in the presence of morphine, in vitro, for 24 h have membrane potentials that are not different from control cells. The acute addition of naloxone reduces membrane potential in these "tolerant" cells to values significantly less negative than control (Johnson and North, 1980). This "depolarizing" effect of naloxone sometimes leads to the firing of action potentials. In naive cells, naloxone, by itself, had no effect on membrane potential, reduced the hyperpolarized state acutely induced by opioids to normal, never lowered potential to levels less negative than control values, and never elicited

action potentials. One logical explanation of these results is that the hyperpolarizing effect of the opioids has been masked by an adaptation in the form of a less negative resting membrane potential. This depolarized state of the tolerant cells is only observed when the hyperpolarizing effect of the opioid is antagonized by naloxone. Thus, the findings of Johnson and North (1980) are consistent with the hypothesis. The excitable state at any moment is a function of membrane potential at that moment and is the sum of resting potential plus any change induced by a drug or transmitter. Preliminary experiments (Leedham et al., 1988) have provided direct evidence of a partially depolarized state in myenteric S neurons during the first few hours after removal of the ileum from morphine-tolerant animals. Thus, there is adequate evidence in support of the hypothesis to give it serious consideration.

VI. Peripheral Nervous System

Tolerance and dependence, induced in experimental animals by chronic pretreatment with morphine, are both prevented by concomitant treatment with an opioid antagonist such as nalorphine, naltrexone, or naloxone (Orahovats et al., 1953; Yano and Takemori, 1977; Bhargava, 1978). This implies that occupation of the receptor by an agonist is a prerequisite for the development of the phenomena. Therefore, knowledge of the acute receptor-mediated actions of opioids is essential for an understanding of the adaptive changes underlying tolerance and dependence. Two peripheral tissues, the isolated ileum of the guinea pig and vas deferens of the mouse, have been extensively exploited for this purpose. The current status of understanding of the acute and chronic actions of opioids in each preparation is reviewed in this section.

A. Guinea Pig Ileum

1. Acute actions. Probably the first observation of a specific opioid effect in vitro was made in the guinea pig ileum. Trendelenburg (1917) showed that morphine inhibited the peristaltic reflex, and numerous investigations confirmed this observation (Schaumann, 1955; Kosterlitz and Robinson, 1957; Green, 1959; Aldunate, et al., 1975; Holzer and Lembeck, 1979). Both the preparatory phase, comprising a contraction of the longitudinal muscle, and the emptying phase, comprising contractions of the circular muscle, that are seen in preparations set up by the Trendelenburg technique are inhibited by opioids (Schaumann, 1955; Kosterlitz and Robinson, 1957), with the preparatory phase being depressed preferentially (Fontaine et al.,1973). Agonists active at both μ- and κ-opioid receptors, including the naturally occurring opioids Met-enkephalin and dynorphin, inhibit the reflex (Van Nueten et al., 1977; Donnerer and Lembeck, 1985; Kromer et al., 1982). Inhibition is due to actions on neurons rather than directly on the muscle layers themselves, because contractions evoked by the direct action of acetylcholine are unaffected by morphine (Kosterlitz and Robinson, 1958), whereas cholinergic contractions evoked by indirectly acting agonists such as nicotine (Kosterlitz and Robinson, 1958; Gaddum and Picarelli, 1957; Schaumann, 1955), 5-HT (Kosterlitz and Robinson, 1958; Gaddum and Picarelli, 1957), and neurotensin (Huidobro-Toro et al., 1984) are inhibited.

a. LONGITUDINAL MUSCLE-MYENTERIC PLEXUS PREPARATION. Because the actions of opioids on the peristaltic reflex were difficult to investigate quantitatively (Kosterlitz et al., 1972), advantage has been taken of the ileal preparation described by Paton (1957). Transmural stimulation of an isolated segment of ileum, by single electrical pulses, evoked contractions of the longitudinal muscle which were due to excitation of neurons in the myenteric plexus and subsequent liberation of acetylcholine. Morphine depressed these nerve-mediated contractions but did not affect contractions evoked by the direct action of acetylcholine on the muscle. This preparation, in which mechanical recordings are made from the longitudinal muscle, either in an intact ileal segment or in a strip comprising the longitudinal muscle and attached myenteric plexus (Rang, 1964), has since been extensively used in investigations of both the acute and chronic actions of opioids.

The inhibition of electrically evoked contractions is due to depression of the release of acetylcholine from the myenteric plexus (Paton, 1957; Schaumann, 1957; Cox and Weinstock, 1966; Paton and Zar, 1968; Szerb, 1982; Vizi et al., 1984). Opioids also decrease the release of acetylcholine evoked by agents that elicit action potentials in myenteric neurons, such as substance P (Vizi and Bartho, 1985; Yau et al., 1986), PGE_1 (Jaques, 1969; Yagasaki et al., 1981), neurotensin (Yau et al., 1983a), and caerulein (Yau et al., 1983b). Agonists active at both μ- and κ-receptors are effective in depressing cholinergic transmission (Hutchinson et al., 1975; Lord et al., 1977; Goldstein et al., 1979; Kosterlitz et al., 1980; Yoshimura et al., 1982; Ward et al., 1986). Although radioligand-binding studies demonstrated the presence of δ-receptors (Leslie et al., 1980), organ bath experiments did not indicate that they were important in the inhibitory actions of opioids (but see the paper by Gintzler and Hyde, 1984). Studies using DADLE to investigate δ-receptors (Egan and North, 1981) must be regarded with caution because of the relatively poor selectivity of DADLE as a δ-agonist (Patterson et al., 1983).

Noncholinergic transmission to the longitudinal muscle is also inhibited. Electrical stimulation of myenteric neurons at higher frequencies (10 Hz) evokes noncholinergic contractions that are probably mediated by release of substance P (Franco et al., 1979). Opioids depress the release of substance P (Holzer, 1984) and reduce the electrically evoked, substance P-mediated contractions (Gintzler and Scalisi, 1982; Bartho et al.,1982).

The myenteric plexus includes complex neuronal circuits that control the activities of both the longitudinal and circular muscles (Furness and Costa, 1987). Opioid receptors could be located at many possible sites, including sensory neurons, interneurons, and motor neurons, as well as on the processes of extrinsic neurons that supply myenteric ganglia. Several observations suggest, however, that the receptors involved in inhibition of cholinergic contractions of the longitudinal muscle evoked by electrical stimulation are located on motor neurons. First, the electrically evoked contractions are largely unaffected by hexamethonium, which blocks intraganglionic cholinergic nicotinic transmission, or by pretreatment with 5-HT (Dingledine and Goldstein, 1976) which depresses all excitatory transmission to myenteric neurons (Johnson et al., 1981c; Galligan et al., 1988). Therefore, the contractions themselves most probably result from electrical stimulation of cholinergic motor neurons. Second, inhibition by morphine is unimpaired in ilia treated with hexamethonium and 5-HT (Dingledine and Goldstein, 1976), indicating that morphine does not act by inhibiting the release of an excitatory transmitter acting at intraganglionic synapses. Third, the inhibition is unaffected by antagonists at α- or β-adrenoceptors (Gyang and Kosterlitz, 1966; Kosterlitz and Watt, 1968) or by pretreatment with 6-hydroxydopamine (Ward and Takemori, 1976b; Ferri et al., 1977). These experiments, and the direct biochemical studies of Henderson et al. (1975), indicate that morphine does not act by releasing norepinephrine present in extrinsic nerves that supply myenteric ganglia. Like morphine, exogenous norepinephrine depresses electrically evoked cholinergic contractions and acetylcholine release by an inhibitory action on myenteric neurons (Kosterlitz et al., 1970). These observations suggest that receptors on excitatory motor neurons could account fully for opioid-induced inhibition of electrically evoked cholinergic contractions.

b. CIRCULAR MUSCLE-MYENTERIC PLEXUS PREPARATION. Far fewer investigators have examined the actions of opioids on the circular muscle and the neurons supplying it. Harry (1963) showed that morphine depressed contractions produced by 5-HT, but not by acetylcholine, suggesting an action on neurons of the myenteric plexus rather than on the circular muscle itself. Morphine did not alter the resting membrane potential of the circular muscle but inhibited cholinergic excitatory junction potentials evoked by electrical stimulation of myenteric neurons (Ito and Tajima, 1980). In strip preparations comprising the circular muscle and myenteric plexus, cholinergic and noncholinergic contractions elicited by electrical stimulation of neurons were depressed by opioids acting on μ- and κ-, but not δ-, receptors (Johnson et al., 1987b). These are the same receptor subtypes as those present on neurons supplying the longitudinal muscle.

One advantage of the circular muscle preparation, compared with the longitudinal muscle strip, is that it enabled the location of the receptors on motor neurons to be better defined. The myenteric plexus may be readily removed from the circular muscle, yielding a preparation comprising the circular muscle and only axonal processes of myenteric neurons, severed from their cell bodies in the plexus (Johnson et al. 1988a). Electrical stimulation of these axons evoked cholinergic contractions that were depressed by μ- and κ-agonists, indicating that some receptors of both subtypes were located on axons of cholinergic excitatory motor neurons.

It should be noted, however, that neither these experiments nor those in the longitudinal muscle preparation exclude the presence of opioid receptors on other types of neurons in the myenteric plexus. Both μ- and κ-agonists have been reported to act presynaptically to depress cholinergic nicotinic excitatory postsynaptic potentials recorded in myenteric neurons (Cherubini and North, 1985; Cherubini et al., 1985). Although this is not a consistent finding (North and Henderson, 1975; North et al., 1979), it suggests that at least some opioid receptors may be located on neurons other than motor neurons. A presynaptic site was also proposed to underlie inhibition of slow noncholinergic excitatory postsynaptic potentials by μ-agonists (Cherubini et al., 1985). Although these receptors are not involved in inhibition of electrically evoked cholinergic contractions of the longitudinal and circular muscles, they could be important in other actions, such as inhibition of the peristaltic reflex.

c. MUCOSA-SUBMUCOUS PLEXUS PREPARATION. Opioids also exert acute inhibitory effects on the neuroeffector circuitry comprising the submucous plexus and the ileal mucosa which transports water and electrolytes across the gut wall (Keast, 1987; Bornstein and Furness, 1988). Electrogenic secretion of Cl^- by mucosal cells is inhibited by opioids by activation of δ-, but not μ- or κ-, receptors (Kachur et al., 1980; Kachur and Miller, 1982; Vinayek et al., 1983). In preparations from rabbit ileum the δ-receptors involved in producing the antisecretory actions are located on neurons in the submucous plexus (Binder et al., 1984).

d. CELLULAR MECHANISMS OF INHIBITION. Opioids could inhibit the release of excitatory transmitters from motor neurons by two basic mechanisms. They could act on the release process itself, decreasing the amount of acetylcholine liberated by each action potential that invades the release site (Paton, 1957; Schaumann, 1957; Paton and Zar, 1968). They could also reduce the excitability of motor neurons, thus reducing the number of action potentials that propagate to sites of release. In the case of μ-agonists, such as morphine, evidence for the second mechanism was provided by electrophysiological recordings from myenteric neurons. Studies using extracellular recording electrodes showed that morphine depressed the discharge of action potentials in myenteric

neurons (Sato et al., 1973; Dingledine et al., 1974; North and Williams, 1977; Karras and North, 1981). Because inhibition persisted after all synaptic transmission in the myenteric plexus was blocked, Dingledine and Goldstein (1975; 1976) postulated a direct action of morphine on neuronal excitability, such as a membrane hyperpolarization. This was verified by intracellular recordings from myenteric neurons, a proportion of which were hyperpolarized by μ-agonists such as normorphine (North and Tonini, 1977). The hyperpolarization, associated with an increase in potassium conductance (Morita and North, 1982; Suprenant and North, 1985), reduced the excitability of these neurons. Principal sites of action were on neuronal processes (North et al., 1979), a hyperpolarization of which prevented the propagation of action potentials (Morita and North, 1981).

The functional identities of neurons were not known in these experiments. However, of the two classes of neurons defined electrophysiologically (S and AH; Hirst et al., 1974), hyperpolarizations were observed predominantly or exclusively in S neurons (North and Tonini, 1977; North et al., 1979; Morita and North, 1982; Johnson et al., 1987c) which comprise both interneurons and motor neurons (Furness et al., 1988a). Hyperpolarization of motor neurons may prevent propagation of action potentials to sites of transmitter release and thus provides a plausible explanation for inhibition of excitatory transmission to the muscle layers (North and Williams, 1983b; North and Egan, 1983).

The mechanisms by which κ-agonists inhibit nerve-mediated cholinergic contractions is less certain. κ-Agonists do not affect the resting membrane potential or conductance of myenteric neurons but have been reported to decrease the inward Ca^{++} current underlying action potentials in the somata of AH neurons (Cherubini and North, 1985). At least part of the inhibitory effect on nerve-mediated cholinergic contractions is due to an action on axonal processes (Johnson et al., 1988a), the action potentials of which do not depend on an inward Ca^{++} current (North and Nishi, 1976). It is, therefore, unlikely that κ-agonists act by interfering with action potential propagation, by modulating Ca^{++} entry in these processes. However, the action potential arriving at sites of transmitter release may promote Ca influx on which release depends. It could be that κ-agonists reduce Ca^{++} entry at these sites and hence decrease transmitter release.

In submucous neurons of the guinea pig ileum, intracellular recordings showed that δ-agonists caused a membrane hyperpolarization accompanied by an increase in K^+ conductance (Mihara and North, 1986; North et al., 1987). A relationship between the hyperpolarization and the antisecretory effects has not been established. It is possible, however, that a hyperpolarization of submucous neurons by δ-agonists, like that produced by μ-agonists in myenteric neurons, could contribute to opioid-induced inhibition of the activity of secretomotor neurons.

The intracellular biochemical mechanisms mediating the inhibitory actions of μ- and κ-agonists on myenteric neurons and of δ-agonists on submucous neurons remain largely unexplored. Receptors for many hormones and neurotransmitters are coupled to a G protein as a first step in transducing receptor occupancy to pharmacological effect (Rodbell, 1980; Graziano and Gilman, 1987; Gilman, 1987; Casey and Gilman, 1988). Pertussis toxin has been used extensively to investigate the role of G proteins in transduction (Ui, 1984). The toxin catalyzes the transfer of an ADP-ribose moiety from nicotinamide adenine dinucleotide to several types of G proteins, including G_i, G_o, and G_p (Sternweis and Robishaw, 1984; Okajima et al., 1985). After ADP-ribosylation, the G protein is inactive as a transducer.

In the ileum, conflicting observations have been made on the effects of pertussis toxin on the inhibitory actions of opioids. Collier et al. (1983) and Lux and Schulz (1986) reported that pretreatment of guinea pigs with pertussis toxin did not alter the depressant effect of normorphine on nerve-mediated contractions of the longitudinal muscle. Tucker (1984) and Lujan et al. (1984), on the other hand, found that the inhibitory effects were prevented after pertussis toxin pretreatment. Purity of batch samples of pertussis toxin may account in part for the discrepancies (Lujan et al., 1984). However, a detailed investigation of the pharmacology of pertussis toxin itself was not undertaken in these studies. This will be necessary in order to interpret the interactions of pertussis toxin with opioid mechanisms and, hence, to determine whether opioid receptors are coupled to G proteins. Of particular importance is the need to establish whether pertussis toxin catalyzes ADP-ribosylation of certain G proteins in myenteric neurons.

More convincing evidence for the involvement of a G protein in opioid actions in the ileum has been obtained by recording electrophysiologically from neurons in the submucous plexus. Hyperpolarization of these neurons by δ-agonists was prolonged by intracellular injection of the nonhydrolyzable GTP analogue, guanosine 5′-[δ-thio]triphosphate (North et al., 1987; Tatsumi and North, 1989) and was prevented by either intracellular injection of pertussis toxin or pretreatment of tissues with pertussis toxin (Tatsumi and North, 1989).

Cyclic AMP has frequently been implicated as an intracellular messenger for the receptor-mediated actions of opioids (Collier and Roy, 1974; Collier, 1980; Schramm and Selinger, 1984; Worley et al., 1987; Neher, 1988). This proposal has been based primarily on biochemical observations that opioids inhibit adenylate cyclase activity or decrease the levels of cyclic AMP in neuroblastoma-glioma hybrid cells and in neurons from a variety of sites in the CNS, principally the rat striatum (section V). More recently, it has been shown that aden-

ylate cyclase activity is also present in guinea pig myenteric neurons (Jeitner and Costa, 1989) and that morphine decreases the activity of the enzyme (T. M. Jeitner, personal communication).

