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PROPERTIES OF CALCIUM CHANNEL INACTIVATION IN ISOLATED GUINEA-PIG MYOCYTES

By

Robert Wayne Hadley

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ABSTRACT

Properties of Calcium Channel Inactivation in
Isolated Guinea-Pig Myocytes

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The Ca current (I_{Ca}) was studied with the whole-cell patch clamp technique in single, isolated guinea-pig myocytes. I_{Ca} was found to inactivate with a biexponential time-course. This process was modulated by Ca, as inactivation was accelerated by elevating external Ca, and was slowed by the introduction of Ca chelators intracellularly. The inactivation curve, the relationship of inactivation to membrane potential, had a partial U-shape. Ca channel inactivation was studied when monovalent cations carried the current (I_{ns}) in the presence of EGTA. I_{ns} inactivation had a smooth, monotonic relationship to membrane potential. It was concluded that a Ca-dependent inactivation mechanism had been separated from another mechanism. This second process was identified as a voltage-dependent inactivation mechanism, as the rate and extent of inactivation of I_{ns} had a monotonic dependence on voltage, and it was demonstrated that inactivation could occur without ion permeation. Voltage-dependent inactivation was found to be the dominant mechanism at very positive potentials, while Ca-dependent inactivation was very important at less positive potentials.

Possible mechanisms for Ca-dependent inactivation were explored. Ca-induced shifts of membrane surface charge seemed unlikely, as the current-voltage relationship of I_{Ca} was unchanged. The lack of effect on inactivation of

calmodulin inhibitors and phosphorylating agents, suggested that dephosphorylation of the Ca channel was not necessarily involved.

The new dihydropyridine derivative Bay K 8644 was found to have concentration- and voltage-dependent inhibitory effects on the Ca channel. It was concluded that this was probably due to a stabilization of the inactivated Ca channel, as Bay K 8644 delayed the removal of inactivation. The drug-induced slow phase of recovery was found to have a voltage-dependence similar to normal recovery. At least some of the inhibitory effects of D600, on the other hand, could be accounted for by a simple model involving the plugging of the pore.

In conclusion, there appears to be two separate mechanisms for turning off Ca channels, one that is Ca-dependent and one that is voltage-dependent. There appears to be some evidence for the preferential binding of some organic Ca channel blockers to these states of the channel.

To my wife, Kaelyn

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

INTRODUCTION

A. General Background on Ca Channels

Ca channels, like Na and K channels, are found in the membranes of a variety of different cell types, including neurons, many endocrine cells, and skeletal, smooth and cardiac muscle. They differ from Na and K channels, however, in that they have more diverse functions. This is principally due to the unique aspects of using Ca as a current-carrier. There are about four orders of magnitude more free Ca ions on the outside of the cell membrane than there are on the inside. This huge gradient for Ca to enter the cell, along with its strong binding properties as a divalent cation, makes Ca capable of being an effective intracellular "second messenger," in addition to its passive role in carrying charge across the membrane. This is important, in view of the role of Ca channels in directly controlling excitation-secretion coupling, excitation-contraction coupling, and membrane permeability, among other functions.

The tissue where Ca channel function has perhaps drawn the most attention is cardiac muscle. The importance of Ca to cardiac function has been appreciated ever since Ringer (1883) demonstrated that frog hearts stopped beating when Ca was omitted from the perfusion fluid. Ca channels are quite important in nodal areas of the heart, as they are essential for generating the upstroke of the action potential in the SA and AV node, and may contribute significantly to the pacemaking properties of the SA node (Brown et al., 1984a). Ca channels are also quite important in the atrial and ventricular muscle of the heart. Their main role in working cardiac muscle is to provide the Ca influx that increases intracellular

Ca, and that may also trigger Ca release from the sarcoplasmic reticulum (Fabiato, 1983). In addition to these roles, the Ca current (I_{Ca}) has been postulated to control the duration of the action potential plateau in some regions of the heart (Reuter and Scholz, 1977a), and to influence the function of membrane proteins such as some K channels (Meech, 1974), and the Ca-activated nonspecific channel (Coloquhoun et al., 1981). It is because of these diverse roles of the Ca channel that the channel, and the factors that modulate its function, have become the subject of intense study.

