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The δ-Opioid Receptor: Molecular Pharmacology, Signal Transduction, and the Determination of Drug Efficacy

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I. Introduction

Although it was long thought that opioid drugs act on specific receptor sites, opioid receptors themselves were not identified until about 25 years ago (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Chemists and pharmacologists suspected the existence of multiple opioid receptors (Portoghese, 1965; Gilbert and Martin, 1976; Martin et al., 1976), and radioligand-binding studies provided evidence to divide opioid receptors into three different types (Goldstein, 1987; Pasternak, 1993). Recent molecular cloning techniques have characterized the nucleotide sequence of at least three distinct opioid receptors, namely, the δ-, κ-, and μ-opioid receptors. It has been suggested by the International Union of Pharmacology Subcommittee on Opioid Receptors that the designations δ-, κ-, and μ-opioid receptors be replaced by the designations OP_1, OP_2, and OP_3, respectively (Dhawan et al., 1996). The OP_1, OP_2, and OP_3 designations, which are based on the order in which these receptors were cloned (Dhawan et al., 1996), have proved to be quite controversial within the research community and will be reconsidered by International Union of Pharmacology in the near future. For this reason, we will use the established δ-, κ-, and μ-opioid receptor nomenclature in this review.

The cloned δ-, κ-, and μ-opioid receptors are highly homologous, and all three interact with heterotrimeric G proteins (Gilman, 1987; Childers, 1991). The G protein-coupled receptor superfamily, which includes numerous neurotransmitter and hormonal receptors, possesses a common three-dimensional structure that spans the cell membrane seven times, forming three extracellular loops and three intracellular loops. The amino terminus is extracellular, whereas the carboxyl terminus is intracellular (Strosberg, 1991). Studies conducted on the cloned opioid receptors demonstrate that the amino acid sequence of the δ-, κ-, and μ-opioid receptors are 65% homologous; hence, it is the other 35% that confer type selectivity (Reisine and Bell, 1993). The domains with the greatest similarity are the transmembrane regions and the intracellular loops, whereas the most divergent regions are the extracellular loops and the amino- and carboxyl-terminals (Fig. 1). Based on results of pharmacological investigations, δ-, κ-, and μ-opioid receptors have been further subdivided into receptor subtypes (Satoh and Minami, 1995); however, the molecular basis for subtypes remains to be resolved.

FIG 1. Human δ-, κ-, and μ-opioid receptor amino acid comparison. The TMs are underlined and numbered (modified from Knapp et al., 1995b).

The existence of a fourth opioid receptor, the ε-opioid receptor, has long been suspected and was initially postulated to explain β-endorphin-mediated inhibition of the electrically induced contraction of the rat vas deferens (Wüster et al., 1979; Schulz et al., 1981). These findings were consistent with the presence of a β-endorphin-binding receptor in the rat vas deferens that was independent of the δ- and μ-opioid receptors. Evidence also suggested that the β-endorphin-binding site in the rat vas deferens is not a κ-opioid receptor because a series of benzomorphan compounds, thought to be κ-selective agonists, were competitive antagonists in the rat vas deferens (Gillan et al., 1981). β-Endorphin activity in the rat vas deferens was antagonized by naloxone (Huidobro-Toro et al., 1982), which is consistent with the identification of the β-endorphin receptor as an opioid receptor.

β-Endorphin-binding sites were also observed in brain tissue (Law et al., 1979; Johnson et al., 1982). These studies suggested the possibility that a portion of the β-endorphin binding in the brain was distinct from enkephalin and morphine-binding sites. Further evidence for brain ϵ-opioid receptors came from competition-binding studies in rat brain membranes with the universal opioid antagonist [^{3}H]diprenorphine (Chang et al., 1981a). Twenty-seven percent of specific [^{3}H]diprenorphine binding was not competitively excluded from receptors in the presence of sufficient [D-Ala^2,D-Leu^5]enkephalin (DADLE)[3] and morphiceptin to block both δ- and μ-opioid receptors. Conversely, benzomorphan drugs such as cyclazocine were able to totally exclude [^{3}H]diprenorphine binding. These authors named the non-δ, non-μ-opioid receptor that bound [^{3}H]diprenorphine as benzomorphan-binding sites. β-Endorphin also inhibited [^{3}H]diprenorphine binding at the benzomorphan site in rat brain membranes with a K_i value of 10 nM (Chang et al., 1984). In the same study, the potencies of β-endorphin, β-endorphin fragments, etorphine, DADLE, and Tyr-D-Ala-Gly-NMe-Phe-Met(O)ol in the contraction of the rat vas deferens correlated with the affinities of these agonists at the benzomorphan-binding site in the rat brain.

In contrast to agonists active at other opioid receptors, β-endorphin-stimulated antinociception is not directly mediated through pertussis toxin-sensitive G proteins (Tseng and Collins, 1995, 1996). Data also indicate that δ-opioid receptors are involved in some ϵ-opioid receptor-mediated antinociceptive pathways (Suh and Tseng, 1990). Hitherto, the greatest impediment to characterization of ϵ-opioid receptor function has been the dearth of selective pharmacological tools. However, a cDNA that may encode the ϵ-opioid receptor was cloned from a human genomic library (O'Dowd et al., 1995). If this proves true, expression of this clone in cell lines should allow further characterization of the ϵ-opioid receptor. The ϵ-opioid receptor was recently reviewed (Narita and Tseng, 1998).

[3] Abbreviations: DADLE, [D-Ala^2,D-Leu^5]enkephalin; AS oligo, antisense oligodeoxynucleotide; BUBU, Tyr-D-Ser[-*O*-C(CH$_3$)$_3$]-Gly-Phe-Leu-Thr-*O*-C(CH$_3$)$_3$; BW373U86 or BWB373, (±)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide; pCl-DPDPE, cyclic [D-Pen^2,4′-ClPhe^4,D-Pen^5]enkephalin; CHO, Chinese hamster ovary; COS, monkey fibroblast; CREB, cAMP response element-binding protein; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; Del-II, [D-Ala^2]deltorphin II; DPDPE, cyclic[D-Pen^2,D-Pen^5]enkephalin; DRG, dorsal root ganglia; DSLET, [D-Ser^2,Leu^5,Thr^6]enkephalin; DTLET, [D-Thr^2,Leu^5,Thr^6]enkephalin; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate; ICI-154,129, *N*,*N*-bisallyl-Tyr-Gly-Gly-ψ-(CH$_2$S)-Phe-Leu-OH; ICI-174,864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; i.t., intrathecal; JOM13, Tyr-c-[D-Cys-Phe-D-Pen]OH; K_D, dissociation constant; K_i, inhibition constant; MAP, mitogen-activated protein; ORL$_1$, opioid receptor-like protein$_1$; PKC, protein kinase C; PKA, protein kinase A; SNC80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; SNC121, (+)-[(4α-*R*)-α(2*S*,5*R*)-4-propyl-2,5-dimethyl-1-piperazinyl-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; TAN67, 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino-[2,3,3-*g*]isoquinoline); TIPP, Tyr-Tic-Phe-Phe-OH; TM, transmembrane domain.

Yet another opioid receptor-like species has been cloned, namely, the opioid receptor-like protein$_1$ (ORL$_1$) receptor (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994; Nishi et al., 1994; Wang et al., 1994a; Wick et al., 1994; Halford et al., 1995; Lachowicz et al., 1995). Like the δ-, κ-, and μ-opioid receptors, with which it shares 50 to 60% sequence homology, the cloned ORL$_1$ receptor is a seven-transmembrane domain (TM)-receptor coupled to G proteins. This naturally occurring receptor is widely distributed in the brain and is responsive to the novel peptide orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995; also known as nociceptin, Rossi et al., 1997). The cloned receptor mediates the inhibition of forskolin-stimulated cAMP production in a naloxone-insensitive manner (Reinscheid et al., 1995). In contrast to the effects of classic opioid receptors, the ORL$_1$ receptor appears to mediate hyperalgesia (Meunier et al., 1995; Reinscheid et al., 1995), and this hyperalgesia is insensitive to the opioid antagonist diprenorphine (Rossi et al., 1997). Further investigation has demonstrated that the ORL$_1$ receptor can also mediate analgesia, although the kinetics of analgesia production differ from those of hyperalgesia and the analgesia is sensitive to the action of opioid antagonists (Rossi et al., 1996, 1997). The ORL$_1$ receptor is thought to be encoded by the same gene that codes the κ$_3$-opioid receptor; however, antisense knockdown experiments suggest that these receptors are splice variants with differing signaling characteristics (Pasternak and Standifer, 1995; Rossi et al., 1997). For a more extensive review, see Meunier (1997).

Compared with the opioid or opioid-like receptors discussed above, the δ-opioid receptor is an attractive target for the development of new drugs to control pain. The κ opioid receptors have previously been shown to mediate dysphoria (Pfeiffer et al., 1986), ORL$_1$ receptors mediate hyperalgesia in addition to analgesia (Rossi et al., 1997), and ϵ-opioid receptors are still poorly characterized. The δ-opioid receptor-selective drugs may possess potential clinical benefits compared with the μ-opioid receptor drugs that are currently in use for the relief of pain. These advantages include greater relief of neuropathic pain (Dickenson, 1997), reduced respiratory depression (Cheng et al., 1993), and constipation (Sheldon et al., 1990), as well as a minimal potential for the development of physical dependence (Cowan et al., 1988).

Reflecting the medical importance of opioid receptors, a number of reviews examining the molecular biology of these receptors have appeared (Reisine and Bell, 1993; Reisine et al., 1994; Kieffer, 1995; Knapp et al., 1995b; Minami and Satoh, 1995; Reisine, 1995; Satoh and Minami, 1995; Dhawan et al., 1996; Raynor et al., 1996; Zaki et al., 1996). The present review, while necessarily covering some of the same ground, will endeavor to 1) emphasize the molecular pharmacology of the δ-opioid receptor and 2) describe the pharmacodynamics of

selected agonists that bind to the δ-opioid receptor. To improve the selectivity of δ-opioid agonists, there needs to be a corresponding increase in our ability to describe drug activity. One such description is efficacy, which is a measure of the ability of an agonist-bound receptor to stimulate a measurable response in a cell or tissue. This review will suggest that efficacy values are a more meaningful measure of drug activity than the traditional dissociation constants and drug potencies commonly used to describe drug activity.

II. Role of δ-Opioid Receptors in Antinociception

Early studies suggested a prominent role for μ-opioid receptors in opioid drug-mediated analgesia. Morphine, the classic opioid agonist, was recognized as the prototypical μ agonist. The affinity of morphine for the μ-opioid receptor is approximately 50 times higher than that for the δ-opioid receptor (Emmerson et al., 1994). In initial experiments, opioid drugs capable of eliciting antinociception in vivo were also potent in suppressing electrically stimulated contractions of the guinea pig ileum; however, these drugs did not inhibit electrically evoked contractions in the isolated mouse vas deferens. Because μ-opioid receptors were found to be highly expressed in the guinea pig ileum and δ-opioid receptors were identified in the mouse vas deferens, it was originally thought that μ receptors were more involved than δ receptors in the mediation of analgesia (Heyman et al., 1988). However, with the discovery of compounds with increased selectivity for δ-opioid receptors, it quickly became clear that these receptors also mediate analgesia. For example, the enkephalin analog Met-kephamide exhibited greater potency than morphine in evoking antinociception and greater in vitro selectivity for the δ-opioid receptor (Frederickson et al., 1981; Burkhardt et al., 1982). The introduction of cyclic [D-Pen^2,D-Pen^5]enkephalin (where Pen = penicillamine; DPDPE; Mosberg et al., 1983a,b) was also significant because it produced antinociception without the usual gastrointestinal effects or Straub tail phenomenon characteristic of μ-selective agonists (Galligan et al., 1984; Porreca et al., 1984). The additional demonstration that acutely morphine-tolerant mice were not cross-tolerant to the δ agonists [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and [D-Thr^2,Leu^5,Thr^6]enkephalin (DTLET) was further evidence that δ-opioid receptors were indeed capable of mediating antinociception (Porreca et al., 1987).

Supporting evidence for δ-opioid receptor-mediated antinociception was provided by the introduction of pharmacological antagonists with relative selectivity for δ-opioid receptors, namely, ICI-154,129 [*N*,*N*-bisallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu-OH; Priestley et al., 1985] and ICI-174,864, [*N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib = α-aminoisobutyric acid; Cotton et al., 1984]. Pretreatment with ICI-174,864 antagonized the effects of DPDPE but not morphine or [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO) in the mouse tail-flick test, and conversely, the μ receptor blocker β-funaltrexamine antagonized the effects of morphine and DAMGO but not DPDPE (Heyman et al., 1987). Additional support for δ receptor-mediated antinociception came from experiments using μ-opioid receptor-deficient CXBK mice. These mice show a 10-fold rightward shift in the potency for morphine-induced antinociception and reduced antinociceptive responsiveness to morphine or DAMGO but unaltered responsiveness to DPDPE compared with control mice (Vaught et al., 1988). The CXBK strain was previously demonstrated to have approximately 30% fewer μ_1-opioid-binding sites than C57BL/6BY progenitors (Moskowitz and Goodman, 1985). Recent antinociception studies in recombinant mice, in which expression of the μ-opioid receptor was disrupted, demonstrate that δ-opioid receptor-selective agonists do not require functional μ-opioid receptors to mediate antinociception (Matthes et al., 1998).

III. The δ-Opioid Receptor

A. Endogenous δ-Opioid Receptors

The δ-opioid receptor was first suggested by the interpretation of studies comparing the effects of morphine and the then newly discovered enkephalins on electrically induced contractions of the guinea pig ileum and mouse vas deferens. The greater potency of morphine in the former bioassay and of enkephalins in the latter suggested that morphine and the enkephalins might act on different populations of opioid receptors (Hughes et al., 1975). The opioid receptor in the mouse vas deferens was assigned the designation "δ-opioid receptor". Thus, recognition of the δ-opioid receptor evolved due to differential drug effects in isolated tissues in vitro (Lord et al., 1977), whereas κ- and μ-opioid receptors were proposed based on differential analgesic drug effects in vivo (Gilbert and Martin, 1976; Martin et al., 1976). Subsequent receptor autoradiographic investigations clearly demonstrated differences in the distribution of opioid receptors within the brain. The pattern of δ receptors was distinctly different from that of μ receptors, and the loci of both δ and μ receptors were unique from that of the κ receptors as well (Sharif and Hughes, 1989; Mansour et al., 1995). As seen for all opioid receptors, the density of δ-opioid receptors varied widely in different brain regions (Table 1).

In addition to these anatomical differences in receptor localization, the development of δ, κ, and μ receptor-selective drugs has provided evidence of a pharmacological difference among opioid receptors. Met-enkephalin and Leu-enkephalin were initially proposed and are still considered the endogenous ligands of the δ-opioid receptor (Hughes et al., 1975). The introduction of drugs with progressively greater selectivity, such as DADLE (Beddell et al., 1977; Belluzzi et al., 1978), Tyr-D-Ser[-*O*-C(CH_3)$_3$]-Gly-Phe-Leu-Thr-*O*-C(CH_3)$_3$ (BUBU; Gacel et al., 1988),

TABLE 1
Distribution of the δ-opioid receptor in the rat brain

Highest concentrations (>500 amol/mm^2)
External plexiform layer of the olfactory bulb
Nucleus accumbens
Olfactory tubercule
Intermediate concentrations (181–313 amol/mm^2)
Layers V–VI of the cerebral cortex
Medial nucleus of the amygdala
Corpus striatum
Basolateral nucleus of the amygdala
Cortical nucleus of the amygdala
Layers I–II of the cerebral cortex
Lateral nucleus of the amygdala
Layers III–IV of the cerebral cortex
Granular layer of the olfactory bulb
Low concentrations (<100 amol/mm^2)
Hippocampus pyramidal layer
Inferior colliculus
Central nucleus of the amygdala
Habenula
Globus pallidus
Hypothalamus
Glomerular layer of the olfactory bulb
Accessory olfactory bulb
Substantia gelatinosa of the spinal cord
Stria terminalis
Central gray
Superior collicus
Substantia nigra
Thalamus
Lateral geniculate bodies
Ventral gray of the spinal cord

These data are taken from Mansour et al., 1988, 1995, and Sharif and Hughes, 1989. Tissues are listed in decending order of receptor density. For δ-opioid receptor density levels in the mouse brain, see Kitchen et al. (1997).