Several studies have suggested, however, that the inhibitory actions of opioids on the excitability of myenteric neurons and on cholinergic transmission to the longitudinal muscle are probably not mediated by decreases in intracellular cyclic AMP. These experiments examined the actions of a variety of agents that elevate or mimic intracellular cyclic AMP, including phosphodiesterase inhibitors, such as isobutylmethylxanthine, membrane-permeable analogs of cyclic AMP, such as 8-bromo cyclic AMP and dibutyryl cyclic AMP, agents such as forskolin which directly activates adenylate cyclase, and cholera toxin which activates G_s, the stimulatory G protein that is coupled to cyclic AMP production. Inhibition of action potential discharge in myenteric neurons by μ- or κ-agonists was unaffected by the phosphodiesterase inhibitor, isobutylmethylxanthine, or the membrane-permeable analogue of cyclic AMP, dibutyryl cyclic AMP (Karras and North, 1979). Neither cholera toxin, dibutyryl cyclic AMP, nor forskolin altered significantly the inhibitory potencies of μ- and κ-agonists on cholinergic transmission to the longitudinal muscle (Lux and Schulz, 1986; Johnson et al., 1989a). The hyperpolarization of myenteric neurons by the μ-agonist, (D-Ala^2,MePhe^4,Gly^5-ol)-enkephalin (DAMGO), was similarly unaffected by forskolin, dibutyryl cyclic AMP, or isobutylmethylxanthine (Johnson et al., 1989a). Although the phosphodiesterase inhibitors, theophylline and isobutylmethylxanthine, prevented the inhibitory effects of morphine on acetylcholine release and on nerve-mediated cholinergic contractions in the experiments of Sawynok and Jhamandas (1976, 1979), nonxanthine phosphodiesterase inhibitors failed to do so. Sawynok and Jhamandas (1979) suggested that the actions of theophylline and isobutylmethylxanthine were unrelated to inhibition of phosphodiesterase but were probably due to mobilization of Ca^{++}.

None of these experiments supports the proposal that acute opioid effects on neurotransmission and neuronal excitability in the ileum are mediated by decreases in intracellular cyclic AMP. The significance of opioid-induced decreases in adenylate cyclase activity, observed biochemically, remains to be determined.

Calcium has also been repeatedly implicated in numerous actions of opioids, principally by the extensive contributions of Way and his associates (Chapman and Way, 1980). In the ileum, elevation of the extracellular Ca^{++} concentration from 1.25 to 5 mM resulted in a 100-fold decrease in potency of normorphine in depressing nerve-mediated contractions of the longitudinal muscle (Huidobro-Toro et al., 1981). Apparent antagonism of morphine by increases in extracellular Ca^{++} was also observed by Sawynok and Jhamandas (1979) and by Opmeer and Van Ree (1980). Conversely, decreases in extracellular Ca^{++} enhanced the inhibitory effect of the μ-agonist, DAMGO (Dougall and Leff, 1987). The antagonistic action of elevated Ca^{++} observed by Huidobro-Toro et al. (1981) was relatively selective insofar as the inhibitory potencies of epinephrine and ATP were reduced to a much lesser extent, approximately 3- to 5-fold (Huidobro-Toro et al., 1981). A similar interaction between Ca^{++} and opioids was reported in electrophysiological experiments. The hyperpolarization of myenteric S neurons by μ-agonists was increased when the external calcium concentration was lowered and reduced when Ca^{++} was raised (Morita and North, 1982). The experiments indicate a close relationship between Ca^{++} concentration and opioid actions, but the mechanism of this interaction within the axoplasm or in membrane ion channels remains to be elucidated. With the rapidly advancing concepts of intracellular effector mechanisms, it might be anticipated that Ca^{++} will reemerge as a major focal point of opioid research.

Receptor-mediated signals may also be transduced by the phosphatidylinositol-phospholipase C pathway (Berridge, 1987). A G protein is involved in the coupling between the receptor and phospholipase C which hydrolyses phosphatidylinositol (4,5)-bisphosphate to form diacylglycerol and inositol (1,4,5)-trisphosphate. These influence ion channels by activation of protein kinase C and mobilization of intracellular Ca^{++}, respectively. The possible role of the phosphotidylinositol-phospholipase C pathway as an intracellular messenger system for opioid actions has not been investigated in the ileum or any other system.

2. Chronic actions. a. EXPERIMENTAL STRATEGIES. Three basic strategies have been used to investigate whether changes characteristic of tolerance and dependence could be demonstrated in the isolated ileum. In two of these, guinea pigs are pretreated with an opioid, either by repeated injections or by subcutaneous implantation of pellets or osmotic minipumps. The ileum is then removed and placed in a modified Krebs' solution. In one strategy, opioid is added to the Krebs' solution at a concentration similar to that in the plasma at the time the tissue was removed. This serves to maintain continuous occupancy of the receptor by the opioid in vitro and, hence, to sustain any changes induced by pretreating the animal. Observations are made subsequently in the presence of the maintenance concentration of the opioid.

In the second strategy, observations are made in the absence of the opioid, after it has been removed from its receptors by washing. The ileum from the pretreated guinea pig is either set up in vitro in opioid-free Krebs' solution or it may be set up in the presence of a maintenance concentration of opioid, which is subsequently removed by washing. Under these conditions, ilia are examined at various times after opioid withdrawal, for up to several hours in vitro.

The third approach uses ileal preparations from untreated guinea pigs. The preparations are incubated in vitro in Krebs' solutions containing an opioid, for periods up to 24 h.

The different experimental strategies may induce or reveal different aspects of tolerance and dependence. For example, tolerance that is present in preparations maintained in opioid may include a phenomenon qualitatively distinct from that observed a few hours after withdrawal. It is essential for an understanding of the chronic actions of opioids that observations made in the different experimental circumstances be adequately reconciled.

b. DEPENDENCE. In longitudinal muscle-myenteric plexus preparations from morphine-pretreated guinea pigs maintained in opioid-containing solutions in vitro, naloxone evokes a powerful contraction of the longitudinal muscle not observed in similar preparations from untreated guinea pigs (Schulz and Herz, 1976). The contraction is due to an abrupt decrease in the occupancy of the opioid receptors by the agonist and is used as an index of morphine dependence. That this phenomenon bears the essential characteristics of dependence in the whole animal was well illustrated by the experiments of Frederickson et al. (1976) and of Schulz et al. (1974). In both studies, the rate of development of dependence in the ileum and the rate of its disappearance after cessation of morphine treatment closely paralleled the onset and offset of behavioral signs of dependence in the whole animal. Agents such as codeine and pentazocine, which induced modest degrees of dependence in the whole animal, as assessed by the frequency of withdrawal jumping, also induced only modest dependence in the ileum, as indicated by the smaller magnitude of the naloxone-induced contractions following pretreatment with these agents (Frederickson et al., 1976).

The contraction of the longitudinal muscle evoked by naloxone is blocked by tetrodotoxin (Schulz and Herz, 1976; Frederickson et al., 1976), indicating that the excitation of the muscle is an indirect consequence of action potential discharge in myenteric neurons. The contraction is also greatly reduced or abolished by atropine (Schulz and Herz, 1976; Frederickson et al., 1976), indicating that it is due to the release of acetylcholine from excitatory myenteric motor neurons supplying the longitudinal muscle. The withdrawal contraction is not, however, affected by hexamethonium, indicating that transmission at excitatory intraganglionic cholinergic nicotinic synapses is not mandatory for the expression of dependence.

In addition to acetylcholine, substance P has also been implicated in the withdrawal response. Part of the naloxone-induced contraction was resistant to hyoscine (scopolamine) and was reduced or abolished after desensitization of the longitudinal muscle to substance P (Gintzler, 1980; Tsou et al., 1982) or after treatment with a substance P antagonist (Tsou et al., 1985). More recently, Wang and Tsou (1989) showed that substance P-like immunoreactivity is released from longitudinal muscle-myenteric plexus preparations during naloxone-precipitated withdrawal. Thus, neurons that release both acetylcholine and substance P may play a role in the nerve-mediated withdrawal contraction of the longitudinal muscle.

Myenteric neurons that supply the circular muscle are activated during withdrawal as well. In preparations comprising the circular muscle and myenteric plexus from guinea pigs pretreated with morphine, naloxone evoked marked phasic contractions of the circular muscle (Johnson et al., 1989b). This response, like that recorded in the longitudinal muscle, was also greatly reduced by tetrodotoxin and by hyoscine, indicating excitation of motor neurons that release acetylcholine onto the circular muscle.

Vinayek et al. (1985) studied changes induced by chronic activation of δ-receptors in the submucous plexus. However, after pretreatment of guinea pigs for 5 days with the δ-preferring agonist, DADLE, isolated segments of ileal mucosa did not exhibit any change in short-circuit current when challenged with naloxone. Prolonged occupation of δ-receptors on submucous neurons apparently does not initiate adaptive changes leading to dependence. Thus, it is possible that the gastrointestinal disturbances such as diarrhea, observed in guinea pigs after morphine withdrawal, may result from enhanced peristalsis (Kromer and Woinoff, 1980) rather than alterations in water and electrolyte transport. It should be noted, however, that in other species, notably the rat, the mucosa may play a major role in opioid withdrawal (Coupar, 1987).

Signs of dependence in the guinea pig ileum can be revealed not only by naloxone but also when the opioid is removed from its receptors more gradually, by repeatedly washing tissues with opioid-free solution. Marked phasic contractions of the longitudinal (Johnson et al., 1987a) and circular muscle (Johnson et al., 1989b) occur after morphine washout. They are prevented by tetrodotoxin and hyoscine but not by hexamethonium and, hence, probably arise from the same mechanism that underlies the contraction evoked by naloxone. Whereas contractions evoked by naloxone reach a peak within about 1 min, those resulting from morphine washout peak more slowly, within 15 to 30 min (Johnson et al., 1987a). The difference in rates of onset is consistent with the relatively slow rate of removal of morphine from its receptors by washing.

After morphine was removed by washing, the ensuing contractions are well sustained, indicating that the cellular changes underlying dependence persist for at least many hours in vitro after morphine withdrawal (Johnson et al., 1987a). Under these conditions, ilia exhibit supersensitivity to any agent or procedure that elicits cholinergic contractions of the longitudinal muscle by excita-

tion of myenteric neurons. Excitatory agonists examined include 5-HT (Takagi et al., 1965; Schulz and Goldstein, 1973; Takayanagi et al., 1974; Ward and Takemori, 1976b; Johnson et al., 1978), PGE_1 (Schulz and Herz, 1976), and nicotine (Johnson et al., 1978). Neurogenic cholinergic contractions produced by raising the extracellular K^+ concentration (Johnson et al., 1978) or by electrical stimulation of myenteric neurons (Schulz and Cartwright, 1974; Johnson et al., 1978; Cox, 1979) are also significantly enhanced. Contractions produced by the direct action of acetylcholine on the muscle are unchanged (Takagi et al., 1965; Haycock and Rees, 1972; Johnson et al., 1978), indicating that the mechanisms underlying nonspecific supersensitivity are located in neurons of the myenteric plexus.

Dependence has also been induced by incubating ilia from untreated guinea pigs with opioids in vitro. Ehrenpreis et al. (1972) first noted that naloxone not only abolished the acute inhibitory effect of morphine but sometimes produced a contraction of the longitudinal muscle similar to that observed in ilia from pretreated guinea pigs. Collier et al. (1981) examined this phenomenon in greater detail. Segments of intact ilia were incubated with normorphine for periods ranging from 1 to 22 h and subsequently challenged with naloxone. Naloxone evoked a contraction of the longitudinal muscle, the magnitude of which increased as the concentration of normorphine in the incubation medium increased and as the time of incubation was increased. A contraction was evoked by naloxone after a 1-h incubation with normorphine and was maximal after a 6-h incubation. Lujan and Rodriguez (1981) made similar observations, demonstrating that a 30-min exposure to normorphine was sufficient for naloxone to elicit a withdrawal contraction.

The contraction was prevented by tetrodotoxin and by either hyoscine or atropine but was unaffected by hexamethonium (Collier et al., 1981; Lujan and Rodriguez, 1981). It, therefore, resembled that evoked by naloxone in ilia from guinea pigs treated chronically with morphine. Chahl (1983, 1986) also showed that naloxone evoked a contraction of the longitudinal muscle in ilia exposed to opioids such as morphine and Met-enkephalin for very brief periods, ranging from 2 to 32 min. Because these contractions were unaffected by atropine but significantly reduced by substance P antagonists, substance P was proposed as the principal mediator of the withdrawal response. The relationship between this phenomenon and that described by Collier et al. (1981) and by Lujan and Rodriguez (1981) is, therefore, uncertain.

Nevertheless, experiments demonstrating withdrawal responses after pretreatment of ilia in vitro provide at least two significant advances in understanding the phenomenon of dependence. They indicate that cellular mechanisms capable of developing dependence reside within the myenteric plexus, independent of the CNS. Furthermore, they demonstrate that these mechanisms may be initiated very rapidly following occupation of opioid receptors. This is consistent with the observation that naloxone evokes withdrawal contractions in ilia removed from guinea pigs 2 h after a single injection of morphine (Chahl and Thornton, 1987; Chahl 1988). It is also consistent with abundant evidence for acute dependence in experimental animals (Martin and Eades, 1964; Kosersky et al., 1974; Eisenberg, 1982; Krystal and Redmond, 1983) humans (Wikler et al., 1953; Bickel et al., 1988) and in single neurons of the CNS (section VII).

c. CELLULAR MECHANISMS OF DEPENDENCE. The experiments discussed above indicate that, irrespective of the experimental procedure used to induce and subsequently reveal dependence, the cellular mechanisms underlying withdrawal excitation reside in neurons of the myenteric plexus. The excitabilities of both the circular and longitudinal muscles are unchanged by chronic morphine treatment.

Earlier hypotheses on the cellular mechanisms of dependence drew attention to the supersensitivity to the excitant effects of 5-HT that was consistently observed after morphine withdrawal (Takagi et al., 1965; Schulz and Goldstein, 1973; Johnson et al., 1978). It was proposed that supersensitivity resulted from an adaptive increase in the number of receptors for 5-HT, a putative excitatory transmitter in the myenteric plexus (Schulz and Goldstein, 1973). This proposal was analogous to the principal mechanism of supersensitivity observed after denervation of skeletal muscle and in accord with the concepts of dependence advanced by Collier (1966). Closer examination revealed, however, that the supersensitivity was not confined to 5-HT; myenteric neurons were also supersensitive to PGE_1 (Schulz and Herz, 1976), nicotine (Johnson et al., 1978) electrical stimulation (Schulz and Cartwright, 1974; Johnson et al., 1978; Cox, 1979), and even K^+ (Johnson et al., 1978). Moreover, supersensitivity to K^+ was apparent in preparations that were desensitized to 5-HT (Johnson et al., 1978). The nonspecific supersensitivity suggested that the underlying mechanism involved a more fundamental change in neuronal excitability, such as a membrane depolarization, rather than changes in receptors for a particular neurotransmitter (Johnson et al., 1978). According to this view, neurons that were partially depolarized were closer to threshold for the initiation of action potentials evoked by the variety of excitants or by electrical stimulation.

Direct evidence for depolarization was obtained by recording intracellularly from myenteric neurons during morphine withdrawal (Johnson et al., 1987c). In a proportion of neurons, abrupt removal of morphine from its receptors by naloxone resulted in a membrane depolarization that was sufficient to initiate action potentials. The membrane depolarization could account for the increase in frequency of action potential discharge recorded

in earlier studies using extracellular suction electrodes (Takayanagi et al., 1974; North and Zieglgänsberger, 1978; Karras and North, 1981).

Of the two electrophysiologically defined classes of neurons in the myenteric plexus (S and AH; Hirst et al., 1974), only one was affected by morphine withdrawal. In AH neurons, which are probably sensory (Nishi and North 1973; Hirst et al., 1974; Furness et al., 1988b; Iyer et al., 1988), the resting membrane properties, action potential configuration and slow spike afterhyperpolarization characteristic of this type of cell, were unaltered after treatment with naloxone. The depolarization and action potential discharge were confined to S neurons, some of which are interneurons and others are motor neurons to the muscle layers (Furness et al., 1988a).