1. Separation of I_{Ca} from other membrane currents

I_{Ca} in the heart was originally studied in multicellular preparations, using either the two microelectrode voltage-clamp technique (Reuter, 1967), or the sucrose-gap voltage-clamp technique (New and Trautwein, 1972). A great deal of progress was made with these methods, but they have severe limitations. The complicated geometry of the multicellular preparations, which have small intercellular clefts, leads to poor spatial and temporal voltage-clamp control, since the clefts act like a resistance placed in series (Johnson and Lieberman, 1971). Additional complications can result from the accumulation or depletion of ions in the intercellular clefts (Attwell et al., 1979). This led to uncertainties in the interpretation of much of the data; at one point even the existence of a Ca current in the heart was questioned (Johnson and Lieberman, 1971).

In addition to the problem of obtaining a good quality voltage-clamp in the multicellular preparations, there was the additional obstacle of separating I_{Ca} from other membrane currents. Currents through K channels produced the most problems, as they often activated in a time-dependent fashion that overlapped, and often obscured, the time course of I_{Ca}. Theoretically, this could be overcome by selectively inhibiting either the K or Ca channels. However, there is no specific, potent K channel blocker that can be applied externally to eliminate

current through all of the different types of K channels. In addition, the available inorganic or organic Ca channel blockers are not completely selective under all conditions (Kass and Tsien, 1975; McDonald et al., 1984a; Hume, 1985).

The difficulites associated with multicellular preparations initially led to a search for smaller multicellular preparations with wider, less troublesome clefts. One such preparation is the rabbit Purkinje fiber, which has had some limited use in studies of the Na current (Colatsky and Tsien, 1979; Colatsky, 1980). However, a more radical approach to this problem was the introduction of single, isolated heart cells as an experimental preparation. Single myocytes from adult heart tissue, which had been isolated with proteolytic enzymes and Ca-free solutions, had been available since the early 1970's (Berry et al., 1970; Vahouny et al., 1970). However, these cells went into contracture when exposed to millimolar concentrations of Ca. This "Ca paradox" prevented electrophysiological studies of single myocytes until the problem was partly overcome by Powell and Twist (1976). They isolated single rat ventricular cells that remained rod-shaped in the presence of millimolar Ca. However, the cells did beat spontaneously, an abnormal property for ventricular tissue. Their method has, for the most part, been modified and refined by subsequent investigators. It has been a general finding that obtaining high yields of quiescent myocytes by this method depends to a great extent on minimizing the exposure to very low concentrations of Ca. Alternative methods of isolating heart cells have also been developed (Hume and Giles, 1981; Isenberg and Klockner, 1982a; Bkaily et al., 1984).

Single myocytes have passive cable properties that allow for a satisfactory clamp of the membrane potential (Hume and Giles, 1981). Therefore, electrophysiological studies in isolated heart cells have proven quite successful in the study of many different aspects of membrane transport. I_{Ca} has been studied in the single heart cells with several different techniques, including the two-

microelectrode voltage-clamp technique (Isenberg and Klockner, 1982b), and a nondialyzing suction pipette technique (Hume and Giles, 1983). However, the development of certain voltage-clamp techniques that allowed for the experimental manipulation of the intracellular milieu was of particular importance in the study of I_{Ca}. One such technique, the suction pipette technique, uses very large diameter pipettes (> 5 μm), and was originally developed for studies of currents in invertebrate neurons (Lee et al., 1980). The other technique, the whole-cell patch clamp technique, usually uses somewhat smaller pipettes, and was originally developed for tight-seal recording of single channel currents (Hamill et al., 1981). Both techniques use pipettes with tip diameters in the μm range, which allows whatever substances are placed in the pipette to diffuse into the cytoplasm. This property has been taken advantage of in reducing the problem of separating I_{Ca} from other membrane currents. In particular, troublesome K currents can be reduced or eliminated by the replacement of intracellular K with Cs ions, as Cs ions are relatively impermeant through K channels (Latorre and Miller, 1983).