DPDPE (Mosberg et al., 1983a,b), the deltorphins (Erspamer et al., 1989; Kreil et al., 1989), (±)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide (BW373U86; Chang et al., 1993) and (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80; Calderon et al., 1994), further demonstrated that δ-opioid receptors were capable of mediating antinociception. Recognition of the pharmacological differences among opioid receptors was also supported by drug antagonism studies. It was initially reported that the dose of naloxone required for blocking the δ receptor was 10 times greater than that needed to block the μ-opioid receptor (Lord et al., 1977). This finding led to the development of progressively more selective δ-opioid receptor antagonists, such as naltriben (Portoghese et al., 1988), naltrindole (Takemori and Portoghese, 1992), Tyr-Tic-Phe-Phe-OH (where Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP; Schiller et al., 1992), and β-methyl-2′,6′-dimethyltyrosine-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Liao et al., 1997).

There is pharmacological evidence of distinct subtypes of the δ-opioid receptor. Initially, inconsistencies in radioligand-binding studies suggested multiple subtypes of δ receptors (Vaughn et al., 1990; Negri et al., 1991). Although an alternative explanation was the existence of a single δ receptor with multiple affinity states, more definitive evidence arrived with the introduction of δ-opioid receptor agonists and antagonists with improved subtype selectivity (Portoghese et al., 1992). The antagonist naltriben shifts the potency of DSLET 4-fold but that of DPDPE by only 1.5 times in the tail-flick assay (Sofuoglu et al., 1991). In addition, there is no development of cross-tolerance between DSLET and DPDPE or between DPDPE and [D-Ala^2]deltorphin II (Del-II; Mattia et al., 1991). More recently, quantitative autoradiographic studies revealed distinctive patterns of [^{3}H]DPDPE and [^{3}H]DSLET binding in rat brain, in some cases, by as much as a 9:1 ratio of δ_2:δ_1 opioid receptors (Hiller et al., 1996). It is now speculated that the putative δ_1 receptor is stimulated by DPDPE and blocked by [Ala^2,Leu^5,Cys^6]enkephalin, whereas the putative δ_2 receptor is stimulated by DSLET and Del-II and blocked by naltrindole-5′-isothiocyanate (Jiang et al., 1991; Vanderah et al., 1994). However, no δ subtypes have been cloned, and there remains no definitive molecular evidence for distinct subtypes of the δ-opioid receptor. We have also observed that DPDPE-mediated analgesia is partly dependent on μ receptors using μ-opioid receptor knockout mice (unpublished results), suggesting the drugs used to establish δ-opioid receptor subtypes may not be sufficiently selective for this purpose.

B. Cloned δ-Opioid Receptors

One major impediment to the characterization of opioid receptors is the fact that there are multiple opioid receptors, and tissues generally possess more than one type of receptor. This obstacle highly complicates the study of the individual types of receptor but, in recent years, has been addressed by the cloning of the first three opioid receptor types and the expression of each in separate cell lines (Miotto et al., 1995; Table 2).

The first opioid receptor to be cloned was the δ-opioid receptor. Two groups independently cloned the mouse δ

TABLE 2
The cloned opioid receptors

Opioid Receptor	Species
δ	Human (9, 19)
	Rat (1, 5)
	Mouse (2, 6, 8)
κ	Human (11, 20, 26)
	Guinea pig (24)
	Rat (5, 10, 12, 14, 16)
	Mouse (17, 25)
μ	Human (18, 23)
	Rat (3, 4, 7, 15, 21, 22)
	Mouse (13)

Sources: 1, Abood et al., 1994; 2, Augustin et al., 1995; 3, Bunzow et al., 1995; 4, Chen et al., 1993a; 5, Chen et al., 1993b; 6, Evans et al., 1992; 7, Fukuda et al., 1993; 8, Kieffer et al., 1992; 9, Knapp et al., 1994; 10, Li et al., 1993; 11, Mansson et al., 1994; 12, Meng et al., 1993; 13, Min et al., 1994; 14, Minami et al., 1993; 15, Minami et al., 1994; 16, Nishi et al., 1993; 17, Nishi et al., 1994; 18, Raynor et al., 1995; 19, Simonin et al., 1994; 20, Simonin et al., 1995; 21, Thompson et al., 1993; 22, J. F. Wang et al., 1993; 23, Wang et al., 1994b; 24, Xie et al., 1994; 25, Yasuda et al., 1993; 26, Zhu et al., 1995.

receptor by preparing an expression library from mouse neuroblastoma x rat glioma hybrid cells of the NG108-15 cell line and transfecting the library into monkey fibroblast (COS) cells (Evans et al., 1992; Kieffer et al., 1992, 1994). The use of the NG108-15 cell line was a critical step because these cells express δ-opioid receptors at a greater density than is normally found in brain tissue (Knapp et al., 1995b) and in the absence of other opioid receptors (Kieffer et al., 1992). Both groups used radioligand-binding assays to detect δ receptors but used different expression screening procedures. A cDNA sequence encoding a 372-amino acid protein was identified. A later isolation of a mouse δ-opioid receptor clone from a brain cDNA library (Yasuda et al., 1993) confirmed the sequence identified by these investigators. The rat δ receptor was cloned by Fukuda et al. (1993) from a rat cerebellum cDNA library by a hybridization screening method using a mouse δ-opioid receptor DNA as a probe. The rat receptor also had 372 amino acids with 97% homology to the mouse δ receptor. The 3% difference lies in the amino acids of the NH_2- and COOH-terminal sequences and one residue in the second extracellular loop.

The next logical step was to clone the human δ-opioid receptor because the human receptor is the ultimate target of therapeutic opioid agents. Our laboratory cloned the cDNA for a human δ receptor using hybridization screening methods (Knapp et al., 1994). cDNA fragments obtained from human striatum and temporal cortex libraries showed a highly homologous nucleotide sequence to the mouse δ-opioid receptor, but neither fragment covered the full open reading frame of the receptor protein. Consequently, the sequence fragments were combined by ligation in their overlapping regions. The reassembled open reading frame encoded a 372-residue protein with 93% homology with both the mouse and rat δ-opioid receptors. Most of the differences in amino acid sequence were in the NH_2- and COOH-terminals, but there were three additional substitutions elsewhere: Met^{80} for Leu in the first cytoplasmic loop, Arg^{190} for Gln in the second extracellular loop, and Asp^{290} for Asn in the third extracellular loop. There were no amino acid differences in any of the TMs. The human δ-opioid receptor was also cloned concurrently by Simonin et al. (1994) from the SH-SY5Y human neuroblastoma cell line. Regardless of species of origin, these cloned receptors uniformly exhibited greater affinity for Met-enkephalin, δ-selective agonists (DPDPE, DSLET) and antagonists (naltrindole), than they did for κ- and μ-selective ligands (Evans et al., 1992; Yasuda et al., 1993).

The use of clonal cells that stably express a recombinant receptor provides a unique system for the study of opioid receptors with several advantages over whole-animal or isolated tissue models (Kenakin, 1996; Mak et al., 1996). First, clonal cells afford a convenient way of inducing a very high level of receptor expression, even to greater levels than might be found naturally. Receptor overexpression in cell systems permits a sufficient density of receptors for clonal characterization (Samama et al., 1993) but may be prone to anomalies in the signaling mechanisms due to supraphysiological receptor densities. These problems can be addressed because recombinant receptors can be stably expressed in a clonal cell line at various densities, including levels comparable to those naturally occurring in tissues. The second advantage of receptor-transfected cells versus tissue is that clonal cells are all identical because they are derived from the same original progenitor cells. Hence, experiments using these cells eliminate experimental variability caused by obtaining tissue samples from different animals or individuals. Finally, clonal cell lines have the added advantage of being transfected to selectively express a given receptor without other receptor types or subtypes that normally coexist in a tissue sample. This eliminates the likelihood of misinterpretation due to the presence of confounding receptors.

IV. Molecular Biology of δ-Opioid Receptors

A. Antisense Oligodeoxynucleotide Gene Knockdown

Gene knockdown is accomplished using short sequences of oligodeoxynucleotide, generally 15 to 25 nucleotides in length, that are complementary to a portion of the mRNA that codes for a particular gene product. Antisense oligodeoxynucleotides (AS oligos) are potentially valuable pharmacological tools, especially in situations where there are no selective antagonists available, and they have been used to inhibit the expression of a specific cannabinoid receptor protein in vivo (Edsall et al., 1996), thus accomplishing essentially the same end as receptor blockade (Albert and Morris, 1994; Weiss et al., 1997).

The pretreatment of experimental animals with AS oligos to the δ-opioid receptor resulted in reduced antinociceptive response to δ- but not κ- or μ-selective receptor agonists (Bilsky et al., 1994; Lai et al., 1994, 1995; Standifer et al., 1994; Tseng et al., 1994). In all of these studies, comparable pretreatment with either sense or mismatch oligodeoxynucleotides was without effect on δ-opioid receptor-mediated antinociception. In rapid order, selective attenuation of κ and μ receptor-mediated antinociception was reported in animals after pretreatment with AS oligos complementary to κ (Adams et al., 1994; Chien et al., 1994)- and μ (Rossi et al., 1994; Chen et al., 1995a)-opioid receptor mRNAs, respectively. AS oligos complementary to opioid receptor mRNAs have also been successfully used to implicate δ, κ, and μ receptors in the development of opioid tolerance and dependence (Kest et al., 1996), β-endorphin-induced antinociception (Tseng and Collins, 1994), and opioid-induced changes in locomotor activity (Mizoguchi et al., 1996) and body temperature (Chen et al., 1995b). Reduced binding of δ-selective radioligands in cultured

NG108-15 cells confirmed the inhibition of δ-opioid receptor expression by treatment with AS oligos (Standifer et al., 1994).

AS oligo knockdown of opioid receptor expression is reversible. Studies using AS oligos have followed various pretreatment regimens, generally involving multiple injections on a daily basis or sometimes on an alternate-day schedule over 5 days (Table 3). Such pretreatment plans imply the importance of an appropriate time sequence to permit simultaneous degradation of existing receptors and inhibition of the synthesis of new receptors. There is a gradual restoration of sensitivity to δ-selective agonist-mediated antinociception 5 days after the final AS oligo treatment (Standifer et al., 1994). This is consistent with estimates of 3- to 5-day turnover times for opioid receptors (Ward et al., 1982).

B. Receptor Knockout Studies in Transgenic Animals

Knockout strategy involves generating transgenic mice possessing a discrete gene deletion that results in failure to express a particular gene product. The availability of such transgenic knockout animals has been instrumental in providing new information on different receptor subtypes, second messengers, transporter proteins, cytokines, hormones, and enzymes. However, the development of knockout mutant animals is likely to have physiological consequences. The absence of a particular gene product may 1) disrupt an intricate system of homeostasis and development resulting in severe pathology or the death of the mutant or 2) result in a deregulated system where alternative systems compensate for the loss of the deleted gene product. In the latter situation, artifacts due to compensatory mechanisms may be introduced that do not reflect the physiological role of the gene under study.

A transgenic μ-opioid receptor knockout mouse has been generated by homologous recombination technology and used to study interactions between δ- and μ-opioid receptors in the central nervous system (Sora et al., 1997a,b). Although the heterozygous knockout mice exhibit about 54% of wild-type levels of μ receptor expression, the homozygous knockout mice displayed 0% receptor expression. Sora et al. (1997a,b) used hot-plate and tail-flick tests and found that DPDPE induced a weaker than expected antinociceptive effect in μ-knockout mice compared with control animals. The implication of this finding is that the antinociceptive effect of DPDPE, a classic δ-selective receptor agonist, appears to be dependent on intact μ receptors. On the other hand, G protein activation by Del-II and SNC80 is unimpaired in membranes prepared from the brains of μ-opioid receptor knockout mice, and Del-II-mediated antinociception is not significantly different from that in control mice (unpublished data). These data indicate that the δ receptor is functional and mediates antinociception in the absence of the μ-opioid receptor. These findings are similar to those reported by Matthes et al. (1998).

C. Identification of δ-Opioid Receptor Domains Mediating Receptor Function

The δ-opioid receptor regions involved in mediating receptor function have been identified primarily by the construction of chimeric receptors containing sequences from κ- and μ-opioid receptors, site-directed mutagenesis of specific amino acid residues within the receptor, and by the construction of truncation or deletion

TABLE 3
Pretreatment regimens to AS oligos to opioid receptors

AS Oligo	Pretreatment Regimen
AS oligos to the δ-opioid receptor	
5′-CGC CCC AGC CTC TTC CTC-3′ (AS oligo to bases 51–70 of the cloned mouse δ receptor)	Rats: 10 μg/1 μl, iPAG[a] at −5, −3, and −1 day (11)
5′-GCA CGG GCA GAG GGC ACC AG-3′ (AS oligo to bases 7–26 of the cloned mouse δ receptor)	Mice: 12.5 μg/5 μl i.c.v. b.i.d. at −3, −2, and −1 day (6, 7)
5′-ATG TAG ATG TTG GTG GCG GT-3′ (AS oligo to bases 459–478 of the cloned mouse δ receptor)	Mice: 12.5 μg/5 μl i.c.v. b.i.d. at −3, −2, and −1 day (8)
5′-AGA GGG CAC CAG CTC CAT-3′ (AS oligo to bases 1–18 of the cloned mouse δ receptor)	Mice: 12.5 μg/5 μl i.c.v. b.i.d. at −3, −2, and −1 day (2)
5′-AGG GCA CCA GCT CCA TGG GG-3′ (AS oligo to bases 25–44 of the cloned mouse δ receptor)	Mice: 1 μg/5 μl i.t. at −3, −2, and −1 day (9, 10, 13, 14, 15)
5′-CGA GCG CAA CAG CTG CAT-3′ (AS oligo to bases 29–46 of the cloned mouse δ receptor)	Mice: 1 or 5 μg/2 μl i.t. at −5, −3, and −1 day (12)
AS oligos to the κ-opioid receptor	
5′-AAT CTG GAT GGG GGA CTC-3′ (AS oligo to bases 226–243 of the cloned rat κ receptor)	Rats: 20 μg/5 μl i.c.v. at −5, −3, and −1 day (1)
5-AAT CTG GAT GGG GGA CTC-3′ (AS oligo to bases 4–21 of the cloned rat κ receptor)	Rats: 20 μg/5 μl i.c.v. at −5, −3, and −1 day (4)
AS oligos to the μ-opioid receptor	
5′-GGT GCC TCC AAG GAC TAT CGC-3′ (AS oligo to bases 761–782 of the cloned rat μ receptor)	Mice: 5 μg i.t. at −5, −3, and −1 day (5)
5′-GCC GGT GCT GCT GTC CAT-3′ (AS oligo to bases 1–18 of the cloned rat μ receptor)	Rats: 20 μg/5 μl i.c.v. at −5, −3, and −1 day (3, 4)

References: 1, Adams et al., 1994; 2, Bilsky et al., 1994; 3, Chen et al., 1995a; 4, Chen et al., 1995b; 5, Chien et al., 1994; 6, Kest et al., 1996; 7, Lai et al., 1994; 8, Lai et al., 1995; 9, Mizoguchi et al., 1996; 10, Narita and Tseng, 1995; 11, Rossi et al., 1994; 12, Standifer et al., 1994; 13, Tseng et al., 1994; 14, Tseng et al., 1995; 15, Tseng and Collins, 1994.

[a] iPAG, injected into the periaquductal region of the brain.

mutants. Following is a discussion of how these techniques have been applied to better understand the: 1) sites that determine ligand binding to the δ-opioid receptor, 2) residues that modulate receptor down-regulation, and 3) receptor regions that interact with G proteins to mediate δ-opioid receptor-dependent signal transduction cascades.