Thus, a depolarization in S neurons may be the electrophysiological basis for the muscle contractions evoked by naloxone and for the nonspecific supersensitivity observed after removal of morphine from its receptors by washout. A withdrawal response was not confined to motor neurons, however, because bursts of nicotinic cholinergic excitatory postsynaptic potentials were recorded in some cells, indicating excitation of cholinergic interneurons that synapsed with the impaled neuron (Johnson et al., 1987c).

The mechanism underlying the depolarization of S neurons has not been determined. In some neurons the magnitude of the depolarization was decreased when synaptic transmission was blocked by reducing the extracellular Ca^{++} concentration (Johnson et al. 1987c). This suggested that the depolarization might be due in part to release of excitatory transmitters that produce slow depolarizations in S neurons (Johnson et al., 1980; Bornstein et al., 1984). An alternate explanation is that the depolarization may be generated by a Ca^{++}-dependent process intrinsic to the impaled neuron. The latter possibility cannot be readily discounted, particularly in view of the close relationship between Ca^{++} and the acute actions of morphine discussed above (section VI, A, 1, d).

The depolarization of S neurons was not associated with a detectable change in membrane conductance. This may indicate that it was generated at a site distant from the soma, in which case any underlying conductance change might not be readily detected by passing current pulses across the soma membrane. It is also possible that the depolarization might result from altered activity of an energy-dependent ion pump. Such a mechanism has precedent in the adaptive supersensitivity of some smooth muscles, in which the membrane depolarization underlying nonspecific supersensitivity results from a decrease in the contribution of the sodium pump to the resting membrane potential (section III). The importance of ion pumps in controlling the resting membrane potential in myenteric S neurons has not been studied in detail.

Both pertussis toxin and cholera toxin have been used to investigate the cellular mechanisms of dependence. In guinea pigs pretreated with pertussis toxin, subsequent chronic exposure to the μ-agonist, fentanyl, failed to induce dependence, as indicated by the failure of naloxone to elicit a withdrawal contraction of the longitudinal muscle (Lux and Schulz, 1986). Similar observations were made when ilia from untreated guinea pigs were incubated with pertussis toxin and normorphine in vitro; the magnitude of the naloxone-induced contraction was reduced by about 50% by pertussis toxin pretreatment (Collier et al., 1983). Unfortunately, these observations are difficult to interpret because of the current disagreement on the effects of pertussis toxin on the acute actions of opioids (section VI, A, 1, d). If, as suggested by the experiments of Lujan et al. (1984) and of Tucker (1984), pertussis toxin prevents the acute agonist action of normorphine, this would readily account for the subsequent failure of naloxone to evoke a withdrawal contraction. It is generally considered that an opioid receptor-mediated agonist action is a prerequisite for the adaptive changes leading to dependence.

The effects of cholera toxin were examined in longitudinal muscle-myenteric plexus preparations from guinea pigs treated chronically with the μ-agonist, fentanyl (Lux and Schulz, 1986). When these tissues were incubated in vitro with cholera toxin for 3 to 5 h, the withdrawal contraction evoked subsequently by naloxone was substantially reduced compared with that observed in similar preparations not treated with the toxin. Cholera toxin also reduced nerve-mediated contractions elicited in control preparations by 5-HT, neurotensin, and PGE_1. It remains to be established whether the depressant effects of cholera toxin in normal and dependent ilia are due to an action on the stimulatory G protein, G_s. The influence of cholera toxin on the induction of dependence has not been investigated.

Several studies have shown that indomethacin, a PG synthesis inhibitor, reduces the magnitude of the withdrawal contraction evoked by naloxone (Ramirez-Solares et al., 1983; Krakow et al., 1986; Johnson et al., 1988b). It was suggested, therefore, that PGs may play a role in the expression of dependence. In experiments of this type it is important to establish the effect of the drug per se on neuronal excitability. In the studies of Ramirez-Solares et al.(1983) and of Johnson et al.(1988b), indomethacin did not alter the amplitude of contractions of the longitudinal muscle evoked by electrical stimulation of myenteric neurons. It is apparent in the records of Krakow et al. (1986), however, that indomethacin substantially reduced the basal tone of the preparation. Irrespective of the mechanisms of action of indomethacin, this effect alone could interfere with the ability of naloxone to evoke a withdrawal contraction.

d. TOLERANCE. Tolerance has been studied using the same pretreatment procedures as those used to investi-

gate dependence. In most cases, it has been assessed by comparing concentration-response curves for the inhibitory effects of the opioid on electrically evoked cholinergic contractions in ileal preparations from untreated and from pretreated guinea pigs. Tolerance is indicated by a shift to the right in the concentration-response curve in pretreated preparations (see the paper by Goldstein and Schulz, 1973).

When continuous exposure is maintained in vitro by addition of opioid to the Krebs' solution, the degree of tolerance observed ranges from about 10-fold to more than 100-fold and is relatively selective for the class of opioid agonist used to promote it (Schulz and Herz, 1976; Cox,1978; Schulz et al., 1981; 1982; 1985). For example, after morphine pellet implantation and subsequent continuous exposure to the μ-agonist normorphine in vitro, tolerance to normorphine was 75-fold, whereas that to the κ-agonist, ethylketocyclazocine was much less, about 4- to 6-fold (Schulz et al., 1981). Conversely, after pretreatment of guinea pigs with ethylketocyclazocine and subsequent maintenance of ilia in vitro in ethylketocyclazocine, tolerance to ethylketocyclazocine was 80-fold, whereas that to the μ-agonist normorphine was much less, about 4-fold (Schulz et al., 1981).

When the opioid was removed by washing, the degree of tolerance decayed very rapidly. In the experiments of Schulz and Herz (1976), tolerance observed before normorphine withdrawal was approximately 20-fold but this declined to about 6-fold within 3 h after withdrawal. A qualitatively similar, but more dramatic, loss of tolerance was observed by Cox (1978). Ilia maintained in the presence of normorphine in vitro exhibited more than 100-fold tolerance to normorphine, and the maximum inhibitory response was markedly depressed. Within 2 h after normorphine withdrawal, tolerance had decayed to between 3- and 10-fold, and there was no evidence of a decrease in the maximum response.

In experiments in which ilia from morphine-pretreated guinea pigs are set up in vitro in morphine-free Krebs', tolerance is studied after an equilibration period of at least 1 h, that is, at least 1 h after morphine withdrawal. Under these conditions, the degree of tolerance observed usually ranges from 3- to 10-fold (Haycock and Rees, 1972; Goldstein and Schulz, 1973; Ehrenpreis et al., 1975; Ward and Takemori 1976a; Cox and Padhya, 1977; Johnson et al., 1978; Gillan et al., 1979; Mayer et al., 1980; Vaught, 1981; Porreca and Burks, 1983; Chavkin and Goldstein, 1984). This is similar to the degree of residual tolerance observed 2 to 3 h after normorphine washout in vitro (Schulz and Herz, 1976; Cox, 1978). Thus, the differences in degrees of tolerance observed using the two different experimental strategies probably reflect a rapid loss of tolerance after morphine withdrawal.

Two factors, however, suggest that the phenomena observed prior to and after morphine withdrawal may be not only quantitatively but also qualitatively different. First, after an initial very rapid decline in tolerance after withdrawal, the residual 3- to 10-fold tolerance decays very slowly, over a period of many days (Cox, 1978; Schulz et al., 1974). Second, whereas tolerance observed before withdrawal is relatively specific for the agonist used to promote it, tolerance observed several hours after withdrawal is nonspecific. Ilia are tolerant not only to morphine or other μ-agonists but also to a variety of other inhibitory agents such as epinephrine (Goldstein and Schulz, 1973; Gillan et al., 1979), norepinephrine (Ward and Takemori, 1976b), clonidine (Gillan et al., 1979; Taylor et al., 1988), dopamine (Goldstein and Schulz, 1973), 2-chloroadenosine (Taylor et al., 1988), ATP (Gillan et al., 1979), and even ethanol (Mayer et al., 1980).

The nonspecific tolerance induced in the ileum by morphine pellet implantation does not manifest itself by qualitatively similar changes in the concentration-response curves of all the affected agonists. For example, the tolerance to morphine and 2-chloroadenosine is demonstrable as parallel shifts of the concentration-response curves of the two agonists without appreciable changes in the maximum response (Taylor et al., 1988). In contrast, the concentration-response curves for two α_2-adrenoceptor agonists, clonidine and xylazine, are characterized by depression of slope and maximum response in morphine-tolerant preparations (Taylor et al., 1988).

These findings are readily explicable in terms of differing relationships between the intrinsic efficacy of each agonist and the density of the receptors specific for that agonist (Taylor et al., 1988 and section I of this review). The nonspecificity of the tolerance suggests an adaptation in cellular function beyond the level of the receptors. Such a reduction in the effectiveness of an agonist-receptor interaction would produce a parallel shift of the concentration-response curve to the right if, in the normal state, maximum response to the agonist occurs when only a small portion of available receptors are occupied. Thus, it appears that there is an excess of the respective receptors for morphine and 2-chloroadenosine on myenteric neurons (spare receptors). Chavkin and Goldstein (1984) and Porreca and Burks (1983), using the irreversible antagonist, β-chlornaltrexamine, also concluded that myenteric neurons contain spare receptors for morphine. In contrast, if an agonist must occupy virtually all of its receptors to reach maximum response (few or no spare receptors), any decrease in the effectiveness of agonist-receptor interaction will result in a depression of slope and maximum of the concentration-response curve, as observed with the α_2-agonists.

More recently, opioid actions have been investigated in ileal preparations comprising the circular muscle and the axonal processes of myenteric neurons, severed from their cell bodies in the myenteric plexus. Nerve-mediated cholinergic contractions were inhibited by a wide variety

of agents, including μ- and κ-agonists (Johnson et al., 1988a). When preparations from morphine-pretreated guinea pigs were examined 2 h after morphine withdrawal, they exhibited reduced sensitivity to both μ- and κ-agonists. They were also tolerant to a variety of other inhibitory agents such as clonidine, 2-chloroadenosine, ethanol, dimethylphenylpiperazinium, and PGE_1, confirming that tolerance is nonspecific (S. M. Johnson, unpublished observations).

Thus, tolerance observed after prolonged pretreatment of guinea pigs with morphine consists of at least two components. One component, most readily recorded when ilia are examined in the presence of the maintenance concentration of opioid (that is, prior to morphine withdrawal), is of high degree, decays within minutes or hours after removal of the opioid from the receptor, and is specific for the class of opioid agonist used to induce it. The other component of tolerance is more modest in degree (3- to 10-fold), decays slowly, over a period of several days at least, and is nonspecific. It is readily apparent when tissues are examined at least 1 to 2 h after opioid withdrawal, at a time when the high degree of specific tolerance has decayed.

It is desirable to examine each component of tolerance separately if possible, particularly in order to investigate their underlying cellular mechanisms. Ilia that are maintained in opioid-containing solutions in vitro exhibit not only a high degree of tolerance to the opioid used for pretreatment of guinea pigs but also a low degree of cross-tolerance to agonists that act on different receptors (Schulz et al., 1981). The magnitude of the latter (4- to 6-fold) is characteristic of the magnitude of nonspecific tolerance observed several hours after opioid withdrawal. It seems likely, therefore, that a complexity of changes underlying both specific and nonspecific tolerance is present prior to withdrawal. However, separation of the two phenomena may be accomplished in part by appropriate experimental design. For example, because of its slow decay, nonspecific tolerance may be investigated between several hours and several days after opioid withdrawal, at a time when specific tolerance has disappeared.

On the other hand, because the rates of decay of specific and nonspecific tolerance are different, it may be that the rates of onset are different as well and that specific tolerance could be induced rapidly. Many studies have, in fact, demonstrated tolerance in the isolated ileum after a single injection of guinea pigs with morphine (Ward and Takemori, 1976a) or after in vitro incubation of ilia from untreated guinea pigs with morphine (Schaumann, 1955; Shoham and Weinstock, 1974; Aldunate et al., 1975; Hammond et al., 1976; Opmeer and Van Ree, 1978; Rezvani et al., 1983; Alguacil et al., 1987), but in most cases the specificity of tolerance was not investigated. The exception was the study of Rezvani et al. (1983) who examined the time course and specificity of this phenomenon in some detail. In their experiments, the concentration of morphine required to produce 50% inhibition of cholinergic contractions evoked by electrical stimulation (IC_{50}) was determined in ileal preparations from untreated guinea pigs. The tissues were then incubated with concentrations of morphine from 0.5 to 2 times the IC_{50}, for periods ranging from 1 to 4 h. After a 1-h incubation in 0.5 times the IC_{50}, the tissues exhibited 5-fold tolerance, which increased to 10-fold after a 4-h incubation.

Important in the context of the present discussion was the observation that tolerance was specific, in that the sensitivities to the inhibitory effects of norepinephrine and AMP were unaltered by morphine pretreatment. Thus, a period of exposure of 4 h was sufficient to promote changes leading to specific tolerance but apparently insufficient for the mechanisms leading to nonspecific tolerance.

e. CELLULAR MECHANISMS OF TOLERANCE. The specific tolerance that predominates prior to opioid withdrawal resembles the phenomenon of receptor desensitization that has been described for a wide variety of neurotransmitters and drugs (section III). Receptor desensitization is characterized by a marked and selective loss of responsiveness to a particular agonist. It is rapidly induced after occupation of the receptor by the agonist and is rapidly reversed when the agonist is removed from the receptor. In opioid-tolerant ilia, however, no changes in the number or affinity of opioid-binding sites were detected (Rubini et al., 1982). A difficulty with this and similar studies (Cox and Padhya, 1977) is that ligand binding was determined after the agonist that induced tolerance had been removed from the receptor. Because selective tolerance decays very rapidly after opioid withdrawal, the receptors may revert rapidly to their normal state. However, even when binding was studied by procedures that did not involve repeated washing that removed the drug from its receptors, no changes in opioid binding were found (Cox, 1978). It was, therefore, proposed that tolerance might result from an uncoupling of the opioid receptor from its intracellular effector system (Rubini et al., 1982). Such a mechanism would not require changes in the recognition site itself. As yet, the intracellular effector systems linked to opioid receptors in the myenteric plexus have not been elucidated.

Porreca and Burks (1983) and Chavkin and Goldstein (1984) investigated the mechanisms of tolerance in ilia that were removed from morphine-pretreated guinea pigs and set up in vitro in morphine-free Krebs' solution. Residual morphine was removed from the tissues by repeated washing during the equilibration period, presumably of 1 h duration at least. By then, the high degree of specific tolerance may have decayed substantially, although perhaps not completely. The degrees of tolerance observed (3- to 10-fold) were consistent with nonspecific tolerance, although in neither study was this

established. In both studies, the irreversible opioid receptor-alkylating agent, β-chlornaltrexamine, was more potent in reducing the maximum inhibitory effect of morphine in tolerant compared with control tissues. These observations implied either a reduction in the fraction of spare receptors or a decrease in the effectiveness of the intracellular mechanism that linked the receptor to the inhibitory action of morphine. Because the number of opioid-binding sites was unchanged, it was proposed that tolerance resulted from reduced effectiveness of the transduction mechanism.

Agonists that act on different receptors may share common intracellular messengers (Neher, 1988; Nicoll, 1988). Therefore, an alteration in the intracellular effector linked to the μ-receptor could result in cross-tolerance to other agonists if the latter acted via the same intracellular effector process. The α_2-adrenoceptor agonist clonidine, for example, has similar effects on myenteric S neurons as does morphine. Both produce membrane hyperpolarizations associated with increases in potassium conductance (Suprenant and North, 1985). If μ- and α_2-receptors were linked to the same potassium channels via a common intracellular effector, an alteration in the common pathway could result in tolerance to both agents. Such a proposal, however, must take into account the observations that nonspecific tolerance extends not only to clonidine but also to a wide variety of other inhibitory agents such as dopamine, 2-chloroadenosine, ATP, dimethylphenylpiperazinium, PGE_1, and ethanol (see above). It is more difficult to conceive that all these agents could produce their inhibitory effects via the same intracellular effector system.