In general, the new single-cell and dialyzing pipette techniques have resulted in more satisfactory studies of I_{Ca} than were possible with the older techniques (see Tsien, 1983; Noble, 1984). These techniques, however, do have their own limitations. The single-cell preparations differ from intact tissue in having been put through the stresses of enzymatic digestion and mechanical agitation. So far, there has been little evidence to suggest any electrophysiological differences between the isolated myocyte and the intact tissue. Dialysis of the cell interior, however, does produce notable changes. The most significant alteration is the phenomenon of Ca channel "rundown," where the magnitude of I_{Ca} gradually decreases during dialysis. This phenomenon has been seen in almost every preparation where Ca channels have been studied (Fenwick et al., 1982; Irisawa and Kokubun, 1983). The precise mechanism of Ca channel rundown is not

known. However, it has been a general finding that rundown can be slowed down considerably by the inclusion of Mg-ATP or cyclic AMP in the pipette solution, which has led to the suggestion that a reduced level of channel phosphorylation (Doroshenko et al., 1982) or an elevated concentration of intracellular Ca due to lowered Ca-ATPase activity (Byerly and Yazejian, 1986) may be involved.

Finally, it should be noted that Ca channels can be studied at the level of the single channel with the patch-clamp technique (Hamill et al., 1981). This approach has many advantages. For example, questions can be addressed at the molecular level, and since single ion channels are under observation, an ideal separation of membrane currents can be achieved. The principal limitation of the technique is that it is restricted to use in unphysiologically high concentrations of divalent cations, 50-110 mM. This is because the single channel conductance is so small under physiological conditions (Akaike et al., 1978; Bean et al., 1984) as to be unmeasureable.

2. Recent studies on Ca channels

a. Multiple Ca channels

The introduction of new biophysical techniques has resulted in the development of several new concepts concerning Ca channels. One of the most important is the classification of Ca channels into different subtypes, and the realization that cardiac tissues may contain more than one subtype.

Many differences exist between Ca channels in different preparations, and for this reason it has long been accepted that there are different types of Ca channels (Hagiwara and Byerly, 1981). However, by the early 1980's, the existence of two different types of Ca channels in a single preparation had been proposed on the basis of various whole-cell recordings for starfish egg cell membranes (Hagiwara et al., 1975), guinea-pig inferior olivary neurons (Llinas and Yarom, 1981) and neuroblastoma cells (Fishman and Spector, 1981). This early

work was expanded upon by the observation that this condition existed for a variety of different preparations, and that quite often, two specific types of Ca channels were found (Carbone and Lux, 1984; Armstrong and Matteson, 1985; Bean, 1985; Nilius et al., 1985). In comparison, one had a more negative voltage-range for inactivation and activation, and turned off more rapidly than the other. The existence of these two types of Ca channels has been confirmed by single-channel studies (Carbone and Lux, 1984; Nilius et al., 1985; Nowycky et al., 1985). These channels have been given a number of different names by different investigators, but the nomenclature of Nowycky et al. (1985) has increasingly been adopted. One channel is seen in patch-clamp records as a "tiny" single channel conductance with a "transient" time course, therefore it has been named the T-channel. The second channel has a larger conductance, and produces longer-lasting records, and therefore has been named the L-channnel. In addition, a third channel, the N-channel (neither T nor L) has been reported in chick doral root ganglion cells (Nowycky et al., 1985), but is not commonly found in non-neural tissue.