1. Identification of Ligand-Binding Domains. Our current understanding of the regions of the δ receptor involved in ligand binding developed from the idea that opioid ligands are bivalent molecules. According to this theory, one portion of the ligand mediates signal transduction while another ligand site determines selectivity toward δ-, κ-, or μ-opioid receptors. These regions are referred to as the message and address regions, respectively. The use of this theory to develop δ- and κ-selective antagonists has been reviewed previously (Portoghese, 1989; Takemori and Portoghese, 1992). The cloning of the three types of opioid receptors has allowed researchers to identify sites in the δ-opioid receptor involved in ligand selectivity and binding. The receptor amino acid sequences showed that δ-, κ-, or μ-opioid receptors demonstrated extensive sequence homology in the seven TMs and divergent sequence in the intracellular tail and extracellular portions of the receptor. Because many opioid drugs exhibit limited selectivity among opioid receptor types, these findings suggest that the highly homologous TMs form a drug-binding pocket that interacts with the message region of the ligand.

Based on drug-binding studies with chimeric opioid receptors, Metzger and Ferguson (1995) proposed a theory to explain the selectivity of drug binding to opioid receptors. These investigators suggest that the extracellular loops act to sterically block binding of some drugs to opioid receptors. As discussed below, when the sixth TM and third extracellular loop of the δ receptor are replaced by the analogous μ sequence, the chimeric receptor binds δ-selective drugs with affinities similar to control μ-opioid receptors. Metzger and Ferguson (1995) would interpret these data to mean that the μ third extracellular loop sequence in the chimeric receptor adopts a conformation that blocks δ-selective agonist binding to sites in the highly conserved TMs of the receptor. They would conclude that the reason δ-selective drugs do not normally bind to the μ receptor is that the μ third extracellular loop excludes these drugs from binding sites in the TMs. The studies cited below are generally consistent with this model. Final determination of the δ-opioid receptor-binding epitopes may have to await determination of the crystal structure of this receptor in the presence of drug.

a. The Third Extracellular Loop of the δ-Opioid Receptor Is Critical to Ligand Binding. Ligand selectivity for δ receptors is thought to depend on recognition sites spanning the fifth through seventh TMs. This conclusion was based on findings from binding studies conducted on chimeric receptors constructed from cloned rat δ- and κ-opioid receptors (Meng et al., 1995). δ-Selective peptides [Met-enkephalin, Leu-enkephalin, Del-II, DSLET, DPDPE, Tyr-c-[D-Cys-Phe-D-Pen]OH (where Pen = penicillamine; JOM13)] all exhibited moderate affinity for κ(1–141)/δ(132–372) and κ(1–227)/δ(215–372) constructs, both of which retain the native fifth through seventh TMs of the δ-opioid receptor. These drugs had virtually no affinity for κ(1–141)/δ(132–214)/κ(228–380) and δ(1–214)/κ(228–380) constructs, which contain the fifth through seventh TMs of the κ-opioid receptor. Consistent with binding results using δ-selective peptide agonists, antagonist ligands (naltrindole, 7-benzylidenenaltrexone, naltriben) bound with high affinity to a κ/δ-chimeric receptor containing δ sequence carboxyl to the second extracellular loop (amino acids 215–372). In contrast to receptor sites required for δ-selective recognition, κ ligands such as dynorphins appear to depend on the second extracellular loop and the top portion of the fourth TM for selectivity of binding (Meng et al., 1995).

In additional chimeric receptor studies, cloned mouse δ-opioid receptor sequence from the N terminus and μ sequence from the C terminus were joined with ligation points at each of the seven TMs. Chimeric receptors exhibited a loss of DAMGO (μ agonist)-binding affinity whenever the first extracellular loop of the μ receptor was lacking and a loss of DSLET binding (δ agonist) whenever the third extracellular loop of the δ-opioid receptor was missing from the chimeric receptor (Wang et al., 1995). Point mutations in the third extracellular loop of the δ-opioid receptor that replaced both Arg^{291} and Arg^{292} with Gln selectively reduced the binding of DSLET but not nonselective opioid agonists (bremazocine and etorphine; Wang et al., 1995). Binding of the δ receptor antagonist, naltrindole, was also unaffected by this double-point mutation (Wang et al., 1995). The results of these studies indicate that 1) the third extracellular loop of the δ-opioid receptor is critically involved in the high-affinity binding of the δ-selective agonist DSLET and 2) the first extracellular loop of the μ receptor plays an important role in high-affinity DAMGO binding.

These data are in general agreement with other work performed on chimeric receptors constructed from cloned rat δ-, κ-, and μ-opioid receptors (Meng et al., 1996). These investigators constructed chimeric receptors combining δ/κ or δ/μ sequences. They found in a δ/κ-chimeric receptor that a fragment containing the sixth TM and the third extracellular loop of the δ-opioid receptor shifted the affinity of the δ-selective peptides Met-enkephalin, Leu-enkephalin, DPDPE, JOM13, and Del-II and the antagonists TIPP, naltrindole, and naltriben toward the values observed for control δ-opioid receptors. A homologous section of the μ receptor shifted the affinity of these drugs to μ values in a δ/μ-chimeric receptor. As a control, the binding affinity of the nonselective opioid ligands ethylketocyclazocine, bremazocine, and

naltrexone was determined for all chimeric receptors to verify that the chimeric receptors were capable of binding opioid ligands. These investigators also introduced a number of point mutations in the third extracellular loop of the δ-opioid receptor. Although some of these mutations reduced the affinity of some δ-selective ligands, none of the mutations were sufficient to ablate the binding of δ-selective ligands. The conclusion of these chimeric studies was that a region composed of the sixth TM and the third extracellular loop is essential in determining selectivity of drugs for δ-opioid receptors.

In research from our laboratory, we substituted the third extracellular loop sequence of the human μ-opioid receptor for that of the cloned human δ sequence [δ(1–282)/μ(304–320)/δ(301–372)] and transiently expressed the chimeric receptor in COS-7 cells (Li et al., 1996; Varga et al., 1996). Binding affinities of the δ antagonist (naltrindole), peptidic δ agonists [cyclic [D-Pen^2,4′-ClPhe^4,D-Pen^5]enkephalin, where Pen = penicillamine (pCl-DPDPE) and Del-II], and nonpeptidic δ agonists (SNC121 [(+)-[(4α-*R*)-α(2*S*,5*R*)-4-propyl-2,5-dimethyl-1-piperazinyl-3-methoxybenzyl]-*N*,*N*-diethylbenzamide] and (−)-TAN67 [2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino-[2,3,3-*g*]isoquinoline)]) to this chimeric receptor were shifted toward higher drug concentrations. Conversely, the affinities of μ-selective ligands (DAMGO and morphine) to this chimeric receptor were comparable to those of the δ-opioid receptor (Li et al., 1996; Varga et al., 1996) indicating that 1) substitution of the human μ-opioid receptor third extracellular loop sequence for that of the cloned human δ sequence was insufficient to confer high affinity toward μ-selective ligands and 2) regions of the δ receptor outside of the third extracellular loop prevent the binding of DAMGO and morphine.

In another study, the binding of three δ agonists (SNC80, DPDPE, Del-II) and the δ-selective antagonist naltrindole were measured in transfected HEK 293S cells expressing wild-type δ- or μ- opioid receptor or one of two δ/μ-chimeric receptors. In these chimeric receptors, the third extracellular loop sequence of δ was replaced by that from the μ receptor (Valiquette et al., 1996). In both chimeric constructs, the binding of all four δ-selective ligands was significantly reduced. Identification of specific key residues in the third extracellular loop region that mediate the binding of selective ligands to the δ receptor was accomplished by substituting Ala or Gly for the wild-type amino acid at 20 different positions between 275 and 312 (sixth TM–seventh TM of the δ-opioid receptor). In most cases, there was no appreciable difference in ligand binding to wild-type versus point-mutated δ-opioid receptors. However, substitution of alanine for Trp^{284}, Val^{296}, and Val^{297} consistently reduced the binding of the δ ligands, suggesting that these three residues participate in the selectivity of these drugs (Valiquette et al., 1996). Concurrent mutation of these three sites reduced δ-selective ligand affinity in a synergistic fashion.

To further investigate the role of the third extracellular loop in ligand binding, our group developed a cloned human δ-opioid receptor mutant in which replacement of Trp^{284} by Leu (W^{284}L) caused a 42-fold shift toward higher drug concentrations in the K_i for binding of SNC121 but not other δ ligands (pCl-DPDPE, Del-II, or naltrindole; Li et al., 1995). This finding suggests that SNC121 interacts with Trp^{284} in a unique manner that is not shared by other δ-selective ligands. Site-directed mutagenesis in this region implicated Val^{281}-Leu^{282} of the δ-opioid receptor in ligand selectivity since their replacement with Ile-Leu (as found in the κ receptor) resulted in a significant reduction in the affinity of Leu-enkephalin, naltrindole, and BWB373. Replacement of Ala^{298}-Ala^{299}-Leu^{300} of the δ receptor with Val-Ser-Trp, respectively (as in the μ-opioid receptor), also caused a marked reduction in the affinity of Leu-enkephalin, naltrindole, and BWB373. Replacement of Arg^{291}-Arg^{292} of the δ receptor with Pro-Glu (as in the μ receptor) reduced the affinity of the three peptide ligands tested (Leu-enkephalin, Del-II, and TIPP) but not bremazocine, naltrindole, or BWB373. However, all of the changes in affinity were less than those observed with the chimeric receptors (Meng et al., 1996).

b. First Extracellular Loop. Other studies have examined the role of the first extracellular loop as a determinant of DAMGO binding to opioid receptors. This was accomplished by replacing the first extracellular loop of the cloned rat δ-opioid receptor for the same region in the cloned rat μ-opioid receptor and construction of a chimeric δ/μ/δ receptor. This substitution conferred high affinity for [^{3}H]DAMGO to the chimeric receptor (Onogi et al., 1995). Because the first extracellular loops of the μ- and δ-opioid receptors differ in only seven amino acids, site-directed mutagenesis was used to individually replace those seven residues in the δ receptor with the corresponding amino acids from the μ receptor and then identify which residues were important in discriminating between μ and δ receptor-selective ligands. Only when Lys^{108} was replaced by Asn was the binding of the μ-selective agonist DAMGO of high affinity (Minami et al., 1996). Lys^{108} was then individually replaced by 19 other amino acids, some with polar hydroxyl or sulfhydryl groups, some with aromatic rings, and others with aliphatic side chains, to further characterize the structural requirement for the residue at position 108. These studies revealed that it was not so much substitution by Asn at position 108 as it was elimination of the more obstructive Lys at position 108 that was responsible for high-affinity DAMGO binding. This finding is consistent with the hypothesis of Metzger and Ferguson (1995) that selectivity of opioid drug binding is the result of amino acid residues of the extracellular loops of opioid receptors sterically excluding drugs from ligand binding sites. In contrast to the role of the first extracellular loop in the binding of the μ-selective ligand DAMGO, a rat δ-chimeric receptor containing the

μ first extracellular loop bound δ-selective ligands with affinity similar to the control δ opioid receptor. This finding indicates that the first extracellular loop does not mediate the selectivity of δ-selective ligands (Meng et al., 1996).

c. Second Extracellular Loop. Studies using chimeric receptors constructed from cloned rat opioid receptors showed that substitution of the second extracellular loop of the δ-opioid receptor for that of either the κ or μ receptor was insufficient to confer selective binding of the δ-selective ligands Met-enkephalin, Leu-enkephalin, DPDPE, JOM13, Del-II, or TIPP (Meng et al., 1996). Consistent with this finding, we found, using a chimera of the human opioid receptors, that δ-selective ligands bind to a second loop chimera, $\delta(1–186)/\mu(208–234)/\delta(213–372)$, with affinity similar to the wild-type δ-opioid receptor. This finding precludes a role for the second extracellular loop in determining δ ligand recognition (Li et al., 1996).

d. Transmembrane Domains. The role of residues in the TMs of the δ-opioid receptor on ligand binding is under active investigation. Asp^{128}, a residue in the third TM, was postulated to be involved in ligand binding. A conserved Asp residue in the third TM has previously been shown to affect ligand binding to other G protein-coupled receptors (Befort et al., 1996a). These investigators anticipated that Asp^{128} would act as a counter ion for the protonated amine of opioid ligands. When this residue was mutated to Ala and the mutant receptor expressed in COS-1 cells, the binding of bremazocine, diprenorphine, naloxone, DTLET, DADLE, DPDPE, Del-II, BW373U86, and naltrindole was unaffected. Conversely, the affinities of DADLE, DTLET, and BW373U86 were shifted toward higher drug concentrations in the presence of NaCl (120 mM) compared with control δ-opioid receptors. An Asp^{128} to Asn mutation shifted the affinity of all agonists tested toward higher drug concentrations by >20-fold. Collectively, these results indicate that 1) Asp^{128} is unlikely to form a salt bridge with opioid drugs, 2) Asp^{128} may be involved in ligand binding under physiological saline concentrations, and 3) Asp^{128} is situated in a region of the receptor that is important to ligand binding as the Asp to Asn mutation shifts the affinity of all opioid ligands tested.

Investigators examined the role of Asp^{95}, located in the second TM of the mouse δ-opioid receptor, in ligand binding (Kong et al., 1993). The rationale for this study was that an Asp in the α-adrenergic receptor had previously been shown to be critical for agonist binding (Horstman et al., 1990). Accordingly, Asp^{95} was replaced with an Asn by site-directed mutagenesis. Wild-type and mutant receptors were transfected into COS-7 cells. The mutated receptor exhibited a selective reduction in the binding of δ-selective agonist ligands (BW373U86, Del-II, DPDPE, DSLET, Met-enkephalin, and 7-spiroindinooxymorphone) without any alteration in the binding of δ-selective antagonists (naltrindole, naltriben, 7-benzylidenenaltrexone) or the nonselective agonists [bremazocine, (−)-buprenorphine]. These findings indicate that Asp^{95} is involved in the binding of highly selective δ agonists yet is not involved in the binding of antagonists and nonselective agonists. These results support the conclusion that there are regions mediating ligand selectivity in addition to the extracellular loops. Interpretation of results in this study are complicated by the fact that Asp^{95} is also the site of sodium regulation of ligand binding to this receptor. Indeed, [^{3}H]DPDPE binding to the wild-type receptor was reduced in the presence of sodium; binding to the mutant was unaffected. However, sodium effects alone do not explain why the Asp^{95} mutation reduces the binding of highly selective agonists more than nonselective agonists.

Molecular modeling of the mouse δ-opioid receptor was used to predict transmembrane amino acids that were likely to mediate ligand binding (Befort et al., 1996c). Based on this model, these investigators mutated residues Tyr^{129} (TM III), Trp^{173} (TM IV), Phe^{218} (TM V), Phe^{222} (TM V), Trp^{274} (TM VI), and Tyr^{308} (TM VII) of the δ-opioid receptor and expressed these mutant receptors in COS-1 cells. They found that mutations of Tyr^{129} caused the greatest shifts in drug affinity toward higher concentrations than the other mutations. Mutations at Phe^{218}, Phe^{222}, and Tyr^{308} had modest effects on the affinity of all agonists tested. Mutation of Trp^{173} and Trp^{274} caused 40-fold affinity shifts for some ligands and had no effect on others. Taken together, these data demonstrate the importance of the TMs to ligand binding and suggest that δ-selective ligands interact at different amino acid residues to mediate binding.

e. N Terminus Domain. A subsequent study revealed that both DPDPE and naltrindole bind to the N terminus chimeric $\kappa(1–78)/\delta(70–372)$ receptor but not to the reverse chimeric $\delta(1–69)/\kappa(79–380)$ receptor; this finding suggests that the N-terminal domain of the δ-opioid receptor is not critical for binding of δ-selective ligands (Kong et al., 1994).

f. Summary. Findings reviewed above are consistent with the interpretation that the third extracellular loop of the δ-opioid receptor is a critical region determining the selectivity of δ receptor ligands. Data also support a role for the TMs of the δ-opioid receptor in ligand binding. In contrast, the N-terminal domain and the first and second extracellular loops do not appear to modulate the binding of δ-selective ligands to the δ-opioid receptor.