We propose an alternate mechanism for nonspecific tolerance. It assumes that both the opioid recognition site and its intracellular effector are unaltered in tolerant ilia. However, myenteric neurons are hyperexcitable after morphine withdrawal, at least in part because of a membrane depolarization (Johnson et al., 1987c). As such, a normal receptor-mediated action of morphine, such as a membrane hyperpolarization, might be less effective in reducing the excitabilities of these neurons and, therefore, less effective in reducing the release of acetylcholine. Tolerance would be apparent to many agents which, like morphine, inhibited the release of acetylcholine by reducing the excitability of myenteric neurons (see section V, B, for further discussion).

f. RELATIONSHIP BETWEEN TOLERANCE AND DEPENDENCE. The above proposal implies that the mechanism underlying nonspecific tolerance is identical with that underlying the nonspecific supersensitivity to excitants which is observed after morphine withdrawal and which is indicative of dependence. An adaptive response, such as a membrane depolarization and increase in neuronal excitability, may facilitate the actions of excitatory agents and oppose the actions of inhibitory agents on myenteric neurons. The observation that supersensitivity and tolerance develop and decline with similar time courses (Schulz et al., 1974) is consistent with this view. The proposal accords in principle with unitary theories of tolerance and dependence, postulating that common mechanisms underlie both phenomena (Shuster, 1961; Goldstein and Goldstein, 1968; Collier, 1980; Martin, 1968). It also accepts that dependence may be unrelated to the phenomenon of specific tolerance (desensitization) and, therefore, that tolerance and dependence may be separable under certain circumstances, as has been observed previously (Schulz et al., 1982; Wüster et al., 1982, 1985).

B. Mouse Vas Deferens

1. Acute actions. Next to the guinea pig ileum, the peripheral tissue most extensively used for studies on opioids has been the isolated mouse vas deferens, originally described as a morphine-sensitive preparation by Henderson et al. (1972). Single electrical pulses elicited contractions of the longitudinal muscle which were reduced or abolished by phentolamine or bretylium, but unaffected by hyoscine, suggesting that they were due principally to the release of norepinephrine contained within nerves supplying the longitudinal muscle. Morphine or normorphine caused concentration-dependent and naloxone-sensitive inhibition of nerve-mediated contractions, indicating an action via opioid receptors (Henderson et al., 1972; Hughes et al., 1975; Henderson and Hughes, 1976; Lord et al., 1977; Waterfield et al., 1977; Marshall et al., 1981; Miller and Shaw, 1983). The equilibrium dissociation constant for naloxone as an antagonist of the effects of morphine was similar to that obtained in the guinea pig ileum, suggesting that the receptors mediating the effects of morphine were similar in both preparations (Henderson et al., 1972; Hutchinson et al., 1975; Lord et al., 1977; Waterfield et al., 1977). Consistent with this were observations that the relative potencies of a range of agonists such as morphine, normorphine, levorphanol, and etorphine correlated closely with those in the guinea pig ileum (Hughes et al., 1975).

Morphine depressed nerve-mediated contractions without affecting contractions produced by exogenous norepinephrine (Henderson and Hughes, 1976; Contreras and Marti, 1979). This observation and the demonstration that μ-agonists depressed the outflow of norepinephrine evoked by electrical stimulation (Hughes et al., 1975; Henderson and Hughes, 1976; Waterfield et al., 1977;) or by K^+ (Sim and Henderson, 1981) indicated that the receptors were located on neurons rather than on muscle cells. The evoked excitatory junction potential recorded in the smooth muscle by an intracellular microelectrode was also depressed by morphine, by an action on nerves that released norepinephrine (Henderson and North, 1976). Numerous investigations confirmed this observation (Bennett and Lavidis, 1980; Illes et al., 1980a; Illes and Schulz, 1980; North and Vitek, 1980a;

1980b; Ito and Tajima, 1980; Illes and North, 1982; Einstein and Lavidis, 1984; Ramme and Illes, 1986).

In addition to μ-receptors, both δ-and κ-receptors are also present on neuronal processes in the mouse vas deferens. Like morphine, δ- and κ-agonists inhibit contractions evoked by nerve stimulation (Hutchinson et al., 1975; Lord et al., 1977; Waterfield et al., 1977; Schulz et al., 1980a; 1980b; Wüster et al., 1980; Cox and Chavkin, 1983; Miller and Shaw, 1983; Schulz et al., 1984; Hayes et al., 1985), decrease the electrically evoked outflow of norepinephrine (Waterfield et al., 1977), and reduce the amplitude of the excitatory junction potential recorded in the smooth muscle (North and Vitek, 1980b; Illes et al., 1980a; Ramme and Illes, 1986).

a. CELLULAR MECHANISMS OF INHIBITION. As in the guinea pig ileum, opioids could inhibit excitatory transmission by two basic mechanisms. They could interfere directly with the Ca^{++}-dependent mechanism of release of norepinephrine from terminal varicosities, or they might reduce the excitability of nerve fibers, thus reducing the propagation of action potentials to sites of norepinephrine release (Illes and North, 1982). A consistent observation is that the potency of morphine or normorphine is related inversely to the concentration of extracellular Ca^{++} (Bennett and Lavidis, 1980; Illes et al., 1980a; Marshall et al., 1981; Milner et al., 1982; Einstein and Lavidis, 1984). It was proposed that morphine inhibited neurotransmission by an action on the Ca^{++}-dependent mechanism of stimulus-evoked release of norepinephrine (Bennett and Lavidis, 1980; Illes et al., 1980a). However, the interaction between Ca^{++} and morphine is also consistent with an effect of morphine on the excitability of axonal processes. For example, the hyperpolarization and decrease in excitability produced by μ-agonists on myenteric neurons of guinea pig ileum exhibits a similar inverse relationship with external Ca^{++} concentration (Morita and North, 1982).

Illes et al. (1982; 1984) examined the release of [^{3}H] norepinephrine from mouse vasa deferentia evoked by high-intensity electrical stimulation. The release was resistant to tetrodotoxin but reduced by omission of Ca^{++} from the incubation medium, suggesting that it involved opening of Ca^{++} channels but not propagation of Na^+-dependent action potentials. Normorphine did not reduce release under these conditions but did inhibit tetrodotoxin-sensitive release of norepinephrine evoked by lower intensity stimulation. Although these observations are consistent with opioid effects on action potential propagation, they do not exclude effects on the action potential-mediated release mechanism, which may differ from the Ca^{++}-dependent release evoked by high-intensity stimulation. The technique of Brock and Cunnane (1988), who recorded concurrently the nerve terminal action potential and the postjunctional current produced by norepinephrine released in the guinea pig vas deferens, might provide a means of determining the mechanism of action of morphine in the mouse vas deferens.

The intracellular messenger systems linked to each receptor subtype have not been determined. Based on the proposal that opioid effects were mediated by reduction in intracellular cyclic AMP, North and Vitek (1980a) examined the interactions between opioids and agents such as cyclic AMP, dibutyryl cyclic AMP, and isobutylmethylxanthine, all of which might be expected to elevate or mimic the intracellular concentrations of cyclic AMP. However, the inhibitory effects of normorphine and DADLE on excitatory junction potentials were not influenced by either cyclic AMP or a combination of isobutylmethylxanthine and dibutyryl cyclic AMP, suggesting that neither μ- nor δ-agonists inhibited transmission by decreasing intracellular cyclic AMP levels. Similar studies in the guinea pig ileum have failed to provide evidence for the proposal, advanced frequently, that changes in cyclic AMP underlie inhibition of neuronal activity by opioids.

2. Chronic actions. a. DEPENDENCE. After chronic pretreatment of mice with morphine, and subsequent exposure of vasa deferentia to an appropriate concentration of morphine in vitro, naloxone does not evoke a withdrawal contraction (Cox, 1978; Gillan et al., 1979; Schulz et al., 1980a; Vargas et al., 1987). This contrasts with the powerful contraction evoked by naloxone in the guinea pig ileum.

The absence of an effect of naloxone in the mouse vas deferens could be due to its low basal activity compared with that of the guinea pig ileum. Myenteric motor neurons in the ileum discharge action potentials, resulting in substantial neurogenic basal contractile activities of the muscle layers. An increase in the excitation of these neurons evoked by naloxone is readily manifest as a tetrodotoxin-sensitive contraction of the muscle. On the other hand, the mouse vas deferens is quiescent compared with the guinea pig ileum. As such, a similar effect of naloxone might not be sufficient to discharge action potentials and so reveal a withdrawal response in mechanical recordings from the muscle.

This difficulty did not arise in the experiments of North and Vitek (1980b) who recorded excitatory junction potentials in muscle cells of vasa deferentia that were removed from morphine-pretreated mice and placed in Krebs' solution containing a low concentration of normorphine (300 nM). Excitatory junction potentials were also recorded in vasa deferentia removed from untreated mice and exposed to the same concentration of normorphine in vitro. Naloxone caused a substantially greater increase in amplitude of the excitatory junction potentials in vasa deferentia from pretreated compared with untreated mice, an observation interpreted as a withdrawal response indicative of morphine dependence. A similar increase in excitatory junction potential amplitude was observed by Einstein and Lavidis (1984).

Illes and Schulz (1980), however, did not detect abnormal excitatory junction potentials when vasa deferentia from pretreated mice were exposed to a higher concentration of normorphine (2 μM), similar to that in the plasma of pretreated mice at the time of removal of the tissues. Moreover, the amplitudes of contractions evoked by nerve stimulation were similar in vasa deferentia from untreated mice and from morphine-pretreated mice after treatment with naloxone, suggesting a lack of dependence (Schulz et al., 1980a).

Vargas et al. (1987) observed that, 5 min after morphine washout, naloxone increased the amplitude of nerve-mediated contractions in vasa from morphine-pretreated mice and suggested that this indicated morphine dependence. It was not clear, however, that the amplitude of contractions after naloxone was significantly different from the amplitude of contractions in vasa from untreated mice. This is important in order to account for the possibility that naloxone merely abolished an inhibitory action of morphine, some of which is likely to remain in the tissue after the 5-min washout period. The enhanced contractions reported in the experiments of Rae and De Moraes (1984) may have been due in part to augmented responses of the muscle to exogenous norepinephrine (Rae and De Moraes, 1983). Other studies have also shown enhanced responses to norepinephrine in vasa from morphine-pretreated mice (Johnson et al., 1977; Contreras and Marti, 1979; Ramaswamy et al., 1981; Marti, 1982; Rae and De Moraes, 1983; McCulloch and Pollock, 1985). These changes were modest, however, and not always statistically significant (North and Vitek 1980b). Taken together, the above observations indicate that dependence is not a reliably reproducible phenomenon in the mouse vas deferens preparation.

b. TOLERANCE. Following chronic pretreatment of mice with an opioid, tolerance can be readily demonstrated in the vas deferens. As in the guinea pig ileum, however, both the presence of tolerance and the degree observed may be dependent upon the experimental conditions. This was well illustrated by the experiments of Cox (1978). Vasa were removed from mice that had been pretreated by morphine pellet implantation for 3 to 5 days. The tissues were placed in Krebs' solution containing 500 nM normorphine, in order to maintain continuous exposure to opioid in vitro. Under these conditions, vasa exhibited 4-fold tolerance to normorphine. This was determined by comparing the concentrations of normorphine required to reduce by 50% nerve-mediated contractions in vasa from pretreated and untreated mice. If, however, the vasa from morphine-pretreated mice were set up in morphine-free Krebs' solution and washed for several minutes, no tolerance was apparent. That is, tolerance decayed rapidly and completely shortly after morphine withdrawal. However, this conclusion should be viewed with caution because of previously discussed problems of interpretation of shifts of concentration-response curves in the presence of preexisting levels of agonist (section IV, B). This is particularly important when the shifts are as small as 4-fold.

Vasa tolerant to the μ-agonist, normorphine, exhibited unchanged sensitivity to the δ-agonist, Leu-enkephalin, indicating that this tolerance was specific for the particular class of agonist used to induce it (Cox, 1978; Illes et al., 1980b). In fact, a characteristic feature of tolerance in the mouse vas deferens is that it is highly specific for the agonist, or particular class of agonists, used to induce it (Gillan et al., 1979; Schulz et al., 1980a,b; 1984; Wüster et al., 1980; 1981a,b; Illes et al., 1980b; Schulz and Wüster, 1981; Rubini et al., 1982). Thus, vasa from mice pretreated with either μ-, δ-, or κ-agonists were highly tolerant in vitro to agonists that acted on the receptors that had been chronically occupied in vivo but were not tolerant to agonists acting on different opioid receptors. Furthermore, vasa rendered tolerant to opioids were not tolerant to agonists that inhibited neurotransmission in the vas deferens by actions on α_2- or adenosine receptors (Gillan et al., 1979; North and Vitek, 1980b; Schulz et al., 1980a).

The highly specific tolerance observed in the mouse vas deferens resembles one of the two components of tolerance observed in the guinea pig ileum. Nonspecific tolerance either does not develop in the mouse vas or requires very prolonged exposure to the opioid. For example, after treatment of mice with large doses of morphine (800 mg/kg) for 23 weeks, McCulloch and Pollock (1985) observed small (approximately 2-fold) tolerance to both morphine and the α_2-adrenoceptor agonist, clonidine.

Following chronic morphine pretreatment, specific tolerance in the mouse vas, like that in the guinea pig ileum, seems to decay rapidly after the opioid is removed from its receptor (Cox, 1978). The rapid loss of tolerance after morphine withdrawal may contribute to the very low degrees of tolerance observed when vasa deferentia are set up in morphine-free Krebs' solution (Johnson et al., 1977; Cox, 1978; Gillan et al., 1979; Marshall et al., 1981; McCulloch and Pollock, 1985;) compared with those observed before morphine was removed from its receptor (North and Vitek, 1980b; Illes and Schulz, 1980; Rae and De Moraes, 1983).

The mechanisms underlying tolerance in the vas deferens have not been elucidated. Because of its high degree of specificity, tolerance was proposed to result from an alteration in opioid receptors analogous to the mechanism of receptor desensitization (Cox, 1978; Schulz et al., 1980a). Rubini et al. (1982) measured the specific binding of [^{3}H]DADLE in homogenates of vasa deferentia rendered tolerant by chronic pretreatment of mice with DADLE. However, no differences in binding in homogenates of pretreated and control vasa were detected, suggesting that the recognition sites, for δ-agonists at least, were unaltered by chronic opioid treatment.

It was suggested, therefore, that tolerance resulted from adaptational changes within the intracellular messenger system that was linked to the opioid receptor. This hypothesis cannot be definitively examined until the intracellular mechanisms coupled to the receptor are understood.

VII. CNS

Many of the important actions of morphine, such as analgesia, are produced by actions on neurons in the CNS. Chronic administration leads to a decreased efficacy of the opioid in producing analgesia, thus implicating central neurons in the development of tolerance. Many of the physiological disturbances observed after morphine withdrawal can similarly be attributed to altered activity of the CNS. For example, characteristic behavioral signs of opioid withdrawal can be elicited when the opioid antagonist, naloxone, is administered into discreet brain regions (Laschka et al., 1976). Also located in the CNS are neurons that underlie opioid self-administration behavior, an essential component of the overall phenomenon of dependence (Smith and Lane, 1983). Therefore, studies on the acute and chronic actions of morphine on central neurons are mandatory in the endeavor to understand the cellular bases of tolerance and dependence.

This section focuses primarily on experiments in which electrophysiological techniques have been used to examine the influence of chronic morphine treatment on the excitability of central neurons. Recordings using extracellular and intracellular electrodes have been made both in vivo, in anesthetized animals, and in vitro, in tissues that are prepared either freshly as a slice or that have been maintained in culture. The advantages and limitations of extracellular and intracellular recordings and of in vivo and in vitro studies have been discussed in detail previously (Duggan and North, 1983; French and Williams, 1989).

A large number of investigators have examined the acute actions of opioids on central neurons (see reviews by North, 1979; Nicoll, 1982; Duggan and North, 1983). The predominant opioid receptor-mediated effect is inhibition of neuronal firing. Excitation is encountered less frequently and, in some cases, such as the excitation of hippocampal pyramidal neurons, is due to opioid-induced inhibition of inhibitory neurons that synapse with pyramidal cells (see below).

Fewer investigators have examined the chronic actions of opioids on the excitation of single neurons. Principal sites investigated are the dorsal horn of the spinal cord and associated dorsal root ganglion (DRG) cells, the locus coeruleus, the hippocampus, the striatum, and the cortex. However, at no site in the CNS have the acute and chronic actions of opioids been investigated as comprehensively as in the guinea pig ileum. In the following discussion, comparisons will be made, where possible, between mechanisms observed in peripheral and in central neurons.

