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PHARMACOLOGY OF CALCIUM CHANNELS AND SMOOTH MUSCLE

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INTRODUCTION

A number of diverse cellular functions (contraction, secretion, etc.) are known to be regulated by fluctuations in the free calcium ion concentration in the cytosol (1). One important source of these divalent ions is the calcium reservoir in the extracellular fluid (1). Calcium ions can be mobilized from this external pool by the operation of calcium channels that are anchored in the plasma membrane (1). The obvious involvement of these membrane-bound ion channels in the regulation of essential cellular functions has generated considerable interest in and exploration of their functional characteristics. This article deals with three aspects of the operation of calcium channels. The first section contains a qualitative description of the functional properties of different types of calcium channels. This is followed by an overview of the manner in which pharmacological agents may modify the functional behavior of calcium channels. Lastly, a discussion of the various types of calcium channels that appear to exist in smooth muscle cells is presented.

FUNCTIONAL CHARACTERISTICS OF CALCIUM CHANNELS

Ion Permeation

A calcium channel residing in the membrane of a eukaryotic cell is viewed as being a macromolecular structure consisting essentially of one or more glycoproteins (2, 3, 4). Its configuration is presumed to be roughly cylindrical with an aqueous pore at its center (2, 3). The permeation of extracellular calcium ions through the pore into the cytosol is accomplished via several discrete steps.

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The initial step consists of a reversible interaction between the divalent ion and a calcium binding site (calcium coordination site) that is thought to be located at or near the surface of the channel (5–8). Alternatively, the initial reaction may be viewed as the passage of the divalent ion over some energy barrier requiring some level of free energy of activation, followed by the descent of the ion into an energy well that is reflected by a net loss of free energy (8, 9). The occupation by the cation of a relatively deep energy well would be analogous to the cation exhibiting a relatively high affinity for the channel coordination site and vice versa.

Because the movement of a cation through the aqueous pore of a calcium channel must be preceded by a reversible interaction with a channel binding site, several dynamic aspects of ion permeation simulate those observed in enzymatic reactions that obey Michaelis-Menten kinetics (5, 6, 7). Thus, the interaction between some particular cation and the channel binding site may be characterized by an apparent dissociation constant that remains invariable as long as other conditions (i.e. membrane potential, temperature, etc.) are not modified. Furthermore, the relationship between the rate of cation permeation through a population of calcium channels and the extracellular cation concentration defines a simple, hyperbolic saturation curve. These functional properties of the calcium channel were first described by Hagiwara and Takahashi (7) who utilized the maximum slope of the action potential as a measure of the maximum current flowing through the calcium channels of barnacle muscle. Beirao and Lakshminarayanaiah (10) obtained similar results with voltage clamp measurements in barnacle muscle.

Based on the manner in which inorganic cations and calcium channels interact, one element of the driving force that energizes the inward movement of permeant cations is the level of saturation of the binding sites in or on the calcium channels. The second element is the interaction between the electric field within the membrane and the charged ion moving across the membrane. Because intracellular concentrations of calcium ions are generally very low, outwardly directed driving forces are correspondingly low. Within the aqueous pore of the calcium channel the driving force acting to propel the permeant cation inward will be countered by steric configurations and reactive groups or, in other terms, by energy barriers that act to impede the flow of ions through the channel. It appears that one of these barriers may be sufficiently high and uniquely structured to permit some types of cations to pass through, but halt any further forward movement of others. Such a barrier has the capacity to confer a selective action on the channel, and thereby restrict the types of ion species that will be permitted to traverse the cell membrane via this pathway. In view of its functional role in the channel, this barrier has been labeled the selectivity filter (3, 9, 11). In addition to establishing the ion specificity of the channel, the selectivity filter, as well as other energy barriers, serve to regulate the speed at

which a permeant cation will move through the aqueous pore of the channel (9, 11). Some cations such as Ca, Ba and Sr penetrate the calcium channel relatively rapidly (5, 6). Others such as Zn, Mn and Cd move through the channel at a much slower speed (5, 6). This rate process will determine the maximum possible electrical current that a particular ion species can generate by moving through a population of calcium channels under a given set of conditions.

When the relationship between current flow and the extracellular ion concentration produces a simple, hyperbolic saturation curve, there is implicit in these dynamics the concept that only one ion may interact with and move through the channel in any given instant. This concept has been challenged by Hess and Tsien (12) who studied calcium channels in single ventricular cells obtained from the hearts of guinea pigs. These workers found that the inhibition of barium currents by calcium ion and the inhibition of sodium currents by calcium ion resulted, in each case, in a very different estimated dissociation constant for the calcium ion-channel binding site complex. Their results, therefore, are inconsistent with the notion that calcium competes with these other cations for a single channel binding site. In addition, they measured the total current produced by mixtures of calcium and barium ions placed in the extracellular medium. The two divalent ions were mixed in various proportions, although their total concentration was held constant at 10 mM. Under these conditions, the total current passing through a population of single ion channels (i.e. channels in which only one ion is present within the pore at any given time) would be expected to be higher than the current produced by the presence of 10 mM of the slower moving cation alone. Hess and Tsien found, instead, that the measured current reached a minimum level when the cation mixture was 70% barium ion and 30% calcium ion.

In order to account for the experimental data they obtained, these investigators envisioned the operation of a single file calcium channel that possesses a sequence of three energy barriers within its aqueous pore. Moreover, the three energy barriers are separated by two distinct energy wells (or two binding sites). Such a channel may contain a single cation that occupies either one of its two energy wells or it may contain two cations simultaneously that occupy both energy wells. When both energy wells are occupied, there is a significant repulsive force between the two cations that facilitates the evacuation of a cation from one or the other of the two energy wells into the surrounding medium (i.e. evacuation into the extracellular medium from one energy well and into the cytosol from the other). The experimental data obtained also dictate that this channel must interact with calcium ions somewhat differently than it does with barium ions. Two differences were built into the hypothetical model of the calcium channel. First, the two energy wells that may be occupied by calcium ions were considered to be deeper than those occupied

by barium ions (i.e. calcium ions have a relatively greater affinity for the two binding sites than do barium ions). Second, the rate of movement of calcium ions through the channel was considered to be lower than that of barium ions (i.e. calcium ions must traverse a higher energy barrier [selectivity filter] during their passage through the channel).

Given these structural and functional properties of the calcium channel, one can readily explain the unusually low level of total current flow in the presence of 7 mM barium ions and 3 mM calcium ions. In qualitative terms, the interaction between these extracellular cations and the calcium channels will produce a variety of cation-calcium channel complexes. One of these complexes, which will be present in some fraction of the total population, will consist of a channel in which the energy well closer to the extracellular medium will be empty whereas the energy well closer to the cytosol will be occupied by a calcium ion. Extracellular barium ions that approach the unoccupied energy well of such a channel will establish a repulsive force between itself and the calcium ion occupying the second energy well or binding site. Since calcium ions have a higher affinity for these binding sites than do barium ions, it is likely that the barium ion, rather than the calcium ion, will be repelled and thus will be unable to react with and occupy the empty site. In effect, the presence of calcium ions will cause the barium ions to exhibit a lesser affinity for *unoccupied* channel sites than they would have had calcium ions been absent. As a consequence, the level of current generated by the 7 mM barium ions, in this situation, will be lower than one would expect from 7 mM barium ions that were merely competing with 3 mM calcium ions for open channel sites in single ion channels. This model of the calcium channel also explains why sodium ion, which exhibits a low affinity for the calcium channel binding sites, appears to be an impermeant ion in the presence of calcium ions, but readily permeates the channels in the absence of calcium ions. (12, 13).