Both the L- and T-type Ca channels have been reported to exist in heart tissue (Bean, 1985; Nilius et al., 1985). Their roles have not been completely assessed, but the L-type Ca channel appears to account for most of the Ca current in ventricular cells, and appears to be the "usual" Ca channel that is associated with the effects of β-adrenergic stimulation and dihydropyridine sensitivity (Bean, 1985; Nilius et al., 1985; Mitra and Morad, 1986). Fortunately, it appears that L-channel current can be studied in ventricular tissue with little contamination from T-type current under the proper conditions.

b. I_{Ca} kinetics

Earlier studies of I_{Ca} kinetics indicated that it was a fairly slowly activating and inactivating channel, especially when compared to the

example of the Na channel (New and Trautwein, 1972; Reuter and Scholz, 1977a; Isenberg and Klockner, 1980). This was one of the reasons why early investigators first called the Ca current "I_{Si}," for "slow inward" current (as well as "second inward"). More recent studies in isolated myocytes have revealed that Ca channel kinetics are much faster than was thought. In particular, I_{Ca} activates completely within a few ms after depolarization (Lee and Tsien, 1982; Isenberg and Klockner, 1982b). Inactivation kinetics are often faster in isolated myocytes as well (Noble, 1984), but this process appears to be more variable and complicated.

c. Open channel properties

The second reason why early investigators termed current through cardiac Ca channels "I_{Si}," was because it was felt that other ions, in particular Na, contributed much of the current. This concept came from some of the early studies where part of the Mn-sensitive current remained after the removal of external Ca (Rougier et al., 1969; Ochi, 1970). This component of the current disappeared when external Na was removed. Therefore it was felt that under physiological conditions, Na ions must carry a large portion of the current. This idea made considerable sense, as channels were usually viewed as being able to discriminate between ions on the basis of charge and size, and there was little reason to suspect that a channel capable of passing Ca ions could reject Na ions.

The importance of Na ions in carrying I_{Si} had been somewhat controversial, however (see Reuter, 1973). Therefore, it had been a matter of great interest to measure the selectivity of the Ca channel directly. Such studies require the measurement of the reversal potential of the current. However, outward current through Ca channels in neuronal preparations was very difficult to demonstrate, primarily because of other overlapping outward currents. This absence of a clear demonstration of outward Ca channel current had given rise to the concept of the Ca channel being a one-way pathway (see Hagiwara, 1981).

There had, however, been several reports of outward Ca channel current in the heart literature. Reuter and Scholz (1977a) measured the reversal potential of I_{Si} in bovine ventricle by examining I_{Si} tail currents, and found it to be about +50 mV in 1.8 mM Ca. This value was much lower than the predicted Ca equilibrium potential of approximately +115 mV. They explained this discrepancy as being due to the contribution of Na and K ions to the current through the channels. These investigators estimated that the Ca channel was a hundred times more permeable to Ca than to Na and K. As Na and K ions predominate over Ca ions in the external and internal solutions, this predicted that they contributed significantly to the current.

The introduction of the single-cell and suction pipette techniques to cardiac electrophysiology led to considerable revisions in concepts regarding the properties of the open Ca channel. The reversal potential was found to be approximately +60 mV under conditions that more stringently isolated the current (Lee and Tsien, 1982). These more accurate measurements of the reversal potential allowed for a calculation of the selectivity of the channel for Ca or Ba ions against K or Cs ions. In solutions containing millimolar concentrations of external divalents, the channel is more than 5000 times more permeable to the divalent cations (Lee and Tsien, 1984). In addition to these findings, it has also been found that the removal of external Na ions does not reduce the size of current through Ca channels (Mitchell et al., 1983; Matsuda and Noma, 1984). What this means is that external monovalent cations contribute little to inward Ca channel current. This observation has been further supported by the finding that inward monovalent currents through single Ca channels can be blocked by micromolar concentrations of Ca (Lansman et al., 1986). However, intracellular monovalent cations can provide outward current, as they are in high concentration, and are driven out by an exceedingly high electrochemical gradient. It is

because of this high selectivity for Ca that the old term "I_{Si}" is slowly being replaced by "I_{Ca}."