A. Spinal Cord

1. Studies in vivo. a. ACUTE ACTIONS. Numerous investigations have shown that morphine, when administered systemically in analgesic doses, depresses the discharge of action potentials in dorsal horn neurons of anesthetized animals. Inhibition was observed whether the neurons were activated by noxious stimuli applied to the skin (Zieglgänsberger and Bayerl, 1976; Tooyoka et al., 1976; Calvillo et al., 1979; Einsphar and Piercey, 1980) or by electrical stimulation of unmyelinated primary afferent fibers that convey nociceptive information into the spinal cord (Duggan et al., 1980; Le Bars 1976a,b). Synaptic excitation of dorsal horn neurons by impulses in the larger A afferents was unaffected by morphine or reduced to a lesser extent.

Administration by microelectrophoresis at discreet sites in the dorsal horn was used to define the sites of action of morphine. When administered near the somata of dorsal horn neurons, morphine depressed the firing of these cells in some studies (Zieglgänsberger and Bayerl, 1976). Other investigators failed to confirm these observations, however (Duggan et al., 1977; 1981), or observed depressant effects that were not consistently antagonized by naloxone (Calvillo et al., 1979). However, when morphine was administered at more dorsal sites, in the region of the substantia gelatinosa, it consistently depressed the firing of neurons in deeper laminae (Duggan et al., 1977). The effect was selective, in that excitation of neurons by noxious peripheral stimuli was depressed, whereas that evoked by innocuous tactile stimuli was unaffected. Furthermore, in contrast to the effects observed when morphine was administered near somata, the inhibitory action in the substantia gelatinosa was reliably antagonized by naloxone administered at the same site. These observations suggested that the opioid receptors in the substantia gelatinosa might be important in mediating the analgesic actions of morphine. Consistent with this was the finding that the depressant effect of morphine, administered systemically in analgesic doses, was antagonized when naloxone was administered microelectrophoretically in the substantia gelatinosa (Johnson and Duggan, 1981b).

Opioid-binding sites are prevalent in the substantia gelatinosa and are reduced in number after dorsal root section, suggesting that at least a proportion of receptors are located on primary afferents. The selective depression by morphine could be explained by an action at opioid receptors located near the terminals of unmyelinated primary afferents that convey nociceptive information to the spinal cord and that synapse in the substantia gelatinosa.

Sastry (1979) and Carstens et al. (1979) measured the excitabilities of single myelinated and unmyelinated primary afferent fibers by positioning electrodes near their

terminations in the upper dorsal horn and recording antidromically conducted action potentials in the dorsal roots. Morphine decreased the excitability of the unmyelinated fibers without consistently affecting myelinated fibers. From these observations it was proposed that morphine caused a hyperpolarization and a reduction in action potential invasion of the terminals of unmyelinated afferents, thus interrupting the synaptic transmission of nociceptive information to dorsal horn neurons.

The intracellular effector mechanisms that couple opioid receptors activated by morphine to the depression of dorsal horn neuron firing have not been determined. However, as in the guinea pig ileum and mouse vas deferens, the effect was not influenced by agents that elevate or mimic intracellular concentrations of cyclic AMP (Duggan and Griersmith, 1979), suggesting that morphine did not act by decreasing adenylate cyclase activity. Consistent with this was the observation that the μ-agonist, DAMGO, did not inhibit adenylate cyclase activity in membrane fractions of rat spinal cord (Attali et al., 1989).

b. TOLERANCE AND DEPENDENCE. The suggestion that opioid receptors in the substantia gelatinosa may be important in morphine-induced analgesia provided incentive to investigate whether prolonged occupation of these receptors produced adaptive changes associated with tolerance and dependence. Extracellular recordings were made of action potential discharge in dorsal horn neurons of cats that were pretreated by repeated injections of morphine for 3 days (Johnson and Duggan, 1981a). Neurons were activated by noxious or innocuous cutaneous stimuli. To circumvene the difficulties of assessing tolerance and dependence (section IV), the numbers of action potentials evoked in dorsal horn neurons by a range of noxious skin temperatures were determined in pretreated and in untreated control cats. From skin temperature-response relationships, the mean temperature that produced half the maximum increase in firing was measured in each group of animals. The resulting values for neurons in untreated and pretreated cats did not differ significantly, despite the high circulating levels of morphine in the pretreated cats. The absence of an inhibitory effect of morphine on neurons of pretreated cats contrasted with the potent depressant effects observed in untreated control cats, indicating that dorsal horn neurons were tolerant to morphine.

Microelectrophoretic administration of naloxone in the substantia gelatinosa did not alter the firing rate of neurons of control cats but markedly increased the firing rate of neurons in pretreated cats. As a consequence, the noxious skin temperature required to evoke 50% of the maximum excitation of dorsal horn neurons was significantly reduced. Hyperexcitability after abrupt displacement of morphine from its receptors in the substantia gelatinosa represents a withdrawal response indicative of morphine dependence. If activation of opioid receptors in the substantia gelatinosa and subsequent depression of the excitation of dorsal horn neurons are involved in morphine-induced analgesia, it follows that the withdrawal hyperexcitability could contribute to hyperalgesia characteristic of morphine withdrawal.

Little is known, however, of the cellular mechanisms of tolerance and dependence in spinal neurons in vivo. Because the acute inhibitory action of morphine may result in part from a hyperpolarization near the terminals or preterminal regions of small diameter primary afferents (Sastry 1979), it would be of interest to determine whether adaptive changes in excitability of these fibers occurred during the chronic administration of morphine. An increase in excitability, for instance, could offset the hyperpolarization produced by morphine, thus resulting in tolerance. After morphine withdrawal, it could also increase the probability that a propagating action potential invaded the fine terminal branches of the myelinated primary afferents. This would lead to the increased excitation of dorsal horn neurons, the dendrites of which make synaptic contacts with primary afferents in the substantia gelatinosa. The enhanced *release* of substance P from the spinal cords of mice during morphine withdrawal (Ueda et al., 1987) is an important observation because substance P has been frequently implicated in nociceptive transmission from primary afferents in the spinal cord. A substantial number of observations, however, have failed to support such a role (see the paper by Bossut et al., 1988) and, therefore, the functional significance of enhanced substance P release is uncertain.

Hendry et al. (1987) identified a neurotrophic factor in the spinal cords of morphine-dependent rats and suggested that this might be produced as an adaptive response to the chronic interruption of neurotransmission by morphine. In addition to the well-established neurotrophic factors, such as nerve growth factor, many other substances have neurotrophic activity (Varon, 1985). The identification of the factor described by Hendry et al. (1987) and studies on its effects on neuronal excitability may provide important new insights into the cellular mechanisms of tolerance and dependence.

Several studies have suggested that adaptive changes may occur during brief exposure to morphine in vivo. Le Bars et al. (1976b) showed that intravenous morphine depressed the excitation of dorsal horn neurons activated by electrical stimulation of unmyelinated primary afferents in the sural nerve of anesthetized cats. Administration of naloxone a few minutes later not only antagonized morphine-induced inhibition but increased the response to electrical stimulation to levels substantially greater than those observed prior to morphine administration. Similar observations were made by Johnson and Duggan (1981b) and by Zhao and Duggan (1987) when the effect of intravenous morphine was antagonized by microelectrophoretic administration of naloxone into the substantia gelatinosa. Administered alone in the substantia ge-

latinosa, naloxone did not alter the firing rate of dorsal horn neurons but produced marked hyperexcitability when administered as soon as 10 min after the intravenous injection of morphine. The enhanced excitation of these neurons was similar to the withdrawal response evoked by naloxone in animals treated chronically with morphine.

These observations in single neurons accord with reports of behavioral signs of withdrawal, precipitated by systemically administered naloxone, as soon as 10 min after a single injection of morphine in mice (Kosersky et al., 1974). Although the relationship between withdrawal hyperexcitability after acute and chronic administration of morphine is unknown, it seems likely that adaptive changes may develop very rapidly following occupation of opioid receptors in the substantia gelatinosa by morphine.

Whereas both tolerance and dependence were observed in spinal neurons of cats pretreated with morphine for 3 days, acute withdrawal hyperexcitability was not accompanied by any apparent tolerance to morphine (Johnson and Duggan, 1984). Comparisons may be drawn between cat spinal neurons and myenteric neurons of the guinea pig ileum, in which naloxone precipitates a nerve-mediated withdrawal contraction of the longitudinal muscle after periods of exposure to opioids of only a few minutes (Ehrenpreis et al., 1972; Lujan and Rodriguez, 1981; Chahl, 1983, 1986). In both spinal neurons (Johnson and Duggan, 1984) and in guinea pig myenteric neurons, acute withdrawal hyperexcitability is observed in the absence of any apparent tolerance to morphine.

Tolerance after brief exposure of dorsal horn neurons to morphine was observed, however, by Zieglgänsberger et al. (1982). In these experiments, morphine was administered microelectrophoretically near the cell bodies of rat dorsal horn neurons and depressed basal and *l*-glutamate-induced firing. Within a few minutes cell firing was restored to normal despite the continuous ejection of the opioid. Neurons that were tolerant to morphine were not tolerant to the inhibitory effect of DADLE. It is possible that this phenomenon represents a type of desensitization of the receptors on the cell bodies, induced rapidly by the very high concentrations of morphine that are likely to be present in the tissue near the tip of the microelectrophoresis pipette.

2. Studies in vitro. a. ACUTE ACTIONS. The actions of morphine have also been investigated in organotypic cultures, prepared from 12- to 14-day-old fetal mice and comprising the spinal cord and attached DRG (Crain, 1984; 1988). The explants are allowed 1 to 4 weeks maturation in culture before experiments are undertaken.

Electrical stimulation in the DRG evoked a complex potential that was recorded using an electrode positioned in the dorsal horn. The response is the sum of synaptically mediated potentials of a population of dorsal horn neurons and is referred to as a dorsal horn network response. Morphine, as well as opioid peptides such as Met-and Leu-enkephalin, caused stereoselective and naloxone-sensitive depression of the dorsal horn network response (Crain et al., 1977; 1978).

Radioligand-binding assays localized opioid receptors in both the dorsal horn and on DRG neurite outgrowths (Hiller et al., 1978), suggesting that morphine could depress the response by an action on DRG neurons or on other neurons in the dorsal horn itself. Intracellular recordings have shown that opioid peptides decrease the duration of the Ca^{++} action potential recorded in the somata of a proportion of DRG neurons (Chalazonitis and Crain, 1986; Crain, 1988). A similar action at the terminals of DRG neurons could contribute to their inhibitory effects on the dorsal horn network response. However, the effect of morphine on these action potentials does not seem to have been examined. Intracellular recordings will also be required to determine whether neurons in the dorsal horn region of the culture are affected by morphine.

Addition of pertussis toxin (10 μg/ml) to spinal cord-DRG cultures for 2 days did not appear to affect the amplitude or duration of the dorsal horn network response but markedly reduced the depressant action of morphine, suggesting that receptors for morphine might be coupled to an inhibitory G protein (Crain et al., 1987). Morphine was also less effective in depressing the dorsal horn network response in cultures treated with forskolin, dibutyryl cyclic AMP, and dioctanoyl cyclic AMP, agents that elevate or mimic the actions of intracellular cyclic AMP (Crain et al., 1986). It was suggested that the depressant action of morphine was mediated by reduction in either adenylate cyclase activity or in cyclic AMP levels and was, therefore, prevented when intracellular concentrations of cyclic AMP were raised. There are, however, several other explanations that could account for the observed antagonism of the effect of morphine by these agents. For example, different intracellular effector mechanisms may converge on the same ion channel, as has been demonstrated in DRG neurons (Grega et al., 1987). Functional antagonism between agents would be expected if each affected the same ion channel, irrespective of whether identical or distinctly different intracellular effector mechanisms were involved.

More recently, Makman et al. (1988) and Attali et al. (1989) provided biochemical evidence for opioid-induced inhibition of adenylate cyclase activity in spinal cord-DRG cultures. Inhibition was mediated predominantly by κ-receptors, whereas agonists acting on μ-receptors were ineffective (Attali et al., 1989). The receptor subtypes that mediate the depressant effects on the dorsal horn network response have not been identified definitively. However, in the experiments of Crain et al. (1978), (D-Ala^2,MePhe^4,Met-(O)^5-ol)-enkephalin was the most potent agonist tested, an observation consistent with a

predominant involvement of μ-receptors. Further studies will be necessary in order to determine the relationship between the biochemical and electrophysiological findings.

b. TOLERANCE AND DEPENDENCE. When cultures were continually exposed to morphine for several days, adaptive changes underlying tolerance and dependence occurred. The dorsal horn network response, which was markedly depressed by morphine acutely, was restored to near normal levels (Crain et al., 1979). Furthermore, addition of concentrations of morphine 10 to 50 times greater than those that depressed the response acutely were relatively ineffective in morphine-pretreated cultures. These observations indicated that tolerance had developed during the prolonged exposure to morphine. Following displacement of morphine from its receptors by naloxone, the amplitude of the dorsal horn network response was increased to levels not normally seen in untreated control cultures. This withdrawal hyperexcitability indicates morphine dependence.

The chronic actions of morphine were prevented by concomitant pretreatment with naloxone (Crain et al., 1979), demonstrating that occupation of opioid receptors by morphine was a prerequisite for the underlying adaptive changes. This is an essential characteristic of tolerance and dependence in intact animals (Orahovats et al., 1953; Yano and Takemori, 1978; Bhargava, 1978). The spinal cord-DRG culture, therefore, provides a valuable experimental model to investigate the chronic actions of opioids on central neurons in vitro.

Some insights into the mechanisms of tolerance may be gained by examining its specificity, as was illustrated in the guinea pig ileum and mouse vas deferens (section VI). A variety of opioid and nonopioid agents inhibit the dorsal horn network response, including morphine, opioid peptides, 5-HT, noradrenaline, carbachol, local anesthetics, and alcohol. Cultures rendered tolerant to morphine were also tolerant to the naloxone-sensitive inhibitory effects of Met-enkephalin and FK-33824 (Crain et al., 1979). Unfortunately, it is not known whether the same or different receptor types are involved in producing the inhibitory effects of the various opioids examined. It is not certain, therefore, whether chronic occupation of morphine receptors, most probably the μ-subtype, results in tolerance to agonists that may act on different opioid receptors. Morphine-tolerant preparations were, however, tolerant not only to opioid peptides but also to 5-HT (Crain et al., 1982), the depressant effects of which are not mediated by opioid receptors. As such, the mechanism underlying tolerance induced by morphine is unlikely to involve a change in opioid receptors. This is fundamentally similar to the nonspecific tolerance observed in the guinea pig ileum, although the actions of other receptor agonists, such as carbachol and norepinephrine, have not been examined in morphine-tolerant cultures.

Crain et al. (1982) speculated that tolerance and dependence in spinal cord-DRG cultures might have common underlying mechanisms. According to this view, withdrawal hyperexcitability impaired the ability of agents such as morphine and 5-HT to decrease the excitability of neurons. Tolerance to their inhibitory effects on the dorsal horn network response ensued as a consequence. In other words, withdrawal hyperexcitability may be not only the indicator of dependence but also the cause of tolerance. A similar relationship has been proposed in the guinea pig ileum, based on the observations that both the neuronal supersensitivity and the tolerance that accompanies it are nonspecific (section VI).

Experiments in vivo and in organotypic culture allow examination of how neuronal circuits involved in the spinal actions of morphine are altered by chronic exposure to morphine. Two other preparations have significant potential for elucidating the cellular mechanisms of tolerance and dependence. First, in fetal mouse DRG neurons maintained in dissociated cell cultures, various opioid agonists have been shown to decrease the duration of the Ca^{++} action potential (Werz and Macdonald, 1982; 1983a,b; Macdonald and Werz, 1986). The presence of μ-, κ-, and δ-receptors on these neurons was demonstrated by the use of selective agonists and antagonists (Werz et al., 1987). μ-Agonists and δ-agonists decreased the duration of the Ca^{++} action potential by activating a potassium conductance (Werz and Macdonald 1983b; Werz and Macdonald 1984; Werz and Macdonald 1985), whereas κ-agonists directly inhibited inward Ca^{++} currents (Werz and Macdonald, 1983b; 1984a,b; 1985; Macdonald and Werz, 1986; Gross and Macdonald, 1987). The effects of chronic exposure of these cultures to morphine remain to be investigated.