Ventricular cells from guinea pig heart are not the only cells that appear to possess multi-ion calcium channels (i.e. a channel that may, at any instant, contain more than one ion in its pore). Evidence for multi-ion calcium channels has also been found in skeletal muscle fibers (13) and rat brain synaptosomes (14). Channels such as those that reside in ventricular cells from the guinea pig heart (multi-ion channels) do not adhere to a simple hyperbolic relationship between the current flow and the extracellular calcium ion concentration. Yet this type of relationship, which is usually characteristic of saturable single ion channels, was observed by several investigators who studied calcium channels in barnacle muscle (7, 10). Hille has pointed out that multi-ion channels may appear to obey such a relationship if the range of extracellular ion concentrations examined is not sufficiently broad (9). On the other hand, there may, indeed, be fundamental differences in ion permeation mechanisms among

various calcium channels that reside in different types of cells or in different animal species.

Types of Calcium Channels

Regardless of the mechanism by which ion permeation occurs in calcium channels, those cations that have the capacity to traverse the selectivity filter and pass through the channel pore can only do so when the channel is in an open conformation. Calcium channels assume this open conformation when conditions are suitable and an appropriate stimulus is applied (see below). In the absence of these essential elements the gating mechanisms of calcium channels maintain the great majority of the channels in a closed conformation. Although it is difficult to generalize, calcium channels found in a number of different types of cells appear to have a gating mechanism that is similar to that of the sodium channel in that it operates to transform the channel into any one of three different conformations or states (15–19). One of these is the open conformation and is referred to as the activated state. The other two are closed conformations and are referred to as: (*a*) the deactivated state and (*b*) the inactivated state.

Based on the type of excitatory stimulus required to convert a channel to the activated state, calcium channels have been divided into two broad groups (20–22). One group is voltage sensitive and will convert to the activated state when the membrane potential has been reduced to an appropriate level. The other group appears to be closely associated with specific receptors in the plasma membrane. Its conversion to the activated state can be brought about by interactions between these receptors and neurotransmitters or hormones that can activate the receptor molecules. Many excitable cells possess both types of calcium channels in their plasma membranes.

Voltage-Dependent Calcium Channels

ACTIVATION By employing an experimental technique and procedure that will rapidly change the membrane potential and hold it to any desired level (voltage clamp technique), it has been possible to examine the relationship that exists between the level of the membrane potential and the magnitude of the current flowing through voltage sensitive calcium channels. Experiments (23–26) have shown that the membrane potential must first be reduced to some threshold point (i.e. brought to some more positive level) before any calcium current becomes detectable. The voltage change required is usually greater than that needed to turn on the membrane depolarizing sodium current (25). As the membrane potential is made increasingly more positive beyond the threshold point, an increasingly larger inward calcium current will be induced (23–25). This progressive increase in calcium current is a consequence of a progressive

increase in ionic conductance (i.e. decrease in electrical resistance) that reflects the activation of increasing numbers of calcium channels (25, 27). At the same time, part of the inwardly directed driving force, namely the interaction between calcium ions and the electric field within the membrane, will become weaker as membrane potential becomes more positive (25, 27). This factor will serve to reduce the flow of inward current. As long as the changes made in the membrane potential are moderate, increases in ionic conductance appear to have a greater influence on current flow than do decreases in driving force. This is evidenced by the progressive increase in calcium current. At some point, however, the change in membrane potential will become large enough to activate a relatively high percentage of the calcium channel population. Further elevations in the positivity of the membrane potential should then have an opposite effect; that is, the continued reduction in driving force should exert a greater influence on current flow than should continued small increases in ionic conductance. In keeping with these concepts, experimental investigations have demonstrated that as membrane potential is made increasingly more positive the inward calcium current will rise progressively until it reaches a peak magnitude (23–25). From that point on, it will fall progressively (23, 25).

The manner in which depolarization (increase in positivity) of the cell membrane increases the conductance of voltage-sensitive calcium channels relates to the stochastic behavior of calcium channels (28, 29). At any given membrane potential all calcium channels in the membrane will fluctuate between the deactivated state and the activated state. In general, a channel will be either in a closed conformation or in a fully activated conformation, although patch clamp studies have demonstrated that channels may, in some cases, exhibit more than one activated state in which there are differing magnitudes of current flow (28). It follows that any single calcium channel observed over some span of time will be in an open conformation for part of that time and in a closed conformation for part of that time. In any single instant there will be some probability that the channel will be in the activated state or in the deactivated state. When dealing with a population of calcium channels in which all individual units behave in the same fashion, the probability that any single channel will be in the activated state translates into an average or mean fraction of the total population that will be in the activated state (28). An adjustment in membrane potential to a more positive level will, therefore, increase the mean fraction of a calcium channel population that exists in the activated state (and the mean ionic conductance) expressly by increasing the probability that individual channels will assume the activated conformation.

The transformation of a channel from the deactivated state to the activated state appears to require the repositioning of charged gating particles or components within the channel (5). The process is both voltage dependent and time dependent (25). Hagiwara and Ohmori (31) determined the rate at which

channel activation rose to a new steady state level following a change in membrane potential to a more positive level. The measurements were made on calcium channels in clonal cells (GH_3) isolated from a rat anterior pituitary adenoma. Their results disclosed a process in which the fraction of activated channels in the total population increased progressively along a complex curve with respect to time. An analysis of the curve showed it to be consistent with a mechanism of activation that included the following series of reversible reactions:

$$C_1 \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} C_2 \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} O$$

where C_1 represents the density of channels (i.e. the number of channels per unit area of membrane) that are in an initial deactivated state; C_2 represents the density of channels in a second conformation, in which the channels are still in a deactivated state; O represents the density of channels in an activated state; and the quantities (α_1), (α_2), (β_1) and (β_2) represent first order rate constants. Moreover (α_1) equals (α_2) and (β_1) equals (β_2). Under these conditions, the mean fraction of the channels that will be in each of the three conformations, under steady state conditions, will depend upon the ratio of the first order rate constants, (α/β). If, for example, (α/β) equalled 2, the mean distribution observed under steady state conditions would be: approximately 14.3% of the population in state C_1; approximately 28.6%, in state C_2, and approximately 57.1%, in state O. Moreover as discussed above, these data indicate that in a uniform population of channels, each individual channel would have a 57.1% chance of being in the activated state in any single instant of time. The increased probability that a calcium channel will be found in the activated state following a reduction in membrane potential is, therefore, the consequence of an increase in the ratio (α/β) that is induced by the voltage change.

Although the probability factor and its underlying determinant, the ratio (α/β), govern the proportion of time that a single channel will be in the activated state, these factors do not reveal the number of times that a channel will fluctuate between states in any designated time frame. The latter process is regulated by the absolute magnitudes of the rate constants (α) and (β) (29). The operation of a large (β), for example, will mean that the transformation of a channel from state O to state C_2 will occur at a rapid rate. Thus, a channel, after assuming the open state conformation, will remain in that state for an average length of time equal to the inverse of (β). Similarly, the state C_1 will have an average time span equal to the inverse of (α) and the state C_2 will have an average time span equal to the inverse of ($\alpha + \beta$). In more general terms, forward and backward transformations that occur rapidly lead to an increased frequency of fluctuations. When considering a whole population of channels,

comparatively large first order rate constants will speed the approach to a new steady state level, once the appropriate stimulus has been applied (29).

Reuter (30) has noted that voltage-dependent calcium channels typically open in bursts and that burst lengths increase with membrane depolarization. However, the mean duration of channel openings is only moderately voltage dependent. This observation fits well with the notion of a three state model in which the calcium channel fluctuates for a time between a closed state (C_2) and an open state (O) (bursts of activity) and then between one closed state (C_2) and another closed state (C_1) (interval between bursts). It is also consistent with the concept that a reduction in membrane potential induces large increases in the magnitudes of the alpha rate constants, but only moderate changes in the magnitudes of the beta rate constants. The resulting increase in the ratio (α/β) increases the probability that the channel will convert to the activated state and thereby increases the mean ionic conductance and the mean inward calcium current that flows through a population of calcium channels. By contrast, the amplitude of the inward calcium current conducted through a single open channel will be reduced somewhat by membrane depolarization because the inward driving force has been diminished.