2. δ-Opioid Receptor Domains Mediating Down-Regulation. Down-regulation of the mouse δ-opioid receptor was examined with truncation mutants (Cvejic et al., 1996). When the terminal 37 amino acids in the intracellular tail of the receptor were deleted, receptor down-regulation in response to chronic (2–48 h) DADLE treatment was blocked in receptor-transfected Chinese hamster ovary (CHO) cells. Conversely, when the murine δ-opioid receptor was truncated by 15 amino acids, the receptor did down-regulate on chronic DADLE

treatment; however, the receptor levels of the 15-amino acid truncation-mutant were not down-regulated to the same extent as the wild-type. Still, these findings indicated that there are amino acid residues in the cytoplasmic tail of the murine δ-opioid receptor that regulate receptor down-regulation. When the cytoplasmic tail residue Thr^{353} was mutated to an Ala in the mouse δ receptor and the mutant receptor expressed in CHO cells, down-regulation was blocked. Although Cvejic et al. (1996) demonstrated that Thr^{353} of the mouse δ-opioid receptor mediates down-regulation, the mechanism of regulation in the human receptor must be different because Thr^{353} is already an Ala in the human δ receptor sequence (Knapp et al., 1994) and the human receptor down-regulates on chronic agonist exposure (Malatynska et al., 1996).

3. δ-Opioid Receptor Domains Mediating Signal Transduction Cascades. Studies examining the regions of the δ-opioid receptor that modulate signal transduction pathways are extremely limited. A role for the carboxyl regions of the cytoplasmic tail in intracellular signaling was precluded by studies in which a 31-amino acid truncation of the tail did not affect DPDPE-mediated inhibition of forskolin-stimulated cAMP production in receptor-transfected CHO cells (Zhu et al., 1997). Merkouris et al. (1996) addressed the question of which δ-opioid receptor regions mediate interactions with G proteins through the use of synthetic peptides. These investigators examined G protein activation in cell membrane preparations as GTPase activity and [^{35}S]GTPγS binding in the presence of peptides (100 mM) homologous to regions of the δ-opioid receptor. They found that peptides homologous to the third intracellular loop inhibited both GTPase activity and [^{35}S]GTPγS binding. Peptides homologous to the second intracellular loop and amino acids 322 through 333 of the cytoplasmic tail did not affect either assay; however, the peptide with homology to the tail slightly enhanced the inhibition of [^{35}S]GTPγS binding mediated by one of the third intracellular loop peptides. Because receptor interactions with G proteins are known to modulate the affinity of agonist binding to G protein-coupled receptors, these investigators also examined the effect of the peptides on binding of the δ-selective agonist [^{3}H]DSLET. Peptides with homologous sequence to the third intracellular loop reduced [^{3}H]DSLET binding, whereas peptides homologous to the second intracellular loop did not. Unexpectedly, the peptide homologous to residues 322 through 333 of the cytoplasmic tail also reduced [^{3}H]DSLET binding. These findings suggest that the cytoplasmic tail may interact with receptor-associated G proteins yet are not vital to signal transduction because a peptide consisting of residues 322 through 333 failed to block either GTPase activity or [^{35}S]GTPγS binding.

Investigators have also shown that δ-opioid receptor regions that are not thought to be in close proximity to G proteins can also modulate receptor-mediated signal transduction. This was demonstrated in a μ/δ-chimeric receptor where the amino terminus of the δ-opioid receptor, through to the beginning of the first extracellular loop was replaced with μ sequence (Claude et al., 1996). Although this receptor-mediated DPDPE-stimulated inhibition of forskolin-stimulated cAMP production in transfected CHO cells with a potency similar to the control δ-opioid receptor, quite unexpectedly a number of opioid antagonists (naloxone, naltrexone, naltrindole, naltriben, TIPP, and *H*-Tyr-Tic[ψ,CH_2NH]Phe-Phe-OH, where Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) also acted as agonists at the chimeric receptor to inhibit cAMP production. On sequencing the chimeric receptor, the investigators found a point mutation that resulted in the mutation of a fourth TM domain Ser residue that is conserved in opioid receptors to a Leu residue. On back-mutation of the Leu to Ser, antagonists no longer behaved as agonists at the chimeric receptor. When the conserved Ser in the fourth TM domains of either δ- or μ- opioid receptors were mutated to Leu, antagonist ligands demonstrated agonist activity in both inhibition of adenylyl cyclase in CHO cells and activation of the G protein-coupled inward rectifying potassium channel in *Xenopus laevis* oocytes (Claude et al., 1996). These findings are consistent with the interpretation that ligand interactions with residues of the fourth TM can alter the conformation of the δ opioid receptor to permit receptor coupling to second messenger systems.

V. Opioid Signal Transduction

Since the initial pharmacological identification of the δ-opioid receptor, considerable effort has been directed toward understanding the signal transduction pathways that couple this receptor to analgesia and other functional responses. It is well established that most δ-opioid receptor-mediated events are dependent on the activity of pertussis toxin-sensitive G proteins. It is also well established that δ receptor-selective ligands inhibit intracellular cAMP levels and modulate the activity of voltage-gated calcium and potassium channels. More recent studies have addressed δ-selective ligand-mediated calcium release from intracellular stores and modulation of a variety of protein kinases. In the sections to follow, δ receptor-selective ligand-mediated effects on second messenger systems is examined followed by a discussion of the significance of these findings to the physiological role of these drugs.

A. G Protein Activity

The role of G proteins in opioid receptor-mediated signaling has been reviewed previously (Childers, 1991; Standifer and Pasternak, 1997). Our present knowledge about the superfamily of seven-helical domain G protein-coupled receptors is based on the work of Lefkowitz and associates on cloned β-adrenergic receptors (Ostrowski et al., 1992). Early evidence supporting opioid receptor coupling to G proteins was that binding of opi-

oid ligands to receptors was guanine nucleotide dependent (Blume, 1978). Opioid drug-mediated inhibition of adenylyl cyclase was found to be pertussis toxin sensitive (Hsia et al., 1984), further supporting G protein coupling of these receptors. As discussed below, later work has shown that pretreatment of cells and tissues with antisera specifically directed against various G protein subunits (Sánchez-Blazquez et al., 1993; Sánchez-Blazquez and Garzón, 1993; Garzón et al., 1994, 1997) or AS oligos against G protein subunits (Standifer et al., 1996) can likewise block opioid drug effects.

The structure and function of G proteins have been extensively reviewed (Gilman, 1994; Rens-Domiano and Hamm, 1995; Strader et al., 1995). G proteins are heterotrimeric, consisting of α, β, and γ subunits. Research to date has shown that there is extensive heterogeneity among G protein subunits with as many as 18 different α, 5 β, and 7 γ subunits that can contribute to the $\alpha\beta\gamma$ G protein heterotrimer (Rens-Domiano and Hamm, 1995). Regardless of the specific α, β, and γ subunits that may comprise a G protein heterotrimer, the activation of G protein-coupled receptors by agonist results in the dissociation of GDP from the α subunit, followed by association of GTP with the open nucleotide binding site (Birnbaumer et al., 1990; Hamm, 1998). The binding of GTP to the α subunit induces a conformational change that results in dissociation of the heterotrimer into α and $\beta\gamma$ subunits. Both the GTP-bound α subunit and the combined $\beta\gamma$ subunits can initiate distal steps in the signaling pathway. These signals are terminated when the endogenous GTPase of the α subunit hydrolyzes the bound GTP to GDP and inorganic phosphate. The α subunit/GDP complex then reassociates with the $\beta\gamma$ subunits to again form heterotrimeric G protein. This sequence of events is reviewed in Fig. 2.

Early classification systems for heterotrimeric G proteins were based on the functional effects of these proteins. G_i proteins were originally named because these G proteins functioned to inhibit intracellular adenylyl cyclase. Conversely, G_s proteins stimulated adenylyl cyclase (Harnett and Klaus, 1988). Pertussis and cholera toxins were also used in classification schemes for G proteins. The cloning of a large number of G proteins now permits the separation of these proteins into subtypes, based on the primary amino acid sequence of the α subunit. Many studies have been conducted to determine the G protein subtypes that mediate the intracellular signaling of drug-bound receptors, including opioid receptor-modulated cell signaling systems (Ueda et al., 1991; Goode and Raffa, 1997; Sánchez-Blazquez and Garzón, 1998).

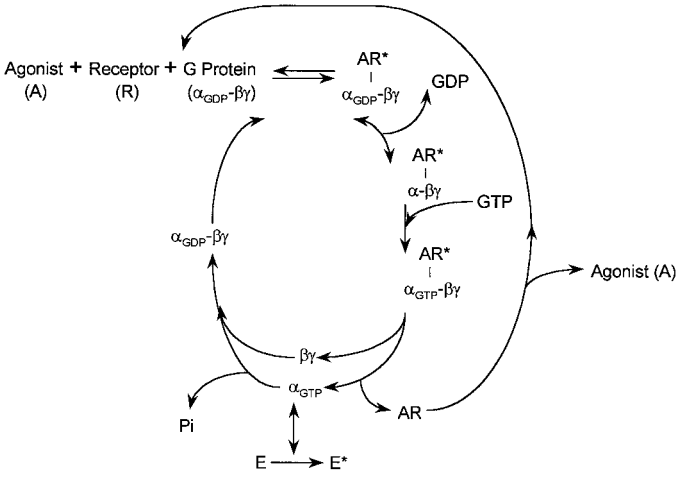

FIG. 2. Activation of the G protein cycle by agonist binding to opioid receptors. *The activated form of the receptor or an effector protein (E), such as adenylyl cyclase, that has bound the α subunit of the G protein. α, β, and γ refer to the subunits of the heterotrimeric G protein.

Extensive evidence supports the conclusion that δ-opioid receptors are linked to G proteins. It has long been known that GTP is required for the inhibition of adenylyl cyclase activity by δ agonists in both brain tissue (Law et al., 1981) and NG108-15 hybrid cells (Blume et al., 1979). δ-Selective agonists were known to stimulate the binding of [^{35}S]GTPγS and reduce the concentration of GTP needed to inhibit adenylyl cyclase activity (Blume, 1978; Chang et al., 1981b). The affinity of δ-selective agonists was reduced by GTP and its guanosine-5′′-(β,γ-imido)triphosphate derivative both in the brain and in NG108-15 cells (Costa et al., 1985a; Law et al., 1985). Finally, pertussis toxin reversed the effects of δ agonists on adenylyl cyclase (Law et al., 1985b) and GTPase activity (Kurose et al., 1983) and shifted the binding affinity of δ-opioid receptors to a low-affinity state for agonists that selectively bind to the receptor (Hsia et al., 1984; Law et al., 1991).

The vast majority of studies concerning the role of G proteins in opioid-mediated signal transduction have focused on the G_α subunits. More recently, some studies have examined the possible contribution of the $\beta\gamma$ subunit complex to opioid effects (Avidor-Reiss et al., 1996). Early evidence of G protein involvement in antinociception came with the observation that pertussis toxin attenuated supraspinal antinociception mediated by opioid agonists (Sánchez-Blazquez and Garzón, 1988). Intracerebroventricular pretreatment of mice with the G_i-G_o activation blocker pertussis toxin antagonized the antinociceptive effects of the δ-selective peptide agonists DADLE, DPDPE, and Del-II (Sánchez-Blazquez and Garzón, 1992); intrathecal (i.t.) treatment with pertussis toxin also antagonized δ-opioid receptor-mediated antinociception in rodents as determined by the tail-flick assay (Przewlocki et al., 1987). Similar pretreatment with cholera toxin, which impairs the ability of G_s to hydrolyze bound GTP to GDP (Spiegel et al., 1992), did not affect the antinociceptive effects of DADLE, DPDPE, or Del-II (Sánchez-Blazquez and Garzón, 1992). These findings suggest that all of the antinociceptive drugs tested activate receptors that are functionally coupled to G_i-G_o.

Substantial evidence has been accumulated to demonstrate which G protein α subunits mediate analgesia on the treatment of animals with δ-selective agonists (Table 4). The i.c.v. pretreatment of mice with antisera against $G_{i\alpha2}$ reduced DADLE-, Del-II-, and DPDPE-induced activation of a low-K_m GTPase activity in membranes prepared from mouse periaqueductal gray (Garzón et al., 1994, 1997). Consistent with this finding, $G_{i\alpha2}$ antiserum inhibited antinociceptive responses of mice to DPDPE, Del-II, and DADLE (Sánchez-Blazquez et al., 1993, 1995). AS oligos complementary to $G_{i\alpha2}$ also inhibited DPDPE- and Del-II-mediated analgesia (Sánchez-Blazquez et al., 1995). Based on these findings, it is concluded that agonist-bound δ-opioid receptors are coupled to $G_{i\alpha2}$.

There is equally strong evidence of involvement of $G_{i\alpha3}$ in δ-opioid receptor-mediated antinociception. When injected i.c.v., antisera against $G_{i\alpha3}$ significantly reduced the antinociceptive effect of DPDPE, Del-II, and DADLE (Sánchez-Blazquez and Garzón, 1993), suggesting that $G_{i\alpha3}$ is coupled to δ-opioid receptors to produce antinociception in mice. In agreement with these findings, when AS oligos were used to block the translation of $G_{i\alpha3}$ protein, the analgesic effects of the δ-selective peptides DPDPE and Del-II were attenuated (Sánchez-Blazquez et al., 1995; Standifer et al., 1996). In these studies, antinociception was determined using the warm water tail-flick assay with mice.

Several investigators have examined the role of $G_{i\alpha1}$ in δ-opioid receptor-mediated antinociception with contrasting results. When the role of this G protein was examined in supraspinal antinociception, by either the i.c.v injection of $G_{i\alpha1}$-specific antisera or AS oligos, no effect on δ receptor-mediated antinociception was observed (Sánchez-Blazquez et al., 1993, 1995; Raffa et al., 1994). Conversely, i.t. injection of AS oligos complementary to $G_{i\alpha1}$ mRNA reduced DPDPE (500 ng/animal)-induced antinociception in CD-1 mice (Standifer et al., 1996). These results suggest that $G_{i\alpha1}$ can mediate δ-opioid receptor-dependent antinociception and that differences exist in the mechanisms responsible for spinal and supraspinal antinociception.

The i.c.v. pretreatment of mice with antisera against $G_{\alpha x/z}$ significantly attenuated the DADLE-activated low-K_m GTPase in membranes prepared from mouse periaqueductal gray (Garzón et al., 1994). In agreement with these findings, antiserum against $G_{\alpha x/z}$ also attenuated the antinociceptive effects of DADLE; however, DPDPE- and Del-II-mediated antinociception was not inhibited (Sánchez-Blazquez et al., 1993, 1995; Garzón et al., 1994). Conversely, i.t. injection of $G_{\alpha x/z}$ AS oligos (5 μg) inhibited DPDPE-mediated antinociception (Standifer et al., 1996). These findings suggest that $G_{\alpha x/z}$ is capable of mediating δ-dependent antinociceptive effects, although evidence suggests that not all δ-selective agonists mediate antinociception through this G protein in the brain.

The i.c.v. pretreatment of mice with an antiserum against G_s did not significantly block the antinociceptive effects of DADLE, DPDPE, or Del-II (Sánchez-Blazquez and Garzón, 1992). However, as with $G_{i\alpha1}$, i.t. injection of G_s-selective AS oligos blocked DPDPE-mediated antinociception in the CD-1 mouse (Standifer et al., 1996). These findings again suggest differences in the mechanisms leading to δ-opioid receptor-dependent spinal and supraspinal antinociception.