The second preparation is the rat spinal cord slice. Intracellular recordings from substantia gelatinosa neurons have shown that normorphine (Yoshimura and North, 1983) or enkephalin (Murase et al., 1982) causes a naloxone-sensitive membrane hyperpolarization associated with an increase in potassium conductance (Yoshimura and North, 1983) and decreases the excitability of these neurons (Murase et al., 1982). Given the importance of opioid receptors in the substantia gelatinosa in dependence in vivo (Johnson and Duggan, 1981a), experiments in slice preparations have the potential to reveal much about the cellular mechanisms underlying these phenomena.

B. Locus Coeruleus

The locus coeruleus is a compact nucleus of norepinephrine-containing neurons located in the anterior pons. It contains more than half of the entire brain content of norepinephrine and sends projections to many brain areas (Swanson and Hartman, 1975; Foote et al., 1983). Investigations on the chronic actions of opioids in this region are particularly important because norepi-

nephrine and the locus coeruleus, in particular, have been implicated in many of the behavioral manifestations of opioid withdrawal (Redmond and Krystal, 1984; Grant et al., 1988).

1. Acute actions. Much of the pioneering research on electrophysiological recording from the locus coeruleus has been undertaken by Aghajanian and his colleagues (Graham and Aghajanian, 1971). Extracellular recordings in vivo showed that neurons discharged action potentials at a quite regular frequency of 1 to 2.5 Hz (Korf et al., 1974). Noxious cutaneous stimuli, such as tail compression, resulted in an increase in firing rate, suggesting a role of these neurons in nociception. Systemic administration of morphine in analgesic doses produced a naloxone-sensitive depression in basal firing rate and in the excitation evoked by a noxious stimulus. The inhibition by morphine was due to a direct action on neurons within the locus coeruleus itself because microelectrophoretic administration of morphine produced a similar naloxone-sensitive depression of basal firing rate (Bird and Kuhar, 1977), consistent with the high density of opioid-binding sites found in the nucleus.

Opioid receptors are also located on fibers of locus coeruleus neurons that project to other areas. Nakamura et al. (1982) measured the excitability of fibers in the rat frontal cortex by recording the current intensity required to antidromically activate their cell bodies, located in the locus coeruleus. Infusion of morphine into the frontal cortex decreased the excitability of fibers projecting from the locus coeruleus, suggesting that this might contribute to inhibition of norepinephrine release by morphine.

To investigate the cellular mechanisms underlying opioid-induced inhibition of cell firing, Pepper and Henderson (1980) developed a brain slice preparation and examined the actions of opioids on guinea pig locus coeruleus neurons by intracellular recording. Opioids, including normorphine, caused a naloxone-sensitive hyperpolarization, associated with an increase in potassium conductance. As a consequence, the basal firing rate was greatly reduced or abolished. The effect of normorphine persisted after blockade of synaptic transmission, indicating a direct action on the impaled neuron rather than a synaptically mediated effect.

These observations have been confirmed and extended by experiments in locus coeruleus slices prepared from rats (Williams et al., 1982; North and Williams, 1983a; Andrade and Aghajanian, 1985; Williams et al., 1988). The opioid-induced hyperpolarization results from an increase in potassium conductance. Opioids also reduce the amplitude and the rate of rise of Ca^{++} action potentials and decrease the amplitude of the spike afterhyperpolarization. These effects are also a consequence of activation of a potassium conductance rather than a direct action on Ca^{++} channels (North and Williams, 1983a). Because all neurons in the locus coeruleus were hyperpolarized in a concentration-dependent and highly reproducible fashion, it was possible to determine accurate concentration-response curves for different opioid agonists. By using agonists and antagonists selective for particular opioid receptor subtypes, and Schild analyzes of concentration-response curves, Williams and North (1984) established that the hyperpolarization resulted from occupation of μ-receptors. For example, the dissociation equilibrium constant for naloxone as an antagonist of hyperpolarizations produced by normorphine and DADLE was 2 to 3 nM, consistent with an action at μ-receptors. These experiments showed that δ- and κ-receptors were not present on cell bodies, although McFadzean et al. (1987) have shown subsequently that κ-receptors are involved in presysnaptic inhibition of excitatory transmission to a subpopulation of locus coeruleus neurons.

Agonists such as clonidine and norepinephrine that act on α_2-adrenoceptors on locus coeruleus neurons also activate a potassium conductance. The outward potassium currents produced by clonidine and normorphine had similar voltage dependencies and sensitivities to potassium channel-blocking agents such as quinine and barium (North and Williams, 1985). Furthermore, maximal activation of potassium conductance by one class of agonist preempted the action of the other (North and Williams, 1985; Andrade and Aghajanian, 1985), indicating that α_2-adrenoceptors and μ-opioid receptors were linked to the same potassium channels.

Aghajanian and Wang (1986) examined the effects of morphine on neurons in locus coeruleus slices prepared from rats that had been treated with pertussis toxin 1 to 3 days previously. Both the hyperpolarization and the outward current were greatly reduced compared with those produced by morphine in slices from untreated control rats, suggesting that μ-receptors may be coupled to G proteins that are ADP-ribosylated and inactivated by pertussis toxin. To consolidate this conclusion, it will be necessary to demonstrate ADP-ribosylation of G proteins in locus coeruleus neurons by pertussis toxin and to examine the effects of pertussis pretreatment on the electrophysiological properties of the neurons under study. Such experiments also need to be undertaken at the variety of other sites where pertussis toxin has been used as a probe to investigate opioid receptor-mediated decreases in neuronal excitability.

North et al. (1987) used a different approach to demonstrate the role of G proteins in opioid-induced hyperpolarizations of locus coeruleus neurons. They impaled cells with electrodes containing the nonhydrolyzable GTP analog, guanosine 5′-(γ-thio)triphosphate. Under these conditions, μ-agonists caused hyperpolarizations that did not disappear with washing. Repeated applications resulted in further hyperpolarization until the resting membrane potential stabilized at the potassium equilibrium potential. These observations suggested that guanosine 5′-(γ-thio)triphosphate diffused from the re-

cording electrode into the impaled neuron and thus prevented the hydrolysis of GTP following occupation of the μ-receptor by an agonist. As a consequence, the potassium conductance was irreversibly activated.

Although it is agreed that μ-receptors on locus coeruleus neurons are linked to a G protein, there appears to be disagreement concerning the subsequent events that lead to opening of the potassium channels. In the experiments of Andrade and Aghajanian (1985), morphine-induced hyperpolarization was inhibited by 8-bromo cyclic AMP or by dibutyryl cyclic AMP. These agents are membrane-permeable analogs of cyclic AMP and would be expected, in effect, to raise the intracellular concentration of cyclic AMP. It was, therefore, suggested that the hyperpolarization produced by morphine resulted from inhibition of adenylate cyclase and a consequent decrease in intracellular cyclic AMP levels. Duman et al. (1988) showed that opioids cause pertussis toxin-sensitive inhibition of adenylate cyclase activity in rat locus coeruleus neurons, an observation consistent with, although not necessarily indicative of, a role of adenylate cyclase in opioid-induced hyperpolarizations. However, North et al. (1987) reported that neither N^6,2′-O-dibutyryl cyclic AMP nor forskolin, a direct activator of adenylate cyclase, altered the hyperpolarization induced by μ-agonists, suggesting that cyclic AMP was not an intracellular effector for opioid actions. In the experiments of Andrade and Aghajanian (1985), 8-bromo cyclic AMP, administered alone, increased the firing rate in half of the cells examined, suggestive of a membrane depolarization. Therefore, the inhibition of the morphine-induced hyperpolarization may have resulted in part from a concomitant depolarization by 8-bromo cyclic AMP, mediated by an unrelated mechanism. The effects of dibutyryl cyclic AMP alone were not examined. In the study of North et al. (1987), no data on the effects of forskolin or of N^6,2′-O-dibutyryl cyclic AMP were provided. Thus, identification of the processes that link μ-receptor occupancy to subsequent opening of potassium channels requires more detailed investigations. More recently, μ-agonists have been shown to open potassium channels in patch-clamp experiments on intact cells of locus coeruleus neurons (Miyake et al., 1989). Responses were recorded only when the agonist was added to the patch pipette and not when administered on the outside of the pipette, implying that coupling between the receptor and the ion channels detected under these conditions did not involve a freely diffusible intracellular effector system.

2. Tolerance and dependence. The effects of chronic pretreatment of rats with morphine have been investigated in locus coeruleus neurons both in vivo and in slice preparations in vitro. Aghajanian (1978) recorded the firing rate of locus coeruleus neurons in anesthetized rats that had been pretreated by subcutaneous morphine pellet implantation for 5 days. Within 1 to 2 h after pellet implantation sufficient morphine had been absorbed from the pellet to stop basal firing. After 2 to 3 days, however, firing rate was restored to normal, despite repeated daily implantation of morphine pellets. Actual mean firing rates were 1.4 spikes/s in control untreated rats and 1.2 spikes/s in morphine-pretreated animals. This difference was not statistically significant. Prolonged exposure to morphine thus resulted in the development of tolerance to its inhibitory effect on cell firing.

In untreated control rats, microelectrophoretic administration of naloxone did not alter basal firing rate. In morphine-pretreated rats, however, naloxone increased the firing rate by more than 2-fold, an observation interpreted as withdrawal hyperexcitability indicative of morphine dependence. Although this interpretation has been questioned (North et al., 1987), the procedures to assess withdrawal hyperexcitability in the experiments of Aghajanian (1978) were adequate (section IV); after displacement of morphine from its receptors by naloxone, neurons of pretreated rats discharged action potentials at a frequency 2-fold greater than the normal frequency observed in untreated control rats. This difference is rather modest compared with the dramatic withdrawal hyperexcitability observed, for example, in cat dorsal horn neurons and in guinea pig myenteric neurons (section VI, A, 2). Nevertheless, because of the very extensive projections of locus coeruleus neurons, even small changes in cell firing could profoundly influence many brain areas and thus contribute substantially to the behavioral manifestations of withdrawal.

The experiments in vivo did not, however, indicate whether withdrawal hyperexcitability was initiated in locus coeruleus neurons themselves or was produced indirectly as a result of excitation via afferent inputs. Observations made in the locus coeruleus in vitro support the second possibility. In the slice preparation, in which many afferent inputs may be disrupted, no withdrawal hyperexcitability was apparent (Andrade et al., 1983). It appears that the somata of locus coeruleus neurons, although powerfully affected by morphine acutely, are not the targets for the changes underlying dependence.

Tolerance, however, was readily apparent in locus coeruleus slices from morphine-pretreated rats. In these experiments, the slice was set up and equilibrated in vitro in artificial cerebrospinal fluid that did not contain morphine. Extracellular recordings of the basal firing rates of locus coeruleus neurons were made between 1 and 8 h later, that is, between 1 and 8 h after morphine withdrawal. Basal firing rates in slices from untreated and from morphine-pretreated rats did not differ significantly (1.3 to 1.5 spikes/s). The concentrations of morphine required to produce a 50% decrease in basal firing rates were 32 nM and 92 nM in slices from control and pretreated rats, respectively, indicating approximately 3-fold tolerance to the depressant effect of morphine. Thus, in neurons of the rat locus coeruleus, chronic pretreat-

ment with morphine leads to tolerance without dependence in vitro.

To determine the specificity of tolerance, Andrade et al. (1983) examined the inhibitory effect of the α_2-adrenoceptor agonist, clonidine. In morphine-tolerant preparations, the inhibitory potency of clonidine was unchanged. The phenomenon of tolerance observed in the rat locus coeruleus, therefore, resembles the specific tolerance observed in the mouse vas deferens and the specific tolerance observed under certain conditions in the guinea pig ileum.

The mechanisms underlying tolerance were investigated by recording intracellularly from locus coeruleus neurons in slices from morphine-pretreated rats (Christie et al., 1987). These experiments confirmed that tolerance to μ-agonists occurred in the absence of dependence in vitro and that tolerance was specific, at least insofar as the sensitivity to the α_2-agonist, clonidine, was unchanged. Some characteristics of the tolerance observed were different for different μ-agonists. The degree of tolerance to normorphine was greater than that to DAMGO, and the maximum outward current produced by normorphine was reduced, whereas that evoked by DAMGO was unchanged. In neurons from control rats, the irreversible receptor-alkylating agent, β-chlornaltrexamine, caused a greater reduction in the effect of normorphine compared with DAMGO, suggesting that normorphine had lower intrinsic efficacy than DAMGO. The differential tolerance to normorphine and DAMGO and the decrease in the maximum response to normorphine may, therefore, have resulted from a lesser receptor reserve for normorphine than for DAMGO. An important advance provided by these experiments is that they localize the mechanisms of tolerance to single neurons bearing opioid receptors.

A reduction in receptor reserve had been proposed earlier by Chavkin and Goldstein (1984) and Porreca and Burks (1983) to account for tolerance to morphine in the guinea pig ileum. However, different mechanisms must underlie tolerance observed in myenteric and locus coeruleus neurons. The tolerance analyzed by Chavkin and Goldstein (1984) and by Porreca and Burks (1983) has been shown to be nonspecific (section VI), whereas that observed in the locus coeruleus by Christie et al. (1987) was specific for agonists acting at μ-receptors. A reduction in the apparent receptor reserve could result from a multiplicity of adaptive processes which could change the receptor itself or uncouple the receptor from its intracellular effector mechanism or reduce the ability of the receptor-mediated signal to influence neuronal excitability. Different adaptive processes may arise in different neurons.

A distinction can also be made between specific tolerance in the locus coeruleus and that component of tolerance in the ileum that is specific. Whereas specific tolerance in the ileum declines rapidly after morphine is removed from its receptor, the specific tolerance observed in the locus coeruleus remained unaltered for many hours after morphine withdrawal (Christie et al., 1987).

There is also evidence for specific tolerance to the inhibitory effects of opioids on norepinephrine release from the terminal fields of locus coeruleus neurons (Werling et al., 1988). Both μ- and κ-agonists inhibited the release of norepinephrine from slices of guinea pig cortex. After chronic treatment of guinea pigs with morphine, cortical slices were tolerant to μ-agonists, whereas the sensitivity to κ-agonists was unaltered.

Biochemical studies have shown increases in adenylate cyclase and in cyclic AMP-dependent protein kinase activities in locus coeruleus neurons from morphine-dependent rats (Nestler and Tallman, 1988; Duman et al., 1988). Such changes might be expected to increase the excitation of locus coeruleus neurons (Wang and Aghajanian, 1987). However, during morphine withdrawal, there is no evidence for increased action potential discharge in locus coeruleus neurons themselves (Andrade et al., 1983; Christie et al., 1987) or for enhanced release of transmitter from terminals of locus coeruleus neurons that project to the cortex (Werling et al., 1988). Hence, the functional consequences of the biochemical alterations remain to be determined. Another important observation that will stimulate further investigation is the increase in the levels of G proteins in locus coeruleus neurons of rats treated chronically with morphine (Nestler et al., 1989).

C. Hippocampus

1. Acute actions. An exception to the general rule that opioids inhibit neuronal firing (North, 1979; Duggan and North, 1983) is found in the hippocampus in which pyramidal neurons are excited by opioids. Although the mechanisms underlying this excitation have been much debated (see the papers by Henderson, 1983; Duggan and North, 1983; Deisz et al., 1988), the generally accepted view is that excitation results from opioid-induced inhibition of inhibitory neurons that synapse with pyramidal cells.

Zieglgänsberger et al. (1979) made extracellular recordings of action potential discharge in hippocampal neurons in anesthetized rats. The increase in firing rate produced by microelectrophoretic administration of Met-enkephalin or D-Ala^2-enkephalin was prevented when synaptic transmission was blocked by microelectrophoretic administration of Mg^{++}, suggesting that excitation was synaptically mediated. Furthermore, opioid-induced excitations were prevented not only by naloxone but also by the GABA antagonist, bicuculline. Similar observations were made by Dunwiddie et al. (1980). Rat hippocampal CA1 pyramidal cells were excited by microelectrophoretic administration of D-Ala^2-Met-enkephalin, an effect that was mimicked by the GABA antagonist, picrotoxin. Microelectrophoretic administration of D-Ala^2-Met-enkephalin also enhanced the population spike

evoked by electrical stimulation of the commissural pathway and often caused the appearance of secondary population spikes.