Calcium channels in tissues other than GH_3 cells appear to be activated via a similar two step process (i.e. transformation of C_1 to C_2 and C_2 to O). Nonetheless, evidence for a two step gating mechanism has not been a consistent finding. Studies carried out on a variety of cell types have characterized the gating mechanism of calcium channels as a process consisting of a single reversible transformation from a closed to an open state up to as many as six reversible transformations (5, 15). Hagiwara and Ohmori (31) contend that these findings may, indeed, indicate that there are several different types of calcium channels in biological tissues or, alternatively, that the experimental procedures used to characterize the activation process did not produce equally reliable data in all cell types.

In a study performed by Fenwick et al (32) on chromaffin cells, the concept of a two step transformation was retained, but the rate constants were found to have different magnitudes. At a membrane potential of -5mV, and an extracellular concentration of 95mM Ba^{++}, the rate constant (α_1) was found to be equal to 61 s^{-1}, (α_2) equalled 345 s^{-1}, β_1 equalled 606 s^{-1} and (β_2) equalled 1230 s^{-1}. This model defines a gating process in which individual channels exhibit bursts of activity, characterized by relatively high frequency fluctuations between a closed and an open state (i.e. fluctuations between C_2 and O). This activity is interspersed with long periods of quiescence (i.e. fluctuations between C_2 and C_1). The comparatively low magnitude of (α_1) is largely responsible for the long periods of quiescence.

Hess et al (33), working with calcium channels in cardiac muscle cells, uncovered three different modes of gating behavior. At a test membrane

potential in the moderately negative range, individual channels were observed to exhibit brief openings which occurred in rapid bursts. The gating mechanism underlying these activation-deactivation events involved the two step transformation process, $C_1 \rightleftharpoons C_2 \rightleftharpoons O$. A probability factor of .3 or less was also noted. This type of channel behavior was labeled mode 1. On occasion, although very infrequently, the channels underwent a spontaneous, short-lived, reversible modification. During this brief period the two step activation process was still operative, but the magnitudes of the first order rate constants were significantly altered. As a result the channels remained in the open state for relatively long periods of time and were closed for only short periods of time. The probability factor rose to a magnitude greater than .7. This type of channel behavior was labeled mode 2. At other times individual channels underwent some sort of modification that caused them to remain closed for an extended period of time. This quiescent pattern was labeled mode 0. Calcium channels in several other cell types have also been found to exhibit a mixture of modes 0, 1, and 2 (33, 34).

Variations in gating behavior detected in calcium channels of GH_3 cells (derived from a rat pituitary adenoma) were interpreted as having an entirely different basis (35). In this case, the differences in gating behavior were attributed to the operation of two distinctly different populations of calcium channels in the same cell. This conclusion was reached, in part, by quickly changing the membrane potential from a level of +10mV to a level of −80mV and measuring the time required for the calcium channels to close. Speed of closure was determined by monitoring the time dependent decay of the calcium "tail" current. It was found that the "tail" current decayed in two phases. One of these, the fast phase, had a time constant (inverse of rate constant) of 110 μsec; the other, the slow phase, had a time constant of 2.7 msec. These data may be interpreted to indicate that some fraction of the calcium channels in the cell membrane closes at a rapid rate while another fraction closes at a relatively slow rate. Further work showed that the fast deactivating fraction differs from the slow deactivating fraction in several respects. Relative to the slow deactivating channels, the fast deactivating channels are activated slightly more rapidly, are activated at more positive voltages, have a greater barium to calcium selectivity ratio, and are considerably more resistant to inactivation by a prolonged depolarizing pulse. These findings were taken as evidence for the existence and operation of two distinctly different populations of calcium channels in the same cell. Multiple types of calcium channels have also been uncovered in chick dorsal root ganglion cells (36), neuroblastoma cells (37, 38), Neanthes egg cells (39), as well as other kinds of cells.

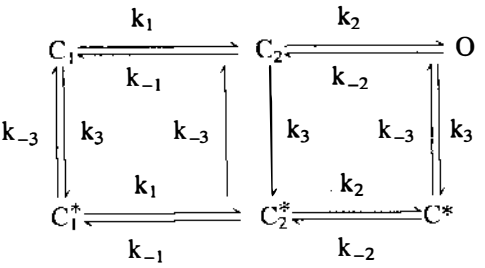
INACTIVATION It is clear from the evidence available that there are a number of different types of voltage-dependent calcium channels, each reacting to a

reduction in membrane potential in its own unique fashion. In all cases, however, a reduction in membrane potential serves to elevate the conductance of a population of calcium channels. The elevation in conductance is a direct consequence of the opening of channel gates. If the reduction in membrane potential is maintained for some period of time, it may not necessarily lead to a sustained high level of channel conductance. The calcium channels may undergo a gradual modification that causes them to exhibit increasingly lower levels of conductance with time. The gradual diminution in ionic conductance seen in the face of a persistent excitatory stimulus is brought about by a mechanism that transforms the channel into a conformation termed the inactivated state. A calcium channel in an inactivated state, like one in a deactivated state, is in a closed conformation and will not permit a flow of ions through its aqueous pore. The specific stimulus that initiates the conversion of a calcium channel from the deactivated or activated state to the inactivated state differs in different types of calcium channels. Current evidence indicates that there are four major types of voltage-dependent calcium channels based on the distinctive factors or conditions that induce or do not induce each of them to assume the inactivated conformation.

In a few cell types the stimulus that initiates the inactivation of calcium channels is the same one that initiates the inactivation of sodium channels. In both cases a time-dependent inactivation occurs when the cell membrane has been adequately depolarized. It has been shown that an increasingly greater reduction in membrane potential, within a restricted voltage range, will generate an increasingly larger rate constant involved in the speed of transformation of calcium channels into the inactivated state (40, 41). At membrane potentials more negative than those in the restricted voltage range (within limits) the rate constant appears to have a very small magnitude; at membrane potentials less negative than those in the restricted voltage range the rate constant has an invariable high magnitude (40, 41). In addition, the mean fraction of the calcium channels that assume the inactivated state during steady state conditions will become increasingly greater as the reduction in membrane potential is increased within a specified voltage range (40, 42). At potentials more negative than this specified voltage range a negligible number of calcium channels assume the inactivated state; at potentials less negative than the specified voltage range essentially all the calcium channels are transformed into the inactivated state (40, 42). In general, the voltage-dependent inactivation of calcium channels exhibits slower kinetics and occurs within a more positive membrane potential range than does the voltage-dependent inactivation of sodium channels (43).

In many tissues, voltage-sensitive calcium channels, although rapidly activated by reductions in membrane potential, are not subsequently inactivated by a maintained low level of membrane potential. The inactivating stimulus in

these channels is free intracellular calcium (43–46). Eckert and Chad (43), in an effort to account for the kinetic behavior of the calcium-dependent inactivation process, have developed a working model of the reactions involved. In their scheme, free intracellular calcium ions react reversibly with calcium channel binding sites. The stoichiometry is 1:1 (no cooperativity). Each calcium ion-calcium channel interaction leads, hypothetically, to an enzymatic dephosphorylation of a channel component and thereby produces an inactivated channel (43, 47). Intracellular calcium ions are assumed to have an affinity for channel binding sites whether the calcium channel is in the activated or the deactivated state. Thus, the following interactions are thought to occur:



where C_1, C_2 and O have their usual meanings (see above), the asterisks denote inactivated states and the various k's represent first order rate constants. The horizontal transitions in the above scheme are voltage-dependent; the vertical transitions are calcium dependent.