TABLE 4
Summary of the involvement of G_α subunits in δ-, κ-, and μ-opioid receptor-mediated drug action

Opioid Antinociception	Inhibited by Antisera or AS Oligo Against	Not Inhibited by Antisera or AS Oligo Against
Supraspinal δ antinociception	$G_{i\alpha2}$ (1, 6, 7) $G_{i\alpha3}$ (5, 7)	$G_{i\alpha1}$ (6, 7) $G_{x/z}$ (1, 6, 7) G_s (4)
Spinal δ antinociception	$G_{i\alpha1}$ (8) $G_{i\alpha2}$ (8) $G_{i\alpha3}$ (8) $G_{x/z}$ (8) G_o (8) G_q (8)	
Supraspinal κ antinociception	$G_{i\alpha1}$ (8) $G_{i\alpha3}$ (8) $G_{x/z}$ (8) G_s (8) G_q (8)	$G_{i\alpha2}$ (8) G_o (8)
Spinal κ antinociception	G_q (8)	$G_{i\alpha1}$ (8) $G_{i\alpha2}$ (8) $G_{i\alpha3}$ (8) $G_{x/z}$ (8) G_o (8) G_s (8)
Supraspinal μ antinociception	$G_{i\alpha2}$ (1, 2, 3, 6, 7, 8) $G_{x/z}$ (1, 6, 7) G_o (8) G_s (4, 8)	$G_{i\alpha1}$ (2, 3, 6, 7, 8) $G_{i\alpha3}$ (2, 5, 7, 8) $G_{x/z}$ (8) G_o (3) G_q (8)
Spinal μ antinociception	$G_{i\alpha2}$ (8) $G_{x/z}$ (2)	$G_{i\alpha1}$ (8) $G_{i\alpha3}$ (8) G_o (8) G_q (8) G_s (8)

References: 1, Garzón et al., 1994; 2, Raffa et al., 1994; 3, Rossi et al., 1995; 4, Sánchez-Blazquez and Garzón, 1992; 5, Sánchez-Blazquez et al., 1993; 6, Sánchez-Blazquez and Garzón, 1993; 7, Sánchez-Blazquez et al., 1995; 8, Standifer et al., 1996.

B. δ-Opioid Receptors Inhibit cAMP Production in Cells and Tissues

By the mid-1970s, prostaglandins had been demonstrated to mediate hyperalgesia as well as to stimulate cAMP production. Because opioid drugs were known to inhibit prostaglandin-stimulated cAMP production, this mechanism was postulated to underlie the antinociceptive activity of opioids (Collier and Roy, 1974). Some investigators demonstrated that injection of cAMP or cAMP analogs, by various routes of administration, antagonized morphine-induced antinociception (Ho et al., 1972, 1973). Antinociception was determined by a tail-flick assay in these studies. Hosford and Haigler (1981) also showed that cAMP and cAMP analogs reversed morphine inhibition of nociceptive stimulus-evoked neuronal firing in the mesencephalic reticular formation in

Sprague-Dawley rats. The i.t. injection of cAMP and dibutyryl-cAMP also reversed morphine- and DPDPE-induced antinociception but not antinociception mediated by the κ-selective agonist dynorphin (J. B. Wang et al., 1993). In contrast to these studies that support a role for opioid-mediated inhibition of cAMP levels in antinociception, Levy et al. (1981) demonstrated that microinjection of dibutyryl-cAMP into either the reticular formation of the caudal brainstem or the periaqueductal gray increased tail-flick latencies, suggesting that dibutyryl-cAMP is analgesic. Other investigators showed that injection of other adenine congeners also blocked morphine effects in mice, calling into question the specificity of cAMP injections (Gourley and Beckner, 1973). Thus, 25 years after the suggestion that opioid-mediated inhibition of cAMP levels regulates analgesia, the exact role of this second messenger molecule is still unclear. Recent studies have suggested that opioid inhibition of cAMP levels mediates respiratory depression in newborn animals (Ballanyi et al., 1997). It is also possible that cAMP may be involved in dependence and withdrawal syndromes (Nestler and Aghajanian, 1997). Thus, multiple physiological effects may be mediated by opioid drug-dependent modulation of intracellular cAMP levels.

In a seminal report, morphine inhibited both basal and prostaglandin E_1-stimulated cAMP production in NG108-15 cells (Sharma et al., 1975). At the time of this report, the δ-opioid receptor had not been characterized; however, it was later shown that opioid drugs interact primarily with the δ-opioid receptor in NG108-15 cells (Garzón et al., 1995; Morikawa et al., 1995). Later studies showed that the δ-selective agonist DADLE inhibited cAMP production in NG108-15 cells. Inhibition was reversed by the nonselective opioid antagonist naloxone (Costa et al., 1985). δ-Selective agonists have also been shown to inhibit basal cAMP levels in rat brain regions (Izenwasser et al., 1993), and studies suggested the involvement of both putative δ_1- and δ_2-opioid receptors in δ-selective agonist-mediated inhibition of cAMP production in rat brain regions (Búzás et al., 1994). δ-Selective inhibition of cAMP production has been verified in transfected cell lines where forskolin-stimulated cAMP production was inhibited by the agonist DPDPE and DPDPE-mediated inhibition was antagonized by naltrindole (Malatynska et al., 1995). In other experiments, pCl-DPDPE, SNC80, and (±)-TAN67 also inhibited forskolin-stimulated cAMP production in human δ-opioid receptor-transfected CHO cells (Knapp et al., 1995a). Inhibition of cAMP production is mediated through the activation of the G_i-G_o family as pertussis toxin blocks opioid effects (Law et al., 1985b; Harnett and Klaus, 1988). The specific G proteins that mediate δ-selective effects on cAMP production have been characterized through the use of IgG fractions specific for G protein α subunits (McKenzie and Milligan, 1990); these investigators found that DADLE-mediated inhibition of forskolin-stimulated cAMP production was $G_{i\alpha 2}$-dependent. Using a similar approach, antibodies specific to $G_{i\alpha 2}$ and G_o blocked DPDPE-mediated inhibition of forskolin-stimulated cAMP production in smooth muscle cells isolated from the circular and longitudinal muscle layers of the guinea pig intestine (Murthy and Makhlouf, 1996).

The δ-opioid receptor-dependent decreases in intracellular cAMP levels have also been shown to be mediated by increased phosphodiesterase activity in NG108-15 cells (Law and Loh, 1993). In these studies, investigators used phosphodiesterase inhibitors to determine that type I phosphodiesterase increased the rate of cAMP degradation after cell stimulation with the δ-selective agonist DADLE. Increased phosphodiesterase activity was insensitive to pertussis toxin treatment that caused >90% ADP ribosylation of pertussis toxin-sensitive G protein substrates. Phosphodiesterase activity was unaffected by the removal of extracellular calcium.

Soon after the finding that opioid drugs inhibit intracellular cAMP levels, investigators found that subsequent to chronic opioid treatment, cells became more responsive to drugs that elevate cAMP levels (Sharma et al., 1977). We have shown that chronic pretreatment of human δ-opioid receptor-transfected CHO cells with agonist caused increased forskolin-stimulated cAMP production versus control after washout of the opioid agonist (Malatynska et al., 1996). The addition of the δ-selective antagonist naltrindole with the forskolin potentiated the forskolin-stimulated cAMP production observed after chronic agonist pretreatment. The significance of this "cAMP overshoot" or adenylyl cyclase supersensitivity has yet to be established; however, evidence suggests that cAMP elevation is involved in opioid withdrawal (Nestler and Aghajanian, 1997). Chronic treatment of NG108-15 cells with the muscarinic cholinergic agonist carbachol, followed by antagonist treatment, resulted in the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) and increased transcription of the c-*fos* gene (Thomas et al., 1995). If δ-opioid receptor-mediated cAMP overshoot induces a similar mechanism, the resultant cAMP-dependent transcription of genes could partially mediate withdrawal syndromes associated with opioid drugs. Support for such a mechanism comes from a recent study in which injection of AS oligos complementary to CREB mRNA into the locus ceruleus of the rat, during chronic morphine treatment, attenuated some withdrawal symptoms induced by the antagonist naltrexone (Lane-Ladd et al., 1997). However, the interaction of CREB with the cAMP-generating system may be quite complex as these investigators show that CREB-selective AS oligos modulate the expression of some subtypes of adenylyl cyclase.

C. Protein Kinases

Recent studies have demonstrated that δ-selective ligands stimulate kinase activity in cell lines that express

the δ-opioid receptor. Specifically, DPDPE was demonstrated to stimulate protein kinase C (PKC) activation in NG108-15 cells in a pertussis toxin-sensitive manner over a matter of minutes (Lou and Pei, 1997). Stimulation was dependent on extracellular calcium as PKC activity was suppressed when extracellular medium was replaced with a calcium-free medium containing EGTA. In this same study, a brief DPDPE (1 μM, 5 min) exposure failed to stimulate protein kinase A (PKA) activity; however, extended incubation with the same concentration of DPDPE (24 h) caused a significant increase in PKA activity. This elevated PKA activity may serve as a homeostatic mechanism during chronic δ agonist exposure as δ-opioid receptor mRNA levels are reduced via a PKA-dependent mechanism by chronic treatments that elevate intracellular cAMP (Búzás et al., 1997). In separate studies, δ-opioid receptors were found to mediate agonist stimulation of mitogen-activated protein kinase (MAP kinase) in receptor-transfected cell lines (Burt et al., 1996; Fukuda et al., 1996). Pertussis toxin blocked MAP kinase activation by δ-selective ligands in both studies. Down-regulation of PKC and the addition of tyrosine kinase inhibitors or dibutyryl-cAMP to these cell lines blocked δ-specific activation of MAP kinase. The δ-opioid receptor-mediated MAP kinase activation was shown to be βγ and Ras dependent in transiently transfected COS-7 cells (Belcheva et al., 1998). G protein-coupled receptor kinases have previously been implicated in the down-regulation of G protein-coupled receptors. This kinase family has been shown to cause the phosphorylation of the δ-opioid receptor in transfected HEK 293 cells because cotransfection of either β-adrenergic receptor kinase 1 or G protein-coupled receptor kinase 5 with the receptor resulted in enhanced phosphorylation of the receptor. In addition, a dominant negative mutant of β-adrenergic receptor kinase-1 ($K^{220}R$) inhibited receptor desensitization after DPDPE pretreatment (5 μM DPDPE, 4 h; Pei et al., 1995).

D. Ion Channels

1. Calcium Flux. The release of neurotransmitters from neurons is dependent on the intracellular concentrations of calcium (Starke, 1977), suggesting that the inhibitory role of δ-selective ligands in nerve function may be explained by this mechanism. The regulation of intracellular calcium levels by δ-opioid receptor-selective agonists has been under study for a number of years and has proved to be a complex issue. The δ-selective agonist DADLE was shown to inhibit calcium currents in a neuroblastoma x glioma hybrid cell line in a pertussis toxin-sensitive manner (Hescheler et al., 1987). Intracellular administration via patch pipet of G_i or G_o, purified from pig brain restored DADLE-mediated regulation of calcium channels in pertussis toxin-pretreated cells. This work was later extended using ω-conotoxin to demonstrate that N-type calcium channels were under the regulation of δ-opioid receptors in NG108-15 cells (Taussig et al., 1992). These investigators demonstrated that G_{oA}-mediated δ agonist inhibition of calcium channels as transfection of a pertussis toxin-insensitive mutant of this G protein reversed pertussis toxin block of δ-opioid receptor-mediated effects on calcium channels. DPDPE was also shown to inhibit N-type calcium channels in a small-cell lung carcinoma cell line (Sher et al., 1996). Inhibition of N-type calcium channels by δ-selective agonists was cAMP independent in all of these studies (Hescheler et al., 1987; Taussig et al., 1992; Sher et al., 1996).

In contrast to these studies, DSLET has been shown to increase intracellular calcium levels in the ND8-47 neuroblastoma x dorsal root ganglion (DRG) hybrid cell line by a pertussis toxin-sensitive mechanism (Tang et al., 1995a). Later experiments using AS oligos demonstrated this effect was mediated through $G_{i\alpha 2}$ (Tang et al., 1995b). Stimulation is blocked by nifedipine and verapamil, indicating that L-type calcium channels mediate the increase in intracellular calcium (Tang et al., 1994). These findings stand in contrast to studies in small-cell lung carcinoma cells where DPDPE inhibited N-type calcium channels but did not modulate L-type calcium channels (Sher et al., 1996). The molecular explanation for these contrasting findings is currently unclear.

The δ-opioid receptor-mediated increases in intracellular calcium levels are not solely dependent on calcium channels. It was reported that etorphine- and DADLE-stimulated release of calcium from intracellular stores was reversible by naloxone in the human neuroblastoma cell line SK-N-BE (Allouche et al., 1996). This increase in calcium was not sensitive to the removal of calcium from the extracellular medium or pertussis toxin pretreatment. Etorphine did not stimulate the release of inositol phosphates from the cell membrane; however, etorphine-stimulated increases in intracellular calcium were attenuated by the addition of the toxin ryanodine. Conversely, DPDPE stimulates inositol-1,4,5-triphosphate production in undifferentiated NG108-15 cells (Smart and Lambert, 1996), as well as stimulating inositol phosphate production in a δ-opioid receptor-transfected mouse fibroblast cell line (Tsu et al., 1995).

In an interesting set of experiments, the stimulatory effect of DADLE on intracellular calcium levels was found to be dependent on the differentiation state of NG108-15 cells (Jin et al., 1992). In differentiated cells (5 μM forskolin, 6–14 days, serum-free medium), δ-opioid receptors mediated transient increases in intracellular Ca^{2+} in addition to blocking Ca^{2+} increases induced by depolarizing stimuli. Both effects were dependent on membrane calcium channels. In undifferentiated NG108-15 cells, the calcium channel blocker nitrendipine did not reduce DADLE-stimulated increases in intracellular Ca^{2+}, in contrast to results with differentiated cells. The removal of extracellular Ca^{2+} only partially attenuated DADLE-stimulated increases in intracellular Ca^{2+}, indicating that

the source of the calcium is intracellular stores in undifferentiated NG108-15 cells. These results were verified in a later study in which the removal of extracellular calcium did not block DADLE-stimulated increases in intracellular Ca^{2+} but U73122 (a phospholipase C inhibitor) and thapsigargin abolished the Ca^{2+} increase (Jin et al., 1994). These results indicate that δ-selective ligands increase intracellular Ca^{2+} levels from an inositol phosphate-sensitive intracellular pool in undifferentiated NG108-15 cells.

2. K^+ Conductance. The δ-opioid receptors in the guinea pig submucous plexus have been shown to increase potassium conductance (North et al., 1987). Neither PKC nor PKA appears to be involved in the modulation of K^+ currents in these studies. In DRG neurons and neuroblastoma x DRG neuron hybrid F11 cells, a biphasic effect of DPDPE was observed for K^+ conductance. At concentrations of <1 nM, conductance was inhibited (Fan et al., 1991), but at higher concentrations, conductance was increased (Fan and Crain, 1995). The decrease in conductance was blocked by cholera toxin (Fan et al., 1993; Fan and Crain, 1995), whereas the increase was blocked by pertussis toxin, suggesting that a multiplicity of G proteins are involved in the coupling of potassium channels to δ-opioid receptors (Fan and Crain, 1995). The physiological significance of the biphasic control of K^+ channels by DPDPE is still unclear, although similar results have been obtained for both κ- and μ-selective agonists (Fan and Crain, 1995).

E. Summary

Inhibition of cAMP generation was the initial observed second messenger effect of opioid receptors. Although the initial hypothesis of Collier and Roy (1974) that inhibition of cAMP would account for the analgesic effects of opioid drugs has not been uniformly supported by subsequent research, i.t. injections of dibutyryl-cAMP blocked both δ- and μ-opioid receptor-mediated spinal analgesia (J. B. Wang et al., 1993). These findings suggest that the inhibitory role of δ-selective drugs on cAMP production may modulate some antinociceptive pathways. In addition, cAMP may still play an important role in other δ-mediated cell functions. The δ-mediated inhibition of calcium channels is important because Ca^{2+} levels influence the release of neurotransmitters and modulate the function of several protein kinase families. The effect of δ-opioid receptors on K^+ conductance is of interest because this current can act to both hyperpolarize neurons, making them less sensitive to neurotransmitters, and restore the membrane potential after a neuron fires. Finally, the study of δ stimulation of G proteins is essential because this represents the first step in opioid-mediated signal transduction. Because G proteins are directly activated by δ-opioid receptors, receptor-mediated G protein activity should provide a more accurate description of receptor coupling to intracellular signaling compared with distal messengers such as cAMP. In addition, the knockdown of specific G proteins may allow the identification of G protein subtypes that mediate beneficial drug effects versus unwanted side effects. Such a possibility is suggested by a report in which i.c.v. injection of AS oligos specific to $G_{i\alpha 2}$ attenuated morphine antinociception but did not block constipation or naloxone-precipitated jumping (a measure of acute dependence; Raffa et al., 1996).