Subsequently, much of the analysis of opioid actions on hippocampal neurons has been undertaken in slice preparations in vitro. Opioid peptides or morphine caused a naloxone-sensitive increase in the amplitude of the pyramidal cell population spike evoked by electrical stimulation of afferent inputs (Corrigal and Linsemann, 1980; Dunwiddie et al., 1980; Nicoll et al., 1980; Masukawa and Prince, 1982), by actions at both μ- and δ-receptors (Dunwiddie et al., 1987).

Excitation could result from several possible mechanisms, for example from a direct excitant effect on pyramidal cells themselves, by facilitation of excitatory synaptic inputs or by inhibition of inhibitory inputs. Intracellular recordings showed that opioids did not alter the resting membrane properties or action potential configuration of pyramidal neurons (Nicoll et al., 1980; Siggins and Zieglgänsberger, 1981; Masukawa and Prince, 1982; Nicoll, 1982) and were likewise without effect on the excitatory postsynaptic potential recorded with extracellular electrodes (Dunwiddie et al., 1980). These observations suggested that opioid-induced excitation resulted from inhibition of inhibitory transmission from interneurons to pyramidal cells. The effects of the inhibitory transmitter GABA on pyramidal cell firing (Dunwiddie et al., 1980) and on resting membrane potential were not altered by opioids, indicating that the latter did not act by interfering with the postsynaptic actions of GABA (Nicoll et al., 1980; Siggins and Zieglgänsberger, 1981; Masukawa and Prince, 1982; Nicoll, 1982). However, opioids reduced the inhibitory postsynaptic potential recorded in pyramidal cells after electrical stimulation of the stratum radiatum or alveus. Depression of the inhibitory postsynaptic potential by opioids resulted in an increase in the probability that excitatory postsynaptic potentials would evoke action potential discharge in pyramidal cells. Thus, these observations suggested that excitation of pyramidal cells by opioids was due to an inhibitory action on inhibitory interneurons.

Lee et al. (1980) tested this hypothesis by recording extracellularly from putative interneurons in the hippocampal slice. The interneurons were identified by their electrophysiological properties and were located in the pyramidal cell layer adjacent to the stratum oriens. In support of the view that opioids acted by disinhibition, D-Ala^2-Met-enkephalin caused a naloxone-sensitive decrease in the firing rate of these cells. More recently, the mechanism underlying the inhibitory action has been investigated by intracellular recording from interneurons in rat hippocampal slices (Madison and Nicoll, 1988). D-Ala^2-Met^5-enkephalinamide did not alter the resting membrane properties of pyramidal cells but caused a membrane hyperpolarization in each of the 7 interneurons impaled. Because interneurons were encountered very infrequently, analysis of the mechanism underlying the hyperpolarization has so far been incomplete. However, the hyperpolarization was associated with an increase in membrane conductance and the extrapolated reversal potential was more negative than the resting membrane potential. It seems possible, therefore, that the hyperpolarization, like that observed in neurons of the rat and guinea pig locus coeruleus (Pepper and Henderson, 1980; North and Williams, 1983a), rat substantia gelatinosa (Murase et al., 1982; Yoshimura and North, 1983), and guinea pig myenteric and submucous plexuses (North and Tonini, 1977; Mihara and North, 1986), results from an increase in conductance to K^+.

The hyperpolarization by D-Ala^2-Met^5-enkephalinamide reduced the excitability of hippocampal interneurons (Madison and Nicoll, 1988). Thus, a hyperpolarization of inhibitory interneurons, with a consequent decrease in the release of GABA, provides a plausible explanation for opioid-induced excitation of pyramidal neurons. Important challenges of future studies will be to determine the receptor subtype and the intracellular effector mechanism that underlie the hyperpolarization.

2. Tolerance and dependence. Both tolerance and dependence have been demonstrated in hippocampal slices from rats treated chronically with morphine. Robinson et al. (1982) made extracellular recordings of the amplitude of the CA1 pyramidal cell population spike that was evoked by stimulation of the Schaeffer collateral-commissural pathway. Increasing stimulus strengths evoked population spikes of increasing amplitudes, so that stimulus intensity-spike amplitude curves were obtained in each slice. In slices from saline-treated control rats, morphine (5 μM) markedly increased the amplitude of the population spike and shifted the stimulus-response curve to the left. A much reduced shift in the stimulus-response curve by morphine was observed in slices from rats treated chronically with morphine, indicating tolerance to morphine.

In slices from control rats, naltrexone did not alter the amplitude of the population spike. However, in slices from morphine-pretreated rats, maintained in 5 μM morphine in vitro, naltrexone decreased the amplitude of the population spike such that the stimulus-response curve was shifted significantly to the right of that obtained in slices from control rats. The diminished response after morphine withdrawal is an indication of morphine dependence.

Zieglgänsberger et al. (1982) also investigated the effects of chronic morphine pretreatment in rat hippocampal slices. Like Robinson et al. (1982), they observed that normorphine enhanced the population spike in slices from untreated rats but not in those from rats treated chronically with morphine, suggesting the development of tolerance. Addition of naloxone to slices from pretreated rats decreased the amplitude of the population spike, an observation interpreted as a withdrawal re-

sponse. In these experiments, however, it is not certain whether the effect of naloxone represents a mere antagonism of an ongoing agonist action of morphine or a true withdrawal response indicative of dependence (section IV). To distinguish between these possibilities, it is essential to demonstrate that, after opioid withdrawal, the population spike is significantly different from that in tissues from untreated control rats. The amplitude of the population spike in these control slices appeared to vary markedly in different experiments (as in figure 1A and B of the paper by Zieglgänsberger et al., 1982). Thus, a quantitative comparison of the amplitude of the population spike in control slices, and in tolerant slices after treatment with naloxone, is not possible. Similar difficulties are encountered in interpreting the experiments of French and Zieglgänsberger (1982).

Corrigal et al. (1981) showed that acute administration of morphine to control hippocampal slices increased the amplitude of the population spike and also induced secondary spikes that were not apparent prior to morphine administration. In slices from rats treated chronically with morphine, tolerance was observed but only to the effect of morphine on the secondary spike. Dependence was not assessed in detail.

Little information is available on the mechanisms underlying tolerance or dependence in hippocampal slices. Zieglgänsberger et al. (1982) examined the specificity of tolerance by studying the responses to Met-enkephalin in morphine-tolerant hippocampal slices. Met-enkephalin increased the amplitude of the population spike, an observation that was interpreted to indicate that tolerance was specific for morphine. No quantitative comparison was made, however, of the magnitude of the effects of Met-enkephalin in slices from control and from morphine-pretreated rats. It is, therefore, uncertain whether morphine-tolerant slices were also tolerant to Met-enkephalin. Corrigal et al. (1981) found that the magnitude of tolerance to morphine (approximately 3.5-fold) was similar in slices that were set up in vitro in solutions containing 0.2 μM morphine and in slices that had been washed in morphine-free solutions for at least 1 h. This contrasts with the specific tolerance in mouse vas deferens and guinea pig ileum, in which removal of the opioid from the receptor in vitro results in a rapid loss of tolerance. On the other hand, nonspecific tolerance in the guinea pig ileum persists undiminished long after opioid withdrawal. This does not infer, however, that tolerance in the hippocampus is nonspecific because the magnitude of specific tolerance in locus coeruleus neurons is not reduced 6 h after morphine withdrawal (Christie et al., 1987). Examination of other agents that have acute actions similar to those produced by morphine and enkephalin will be necessary to determine the specificity of tolerance in the hippocampus.

Gahwiler (1981) made intracellular recordings from hippocampal pyramidal cells that were taken from 4- to 10-day-old rats and maintained in cultures for 3 weeks. Bath application of the enkephalin analog and μ-agonist, FK33824, caused a membrane depolarization and discharge of action potentials. The excitant effect subsided within a few minutes despite the continued application of the opioid, suggesting the development of acute tolerance. Preparations that were tolerant to FK33824 were also tolerant to DADLE. It was proposed that both agonists acted on μ-receptors, but no conclusion was made on the possible mechanism of tolerance. Such rapid loss in responsiveness resembles the phenomenon of receptor desensitization, although studies with more recently developed agonists of very high selectivity for different opioid receptor subtypes are necessary to accurately define the specificity of the phenomenon studied. Cells rendered tolerant to FK33824 were not tolerant to the GABA antagonist, bicuculline, which also excited pyramidal cells. Lack of cross-tolerance to bicuculline was explained on the basis that bicuculline did not act on opioid receptors. It should also be noted that the opioids and bicuculline probably act upon different neurons to produce excitation. Whereas the opioids inhibit inhibitory interneurons that release GABA onto pyramidal cells, bicuculline presumably excites pyramidal cells by antagonizing the postsynaptic actions of GABA. Studies on the specificity of tolerance in the hippocampus need to include examination of agents that act on opioid-sensitive neurons in the circuitry involved in generating the pyramidal cell population spike.

Acute tolerance was also observed in the hippocampal slice by Dingledine et al. (1983). Upon continuous perfusion with morphiceptin, the initial excitant effect decayed after 1.5 h. Similar loss in responsiveness was found after 4.5 h of continuous perfusion with DADLE. In contrast to Gahwiler (1981), Dingledine et al. (1983) concluded that DADLE acted on δ-receptors and that acute tolerance was due to down-regulation of the receptor. Ligand-binding studies showed a 53% decrease in the amount of DADLE bound to receptors after a 4-h perfusion. However, in preparations perfused for the same period but without DADLE present in the bath, there was a 35% decrease in DADLE binding compared with that in freshly prepared untreated preparations. The difference in binding that may be attributed to constant occupation of the receptor by DADLE is, therefore, small and unlikely to account for the tolerance observed (section III). The decline in the excitant effect following a 1.5-h perfusion with morphiceptin was not associated with changes in morphiceptin receptor binding. Thus, the mechanisms of acute tolerance need to be examined further.

Acute tolerance in the hippocampus (Dingledine et al., 1983) was not associated with dependence; naloxone merely antagonized the excitant effect of the agonist without any withdrawal response. In the experiments on acute tolerance in cultured hippocampal neurons (Gah-

wiler, 1983) discussed above, the effects of naloxone were not examined.

D. Striatum

1. Acute actions. As in most other brain areas, opioids decrease the discharge of action potentials recorded extracellularly in neurons of the rat striatum in vivo (Zieglgänsberger and Fry, 1976; Frederickson and Norris, 1976; Nicoll et al., 1977; Perkins and Stone, 1980; Fry et al., 1980a). Both morphine and enkephalins inhibited the firing rate of these neurons, although the inhibitory effects of morphine were rather resistant to antagonism by naloxone (Perkins and Stone, 1980). This is consistent with a predominance of δ-receptors, although the receptor subtypes involved in depression of cell firing have not been accurately defined.

The mechanisms underlying opioid-induced depression of striatal neuron firing have not been determined. Numerous biochemical studies have shown that opioids decrease adenylate cyclase activity or cyclic AMP production in rat striatum (Law et al., 1981; Kamikubo et al., 1981; Cooper et al., 1982; Barchfeld et al., 1982; Abood et al., 1985; Kelly and Nahorski, 1986; O'Shaughnessy and Headley, 1986; Childers, 1988). The inhibitory effects are GTP dependent (Law et al., 1981; Kamikubo et al., 1981; Cooper et al., 1982; Bhoola and Pay, 1986) and are prevented by pretreatment with pertussis toxin (Abood et al., 1985; Childers, 1988), suggesting that inhibition of adenylate cyclase is produced via coupling of the opioid receptors to the inhibitory G protein, G_i.

Morphine and enkephalins also altered the phosphorylation of synaptic membrane proteins by kinase enzymes in rat striatal neurons (Williams and Clouet, 1982; Clouet and Yonehara, 1984). An initial increase in phosphorylation of some proteins was followed by a more substantial reduction in phosphorylation of the same proteins. Both effects were antagonized by naloxone. The importance of these observations is that phosphorylation reactions participate in many cellular functions, including receptor-mediated regulation of ion channnels (Kaczmarek, 1987; Rosenthal and Schultz, 1987). In the experiments of Williams and Clouet (1982), opioids altered the phosphorylation of a range of proteins that resembled those phosphorylated by calcium-dependent protein kinase rather than those phosphorylated by cyclic AMP-dependent protein kinase. Thus, the relationship between the changes in opioid-induced decreases in adenylate cyclase activity and the altered pattern of synaptic plasma membrane phosphorylation is unclear. Moreover, it remains to be established which, if any, of these biochemical changes are important in the opioid-induced decrease in excitability of striatal neurons.

2. Tolerance and dependence. Electrophysiological studies in striatal neurons of rats pretreated chronically with morphine illustrate some of the difficulties encountered in measuring tolerance and dependence in experiments on single neurons (section IV). In untreated control rats, the majority of striatal neurons (36 of 62) decreased their firing rates upon microelectrophoretic administration of enkephalin, whereas in morphine-pretreated rats the majority of cells (34 of 43) were unresponsive (Zieglgänsberger and Fry, 1976). The lower proportion of opioid-sensitive neurons in the pretreated rats may indicate tolerance to the inhibitory effects of enkephalin. However, more than 2 g of morphine had been implanted in these animals during the preceding 10 to 12 days. Under these conditions, enkephalin may have been relatively ineffective, not because of a true decrease in potency (tolerance) but because its receptors were substantially occupied by very high concentrations of circulating morphine present in the animals when the effects of enkephalin were measured (section IV).

A similar difficulty applies to the assessment of dependence. The proportion of striatal neurons that were excited by microelectrophoretic administration of naloxone was greater in morphine-pretreated than in untreated control rats (Fry et al., 1980b). Excitation by naloxone was taken as a withdrawal response indicative of dependence. As pointed out above, however, high concentrations of morphine were probably present in the circulations of these rats, as a result of the intensive morphine pretreatment schedule. Any ongoing receptor-mediated inhibitory effects of morphine would be antagonized by naloxone, thus readily accounting for the higher incidence of naloxone-induced excitations in pretreated compared with untreated control rats. Excitation by naloxone, therefore, does not, per se, indicate withdrawal hyperexcitability but may indicate merely that excitability has been restored to normal (section IV).

E. Cortex

1. Acute actions. The majority of studies on the effects of opioids on cortical neurons have used extracellular electrodes to record action potential discharge in anesthetized animals in vivo. Microelectrophoretic administration of morphine or enkephalin caused naloxone-sensitive decreases in the basal firing rates of these neurons (Satoh et al., 1974; 1975; 1976a; Frederickson and Norris, 1976; Palmer et al., 1978). Excitations produced by microelectrophoretic administration of *l*-glutamate or acetylcholine were also depressed, suggesting direct inhibitory actions of opioids on the neurons from which recordings were made. Intracellular recordings in slice preparations revealed that DADLE did not alter the resting membrane potential or input resistance of rat cortical neurons but depressed the excitatory postsynaptic potential evoked by electrical stimulation of afferent inputs (Sutor and Zieglgänsberger, 1984). Depolarization produced by microelectrophoretic administration of *l*-glutamate was also depressed. DADLE depressed glutamate-induced depolarizations after synaptic transmission was blocked, indicating a location of opioid receptors on the impaled neuron. In these experiments, the

effects of morphine were not examined. It is possible that morphine and DADLE reduced the excitation of cortical neurons by actions on different receptors (Williams and Zieglgänsberger, 1981). Further studies are necessary to establish this and to determine the cellular mechanisms underlying the inhibitory effects.

2. *Tolerance and dependence.* Several investigators have examined the excitation of cortical neurons in rats treated chronically with morphine. In contrast to its consistent inhibitory effects on the firing of cortical neurons in control rats, morphine failed to depress firing of cortical neurons in pretreated rats, an observation taken as evidence for morphine tolerance (Satoh et al., 1974; 1975; 1976b). Difficulties in interpreting experiments of this type have been discussed above (section IV). Because the animals were pretreated with an intensive schedule of morphine pellet implantation, it is likely that high concentrations of morphine were present in the circulation when the effects of microelectrophoretic administration of morphine were examined. If most of the morphine receptors were already occupied, further administration of morphine might be incapable of inhibiting cell firing, irrespective of whether there was a shift to the right in the concentration-response curve indicative of tolerance. Thus, the absence of an effect of morphine does not, per se, indicate tolerance. To assess tolerance under these conditions, it is necessary to compare the actual firing rates of cortical neurons in untreated and pretreated rats. Because morphine depressed firing when administered acutely, a return of the firing rate to normal in pretreated animals would indicate morphine tolerance. In the experiments of Satoh et al. (1974; 1975; 1976b), no obvious differences in basal rate of firing of cells in untreated and pretreated rats were observed, consistent with the development of tolerance. A *quantitative* comparison was not made, however, presumably because of the great variability in the firing rates of different neurons. Tolerance is, therefore, difficult to assess in experiments in which an unknown concentration of morphine is already present at receptors and in which the firing rate of neurons cannot be quantified accurately. Large variations in firing rates are common in studies on single neurons at most sites in the CNS. An exception is the locus coeruleus (section VII, B) in which neurons discharge action potentials at rates that are sufficiently constant to permit a mean firing frequency to be determined (Aghajanian, 1978).