Since the inactivation of calcium channels is purported to be a consequence of a reversible interaction between calcium channel binding sites and intracellular calcium ions, the degree to which a calcium channel population will become inactivated should be dependent upon the concentration of free calcium ions in the cytosol. The mean fraction of the channel population that assumes the inactivated state, in this case, will be a hyperbolic function of the free calcium ion concentration in the cytosol. Although the intracellular calcium ion concentration of excitable cells in the resting state is usually very low, one can induce a large increase in the intracellular level of calcium ions by lowering the membrane potential sufficiently to initiate a substantial influx of calcium into the cell. If the resultant influx of calcium ions produces a progressive rise in the intracellular calcium ion concentration that, ideally, is linear with respect to time, then the mean fraction of the calcium channel population that becomes inactivated will increase as a hyperbolic function of time. This means that the rate of inactivation of calcium channels, shortly after the cell membrane has been depolarized, will be relatively rapid. However, as time passes and the free calcium ions in the cytosol reach a high level, further inactivation of the calcium channels will proceed relatively slowly.

The hyperbolic increase in the fraction of inactivated calcium channels that occurs with the progression of time may be viewed as the basic dynamics of the calcium-dependent inactivation process. It can, however, be modified by a number of factors. One such factor is the temporal variation in the inward calcium current. For a comparatively short span of time immediately following an excitatory reduction in membrane potential a time-dependent elevation will occur in the average number of calcium channels that assume the activated state. During this period, the inward calcium current will quickly rise from a negligible level to a relatively high level. Once this change has been achieved the current flow, in the absence of any other modifying influence, will display a constant amplitude, signifying the attainment of new steady-state conditions for the activation of the calcium channels. The attainment of these steady-state conditions cannot be detected experimentally because the calcium-dependent inactivation reaction, superimposed on the activation process, causes a continuous diminution of the inward calcium current. As a result, the calcium current, following membrane depolarization, will exhibit a sharp rise in amplitude, reach a peak (below the steady state level for activation) and then exhibit a relatively slow fall in amplitude. This swing in the calcium current is unlikely to produce an increase in the intracellular calcium ion concentration that is linear with respect to time. In addition, there are several intracellular processes and reactions that undoubtedly alter the free calcium ion concentration in the cytosol. These include: (*a*) diffusion of calcium ions away from their sites of interaction with calcium channels, (*b*) interactions between calcium ions and intracellular binding sites, and (*c*) sequestration and/or extrusion of calcium ions by energy-dependent calcium pumps (43). Despite the influence of these modifying factors, the rate of the calcium-dependent inactivation process has been observed to be biphasic, i.e. characterized by two different time constants (43, 48, 49). Initially, one sees a fast phase of inactivation that appears to reflect, at least in part, the steep limb of a hyperbolic curve and, subsequently, a slow phase of inactivation that seems to reflect, in part, the shallow limb of a hyperbolic curve. If, as a result of the progressive inactivation of calcium channels, the inward calcium current dwindles to a point that the rate of entry of calcium ions equals the rate of removal of calcium ions from the site of action, the calcium-dependent inactivation process will display a steady state mode (44).

Studies have shown that the entry of strontium ions or barium ions into the cytosol of various cell types is less effective in producing calcium channel inactivation than is the entry of calcium ions. Barium ion produces much less inactivation than does calcium ion and strontium ion is intermediate (43, 46, 50–53).

Calcium channels in which inactivation is both voltage-dependent and calcium-dependent have also been uncovered. The channels of snail neurons (23)

and possibly cardiac muscle (15) fit into this category. Eckert and Tillotson (52) have speculated that in cells that contain this type of calcium channel, the intracellular calcium ion, by binding to the inner surface of the cell membrane, may change the surface potential. The change in surface potential would then bring about a hyperpolarizing displacement of a voltage-sensitive gating process. Such a mechanism would be both voltage dependent and calcium dependent.

Lastly, there is a group of voltage-dependent calcium channels in which neither the voltage-dependent nor the calcium-dependent inactivation process operates or operates effectively. The channels in this group are, at most, only slightly inactivated or very slowly inactivated. Channels that exhibit these characteristics have been found in squid synaptic terminals (55, 56), adrenal chromaffin cells (57), photoreceptor inner segments (58), nerve cell bodies of Helix (59, 60), frog skeletal muscle fibers (61), type II channels in the egg cell of the worm, *Neanthes arenacedentata* (39) and other cell types.

Receptor-Operated Calcium Channels

Those calcium channels closely linked to membrane receptors and activated by agonist-receptor interactions have been labeled receptor-operated channels. Although they play an important role in the functional behavior of smooth muscle and many secretory cells, the receptor-operated calcium channels have received much less scrutiny than have calcium channels activated by membrane depolarization. Because of the lack of experimental data characterizing receptor-operated calcium channels, their specific properties remain largely unknown.

PHARMACOLOGY OF CALCIUM CHANNELS

Indirect Acting Agents

A wide variety of chemical substances can affect calcium channels indirectly by modifying the conditions that regulate calcium channel function. These include drugs that induce or prevent changes in membrane potential, drugs that alter the extracellular and/or intracellular concentrations of calcium ion, drugs that modify biochemical reactions involved in the operation of calcium channels (i.e. phosphorylation and dephosphorylation reactions, etc.) and drugs that activate or prevent the activation of membrane receptors closely linked to calcium channels. Often a single agent will elicit a series of cellular responses affecting more than one type of calcium channel. For example, acetylcholine, by complexing with and activating cholinergic receptors in a smooth muscle cell, will activate the receptor-operated channels that are directly associated with these membrane receptors. The resulting increase in inward calcium current as well as increases in other ionic currents induced by the activation of

cholinergic receptors serve to reduce the membrane potential (20). The latter effect may, in turn, be of sufficient magnitude to initiate the activation of voltage-dependent calcium channels that reside in the same smooth muscle cell (20). It is of interest to note that the indirect modification of voltage-dependent calcium channel behavior by various neurotransmitters and hormones constitutes, in many instances, the normal physiological mechanism for regulating calcium channel function.

Inorganic Ions

A number of inorganic cations can interact with calcium channels in a direct and reversible manner. As a consequence, they exert a strong influence on the operation of activated channels. The type of influence that a particular inorganic cation will exert depends upon two factors. One is the affinity of the cation for the channel binding site involved in the first step in ion permeation (see above). The other is the speed at which the cation travels through the aqueous pore (i.e. the height of the energy barrier [selectivity filter] that the cation encounters) to reach the cytosol. Divalent cations, such as strontium and barium, which exhibit reasonably high affinities for the channel binding site and can traverse the channel pore more rapidly than calcium ion, act as excellent calcium substitutes (25). Depending on the extracellular concentrations employed, they can generate currents either equal to or greater than the one produced by calcium ion. Divalent cations, such as cobalt, nickel, cadmium, and manganese, exhibit reasonably high affinities for the channel binding site, but traverse the channel pore at low or even negligible speeds (25). These cations behave as potent competitive inhibitors of ion permeation through calcium channels. Monovalent cations, such as sodium (62–64), potassium (62–65), and caesium (65) also exhibit an affinity for the calcium channel binding site and can penetrate its aqueous pore. They have the capacity, therefore, to carry substantial currents through calcium channels. The trivalent ion, lanthanum, interacts rather strongly with calcium binding sites and has been found to be an excellent inhibitor of ionic currents conducted by calcium channels (5, 7, 25).

Organic Calcium Channel Blockers and Stimulators

In 1964, Fleckenstein reported that the chemical agents, verapamil and prenylamine, had the same inhibitory effect on cardiac muscle as did the withdrawal of extracellular calcium ions (66). Since that time a large number of organic compounds, of widely different chemical structures, have been found to have a similar inhibitory effect (67, 68). This effect stems, ultimately, from the direct actions of these compounds on calcium channels. The members of this group of compounds were originally called calcium antagonists because their inhibitory effects could be reversed by increasing the calcium ion concen-

tration in the extracellular medium. More recently, they have also been referred to as calcium entry blocking agents or calcium channel blockers.