These results were intriguing, as the Ca channel prefers Ca ions to Na or K, despite the fact that the latter have smaller diameters. Insight into this question has been gained by the observation that the Ca channel becomes permeable to monovalent cations when Ca chelators, such as EDTA or EGTA, are added to the external solution (Rougier et al.,1969; Kostyuk and Krishtal, 1977; Kostyuk et al., 1983). In other words, the selectivity of the channel pore depends on the presence of divalent cations. In fact, in the absence of external divalent cations, the Ca channel is somewhat permeable to cations as large as tetramethylammonium (McCleskey et al. 1985). Such observations have been explained with a model containing two binding sites for Ca in the pore (Almers and McCleskey, 1984; Hess and Tsien, 1984). This model is shown schematically in Figure 1. Ca ions enter the pore from the outside, and bind with great affinity to the first anionic site. The Ca ion can then move back out of the channel, or on to the next site. If another Ca ion then enters the pore, mutual repulsion increases the chance that the first Ca ion will move on into the cell. This model can explain why micromolar concentrations of Ca can block the flux of monovalent cations through the channel, but millimolar concentrations are required for significant influx of Ca itself. Block of the channel by Ca ions has been attributed to the occupancy of a single site at low concentrations, while Ca influx is supposed to require double occupancy of the pore, which would only occur at high Ca concentrations.

Figure 1. Model of Ca channel pore. Taken from Almers and McCleskey (1984). Panel A shows a schematic drawing of the pore with two binding sites, and with one Ca ion bound. Panel B shows the energy profile of the channel. The y-axis plots energy (J/mol) divided by RT, the x-axis plots the fraction of the transmembrane potential experienced at any given point. Profiles are shown for Ca (continuous curve) and Na (dashed curve). Further details in text.