VI. δ-Opioid Receptor-Selective Agonist Efficacy

Various parameters are presently used to characterize the interaction between drugs and receptors. The most direct determination of drug-receptor interaction is the dissociation constant (K_D) of a radiolabeled drug. However, a major shortcoming of the dissociation constant is the failure of this parameter to describe the functional responses mediated by drug binding to a receptor. For instance, determination of K_D alone does not distinguish among drugs with agonist, partial agonist, inverse agonist, or antagonistic properties. An alternative pharmacodynamic measure is drug potency (e.g., EC_{50} value), which effectively describes drug-stimulated cell function. However, drug potencies are dependent on receptor concentration, which may differ from tissue to tissue. If the expression of receptors in a particular tissue, for example, is uniquely high or low, the potency value may be shifted to lower or higher drug concentrations, respectively (Nickerson, 1956). In the former situation, there may be an excess of receptor sites beyond that required for a maximal functional response, and these are referred to as a "receptor reserve" or "spare receptors" (Ruffolo, 1982). Unless these spare receptors are eliminated, determination of drug potency values are unlikely to accurately reflect the K_D value of drug-receptor interaction.

In addition to tissue-specific factors such as receptor densities, drug potency values are also dependent on 1) the affinity of drug for a receptor and 2) the ability of a drug to induce a conformation of the receptor that favors production of a measurable effect. It is not possible to distinguish the contribution of these latter two factors to a drug effect from potency values alone. Efficacy, conversely, is a measure of the ability of an agonist bound receptor to stimulate cell functions. Because efficacy values allow an investigator to separate the contributions of 1) agonist affinity and 2) cell-stimulating activity to drug potency, efficacy values are potentially useful for new drug development. The concept of efficacy arose from the realization that the relationship between receptor occupancy by a drug and a functional response mediated by the drug-bound receptor does not always follow a linear relationship. Thus, a highly efficacious drug is able to stimulate a maximal response in a functional assay while occupying only a small fraction of the available receptors. Conversely, a drug with low efficacy may stimulate a submaximal response even at 100% receptor occupancy. To understand efficacy, it is necessary to understand the development of receptor occu-

pancy theory. Stated simply, this theory assumes that biological responses are initiated by drugs binding to receptors. A brief discussion of the seminal events in the development of this theory follows; the reader is also referred to two excellent reviews of the topic (Mackay, 1966; Ruffolo, 1982).

A. Evolution of the Concept of Efficacy

The mechanism by which drugs produce their effects has long been a fundamental question of pharmacology. The emergence during the early 20th century of the concept of drug receptors (Langley, 1905; Clark, 1937) was accompanied by a need for a better description of the interaction of drug molecules at receptors to produce a functional response. During the 1920s, A. J. Clark examined the concentration-dependent effects of acetylcholine and atropine to modulate muscle contractions in various tissue preparations (Clark, 1926a,b). When Clark attempted to describe his data mathematically, the results suggested "that a reversible monomolecular reaction occurs between the drug and some substance either in the cell or on its surface" (Clark, 1926a). Clark also observed that drug-mediated effects were described by mass action relationships (Clark, 1937). He assumed that drugs act at receptor molecules and that "there is some simple relation between the amount of drug fixed by these receptors and the action produced" (Clark, 1933). A fundamental assumption implicit in Clark's writings was that the intensity of the drug effect was in direct proportion to the number of receptors occupied by the drug. Accordingly, Clark assumed the maximum drug response resulted from occupation of all possible receptors by a drug, a 50% maximal response resulted from drug occupation of 50% of the available receptors, and so on.

Clark's assumptions have been previously used to describe the relation between receptor occupancy and a functional response (Goldstein et al., 1974; Ruffolo, 1982). According to these assumptions, the functional effect of a drug in a tissue or cell system is related to receptor binding by the equation

$$E = E_{max}[A]/(K_D + [A]) \quad (1)$$

where E is functional effect, E_{max} is maximal functional effect, [A] is agonist concentration, and K_D is dissociation constant.

Clark (1937) theorized that two factors governed whether a drug effect would result from receptor occupation by the drug: 1) fixation, or the actual binding of the drug to the receptor; and 2) the power of the drug to produce an effect after fixation. However, this second factor remained unaddressed by the occupation theory based on Clark's assumptions.

1. Ariëns' Concept of Intrinsic Activity. Ariëns (1954) directly addressed this deficiency in Clark's theory and labeled the first factor affinity and the second factor intrinsic activity. Hence, affinity was a measure of the attachment or binding of the drug to the receptor; affinity was governed by the law of mass action. Intrinsic activity described the ability of the drug to evoke an effect after receptor binding. Ariëns envisioned intrinsic activity as describing the relative maximal responses elicited by drugs in a functional assay. The intrinsic activity of a full agonist was defined as equal to 1, whereas drugs that stimulated less than maximal response at receptor saturation had intrinsic activities of <1. Thus, a drug giving only 40% maximal effect had an intrinsic activity equal to 0.4, whereas the intrinsic activity of an antagonist was 0.

According to Ariëns

$$E = \alpha[AR] \quad (2)$$

and

$$E/E_{max} = \alpha[AR]/[R_T] \quad (3)$$

where α is intrinsic activity, $[AR]$ is agonist-receptor complex, and $[RT]$ is total number of receptors.

Both the hypotheses of Clark (1937) and Ariëns (1954) assumed that maximum effect required maximal occupation of receptors. The possibility of a nonlinear relationship between receptor occupancy and drug response was not addressed. For example, two full agonists can elicit a maximal response while occupying different proportions of available receptors. Clearly, these two full agonists activate receptors to different extents, yet according to Ariëns, the agonists would be defined as having the same intrinsic activity.

2. Stephenson's Concept of Efficacy. Building on the earlier work of Clark (1926a,b) and Ariëns (1954), Stephenson (1956) focused on the apparent property of drugs to mediate maximal functional responses while occupying different fractions of available receptors. Stephenson was also the first to define partial agonists as drugs with mixed agonist and antagonist effects. In an effort to describe the apparent nonlinear relationships between receptor occupancy and drug response, Stephenson introduced the concepts of stimulus and efficacy. Stimulus was defined as "the stimulus given the tissue" when exposed to drugs and was defined as being proportional to receptor occupancy. Thus, stimulus was a description of the relative strength of the response-inducing signal mediated by agonist-bound receptors. Efficacy was the property of the agonist that would permit two drugs to occupy different proportions of receptors yet produce equal responses.

According to Stephenson (1956),

$$S = e[AR]/[R_T] \quad (4)$$

and

$$E = f(S) \quad (5)$$

where S is stimulus, e is efficacy, $f(S)$ is function of stimulus, and $[AR]/[RT]$ is receptor occupancy.

Efficacy and intrinsic activities remain two entirely different concepts. For example, it is theoretically possible for two full agonists to possess the same intrinsic activity (i.e., $\alpha = 1$ for both) but to display different efficacies. By definition, Stephenson made $S = 1$ at 50% of the maximum response the drug was capable of eliciting. Thus, if an investigator determines the fractional receptor occupancy at the drug concentration eliciting 50% maximal response (the potency of the drug), Stephenson's efficacy value can be calculated with the following equation:

$$e = 1/y \quad (6)$$

where y is fractional receptor occupancy at the drug concentration eliciting 50% maximum response.

3. Furchgott's Concept of Intrinsic Efficacy. Furchgott (1966) used irreversible receptor antagonists to obtain accurate K_D values for agonists. He observed that as spare receptors were blocked with irreversible antagonist followed by agonist stimulation, agonist efficacy was reduced in tissues. This led him to propose that Stephenson's efficacy (e) was the product of the intrinsic efficacy (ϵ) of the drug and the concentration of receptors in the target tissue.

$$e = \epsilon[R_T] \quad (7)$$

By doing this, Furchgott recognized that, as with potency values, the observed efficacy depended on the receptor concentration in the test tissue in addition to the stimulus delivered by the drug-bound receptor to second messenger systems. Because of the role of the receptor concentration and second messenger systems in defining efficacy, observed efficacy values vary from tissue to tissue.

4. Estimation of Relative Efficacy Using the Formula of Ehlert. Furchgott first showed that once the dissociation constants and concentration-response curves of the two agonists are known, it is possible to calculate the intrinsic efficacy of one agonist relative to that of the other (Furchgott and Bursztyn, 1967). This technique involved plotting the responses of the agonists against their respective receptor occupancies and graphically estimating the ratio of receptor occupancies that yielded equivalent responses. Ehlert (1985) developed an equation to determine the relative efficacy from the K_D and EC_{50} values and the ratio of the maximal response of an agonist versus the maximal response induced by a full agonist. This simple method 1) does not require a graphic analysis and 2) estimates the relative efficacies of agonists from published K_D, EC_{50}, and E_{max} values without having access to the data for the concentration-response curve. In this review, we are referring to efficacy values calculated using the Ehlert equation as relative efficacy values to emphasize the fact that 1) these values describe the relative ability of a set of drugs to activate intracellular signaling pathways (induce stimulus) in a test system and 2) the numerical values for Stephenson's efficacy and Ehlert's relative efficacy are different. However, the ratios of efficacy values calculated by the Stephenson equation (eq. 6) and the relative efficacy values, as calculated below, are the same for a set of drugs in a defined test system.

The equation of Ehlert (1985) is based on two assumptions: one regarding the stimulus and the other regarding how the stimulus is converted into the response. The first assumption recognizes the stimulus as the measure of agonist-receptor activation and defines this parameter in a manner similar to that of Stephenson (Stephenson, 1956; Furchgott, 1966). The second assumption arises from the logistic behavior of most agonist-concentration response curves. This mathematical behavior dictates that the stimulus-response relationship is also a logistic function (Black and Leff, 1983), which causes the response to be nearly proportional to the stimulus at low levels of receptor occupancy. The formula of Ehlert (1985) is based on this latter assumption. Specifically, the response is assumed to be proportional to the stimulus up to the half-maximal response (Fig. 3). Note that the response and stimulus curves are equal up to the EC_{50} value.

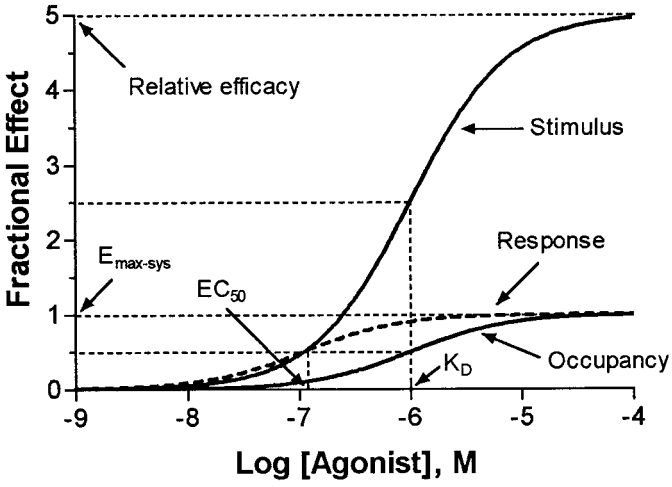

FIG. 3. The relationships among stimulus, response, and occupancy for a highly efficacious agonist with a relative efficacy of 5. The stimulus, response, and occupancy are plotted against the logarithm of the agonist concentration. Fractional effect is the response of a system to a drug where a fractional effect value of 1 is the maximal response the test system is capable of producing. The stimulus is proportional to the receptor occupancy; hence, the K_D value of receptor occupancy and the concentration of half-maximal stimulus are the same. Because the agonist is of high efficacy (i.e., the drug efficiently mediates a response on binding to the receptor), the ability of the test system to respond to the drug is exceeded at submaximal receptor occupancy. Hence, even though the stimulus continues to increase with drug concentration, the response plateaus; this is referred to as the ceiling effect. Because a maximal response requires only partial receptor occupancy, the response curve is shifted toward lower drug concentrations compared with the occupancy and stimulus curves. This shift of the response curve relative to the receptor occupancy curve is observed with highly efficacious drugs. The maximal stimulus reflects the maximal response that would be possible if the ceiling effect did not exist.

Figure 3 shows the assumed relationships among the stimulus, response, and occupancy for a full agonist. In this example, the EC_{50} value of the concentration-response curve (0.11 μM) is approximately one-tenth the K_D value (1.0 μM), indicating that the agonist is capable of eliciting a maximal response at a submaximal level of receptor occupancy. The fractional response is expressed relative to the maximal response of a full agonist. Because the stimulus is equivalent to the product of receptor occupancy and efficacy (eq. 4), the stimulus function is congruent with the occupancy function, and the concentration of agonist generating a half-maximal stimulus is equal to the K_D value. In addition, the maximum value of the stimulus is equivalent to efficacy (i.e., at receptor saturation by drug, when occupancy equals 1, then the product of occupancy and efficacy is equal to efficacy; see eq. 4). In molecular terms, the stimulus is a measure of agonist-receptor complex in the active conformation, which would be difficult to measure at a G protein-linked receptor such as the δ-opioid receptor. Nevertheless, we can consider a relative measure of the stimulus by taking advantage of the proportional relationship between the stimulus and response at low levels of receptor occupancy (see prior discussion). Specifically, we can assume that the half-maximal response and the relative stimulus are equal at the EC_{50} concentration of the agonist. Therefore, in Fig. 3, the function for the stimulus intersects the concentration-response curve at the half-maximal response, and it continues to overlap the concentration-response curve at lower agonist concentrations. To estimate relative efficacy, all one has to do is extrapolate to the maximum value of the relative stimulus. The algebraic method for doing so is described next.

According to Stephenson (1956) and Furchgott (1966), the stimulus is equivalent to the product of receptor occupancy and efficacy (eq. 4). Substituting a mass action equation ($[AR/R_T] = [A]/K_D + [A]$; Goldstein et al., 1974) for occupancy yields:

$$S = [A]e/([A] + K_D) \quad (8)$$

Because the numerical value of S is equivalent to the fractional response at low receptor occupancy, we can substitute the EC_{50} value for $[A]$ and the half-maximal response for S in eq. 8 and then solve for e. The half-maximal response of an agonist is defined by the equation:

$$\text{Half-maximal response} = 0.5 \times E_{max}/E_{max\text{-}sys} \quad (9)$$

where E_{max} denotes the maximum response of the agonist, $E_{max\text{-}sys}$ denotes the maximum response of the system or that of a full agonist. Substituting $0.5 \times E_{max}/E_{max\text{-}sys}$ for S and EC_{50} for A in eq. 8 yields:

$$0.5 \times E_{max}/E_{max\text{-}sys} = EC_{50}e/(EC_{50} + K_D) \quad (10)$$

Upon replacement of the term *efficacy* (e) with *relative efficacy* (e_{rel}), to reflect that efficacy values calculated using this method are indicative of the relative ability of drugs to stimulate a given test cell system, rearrangement of eq. 10 yields an expression equivalent to that given by Ehlert (1985) for the estimation of relative efficacy:

$$0.5 \times E_{max}/E_{max\text{-}sys} \times (1 + K_D/EC_{50}) = e_{rel} \quad (11)$$

According to eq. 11, an agonist with a relative efficacy of 1 is nearly capable of eliciting a full maximal response and exhibits an EC_{50} value that is approximately equal to the K_D value. Such an agonist could be considered as a theoretical standard, and the efficacies of other agonists are expressed relative to this standard. However, after the use of eq. 11 to calculate the relative efficacies of a series of agonists, one of the agonists can be designated as the standard, and the efficacies of the other agonists can be normalized relative to the designated standard.

Several criteria must be met to use eq. 11 to accurately estimate the relative efficacies of agonists. First, the functional response being measured must be mediated through a single receptor type, and the binding of agonist to the receptor must follow the law of mass action. This criterion is met if the pseudo-Hill coefficient (n_H) of the agonist binding isotherm is equal to 1. Second, the binding constant used in eq. 11 to estimate relative efficacy must accurately reflect the K_D value of the agonist for the receptor under the conditions of the functional assay. As indicated in the next section, this concern is critical for G protein-coupled receptors where buffer concentrations of Mg^{2+}, Na^+, and guanine nucleotide can greatly influence agonist affinity (Rosenberger et al., 1980; Wong et al., 1994; Quock et al., 1997). This K_D value can be estimated using a radioligand binding assay or by Furchgott's method of partial receptor inactivation.