SUBGROUPS The calcium entry blocking agents have been divided into three subgroups (67). Group I consists of nifedipine and related 1,4 dihydropyridines. Group II contains the calcium blockers verapamil, D600, diltiazem, and diclofurime which, for the most part, have unrelated chemical structures. Group III is made up of diphenylalkylamine compounds such as cinnarizine, fendiline, flunarizine, and prenylamine. The compounds in groups I and II exhibit potent and selective actions on calcium channels in cardiac muscle, whereas group III compounds appear to be less selective, causing a diminution in activity of both calcium and sodium channels in cardiac muscle (69). It has been shown, moreover, that verapamil, D600, and diltiazem, namely group II compounds, have approximately equiactive effects on calcium channels in cardiac muscle and vascular smooth muscle (69), while nifedipine and related dihydropyridines, as well as flunarizine and cinnarizine, exert preferential effects on vascular smooth muscle (68, 69).

SITES OF ACTION The manner in which the organic calcium entry blocking agents inhibit voltage-dependent calcium channels in cardiac cells and other types of cells (nerve, smooth muscle, secretory, etc.) does not appear to be unique. Local anesthetic agents inhibit sodium channels in a similar fashion (70, 71). As a first step, the calcium channel blocking agent presumably enters or crosses the cell membrane in order to gain access to the appropriate site of action in or on the channel (70). This concept is supported by several lines of evidence. First, the calcium blockers were found to be sufficiently lipophilic to penetrate the cell membrane and enter the cytosol (72). Second, a highly polar N-methyl quaternized derivative of D600 was observed to have no effect on the action potential of guinea-pig ventricular myocytes when applied externally, but lowered and shortened the calcium-dependent plateau of the action potential when applied intracellularly (73). On the other hand, D600 produced the latter effect when applied either internally or externally. It was inferred, therefore, that externally applied D600 had to cross the cell membrane before acting (73).

Although the inhibitory effects of the organic calcium channel blockers can be reversed by increasing the extracellular calcium ion concentration, a simple competition between the blocking agent and extracellular calcium for the channel coordination site involved in ion permeation is not a likely possibility. This conclusion is based on experiments that show that cadmium ion blocks barium currents more strongly than it does calcium currents, as would be expected from the relative affinities of these two current-carrying ions for the

coordination binding site (see above), whereas the organic calcium blockers have the opposite effect (74).

Numerous radioligand binding studies have been performed for the purpose of characterizing the channel binding sites that interact with the organic calcium blockers. Often the calcium blockers have been observed to complex with two specific groups of membrane sites; a low affinity-high capacity group and a high affinity-low capacity group (75, 76). In many cases, particularly in those studies performed on smooth muscle, the correlation between the concentrations of blocking agent required to produce functional blockade of calcium channels and the concentrations at which substantial binding takes place was found to be very good (68). Other studies have disclosed a poor correlation. In cardiac muscle, skeletal muscle and brain tissue (68, 4) binding of the inhibitory agent to a high affinity site occurs at concentrations well below those required to affect ion permeation through calcium channels. This finding has been rationalized, to some extent, by the proposal that calcium channels in membrane fragments frequently used for binding studies are in an inactivated state, the conformational state that displays the highest affinity for calcium blockers (67, 70). This proposal seems to be borne out by work performed by Kunze and Hawkes (75). These investigators measured the binding of ^3H nitrendipine to membrane preparations of PC12 (pheochromocytoma) cells. They found that the apparent dissociation constant (K_D) and the total number of binding sites (B_{max}) were similar to values obtained in whole cells. When a calcium current was produced in the whole cell by a voltage step to + 10 mV from a holding potential of −100mV, the inhibitory dose 50 (IC_{50}) of nitrendipine was 10^{-6} M. When the holding potential was lowered from − 100 mV to −20 mV, a membrane potential at which the steady state inactivation of calcium channels rises to 50%, the inhibitory dose 50 (IC_{50}) of nitrendipine decreased to 130 nM. They also calculated the dissociation constant for the binding of nitrendipine to inactivated channels and found it to be 8 nM, a value very similar to the K_D for the nitrendipine-high affinity site complex. Moreover, the total number of calcium channels calculated from electrophysiological studies agreed with the value calculated from data delineating the binding of nitrendipine to high affinity sites. On the other hand, a study carried out on intact frog sartorius muscle led to the conclusion that this muscle contains many more dihydropyridine binding sites than it does calcium channels (78).

The binding of dihydropyridine derivatives to membrane sites may be altered by introducing other organic calcium blocking agents. A number of calcium blockers including verapamil and diltiazem appear to interact with a common membrane site different from but allosterically linked to the dihydropyridine binding site (79). By interacting with the common site verapamil can decrease, whereas diltiazem can increase, the binding of a dihydropyridine blocking agent to its membrane sites (79).

Despite the functional antagonism that exists between extracellular calcium ions and calcium blockers, the binding of ^3H dihydropyridine derivatives to membrane sites is not reduced by low to moderate concentrations of calcium ion. Only very high levels of inorganic divalent ions have been observed to reduce drug binding (80, 81). In addition to these observations, Krafte et al (82) have demonstrated that an elevation in the concentration of Ca^{++}, Sr^{++}, Ba^{++}, or Mg^{++} will cause a depolarizing shift in the calcium channel inactivation-voltage curve both in single cells and in multicellular Purkinje fibers; that is, the fraction of the channel population found in the inactivated state at a given membrane potential is reduced. The divalent ion-induced shift in the inactivation curve also occurs in the presence of nisoldipine. Since the magnitude of the shift is the same order whether or not nisoldipine is present, these investigators concluded that the antagonistic actions of the divalent ions are due, in part, to a nonspecific modification of a negative membrane surface charge.

MODES OF ACTION

Use-dependent blockade A prominent feature of the blockade produced by calcium channel blocking agents is use dependence (70, 71, 74, 83–85). In a cell stimulated repeatedly (i.e. one whose membrane is depolarized repeatedly for short periods), a drug exhibiting use dependence will induce an increasingly greater degree of inhibition with each successive stimulation until a steady-state level of inhibition is reached. Use dependence will theoretically be observed when an inhibitory agent exhibits little or no affinity for an ion channel in the deactivated state, but interacts to a significant degree with an activated or an inactivated ion channel or both (70, 71, 86). The activation and inactivation of ion channels initiated by membrane depolarization will, therefore, lead to an inhibitory agent-ion channel interaction and a consequent blockade of ion permeation. If the duration of the depolarization is very brief, a state of equilibrium for the interaction may not be achieved. Moreover, a return to resting conditions will be followed by a dissociation of bound inhibitory agent that may or may not reach completion before the next membrane depolarization is elicited. In the latter instance some bound inhibitory agent will be retained and more will become bound during the second depolarization. This will result in a greater degree of blockade than had occurred during the first depolarization. The process will continue until the quantity of inhibitory agent that becomes bound to activated and/or inactivated channels during a period of activity (i.e. when the membrane is depolarized) is equal to the quantity dissociating from deactivated channels during a period of rest (i.e. when the membrane is polarized to resting level). At that point the blockade of ion permeation will remain at a constant level.

Given these circumstances, a prerequisite for demonstrating use-dependent

blockade should be the selection of a frequency of stimulation high enough to prevent the complete dissociation of the inhibitory agent-ion channel complex during a period of rest. The selection of a suitable frequency will obviously depend upon the rate at which the blocking agent in question reacts with and dissociates from the ion channels under the conditions that prevail. There are reports in the literature that do, indeed, show that an increase in the frequency of stimulation enhances the progression of use-dependent blockade of calcium channels induced by several different kinds of organic calcium blockers (83, 84, 85) and, further, that the use of very low frequencies essentially abolishes this type of blockade (83, 85).