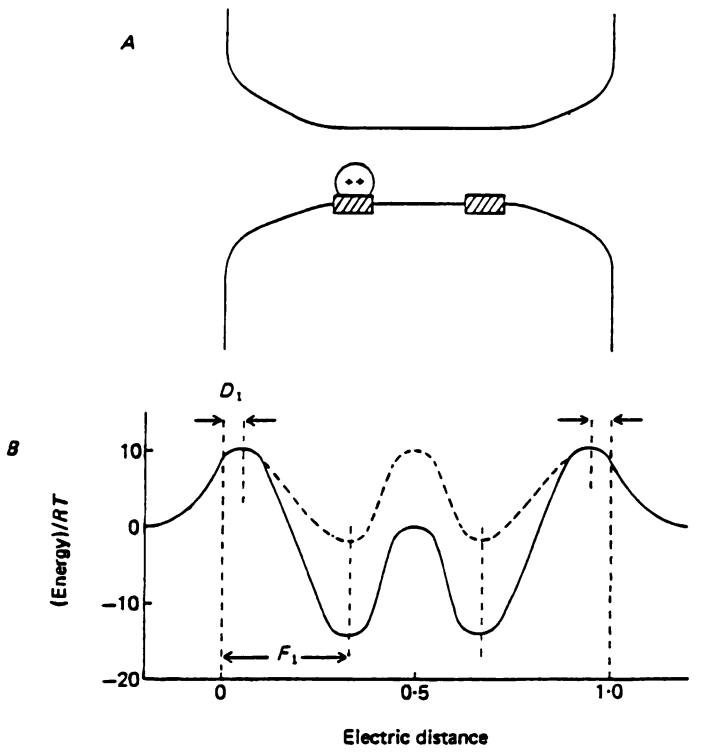


Figure 1

B. Ca channel inactivation

1. Traditional views of Ca channel inactivation

Early on in the studies of the Ca current, it was recognized that after the current was activated, it then decayed, or became inactivated, with a certain time course (see Reuter, 1973; Trautwein, 1974). Obviously, this is essential from a functional viewpoint, so as to allow for repolarization of the cell, and to prevent Ca overload of the cell. All of the early studies interpreted the current decay as being due to a voltage-dependent inactivation gate, analogous to the h gate of the Na channel. In fact, in analogy to Hodgkin and Huxley's (1952b) m^3h model of the Na channel, the Ca current was often described as being due to a membrane conductance g_{Ca}, which could be described mathematically as being equal to the product $\bar{g}_{Ca}$ d f (Reuter, 1973). In this equation, $\bar{g}_{Ca}$ is the maximal conductance when all Ca channels are open, d is a factor which varies between 0 and 1, and represents the role of the activation gate, and f is a similar factor representing the role of the inactivation gate. In this analysis, f would vary between 0 and 1 in a manner dependent on both voltage and time. The voltage- and time-dependence of f can be further analyzed in terms of f infinity, the steady-state value of f at a given potential, and tau_f, a time constant that describes the rate at which f approaches f infinity at a given potential. The voltage-dependence of f infinity was thought to closely resemble that of h infinity, the inactivation variable of the Na channel, except that the f infinity curve was shifted to somewhat more positive potentials (Reuter, 1973). F infinity was approximately 1 at -50 mV, representing no inactivation of Ca channels, and approached 0 at about +10 mV. The potential at which half of the Ca channels were inactivated was usually about -25 mV.

Although f infinity had a similar voltage-dependence to h infinity, it was noted early on that the voltage-dependence of tau_f differed from tau_n, the

rate at which Na channels inactivated. Both nerve (Hodgkin and Huxley, 1952a) and cardiac Na channels (Brown et al., 1981a) inactivate faster with more positive potentials. It was found, however, that tau_f actually increases at very positive potentials (New and Trautwein, 1972; Reuter and Scholz, 1977a). In general, tau_f was found to decrease over the range of potentials from -50 to 0 mV, and then to increase at potentials beyond 0 mV. In addition to this peculiarity, it has been noted that the d and f curves overlap to some extent, permitting the steady entry of Ca into the cell at some potentials (Reuter and Scholz, 1977a). These results suggested that the inactivation properties of Ca channels may play an important role in maintaining the plateau of the cardiac action potential.

2. Inactivation of Ca channels in other tissues

Although it had always been accepted that I_{Ca} inactivated in cardiac tissues, for a long time there was considerable disagreement about the rate and extent to which I_{Ca} inactivated in other tissues (see Hagiwara and Byerly, 1981; Chad and Eckert, 1984). It was often quite difficult to distinguish the decay of I_{Ca} from the development of various outward currents. This problem was resolved when techniques were developed that allowed Cs to be introduced intracellularly. It was then apparent that I_{Ca} still decayed in most preparations, even when severe measures had been taken to suppress outward currents. Furthermore, tail current measurements, which give a better estimate of the true Ca conductance, indicated that Ca channel conductance did decay with time in both neuronal (Eckert and Ewald, 1983) and cardiac preparations (Lee et al., 1985).

Studies of I_{Ca} inactivation indicated that it varied considerably between different preparations, and that it was usually much slower than inactivation of the Na current. Nevertheless, I_{Ca} inactivation was usually assumed to be a comparable process. This concept was challenged by Brehm and

Eckert (1978), who demonstrated that I_{Ca} inactivation in Paramecium did not have a simple dependence on the membrane potential. The most inactivation occurred at approximately 0 mV, and progressively less inactivation was left behind by more positive depolarizations. Extremely strong depolarizations (> ±50 mV) seemed to leave behind little or no inactivation. It was noticeable that this unusual, U-shaped, inactivation curve had a close resemblance to the current-voltage relationship of I_{Ca}. That is, the most inactivation occurred at the potential where there was the largest I_{Ca}, suggesting that it was the Ca entering the cell, and not the membrane potential itself, that inactivated the channels. The existence of a U-shaped inactivation curve for I_{Ca} was subsequently confirmed in a number of preparations, such as neurons from Aplysia californica (Tillotson, 1979) and from the snail, Helix aspersa (Brown et al., 1981b). Furthermore, it has been found that the degree of inactivation left behind at different potentials is a linear function of the amount of Ca entry in Aplysia californica neurons (Eckert and Tillotson, 1981).