To investigate whether chronic pretreatment of rats with morphine induced signs of dependence in cortical neurons, Fry et al. (1978; 1980) compared the effects of naloxone in untreated and in chronically pretreated rats. In the pretreated rats, microelectrophoretic administration of naloxone caused an increase in *l*-glutamate-induced excitation in 6 of 17 cortical neurons. In untreated animals, excitation by naloxone was observed with only 1 of 12 cells (Fry et al., 1980). The higher incidence of excitations by naloxone was considered as evidence for a withdrawal response in these neurons. However, excitation by naloxone may merely represent antagonism of an ongoing inhibitory action of morphine that is present in the circulations of pretreated, but not of untreated, rats. To distinguish between a simple antagonism of an agonist action of morphine and a withdrawal response indicative of dependence, it is essential to establish that the neurons are hyperexcitable after naloxone administration. An excitatory effect of naloxone does not, per se, acccomplish this distinction (section IV).

Hyperexcitability of cortical neurons was, however, demonstrated by the approach taken by Satoh et al. (1976b). They compared the excitant effects of *l*-glutamate on cortical neurons in untreated and in morphine-pretreated rats. *l*-Glutamate was administered microelectrophoretically and an estimate made of the ejection current required to cause a 50% increase in firing rate. Neurons in pretreated rats were supersensitive to *l*-glutamate as indicated by the 3-fold decrease in the effective microelectrophoretic current. Supersensitivity was nonspecific in that the excitant effects of acetylcholine were also enhanced to a similar degree.

To account for nonspecific supersensitivity, the authors proposed that adaptive changes occurred beyond the level of the agonists' receptors. Thus, adaptive supersensitivity in cortical neurons bears a fundamental resemblance to nonspecific supersensitivity described in myenteric neurons of morphine-dependent guinea pig ileum. Intracellular recordings from cortical neurons will be required to determine whether the similarities extend to the membrane depolarization observed in myenteric neurons. For this purpose, the cortical brain slice preparation described by Sutor and Zieglgänsberger (1983) could prove valuable in investigations of the cellular bases of adaptive supersensitivity.

VIII. Summary and Conclusions

It is generally accepted that the phenomena of tolerance to and dependence upon opioids are a reflection of cellular adaptations. It follows that the broad literature on cellular adaptive processes may provide useful clues to a better understanding of opioid tolerance and dependence at the cellular levels. The background literature on measurement of sensitivity and adaptive processes is provided in sections I to III.

Excitable cells of many types have the capacity to adapt to acute or chronic overstimulation or understimulation. A number of cellular mechanisms have been associated with adaptive sensitivity changes. Most of those mechanisms can be divided into two categories.

The first category includes acute changes initiated by relatively short-term exposure to high concentrations of an agonist. The changes are frequently known collectively as desensitization and are characterized by rapid onset and rapid recovery, ranging from seconds to minutes and, occasionally, a few hours. A number of specific

molecular changes have been identified with desensitization. These include alterations in affinity and configuration of the receptor, uncoupling of the receptor from its second-messenger system, internalization of receptors, actual loss of receptors, and loss of responsiveness of the second-messenger system itself. With the exception of loss of responsiveness of the second messenger, all of these forms of desensitization are homologous, that is, they are specific for one receptor system. When the second-messenger system, such as adenylate cyclase, becomes desensitized, the effect is heterologous, that is, responses mediated via all receptors that are coupled to the cyclase are depressed.

Some cell types display most or all of these molecular changes in a temporal sequence, whereas other cells display only some of them. The degree of their interdependence is still uncertain.

The second category includes molecular changes that come on slowly and regress slowly, over a period of days to weeks. These changes come about in compensation for long-term changes in the net stimulus a cell receives (and, in some instances, perhaps, the influence of trophic factors). Each change appears to involve an altered concentration of a specific protein and may represent an altered genetic expression or repression of that protein. These slow adaptive sensitivity changes are always in a direction to compensate for the chronic alteration in net stimulus received by the cell. Because opioids usually inhibit the neurons upon which they act, chronic exposure to an opioid represents a chronic decrease in the net stimulus to those neurons.

Two molecular alterations have been firmly established as factors Underlying slow adaptive sensitivity changes. These are increases or decreases in receptor density and decreases in Na^+/K^+ pump sites. Two other cellular changes may also contribute in some cells, increased or decreased activity of adenylate cyclase and altered calcium dynamics. However, these latter mechanisms are ill defined.

The increase in receptors (up-regulation) is a major component of supersensitivity in skeletal muscle. However, the increases and decreases in receptors associated with supersensitivity and subsensitivity in other cell types are generally small and unlikely to fully explain the sensitivity changes by themselves. Whether or not the loss of receptors reported in association with slow adaptive sensitivity changes (days to weeks) in some cells is identical with the rapid loss of receptors (hours) demonstrated as part of desensitization in other cells is unknown. In either case, the loss of sensitivity is specific (i.e., homologous).

The association of a decrease in Na^+/K^+ pumping and partial membrane depolarization with supersensitivity has been identified only in smooth muscle but has not been investigated in many other tissues. Few attempts have been made to correlate sensitivity changes with increases or decreases in pump sites. The potential importance of decreases or increases in Na^+/K^+ pumping is that such changes produce nonspecific sensitivity changes.

If opioid tolerance and/or dependence were due to similar mechanisms as those summarized above, one may predict some experimental results. Desensitization via altered affinity, uncoupling, and/or internalization of receptors would cause subsensitivity (tolerance) specifically to opioids acting on those receptors involved in responses to the desensitizing opioid. For example, a highly μ-selective opioid would induce desensitization only to opioid effects mediated by μ-receptors. Such desensitization would not lead to dependence because dependence reflects enhanced sensitivity to other transmitters, those acting in opposition to the effect of the opioids. Such desensitization would develop rapidly and disappear rapidly with the removal of the opioid.

Down-regulation of opioid receptors (destruction of receptors) would have intermediate to long onset and offset times (hours to days) and would be manifest as subsensitivity (tolerance) specific for opioids acting through the down-regulated receptor. Again, because of the location of the molecular defect, such receptor-related tolerance would not be associated with dependence.

Changes in a second-messenger system, such as adenylate cyclase, would also have intermediate to long onset and offset rates. The sensitivity would be changed to a wider selection of agonists. For example, if opioids acted by inhibiting the second-messenger system, subsensitivity (tolerance) might occur through enhanced activity of the second-messenger system. This would lead to cross-tolerance to all substances that inhibited that second-messenger system and supersensitivity to all substances that activate the system. Supersensitivity to the activation of the second messenger would cause the withdrawal response associated with dependence.

A partial depolarization of basal membrane potential of the opioid target neurons could readily explain both tolerance and dependence. A partial depolarization of neurons could render them more resistant to all agonists, including opioids, that inhibit the neurons by hyperpolarization and supersensitive to all agonists that stimulate the neurons by depolarization. Thus, one would expect nonspecific subsensitivity to all hyperpolarizing substances, as well as nonspecific supersensitivity to depolarizing substances. These are indices of tolerance and dependence, respectively.

To fit the large body of data on opioid tolerance and dependence into the above scheme, a number of pitfalls in experimental design must be recognized. This topic is discussed in section IV of this review. The principles developed are then applied to evaluate data in subsequent sections.

In 1974 it was reported that opioids inhibited the formation of cyclic AMP in rat brain homogenates (sec-

tion V). Out of this grew the hypothesis that the adenylate cyclase system may contribute to tolerance and dependence. The concept received momentum from the discovery of a "quasimorphine abstinence syndrome," in which certain substances that elevated cyclic AMP in the brain caused an acute behavioral response similar to the abstinence syndrome evoked by naloxone.

Much is known about the relationship of δ-receptors and adenylate cyclase in pure cell lines, especially the hybrid strain of neuroblastoma × glioma cells (NG108–15). These cells have a homogeneous population of δ-opioid receptors. All of the classical components of the adenylate cyclase system are present, including G_s and G_i. Stimulation, such as by PGE_1, produces the usual cascade of events, including the activation of cyclic AMP-dependent kinase.

Exposure of NG108–15 cells to δ-agonists causes an uncoupling of the receptor from G_i with a half-life of 7 min followed by internalization. Prolonged exposure (12 to 24 h) leads to down-regulation (loss of receptors). Exposure to agonists for 1 to 3 days results in an increase in maximum activity of the adenylate cyclase. Thus, there is demonstrated both homologous desensitization and a heterologous change, the latter with the potential of nonspecific subsensitivity to inhibitors and supersensitivity to stimulants.

Similar findings have been reported for 7315c pituitary tumor cells. These cells contain only μ-type opioid receptors. Thus, the sequence, desensitization followed by down-regulation in cultured cells, extends to both μ- and δ-receptors. The degree to which these findings pertain to opioid tolerance in normal neurons is still unknown.

In vivo exposure to opioids leads to increased affinity of receptors for naloxone in the CNS. However, this occurs prior to measurable tolerance and without a change in affinity to agonists. Its significance, therefore, is unclear. Chronic administration of opioids to animals has also led to a decrease in maximum binding of opioid ligands. However, once again, the results do not correlate well with tolerance.

Another possible cellular explanation for tolerance and dependence is a chronic partial depolarization of the neurons upon which opioids act. Such a depolarization could nonspecifically counteract the hyperpolarizing effects of inhibitors and nonspecifically enhance the depolarizing effects of stimulants. This concept has received relatively little consideration in the past. However a growing body of evidence indicates that it is worthy of more attention.

Section VI is devoted to experiments with the guinea pig ileum and the mouse vas deferens. Both the longitudinal and the circular muscle of the guinea pig ileum are stimulated via motor neurons from the myenteric plexus via the release of acetylcholine and, to some extent, substance P. Opioids, acting upon μ-receptors, hyperpolarize myenteric neurons and thereby inhibit the activation of those neurons by either electrical stimulation or depolarizing substances. The hyperpolarization is associated with an increase in potassium conductance. Opioids also inhibit transmitter release from myenteric neurons via κ-receptors. However, this mechanism probably involves inhibition of calcium entry into the neurons rather than hyperpolarization.

The mechanisms of coupling of opioid receptors to neuronal inhibition in the myenteric plexus remain unknown. Although opioids decrease the activity of adenylate cyclase in the neurons, the weight of evidence does not support a mediation by cyclic AMP of the acute effects of opioids on neurotransmission or neuronal excitability in the plexus. A potential role of the phosphatidylinositol system has not been investigated. Although there is an intimate relationship between extracellular Ca^{++} concentration and opioid action, the mechanism of the interaction is not understood.

Chronic treatment of guinea pigs with opioids produces tolerance and dependence in the ileum. This is observed, for example, in the isolated longitudinal smooth muscle-myenteric plexus preparations. What appear to be conflicting results can be resolved by the suggestion, supported by the data, that the tolerance is composed of at least two separate phenomena. There is a subsensitivity to opioids that is very marked, often greater than 50-fold, and that disappears rapidly, within 1 to 2 h. This phenomenon is not associated with dependence. It, thus, has characteristics that would be predicted from a desensitization reaction.

The second type of tolerance coexists with the first but is more modest in magnitude (3- to 10-fold) and is nonspecific, involving cross-tolerance (subsensitivity) to nonopioid inhibitors such as α_2-adrenoceptor agonists and adenosine analogs. This nonspecific subsensitivity disappears slowly over several days. The nonspecific subsensitivity to inhibitory agonists coincides with a nonspecific supersensitivity to a variety of stimulatory agonists. This supersensitivity is an index of opioid dependence.

Experiments with ligand binding have not produced any evidence that either of the two types of tolerance involve changes in the availability of receptors. The short half-life of the specific tolerance is inconsistent with the well-established longer time course of receptor down-regulation and recovery. An uncoupling of the opioid receptor from its cellular response is an explanation that fits all of the available data.

At the present time two theories have been advanced regarding the mechanism of the slowly disappearing nonspecific tolerance and dependence. (*a*) If opioid receptors are coupled by an inhibitory G protein (G_i) to adenylate cyclase, then an increase in adenylate cyclase capacity would produce subsensitivity to the opioids and other inhibitory agonists acting through adenylate cyclase and supersensitivity to all excitatory agonists which activate

adenylate cyclase. There is evidence that cholera toxin or pertussis toxin alter the development and/or the demonstration of dependence in the ileum. However, as discussed in section VI, the results are difficult to interpret. Furthermore, for the adenylate cyclase hypothesis to be correct, all inhibitory agonists to which tolerance develops and all excitatory agonists to which supersensitivity develops in the myenteric neurons must act through G_s or G_i, i.e., via activation or inhibition of adenylate cyclase. Given the variety of those agents, this seems unlikely. Indeed, one important agonist, nicotine, is known to directly open sodium channels without the intervention of a G protein. (*b*) A partial depolarization (decreased magnitude of the resting membrane potential) of myenteric neurons would result in nonspecific subsensitivity to inhibitory agonists and nonspecific supersensitivity to excitatory agonists. Such an adaptive depolarization is a well-established phenomenon in skeletal muscle and smooth muscle. It has been a neglected possibility in neuronal adaptation. Evidence in support of the depolarization hypothesis of tolerance and dependence has been obtained in the guinea pig ileum. In in vitro experiments, opioids induce a hyperpolarization of S neurons in the myenteric plexus. Addition of naloxone antagonizes this hyperpolarization, returning the membrane potential to the preopioid (resting) level. Naloxone has no effect in the absence of opioid. After prolonged exposure to opioid, the membrane potential declines to the preopioid level, in spite of the continued presence of the agonist. The addition of naloxone now produces a depolarization similar in magnitude to the hyperpolarization produced acutely by the opioid agonist. This depolarization often leads to action potentials. In preliminary experiments in ilia removed from guinea pigs in which tolerance is induced in vivo by morphine pellet implantation, the resting membrane potential of S neurons is also less negative than that in neurons from naive animals. Furthermore, the magnitude of the hyperpolarization induced by morphine in those experiments was not different between tolerant and naive neurons.

Neurons in the mouse vas deferens stimulate the smooth muscle via the release of norepinephrine. Opioids inhibit the release of transmitter via μ-, κ-, and δ-receptors. Attempts to link this inhibition to cyclic AMP have been unsuccessful. Chronic treatment of mice with opioids induces a highly specific tolerance which disappears rapidly when the opioid is removed from the receptor. There is little or no indication of dependence. Experiments with ligand binding have detected no changes in receptors. Thus, the tolerance appears similar to the short-term, specific tolerance observed in the guinea pig ileum and is probably due to a desensitization reaction such as uncoupling of the receptor from its second-messenger system.

Section VII surveys results in the CNS. A considerable amount of information is available for the spinal cord, locus coeruleus, and hippocampus. Data from these regions are enhanced by the inclusion of experiments with cultured spinal cord cells and in vitro brain slices including locus coeruleus or hippocampus. Information is much more limited and interpretation compounded by methodological problems in the striatum and cortex. Results in the spinal cord, locus coeruleus, and hippocampus will be summarized collectively.

As in peripheral neurons, the primary acute effect of opioids in CNS neurons is inhibitory. The excitatory effect in hippocampal pyramidal cells is due to inhibition of inhibitory interneurons. Where determined in individual neurons, the opioids have often been shown to induce neuronal hyperpolarization linked to an increase in potassium conductance. A role of cyclic AMP has not been clearly established, but in some neurons, a G protein may be involved in opioid action. It is possible that some of the opioid receptor-mediated actions occur through a coupling by a G protein directly to ion channels rather than to adenylate cyclase.

Tolerance has been demonstrated in all three regions. Only a modest effort has been made by investigators to address the question of specificity of the tolerance. There is some evidence of cross-tolerance to nonopioid inhibitors in spinal cord preparations. On the other hand, tolerance in the locus coeruleus appears to be specific. Dependence has been established to occur in the spinal cord and hippocampus but not locus coeruleus. Data are limited and inconclusive regarding mechanisms of tolerance and dependence in the CNS.

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