Use-dependent blockade is also influenced by the magnitude of the membrane potential during periods of rest. As the negativity of the membrane potential is reduced, within a limited voltage range, the extent to which inactivated and activated cardiac calcium channels are transformed to the deactivated state will be reduced (5, 23–25, 30, 31, 40, 42). In addition, the presence of a calcium blocking agent that exhibits a preferential affinity for activated and/or inactivated channels will produce a further shift in the steady-state distribution of ion channels between the deactivated state and other conformational states; that is, the fraction of ion channels found in the deactivated state at a given membrane potential will be reduced (67, 86). Consequently, the dissociation of an inhibitory agent from ion channels should be more rapid in a strongly polarized membrane (one with a large negative membrane potential) during periods of rest than in a membrane brought to a less negative potential during periods of rest (71). In keeping with these concepts, hyperpolarization of the membrane has been shown to reduce or abolish use-dependent blockade of calcium channels induced by calcium entry blocking agents (83, 85, 87).

An effort has been made to determine which conformational states of the calcium channel form firm complexes with the channel blockers. In a study carried out by Lee and Tsien (74), a barium current was elicited in ventricular myocytes by a prolonged depolarizing pulse (600 msec). In the absence of any inhibitory agent this current underwent a slight inactivation. The presence of nitrendipine or D600 hastened the decay of the barium current. This effect was attributed to a blockade of activated channels. Diltiazem did not hasten the decay of the barium current and was judged to have little effect on activated calcium channels. Kanaya et al (84) showed that diltiazem and verapamil produced a stronger block of calcium current when the duration of a depolarizing conditioning impulse was increased from 100 msec to 2–3 sec. Moreover, the diltiazem-induced block could not be enhanced by increasing the voltage of a 30 msec conditioning pulse (too little time for much inactivation to occur) from −30 mV to +80 mV. Based on these data, the investigators concluded that the affinity of diltiazem for inactivated channels is greater than its affinity

for activated channels. These studies as well as others indicate that some calcium blockers such as diltiazem interact primarily with inactivated calcium channels, whereas blocking agents such as verapamil, D600, and nitrendipine also interact with activated calcium channels.

Actions of dihydropyridine compounds In some respects, the pharmacological actions of the dihydropyridine compounds differ sharply from those of other blocking agents. Members of the dihydropyridine group such as nitrendipine (74), nisoldipine (88), nifedipine (89), and nimodipine (89) have been observed to produce a blockade of calcium current that fails to exhibit more than minimal use dependency. In addition, Kass (88) has reported that the blockade of calcium current in cardiac tissue induced by nisoldipine, unlike that produced by D600, could not be removed by holding the membrane at a relatively negative potential for up to 2 minutes. There is, however, an indication that the dihydropyridine blockers dissociate from calcium channel sites relatively quickly (74). On this basis, the suggestion has been made that stimulation frequencies employed in past studies may have been too low to demonstrate use-dependent block with the dihydropyridines (70). Additional work will be needed to ascertain the validity of this contention.

The dihydropyridine derivatives, Bay K 8644 and CGP 28392, have been shown to have the unique capacity to enhance rather than block current flow through calcium channels (90, 91). In Purkinje fibers, Bay K 8644 induced a shift in the peak inward current-voltage curve (see above) toward a more negative voltage range and increased peak inward currents (92). Kokubun and Reuter (91) found that this type of drug increases single channel current somewhat, but the primary basis for its excitatory effect is a prolongation of the mean open time of the calcium channel. The characteristic burst behavior seen in control experiments was transformed, in the presence of the drug, to a much longer channel opening with only a few, brief interruptions. As a result, the fraction of the total calcium channel population that assumed the open channel conformation at some specified membrane potential was substantially increased (i.e. the probability factor was increased). The presence of Bay K 8644 or CGP 28392 also shortened the time constant for the brief closures that occur during bursts and lengthened slightly the time constant for the long interval between bursts. It is of interest that the dihydropyridine blocking agents, nimodipine, nitrendipine, and nifedipine, like Bay K 8644 and CGP 28392, increase the time constant for the long intervals of closure between bursts. With the former agents, however, the increase in the duration and number of long intervals of closure appear to be more pronounced than any modification induced in the open state of the channel. The net outcome, therefore, is a reduction in overall calcium current (91). If a cardiac cell preparation is depolarized or stimulated at a high rate the excitatory agent Bay K 8644 will

exert an inhibitory effect on the calcium channels (92). High concentrations of the drug will also inhibit the channels (92). Consequently, it has been referred to as a partial agonist (92).

Hess et al (33) consider these effects of the dihydropyridines to be a reflection of their modulating action on the naturally occurring behavior of calcium channel gates. As indicated previously (see above) these investigators discovered that the gating mechanism of a calcium channel may undergo spontaneous transitions among three distinctly different modes. Mode 1, characterized by brief openings that occur in rapid bursts, is the most probable gating form that calcium channels assume. However, in the presence of an inhibitory dihydropyridine such as nimodipine, the channel gates display an increased likelihood of converting to mode 0 which leads to prolonged periods of channel closure. By contrast, Bay K 8644, an excitatory dihydropyridine, enhances the likelihood that a channel will exhibit mode 2 behavior, namely long lasting channel openings and very brief channel closings. They also noted that any particular drug such as nitrendipine or Bay K 8644, at an appropriate concentration, may increase the occurrence of both mode 0 and mode 2 behavior. Whether the resultant effect is inhibitory or excitatory will depend upon which mode change is the more dominant. These observations led to the contention that dihydropyridine compounds cannot possibly modify calcium currents by physically plugging the channel pore. Such a mechanism would not explain the agonist-like (excitatory) component of nitrendipine's net inhibitory action. The idea that agonist and antagonist actions of nitrendipine may occur at different binding sites seems improbable because Bay K 8644 competes for all nitrendipine binding sites (93). The experimental results point, instead, to a mechanism of action involving the modulation of calcium channel gating. It would appear, moreover, that the antagonistic actions of the dihydropyridine agents simulate the action of the voltage-dependent inactivation process (conversion to mode 0) and serve to reinforce and to be reinforced by the action of this potential-dependent inactivation process.

The effects of dihydropyridine agents have also been studied in GH4Cl pituitary cells that possess two different populations of calcium channels. One set of calcium channels undergoes little or no inactivation, while the other set inactivates rapidly. It was found that nimodipine preferentially blocked and Bay K 8644 preferentially enhanced the current in the noninactivating calcium channels (94).

Neurohormones

The membranes of cardiac cells contain neurohormone receptors known to play an important regulatory role in the operation of voltage-dependent calcium channels. Excitation of beta adrenergic receptors in these membranes increase (95), whereas excitation of muscarinic cholinergic receptors decrease (96, 97)

the amplitude of calcium currents initiated by membrane depolarization. Drugs such as norepinephrine, epinephrine, and isoproterenol which excite beta adrenergic receptors are, therefore, powerful calcium channel stimulants. The sequence of events that leads to an increase in the calcium current by these drugs begins with a reversible interaction between the drug and the membrane receptor. This interaction induces the activation of adenylate cyclase. As a consequence, the level of cyclic AMP in the cytoplasm increases. The elevation in the level of cyclic AMP activates a cyclic AMP-dependent protein kinase resulting in the phosphorylation of membrane proteins presumed to be part of or linked, in some manner, to potential calcium channels (31, 98, 99). In ventricular heart cells of the frog, phosphorylation of these critical proteins increased the average number of voltage-sensitive calcium channels that could be transformed to the open state. The net result was a larger inward calcium current. Single channel current remained unaltered and the probability that a channel would assume the activated conformation at some given membrane potential increased to a moderate degree (about a 50% increase). The major change was an increase in the average number of functional calcium channels in the cell (about a threefold increase at a membrane potential of +10 mV) (100).