Additional evidence for Ca-dependent inactivation of Ca channels was obtained with many different approaches. It was found that substituting Sr or Ba for external Ca often had profound effects on Ca channel inactivation. It had earlier been noted that Ba currents decayed much more slowly than Ca currents in Helix pomatia neurons, but this was attributed to the enhancement of a inherently slow component of current decay (Magura, 1977). Sr or Ba substitution was also found to reduce and slow Ca channel inactivation in Paramecium (Brehm and Eckert, 1978) and Aplysia neurons (Tillotson, 1979), but these investigators attributed the effect to an action on one type of Ca channel. Presumably, Sr and Ba ions interact less effectively with whatever intracellular site Ca acts upon.

If Ca channels are inactivated by a rise in intracellular Ca during the time course of I_{Ca}, then this effect should be mimicked by other approaches that

increase intracellular Ca. Kostyuk and Krishtal (1977) used a dialysis method to change internal solutions during voltage-clamp studies of I_{Ca} in molluscan neurons. They found that raising the free Ca of the internal solution to as little as 60 nM completely blocked I_{Ca}. This basic observation has been confirmed by iontophoretic injection of Ca into Helix aspersa neurons (Standen, 1982; Plant et al., 1983) and by using the suction pipette technique to dialyze neurons from the snail, Lymnaea stagnalis with different Ca-containing solutions (Byerly and Moody, 1984). In addition, the latter investigators also monitored the free Ca-concentration inside the cell with a Ca-sensitive microelectrode. They found that the intracellular Ca concentration had to approach 1 μM to completely block I_{Ca}, a result quite different from that of Kostyuk and Krishtal (1977). This discrepancy was likely due to the great difficulty in properly controlling the free intracellular Ca concentration.

In addition to the effect raising intracellular Ca has on I_{Ca}, it should be possible to oppose Ca-dependent inactivation by introducing EGTA or other Ca chelators intracellularly. Iontophoretic injection of EGTA was found to reduce the rate and extent of inactivation in Paramecium caudata (Brehm et al., 1980), and in Helix aspersa neurons (Plant et al., 1983). However, EGTA could never completely eliminate I_{Ca} inactivation.

Although there seemed to be much evidence for Ca-dependent inactivation in many preparations, I_{Ca} in other preparations can display considerably different behavior. Little or no inactivation of Ca channels appears to occur in the presynaptic terminal of the squid stellate ganglion (Katz and Miledi, 1971; Llinas et al., 1981), or in some bovine chromaffin cells (Fenwick et al., 1982). Furthermore, Ca channels in other preparations appear to inactivate in a voltage-dependent manner. Substitution of Sr or Ba for Ca did not affect the inactivation rate of I_{Ca} in mouse myeloma cells (Fukushima and Hagiwara, 1983) or Neanthes

arenaceodentata egg cells (Fox, 1981). In the latter study, Ca channel inactivation was also found to have a monotonic relationship to membrane potential. In addition, it has been suggested that Ca- and voltage-dependent inactivation can occur in the same preparation, in this case, Helix aspersa neurons (Brown et al., 1981b). All of these results further emphasize the heterogenity of Ca channels.