5. Summary. Efficacy values reflect the ability of drugs to activate cells and tissues through receptors. In his extension of receptor occupancy theory, agonist activation of tissue was denoted as "stimulus" by Stephenson (1956). Stimulus can be thought of as the driving force resulting in a measurable cellular or tissue response and is induced by agonist binding to a receptor. On a molecular level, we assume that 1) stimulus is an agonist-dependent change in the conformation of a receptor that favors receptor interactions with distal components of signal transduction cascades and 2) stimulus is directly proportional to the concentration of receptors that assume an active state on agonist binding. It is further assumed that a functional response or effect is some function of stimulus (eq. 5) but that this function departs from linearity for highly efficacious agonists. All methods for the calculation of stimulus and efficacy share a fundamental assumption; namely, that a mea-

surable cellular or tissue response (effect) is an accurate measurement of the stimulus at submaximal response levels. For this reason, Stephenson defined $S = 1$ at a 50% maximal response and the Ehlert equation (eq. 11) includes EC_{50} values.

When agonists are highly efficacious, namely, are capable of inducing high levels of stimulus, the ability of a cell or tissue to respond to that stimulus may be exceeded. In this case, a maximal effect, or ceiling, may be reached beyond which the cell cannot respond. The maximum response occurs despite the fact that only a fraction of the available receptors are occupied by drug and increasing agonist concentration will increase both receptor occupancy and stimulus. Efficacy values can reflect differences in the strength of the stimulus induced by full agonists (agonists with an intrinsic activity = 1). Highly efficacious agonists induce greater levels of stimulus at lower fractional receptor occupancies. This is observed as a shift of functional response curves toward lower agonist concentrations compared with receptor occupancy curves; the shift results in a higher numerical value when efficacy is calculated.

Stephenson initially defined efficacy according to eq. 6. Furchgott (1966) observed that Stephenson's efficacy value was dependent on both agonist-mediated stimulus at the receptor and the concentration of the receptors in the tissue. He thus defined the tissue-independent measure of agonist-mediated stimulus as intrinsic efficacy. This observation was of fundamental importance and explained how the efficacy of agonists could vary between tissues. In the studies discussed below, we determined the efficacy of δ agonist-stimulated G protein activation using the Ehlert efficacy equation. These efficacy values should be considered relative efficacy values for the agonists tested as these values would change in another δ-opioid receptor-transfected CHO cell line that expressed receptor at a different level. We would expect the ratio of the efficacy values to remain the same, however.

B. Relative Efficacy of δ-Selective Drugs in Transfected Cells That Stably Express the Human δ-Opioid Receptor

For the purposes of this discussion, relative efficacy describes the relationship between receptor occupancy and a functional response as calculated by the equation derived by Ehlert (1985). The term "efficacy" has several different uses. According to federal law, efficacy is proof of therapeutic effectiveness (McPhillips, 1994) and should, in this context, be identified as clinical or therapeutic efficacy in patients. Unfortunately, Ariëns' concept of intrinsic activity is often incorrectly referred to as efficacy in the pharmacological literature.

The original concept of efficacy, as introduced by Stephenson (1956), focused on the apparent property of drugs to produce comparable magnitudes of drug effect while occupying different numbers of receptors. Pharmacological efficacy, therefore, describes the relationship between receptor occupancy by a drug and the magnitude of the response to the drug. Based on this definition, our laboratory determined the relative efficacy of drugs with agonist properties at the cloned human δ opioid receptor (Quock et al., 1997) using the formula of Ehlert (1985). The present review includes an expanded discussion of those preliminary observations to more thoroughly characterize agonist efficacy at the δ-opioid receptor. These studies were conducted in a CHO cell line that was stably transfected with the wild-type cloned human δ-opioid receptor. These cells express a homogeneous population of receptors that permit the study of receptor function without the potentially confounding interference of other receptors. The δ-selective agonists tested were the nonpeptidic compounds SNC80 and (−)-TAN67 and the peptidic agonists DPDPE, Del-II, and biphalin [(Tyr-D-Ala-Gly-Phe-$NH)_2$; Fig. 4].

Because G proteins are the first intracellular proteins to be activated after the binding of agonists to δ-opioid receptors, we chose to determine the efficacy of δ-selective agonist-mediated G protein activation by using [^{35}S]GTPγS binding (Traynor and Nahorski, 1995; Befort et al., 1996b) as a sensitive measure of receptor coupling to signal transduction according to our previous protocol (Quock et al., 1997). Earlier studies established that an increase in [^{35}S]GTPγS binding was an index of G protein activation by muscarinic (Lazareno et al., 1993), α_2-adrenergic (Tian et al., 1994), adenosine (Lorenzen et al., 1993), and cannabinoid (Sim et al., 1995; Selley et al., 1996) receptor agonists. Traynor and Nahorski (1995) reported previously that opioids could stimulate [^{35}S]GTPγS binding in membranes from the SH-SY5Y neuroblastoma cell line, thus demonstrating the applicability of this method to measure G protein activation by opioids.

Efficacy values require calculation of the affinity of the agonist for the receptor. We determined agonist K_i values by competitive inhibition of the δ-selective antagonist ([^{3}H]naltrindole) binding at the δ-opioid receptor (Yamamura et al., 1992; Contreras et al., 1993) as previously described (Quock et al., 1997). The results of the [^{3}H]naltrindole competitive inhibition study indicate that of the δ receptor agonists tested, (−)-TAN67 possessed the greatest affinity for the cloned human δ-opioid receptor, at least 10 times greater than the other δ-selective agonists tested (Table 5). The five δ receptor agonists competitively inhibited [^{3}H]naltrindole binding and demonstrated the following order of affinity (based on calculated K_i values): (−)-TAN67 > Del-II ≈ biphalin > SNC80 > DPDPE. (−)-TAN67 had 13-, 15-, 19-, and 27-fold greater affinity for the cloned human δ opioid receptor than Del-II, biphalin, SNC80, and DPDPE, respectively. The K_i value was determined using the equation of Cheng and Prusoff (1973), $K_i = IC_{50}/(1 + L/K_D)$, where the IC_{50} is the concentration that inhibits binding by 50%, L is the concentration of the radioli-

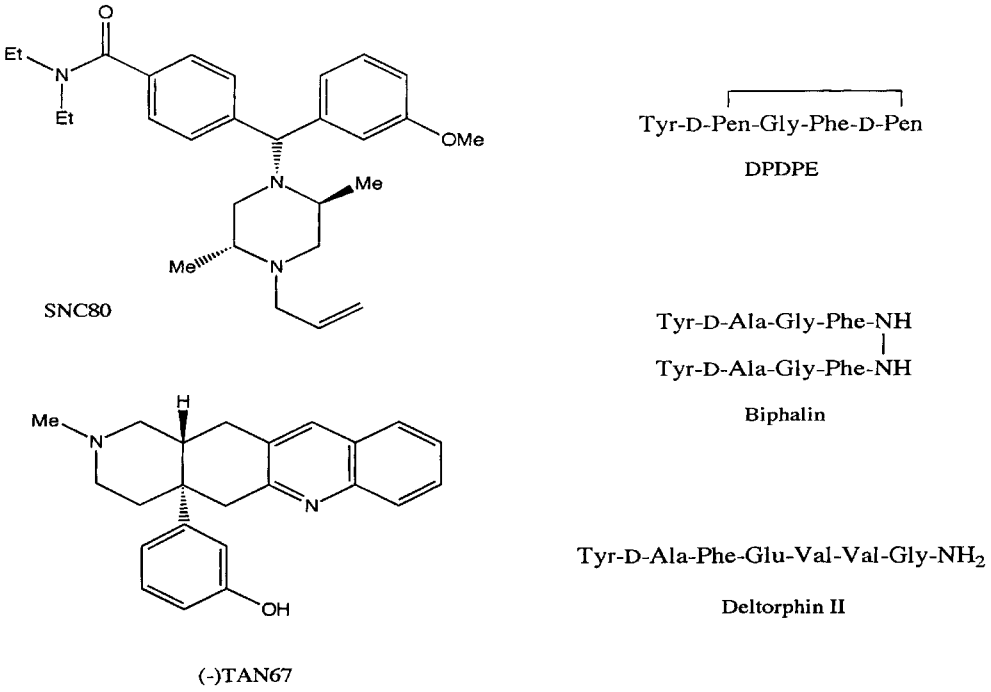

FIG. 4. Chemical structures of the δ-opioid receptor agonists SNC80, (−)-TAN67, DPDPE, Del-II, and biphalin.

TABLE 5
Relative efficacies of δ-selective agonists at the wild-type cloned human δ-opioid receptor

Agonist	K_i[a]	EC_{50}[a]	E_{max}[a]	Relative Efficacy	Efficacy Ratio
	nM		*%*		
Del-II	42.7 ± 9.7	9.3 ± 4.2	96 ± 2	2.80	1.00
DPDPE	85.5 ± 7.2	19.1 ± 7.2	82 ± 2	2.74	0.98
SNC80	59.3 ± 10.2	15.7 ± 6.8	100 ± 0	2.39	0.85
(−)-TAN67	3.2 ± 0.3	1.4 ± 1.2	83 ± 5	1.64	0.59
Biphalin	46.5 ± 1.5	34.0 ± 13.1	98 ± 10	1.18	0.42

The K_i values were determined from [^{3}H]naltrindole competitive inhibition experiments using the equation of Cheng and Prusoff (1973), and the EC_{50} values were determined from the [^{35}S]GTPγS stimulation experiments as previously determined (Quock et al., 1997). Relative efficacy was determined according to Eq. 11 [$0.5 \times E_{max}/E_{max\text{-}sys} \times (1 + K_D/EC_{50}) = e_{rel}$] with $E_{max}/E_{max\text{-}sys} = 1$ as differences between the E_{max} values for these drugs did not reach statistical significance. The K_i and EC_{50} values for each drug were compared for statistical significance with use of a t test. These values were significantly different for Del-II, DPDPE, and SNC80 but not for (−)-TAN67 and biphalin. Hence, the relative efficacy values calculated for these latter two drugs are not significantly different than 1, the value expected for a full agonist in the absence of spare receptors.

[a] Values are mean ± S.E.

gand, and K_D is the dissociation constant of [^{3}H]naltrindole. The K_i values reported in Table 5 are of lower affinity than those reported in the literature (Raynor et al., 1994; Knapp et al., 1995b; Varga et al., 1996; Misicka et al., 1997). These lower affinity values are due to the presence of GDP (50 μM) and NaCl (150 mM) in the assay buffers. Guanine nucleotide and sodium have previously been shown to reduce the affinity of agonists at G protein-coupled receptors (Pert and Snyder, 1974; Blume, 1978; Rosenberger et al., 1980). When we compare the K_i values in Table 5 with K_i values previously obtained in Tris (50 mM)/$MgCl_2$ (5 mM) with membranes from the same human δ opioid receptor transfected cell line, we observe a shift in affinity of 50-, 2.1-, and 1.3-fold for SNC80, Del-II, and (−)-TAN 67, respectively. We believe the lower-affinity K_i values are physiologically relevant due to the high sodium content in body fluids and the presence of intracellular guanine nucleotides.

The results of the [^{35}S]GTPγS stimulation study reveal that the δ-selective agonists tested to have EC_{50} values in the nanomolar range. Table 5 shows the K_i, EC_{50}, and calculated relative efficacy values of the agonists tested. Results show the calculated relative efficacy values in the following order: Del-II ≈ DPDPE ≥ SNC80 > (−)-TAN67 ≥ biphalin.

Graphing receptor occupancy curves with the dose-response curve for [^{35}S]GTPγS binding on the same axes helps clarify efficacy. For drugs with greater efficacy, the dose-response curve is shifted farther to the left of the occupancy curve compared with drugs with low efficacy. This is illustrated here for the two drugs that yielded the greatest and least efficacy in the [^{35}S]GTPγS-binding assay for G protein activation, namely, Del-II and biphalin, respectively (Figs. 5A and 6A). For Del-II, the dose-response curve for G protein activation is shifted >4-fold toward lower drug concentrations compared with receptor occupancy curves. This indicates that a Del-II-bound

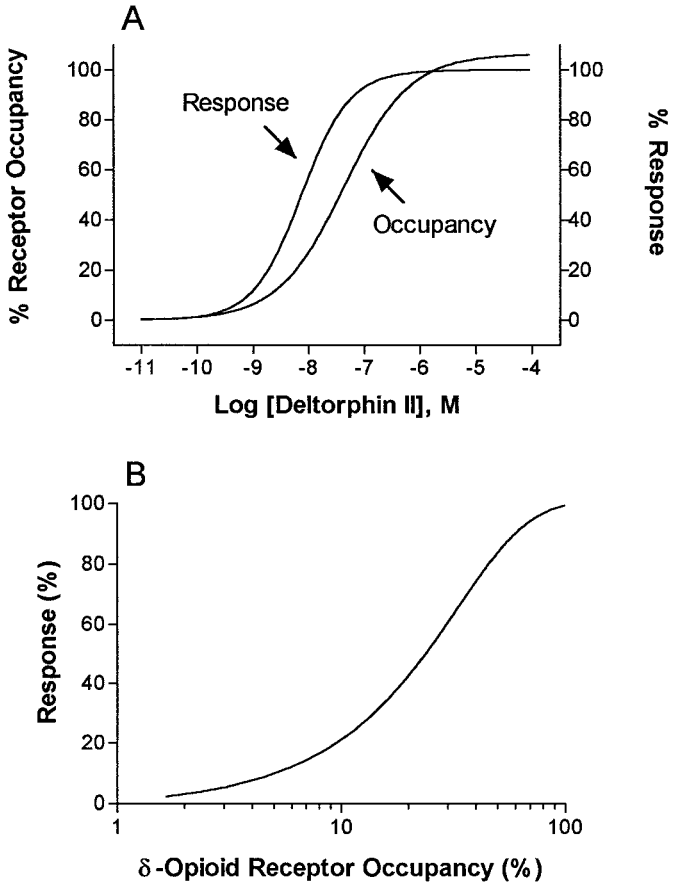

FIG. 5. A, Del-II dose-response and dose-receptor occupancy curves. The dose-response curve, with the Hill slope fixed to a value of 1, was generated from experimental data (see Table 5) using Prism Version 2 (GraphPAD, San Diego, CA). The maximal response was set at 100% because the maximal responses induced by the δ-selective agonists (Table 5) were not significantly different. The dose-receptor occupancy curve was generated as a theoretical curve in Prism Version 2 using the K_i value for Del-II obtained from competitive inhibition experiments with the antagonist [^{3}H]naltrindole (Table 5). B, semilogarithmic receptor occupancy-versus-response plot for Del-II.