The reduction in calcium current brought about by the interaction of cholinergic agonists, such as acetylcholine, and cholinergic receptors in cardiac cells may possibly involve cyclic GMP (101–103). However, the series of reactions that lead to this inhibitory effect is not yet clearly understood.

CALCIUM CHANNELS IN SMOOTH MUSCLE

Evidence for Two Calcium Channel Types

The calcium ions that initiate a mechanical response in smooth muscle cells may be mobilized from calcium reservoirs within the cell and/or from the calcium pool (both free and loosely bound calcium) in the external environment (22). There are two major routes by which calcium ions from the latter pool may traverse the cell membrane and thereby gain access to the contractile apparatus. One pathway is the receptor-operated calcium channel system; the other is the voltage-dependent calcium channel system (20–22). To some extent, extracellular calcium ions may also reach the cytosol via a small membrane leak (22) and perhaps via a sodium-calcium exchange mechanism (104).

Several lines of evidence support the view that receptor-operated calcium channels in smooth muscle cells constitute a separate group of membrane elements that can be distinguished from voltage dependent-channels. In 1958 Evans et al (105) reported that isolated smooth muscle preparations that were completely depolarized in a bathing medium containing a high concentration of K_2SO_4 could still undergo contractions in response to an agonist. Durbin and Jenkinson (106) observed that the size of the contractions induced were directly

related to the concentration of calcium ions in the bathing medium. In addition, Robertson (107) noted that in the depolarized longitudinal muscle from rabbit ileum ^{45}Ca uptake could be markedly increased by acetylcholine. Subsequently, several groups of investigators (108–110) showed that certain normally polarized vascular tissue (i.e. rabbit ear artery, rabbit main pulmonary artery, porcine coronary artery and others) could be stimulated to contract by concentrations of an agonist that did not produce any significant change in membrane potential. However, the extent to which the agonist-induced contractions, under these conditions, resulted from the influx of calcium ions through activated voltage-independent calcium channels or the release of calcium ions from internal stores is not clear. Using a pharmacological approach Meisheri et al (21) demonstrated that, in the rabbit aorta, calcium influx stimulated by a depolarizing high potassium medium could be preferentially inhibited by D600, whereas calcium influx stimulated by norepinephrine could be preferentially inhibited by amrinone. They also observed that the calcium fluxes stimulated by the high potassium medium and by norephinephrine were additive. In sum, these experimental results favor the proposal that receptor-operated calcium channels constitute one population and that voltage-dependent calcium channels constitute another population of membrane elements. Whether or not membrane potential exerts any essential or modulating influence on the activation of receptor-operated calcium channels is still unresolved.

Receptor-Operated Calcium Channels

Golenhofen and colleagues have reported the existence of two different types of agonist- (i.e. acetylcholine, epinephrine) controlled calcium activation systems (111). One, which they labeled the P system, is activated in association with regenerative potential changes in the membrane; the other, labeled the T system, seems to be less dependent upon electrical activity in the membrane. Agonist-activation of the P system elicits phasic contractions in smooth muscle cells; agonist-activation of the T system elicits tonic contractions in smooth muscle cells. The two systems can also be distinguished by their differing responses to various inhibitory agents. The P system is preferentially suppressed by verapamil, D600, and nifedipine, whereas the T system is more strongly inhibited by sodium nitroprusside. Golenhofen notes that the molecular basis of the P-T differentiation is not yet clear. There is evidence to indicate that both systems can operate with calcium ions from a variety of sources. It seems reasonable to assume, however, that the phasic contractions they observed are induced, at least in part, by action potentials that reflect the operation of voltage-dependent calcium channels. These channels are presumably activated by the increase in ionic conductance (supposedly Na and Ca conductance) and consequent membrane depolarization brought about by the agonist.

Little is known about the functional characteristics of receptor-operated calcium channels or about the manner in which they are linked to membrane receptors in the smooth muscle cell. The proposal has been made that a receptor-induced breakdown of inositol phospholipids is responsible for the opening of calcium channel gates (112). The specific membrane substrates that are broken down following an agonist-receptor interaction are phosphatidylinositol and polyphosphoinositides (113). Much experimental data have been generated, some of which support (113–116) and some of which question the validity of (117–119) this proposal. Recently, the metabolic degradation of a fraction of the total inositol phospholipid pool, namely the polyphosphoinositides, has been considered as a possible intermediary event that links receptor activation to a physiological response (113). The polyphosphoinositides of interest are phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5 biphosphate, particularly the latter (113). The smooth muscle of the iris is an example of a cell in which activation of muscarinic, cholinergic, and alpha$_1$ adrenergic receptors induce an extremely rapid breakdown of phosphatidylinositol 4,5 bisphosphate but not phosphatadylinositol or phosphatidylinositol 4 phosphate (120–122). It should be noted, however, that interest in the relationship between the rapid breakdown of phosphatidylinositol 4,5-bisphosphate and calcium mobilization is focused primarily on the release of calcium ions from internal cellular stores (113, 123–125). It stems from observations that inositol 1,4,5-trisphosphate, a hydrolytic product of phosphatidylinositol 4,5-bisphosphate (113), can induce the release of calcium from nonmitochondrial stores in permeabilized leaky pancreatic acinar cells (123) and saponin-treated hepatic cells (124, 126, 127) and from microsomal fractions of rat insulinoma (128). The role of inositol phospholipid turnover, if any, in the external calcium influx often required to sustain a physiological response, is still not understood (113).

Agonist-receptor interactions that lead to an elevation in cyclic nucleotides also have a questionable influence on the influx of calcium ions in smooth muscle cells. Ousterhout and Sperelakis (103) have examined the effects of dibutyryl cAMP, dibutyryl cGMP, and 8-bromo-cGMP on calcium channel function in vascular smooth muscle. Cultured smooth muscle cells were prepared from rat aortas by enzyme dispersion and reaggregation. Calcium-dependent action potentials were elicited in these aortic reaggregates by electrical stimulation in the presence of tetraethylammonium (TEA). They found that .1 mM dibutyryl cAMP depressed and .5–1.0 mM dibutyryl cAMP abolished the TEA-induced action potentials. By contrast, 1 mM dibutyryl cGMP and .1–1.0 mM 8-bromo-cGMP had no appreciable effect on these action potentials. In an intact preparation of rabbit pulmonary artery, however, .1–2.0 mM dibutyryl cAMP was observed to have little or no effect on TEA-induced action potentials. Based on these results, Ousterhout and

Sperelakis suggest that cAMP, but not cGMP, may regulate the function of voltage-dependent calcium channels in rat aortic smooth muscle cells. Obviously, more work is needed to clarify the role that cyclic nucleotides may play in the operation of both receptor-operated and voltage-dependent calcium channels in smooth muscle cells.

Voltage-Dependent Calcium Channels

A number of studies have been performed that collectively indicate that several different types of voltage-dependent calcium channels reside in smooth muscle cells. Working with uterine smooth muscle of the guinea pig, Hamon and Vassort have characterized an inward current that appears to flow through calcium channels that are both activated and inactivated by reductions in membrane potential (129). This inward current has been shown to be sensitive to the inhibitory actions of Mn, Co, La (130–132), and D600 (131, 132) and to be unaffected by drastic reductions in the extracellular sodium ion concentration (131, 133, 134) or by the presence of the sodium channel blocker, tetrodotoxin (131, 135, 136). A relatively small diminution in membrane potential was sufficient to induce this calcium channel current to attain a detectable amplitude, indicating a low threshold of activation. Peak current amplitude occurred at 50 mV depolarization (i.e. a reduction in membrane potential from a resting level of −55 mV to a level of −5 mV). Inactivation of the channels conducting this inward current was investigated by noting the effect that a prior conditioning impulse of one second duration had on the amplitude of the inward current induced by a 38 mV depolarization. These investigators found that conditioning impulses of increasingly greater positive potential levels caused the subsequent current initiated by the 38 mV depolarization to exhibit increasingly smaller amplitudes. Their results demonstrated that the inward conductance underwent steady-state inactivation the level of which was dependent on the membrane potential. The relationship between membrane potential and inactivation of these calcium channels was observed to have a form similar to that used by Hodgkin and Huxley (137) to describe inactivation of sodium channels. Calcium channels in smooth muscle that are rapidly activated and inactivated by reductions in membrane potential are assumed to be involved in the development of action potentials (and consequent increases in muscle tension) in those smooth muscles that exhibit this kind of electrical activity. There is ample evidence to indicate that action potentials in smooth muscle cells can be inhibited by organic (138, 139) and inorganic (140–142) calcium blockers, can be modified by variations in the extracellular calcium ion concentration (140–144), and are not greatly affected by drastic reductions in the extracellular sodium ion concentration (140, 143–146) or by the sodium channel blocker, tetrodotoxin (147, 148).

It has been known for many years that many smooth muscle preparations can

also be stimulated to contract by exposing them to a membrane-depolarizing high potassium bathing medium. Some smooth muscles characteristically undergo a monophasic mechanical response; others undergo a biphasic or even more complex mechanical response in the presence of a high potassium medium (20). The longitudinal muscle of the guinea pig ileum, for example, exhibits a biphasic response (149–151). The initial component of the response, termed the phasic response, consists of a very rapid but transitory increase in muscle tension. This tension change is usually followed by some degree of relaxation. Subsequently, the longitudinal muscle will undergo a second, less rapid, but more sustained increase in muscle tension. The second tension change, termed the tonic response, after reaching a peak level, will gradually diminish in amplitude over a prolonged period of time (90–120 min).

Whether a smooth muscle preparation exhibits a monophasic or more complex contraction, experience has shown that these potassium-induced tension changes are usually quite sensitive to the inhibitory actions of both inorganic (149, 151, 152–154) and organic (150, 155–161) calcium blockers. The mechanical response can be prevented by adding a calcium entry blocking agent to the high potassium bathing solution (151, 160) or aborted by adding such an agent after the contraction has been induced (151, 162). It can also be abolished by removing calcium ions from the high potassium bathing solution and reinstated by adding calcium ions back to the solution (150). These findings support the notion that an essential step in the development of a potassium-induced contraction is the influx of calcium ions from the extracellular environment. Additional support for this notion stems from experiments demonstrating that the potassium-induced biphasic mechanical response of the longitudinal muscle from the guinea pig ileum is associated with a cobalt-sensitive biphasic influx of calcium ions. The initial phase of the calcium influx is rapid and very transient; the latter phase is considerably slower and more sustained (152). Data showing that calcium fluxes accompany potassium-induced tension changes have also been obtained in other types of smooth muscle (160, 163).

Based on experiments performed on the longitudinal muscle of the guinea pig ileum, Hurwitz et al (150, 151) have advanced the proposal that a high potassium bathing medium activates at least two different types of voltage-dependent (or potassium-activated) calcium channels in the smooth muscle cell. The data that led to this proposal was obtained, to a large extent, by monitoring changes in muscle tension. A significant disclosure was the finding that a relaxed longitudinal muscle that had been incubated in a calcium-deficient, high potassium bathing medium (140 mM KCl) for 10 minutes or more could be induced to undergo a characteristic biphasic mechanical response by introducing 1.8 mM $CaCl_2$ into the bathing medium. This finding demonstrated that both components of the mechanical response can be elicited in smooth muscle cells that have been depolarized for an extended period of

time. The inference drawn is that the calcium channels associated with this complex mechanical response cannot be rapidly inactivated by large reductions in membrane potential. A series of experiments was also performed to test the relative effects of various interventions on the biphasic mechanical response. The results obtained showed that both components could be inhibited by organic and inorganic calcium blocking agents, but could not be modified by the anticholinergic agent, atropine. However, the initial phasic component exhibited a preferential sensitivity to the inhibitory effect of small concentrations (6–20 μM) of lanthanum ion and to an inhibitory action exerted by moderate concentrations (1.8 mM) of calcium ions. Indeed, the relatively brief duration of the phasic component was attributed to the somewhat latent inactivating effect of calcium ions present in the bathing medium. By contrast, the tonic component displayed a preferential sensitivity to the inhibitory effect induced by incubating the muscle for a very long period (70–80 min) in a calcium-deficient, high potassium bathing medium before adding 1.8 mM $CaCl_2$ to elicit the biphasic response. The influx of calcium ions associated with the tonic component of the mechanical response was also inhibited more strongly by this procedure than was the influx of calcium ions associated with the phasic component (152). Other data pointing to differences between these two components have also been reported. Cameron and Lewis (164) found that low doses (15 μg/ml) of a toxin obtained from the stonefish produced an appreciable inhibition of the phasic response but had a lesser effect on the tonic response. Rangachari et al (165) have noted that a selective inhibition of the phasic response could be achieved by replacing chloride ion in the bathing medium with sulphate ion. Taken as a whole, the available evidence favors the contention that the biphasic mechanical response induced by a high potassium medium in the longitudinal muscle of the guinea pig ileum reflects the operation of two different voltage-dependent calcium channel systems. Those channels associated with the initial phasic component of the response permit a rapid, but transient inward movement of calcium ions and are quickly inactivated by the presence of calcium ions. Those channels associated with the subsequent tonic component of the response permit a much slower but more sustained inward movement of calcium ions and are slowly inactivated by prolonged exposure to the high potassium bathing medium. Hogestatt and Andersson (166), working with cerebral arteries from the rat, have suggested that the operation of two different voltage-dependent calcium channel systems may also be responsible for the potassium-induced biphasic contractions seen in their vascular smooth muscle preparation.

Although the physiological role of the calcium channels that become functional in a high potassium bathing medium is uncertain, Ratz and Flaim (167) raise the possibility that calcium channels of the type encountered in a high potassium medium may play an important role in smooth muscle cells that

normally contract in response to graded depolarizations of the plasma membrane. A channel that exhibits an increasingly greater probability of assuming the activated state as membrane potential is reduced, and does not readily undergo inactivation in a depolarized cell, would be well suited to deliver calcium ions to the cytoplasm during graded, well sustained depolarizations of the cell membrane.

Summary of Multiple Calcium Channel Types in Smooth Muscle

It would appear, therefore, that the types of voltage-dependent calcium channels that may be present in smooth muscle cells include those that are rapidly inactivated by reductions in membrane potential, those that are inactivated by the presence of calcium ions and/or those that are not readily inactivated by a diminution in membrane potential or the presence of calcium ions. A number of observations favor the assumption that any single smooth muscle cell may harbor several different types of voltage-dependent calcium channels. Visceral smooth muscles (uterine, intestinal, etc.), for example, will, under normal physiological conditions, undergo an increase in muscle tension following a stimulus-induced initiation or increase in the frequency of trains of action potentials (20, 168). These same cells can also be stimulated to develop complex, well sustained increases in muscle tension by immersing them in a membrane-depolarizing, high potassium bathing medium (20). In addition to harboring multiple types of voltage-dependent calcium channels, smooth muscle cells contain receptor-operated calcium channels, the functions of which are regulated, at least in part, by agonist-receptor interactions. In early experiments performed on various smooth muscle preparations, voltage-dependent calcium channels, as a group, seemed to differ from receptor-operated calcium channels in being much more sensitive to the inhibitory actions of calcium channel blockers (21, 169, 170). More recent work has uncovered a number of exceptions to this general observation. Examples of receptor-operated channels that are equally sensitive or even more sensitive to the actions of calcium blockers than are voltage-dependent channels have been noted in the literature (171–173).

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