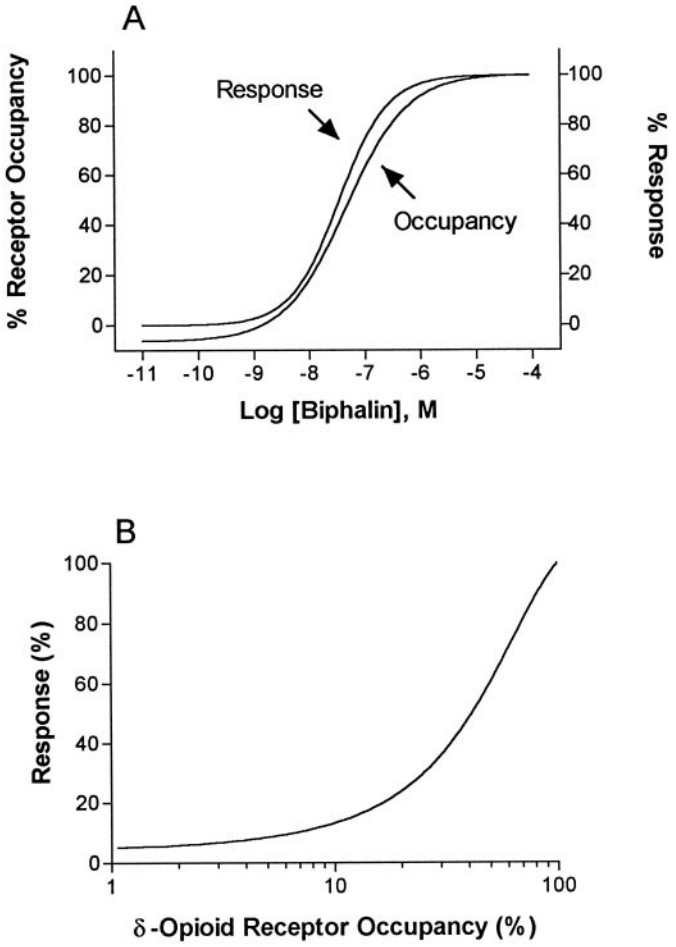

FIG. 6. A, biphalin dose-response and dose-receptor occupancy curves. Curves were generated by the same method as the Del-II curves in Fig. 5. B, semilogarithmic receptor occupancy-versus-response plot for biphalin.

receptor mediates high levels of stimulus and thus effectively activates G proteins when occupying only a fraction of functional δ-opioid receptors. This is characteristic of a highly efficacious drug. When the same data are represented on a semilogarithmic receptor occupancy-versus-response plot (Fig. 5B), it is clear that ~25% receptor occupancy corresponds to >50% response and that 70% receptor occupancy approaches a maximal response. Hence, for Del-II-stimulated G protein activation in this transfected cell system, there are more receptors present than necessary to achieve a maximal response. These extra receptors are referred to as "spare receptors". The number of spare receptors is unique for each drug, and this number is determined by the strength of the stimulus delivered to the cell or tissue by drug binding to receptors.

In marked contrast to Del-II, the biphalin data yielded essentially overlapping sigmoidal dose-response curves for G protein activation and receptor occupancy (Fig. 6A). These data are consistent with biphalin demonstrating lower efficacy compared with Del-II. Plotted on a semilogarithmic receptor occupancy-versus-response plot (Fig. 6B), it is evident that there are no spare receptors for biphalin and total receptor occupancy is required for a maximal response.

1. δ-Opioid Receptor-Selective Agonists. A comprehensive discussion of δ-selective agonists is beyond the scope of this review. Instead, the agonists used in the studies cited above are described, and the efficacy of each agonist is discussed.

a. [D-ALA^2]DELTORPHIN II. The Argentinian frog species *Phyllomedusa sauvagei* has proved to be an excellent source of peptide ligands with selectivity for opioid receptors (Erspamer et al., 1989; Kreil et al., 1989). Three different δ-selective heptapeptides have been isolated from the skin of *P. sauvagei* and named deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2). All three of these peptides demonstrate >1000-fold selectivity for the δ- versus μ-opioid receptor as measured by inhibition of contraction of the mouse vas deferens and guinea pig ileum. Del-II was the most efficacious agonist tested

and was of similar relative efficacy to DPDPE in the assays reported in Table 5, indicating that this drug caused maximal G protein activation at fractional δ-opioid receptor occupancy.

b. CYCLIC [D-PEN^2,D-PEN^5]ENKEPHALIN. Initial studies of the δ-opioid receptor were hampered by the fact that enkephalin peptides were not particularly selective for this receptor over μ opioid receptors. Mosberg et al. (1983b) reasoned that if the enkephalin pentapeptide could be restrained into a cyclic structure, it might demonstrate more selectivity for the δ receptor as it would lose conformational flexibility that might be necessary to bind to other receptors. One of the cyclized enkephalin analogs synthesized by these investigators, DPDPE, showed great selectivity for the δ-opioid receptor. In the isolated mouse vas deferens and guinea pig ileum preparations, DPDPE proved to be 3000 times more potent at the δ- than at the μ-opioid receptor (Mosberg et al., 1983b). In radioligand binding studies, the binding affinity of DPDPE for the δ receptor was 175 times greater than that for the μ receptor in rat brain membranes (Mosberg et al., 1983a). DPDPE is able to stimulate maximal G protein activation at fractional receptor occupancy, indicating that this drug induces efficient coupling of its receptor to second messenger systems (Table 5). Preliminary studies are being conducted that may lead to clinical trials of DPDPE as an analgesic in humans. If approved by the Food and Drug Administration, DPDPE will be the first δ receptor-selective agonist to be used clinically (V. J. Hruby, personal communication).

c. SNC80. SNC80 is the dextrorotatory methylether analog of BW373U86, a novel nonpeptidic δ-selective agonist (Calderon et al., 1994). Pharmacological and neurochemical experiments demonstrate that this analog retains selective action at δ-opioid receptors. In mice, SNC80 produces a dose-related antinociception that is sensitive to antagonism by δ but not μ receptor antagonists (Bilsky et al., 1995). Radioligand-binding inhibition studies show that the K_i value for SNC80 at the δ receptor (1.78 nM) was of greater affinity than that for the κ (442 nM)- or μ-opioid receptors (882 nM; Bilsky et al., 1995). It must be noted that these K_i values were determined in the absence of Na^+, which explains the difference between the value reported by Bilsky et al. (1995) for SNC80 binding and those in Table 5.

d. TAN67. (±)-TAN67 is a nonpeptidic compound that is highly selective for the δ-opioid receptor in vitro (Nagase et al., 1994). In the rat brain, it shows a high affinity for δ receptors (K_i = 1.12 nM) but poor affinity for κ and μ receptors (K_i = 1790 and 2320 nM, respectively). In our laboratory, (±)-TAN67 showed high binding affinity (K_i = 0.647 nM) in CHO cells stably transfected with the cloned human δ-opioid receptor, high δ-binding selectivity (>1000 times relative to the human μ opioid receptor), high potency (EC_{50} = 1.72 nM) for inhibiting forskolin-stimulated accumulation of cAMP at human δ receptors, and extremely low potency (EC_{50} = 1520 nM) at human μ-opioid receptors expressed by B82 mouse fibroblast cells (Knapp et al., 1995a).

The pharmacological profile of (±)-TAN67 that was characterized at a number of laboratories was conspicuous by the absence of a strong antinociceptive effect in animals. Despite this high affinity and selectivity for the δ-opioid receptor, (±)-TAN67, when administered alone, produced little or no antinociceptive activity in the 51°C warm plate test in mice (Suzuki et al., 1995). However, coadministered with morphine, (±)-TAN67 potentiated morphine-induced antinociception similar to the putative δ_1 agonist DPDPE; putative δ_2 agonists like Del-II are lacking in this property (Suzuki et al., 1995). An antinociceptive action of (±)-TAN67 was more prominent in diabetic mice, which exhibit a greater responsiveness to putative δ_1-opioid agonists (Kamei et al., 1995). More recent studies indicate that antinociceptive activity resides in the (−)-enantiomer, whereas the (+)-form of TAN67 appears to be hyperalgesic, especially after i.t. administration (Tseng et al., 1997).

e. BIPHALIN. Biphalin is unique in structure, consisting of a pair of biologically active pharmacophores (enkephalin sequences) linked through a hydrazide bridge (Fig. 4; Lipkowski et al., 1982, 1987). Antinociceptive testing indicates that biphalin is highly potent. Biphalin administered i.c.v. is 6.7- and 257-fold more potent than etorphine and morphine, respectively (Horan et al., 1993). Biphalin administered i.t. is also more potent than morphine (Silbert et al., 1991). The antinociceptive effect of biphalin is reduced by antagonist blockade of δ- or μ-opioid receptors (Pasternak et al., 1980; Takemori et al., 1980; Heyman et al., 1987; Jiang et al., 1991) but not κ-opioid receptors (Portoghese et al., 1986). Biphalin has also been found to competitively inhibit binding of [^{3}H]D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 and [^{3}H]pCl-DPDPE binding at the μ- and δ-opioid receptors, respectively (Misicka et al., 1997). These findings suggest that biphalin exerts agonist activity at both δ- and μ-opioid receptors.

When the efficacy of biphalin-stimulated G protein activation was examined in δ-opioid receptor-transfected CHO cells, an efficacy ratio of 0.42 was determined as compared with Del-II. This efficacy value indicates that biphalin does not efficiently stimulate G proteins through the δ receptor. These results are inconsistent with the degree of antinociception observed experimentally (Horan et al., 1993). The contrast between the relatively low agonist efficacy of biphalin in this study, the exceptional potency of biphalin in antinociceptive testing, and the fact the drug binds to the μ receptor suggests that the antinociceptive response to biphalin may be partly attributed to activation of the μ-opioid receptors.

2. Comparison of Stephenson Efficacy and Ehlert Relative Efficacy Calculations. We reanalyzed the data in

Table 5 for efficacy using the formula of Stephenson (1956; Eq. 6). Receptor occupancy for each drug was calculated at the EC_{50} value using the K_i value reported in Table 5. The calculated efficacy values determined using the Stephenson equation (eq. 6) were 5.59, 5.48, 4.78, 3.29, and 2.37 for Del-II, DPDPE, SNC80, (−)-TAN67, and biphalin, respectively. Although the Stephenson efficacy and Ehlert relative efficacy values were different in absolute magnitude, the efficacy ratios for these drugs, as calculated using these equations were, nevertheless, identical. This suggests that these treatments are consistent assessments of δ-selective agonist efficacy relative to one another. The Ehlert method seemingly has the advantage of determining relative efficacy by using directly measurable parameters. It should be noted that all drugs in this study were treated as full agonists because there were no statistically significant differences between E_{max} values as determined by ANOVA and the Tukey test (Table 5).

3. Summary: Drug Efficacy Determinations in Transfected Cell Lines. In most tissues, multiple receptors can be targeted by a given drug, resulting in ambiguity over the relative contributions of each receptor to an observed functional response. One approach to overcome this problem is to study receptor function in cell lines that express only a single type of receptor. Although this assay system may have limitations, receptor-transfected cell lines nevertheless permit in-depth examination of a particular receptor with its associated second messenger systems in isolation. In addition, the diversity of G proteins involved in coupling receptors to signaling pathways is more amenable to investigation in cell lines. For example, G protein expression levels can be reduced by AS oligo knockdown procedures. Alternatively, the composition of G proteins coupled to a receptor can be selectively altered by cotransfecting cells with cDNA molecules for a given G protein. Experiments can be conducted in cells lines expressing different densities of receptors to determine the effect of receptor density on function that would never be possible in vivo. All in all, cell lines stably transfected with wild-type or mutant receptors are well suited to elucidating the role of G proteins in receptor function and identifying the molecular determinants of efficacy.

The ultimate objective of efficacy calculations is to predict the in vivo effectiveness of agonist drugs. However, inconsistencies arise when comparing in vitro efficacy determinations with actual assessment of in vivo effectiveness of drugs. For instance, (−)-TAN67 was predicted to be a poor antinociceptive agent (Quock et al., 1997); however, (−)-TAN67 has recently been demonstrated to evoke a strong antinociceptive effect in the mouse tail-flick test after i.t. administration (Tseng et al., 1997). Biphalin was likewise predicted to be a poor antinociceptive agent, yet there is obvious evidence of a potent antinociceptive effect in experimental animals. A number of explanations exist for these discrepancies. For example, efficacy is dependent on the level of receptor present on a given tissue. In the case of δ-opioid receptors, various brain regions have different levels of receptor (Kitchen et al., 1997). Thus, the magnitude of two in vivo effects of a drug, such as analgesia and respiratory depression, may be very different if the effects are not mediated by the same brain region. Drug efficacy is also dependent on the complement of intracellular signaling molecules within the tissue. In the example of δ opioid receptors, if a brain region mediating a functional response in vivo has different G protein levels or subtypes compared with a δ receptor-transfected cell line, the effects of a drug in the tissue and cell line are likely to differ.

Despite these limitations, in the study of δ-opioid receptors, efficacy calculations do provide a measure of cellular activation in response to drug binding. This measure allowed us to examine drug-mediated effects in a defined system. The predictive value of in vitro efficacy calculations to drug effects in vivo will be dependent on how closely the in vitro system models the second messenger systems of an animal and whether the drug is capable in vivo of reaching the tissue in sufficient quantity to exert a pharmacological effect. In vitro efficacy studies should be another valuable measure of drug activity that will aid in the better design and development of drugs for clinical use.

VII. Conclusions and Future Directions

The molecular biology of opioid receptors remains an area of active research. The successful cloning of the δ-, κ-, and μ-opioid receptor cDNAs has permitted the use of both molecular biology techniques and classic pharmacology to examine δ-opioid receptor regions that mediate 1) ligand binding and 2) receptor-mediated functional responses. Cloning of opioid receptor cDNAs now permits the localization in the nervous system of different opioid receptors by immunohistochemistry and of cells that express receptor mRNAs by in situ hybridization. Molecular techniques will permit identification and localization of the signal transduction pathway components that are coupled to and activated in the presence of an opioid agonist. Studies with chimeric and point-mutated opioid receptors will continue to identify the essential domains and amino acid residues that determine drug binding, efficacy, and receptor desensitization.

Efficacy calculations are a valuable pharmacodynamic measurement that will aid in the development of clinically useful δ-selective drugs and are a direct measure of the ability of a drug bound receptor to mediate a given functional response. In other words, efficacy describes the relationship between the fractional receptor occupancy by a drug and a given level of functional response. Highly efficacious drugs occupy only a small fraction of available receptors to stimulate a response, whereas drugs with low efficacy may not mediate a

maximal functional response even at receptor saturation. In contrast, drug potency values, which are widely used to characterize new drugs, are dependent both on the affinity of the drug for its receptor and the coupling efficiency of the drug-bound receptor to the effector system under study. Indeed, the principal weakness of drug potency values as a pharmacodynamic measurement is that the contributions of drug affinity and coupling efficiency to an observed effect cannot be separated. On the other hand, efficacy values can help the investigator distinguish between these mechanisms. For example, when two drugs with selectivity for the δ-opioid receptor are tested in the mouse tail-flick assay, it is possible that they would have similar potency. However, the first drug might have high efficacy and poor receptor affinity, whereas the second may have the opposite. When side effects are a problem with a given class of drug, we believe the preferable drug will likely be the one with high receptor affinity and low efficacy. The high affinity allows the drug to demonstrate pharmacological activity at lower doses, whereas the low efficacy may mediate fewer side effects. Indeed, this may be the case with morphine. After >100 years of use, morphine is still the gold standard against which other analgesic agents are measured, yet we have shown that morphine has modest efficacy in assays of G protein activation (Hosohata et al., 1998). Although morphine certainly has problematic side effects, perhaps the poor coupling efficiency of morphine-bound μ-opioid receptors to G proteins actually tempers the intensity of morphine toxicity and restricts the side effects to manageable or tolerable levels. In addition, the cloned rat δ-opioid receptor has been demonstrated to down-regulate to a greater degree in the presence of full agonists compared with partial agonists (Remmers et al., 1998). These findings suggest that partial agonists at the δ-opioid receptor may induce less tolerance than full agonists when given to animals chronically.

Recent findings regarding 1) receptor regions or amino acid residues that modulate ligand binding and 2) receptor coupling to second messenger systems are providing novel opportunities for the design of pharmaceuticals. With improved knowledge of the ligand binding sites on receptors, medicinal chemists will be able to rationally design drugs that bind to these sites with improved affinity. Using site-directed mutagenesis and chimeric receptors, it may also be possible to distinguish drug-binding domains that mediate high efficiency coupling to second messenger systems. From the studies cited above, it is clear that δ-selective ligands do not bind to identical amino acid residues in the receptor; however, residues responsible for high-efficiency coupling of drug-bound receptors to functional responses remain to be determined. The Ehlert relative efficacy equation is a sensitive tool that may be used to determine whether new pharmaceutical agents, based on molecular modeling of δ-selective ligand binding, have improved cell activation characteristics compared with older drugs. The use of K_D and potency values in the Ehlert equation make this relative efficacy expression simpler to use than equivalent equations that require the calculation of fractional receptor occupancies (Stephenson, 1956; Black and Leff, 1983). Thus, a new generation of opioid receptor research that uses the tools of molecular biology and improved pharmacodynamic measurements should facilitate the design and synthesis of drugs 1) with greater selectivity and efficacy for the δ-opioid receptors and 2) that display reduced toxicity and abuse potential coincident with therapeutic use.

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