3. Inactivation of cardiac Ca channels

As it was obvious that there was considerable variability of channel inactivation between Ca channels from different tissues, it was of great importance to examine I_{Ca} in cardiac preparations more closely. Several investigators have found that the I_{Ca} inactivation curve has the U-shape that is characteristic of Ca-dependent inactivation (Marban and Tsien, 1981; Hume and Giles, 1982; Mitchell et al., 1983). However, other investigators have not observed this phenomenon, but instead found a monotonic relationship of inactivation and membrane potential (Campbell et al., 1983; Kass and Sanguinetti, 1984). Nevertheless, there is other evidence that suggests that Ca entry is important in turning off cardiac Ca channels. It has been a common finding that I_{Ca} decays faster when the external Ca concentration is raised (Kohlhardt et al., 1975; Lee et al., 1985), and is slowed when Sr and Ba are substituted for Ca (Josephson et al., 1984; Kass and Sanguinetti, 1984). In addition, pressure injection of EGTA into rat ventricular cells slowed I_{Ca} inactivation considerably (Josephson et al., 1984), and a similar result was obtained when cultured guinea-pig atrial cells were dialyzed with Ca chelators (Bechem and Pott, 1985).

Such results prompted Mentrard et al. (1984) to propose that the inactivation of I_{Ca} in the heart was a strictly Ca-dependent phenomenon. They supported this idea by demonstrating that the degree of Ca channel inactivation in frog atrial muscle was a linear function of the preceeding amount of divalent cation entry. This relationship held up whether the divalent cation was Ca or Sr,

although Sr was considerably less effective. However, this strict model of I_{Ca} inactivation is questionable in view of subsequent experimental results. First, the relationship between the amount of Ca entry and subsequent I_{Ca} inactivation has been found to be quite nonlinear in isolated rat and guinea-pig ventricular cells (Josephson et al., 1984). Second, it has been found that outward current through Ca channels does inactivate, although perhaps not completely (Lee and Tsien, 1982; Lee and Tsien, 1983).

These complicated results have led to the suggestion that cardiac Ca channels may have two mechanisms of inactivation, one that is voltage-dependent and another that is Ca-dependent (Kass and Sanguinetti, 1984; Lee et al., 1985). However, the relationship and relative importance of the two mechanisms is unknown, and it has proven difficult to study them independently. In addition, no evidence supporting a dual mechanism of inactivation has been found at the level of the single channel. All studies of single Ca channels have so far interpreted inactivation as being strictly voltage-dependent, with little evidence for Ca-dependent inactivation (Cavalie et al., 1983; Lux and Brown, 1984; Trautwein and Pelzer, 1985; Cavalie et al., 1986).

4. Possible mechanisms of Ca-dependent inactivation

One of the most important and controversial questions about Ca-dependent inactivation is the precise mechanism by which it prevents flux through the Ca channel. Perhaps the first possible mechanism to draw attention was that a decreased driving force for Ca might result from accumulation of intracellular Ca (see Eckert and Chad, 1984). This concept arose from the tremendous disparity between the extracellular and intracellular concentrations of Ca. The extracellular Ca concentration is usually about 2 mM, while free intracellular Ca (Ca_i) can be as low as 100 nM. This raises the possibility that Ca influx through the channel can greatly increase Ca_i, and diminish I_{Ca} by decreasing the driving

force for Ca to cross the membrane. Most investigators who have studied this problem have rejected this hypothesis, both on theoretical and experimental grounds. First, calculations of Ca permeability using the constant-field equation indicate that current through Ca channels will be quite insensitive to the electrochemical effects of Ca (Hagiwara and Byerly, 1981; Eckert and Chad, 1984). An increase in Ca_i up to 1 μM should only produce a 1% decrease in I_{Ca} by this mechanism. This idea is supported by the observation that Ba currents through Ca channels, which are larger than Ca currents and should accumulate to a greater extent, inactivate quite slowly (Brehm and Eckert, 1978; Kass and Sanguinetti, 1984). It has also been found that the reversal potential of Sr currents through cardiac Ca channels was not affected by previous large Sr currents (Kass and Sanguinetti, 1984), as would be expected if inactivation was due to a diminished driving force.

Although a change in the driving force for Ca due to intracellular accumulation does not appear to be important, the depletion of extracellular Ca may be. The gradual decay of I_{Ca} in frog skeletal muscle was found to be related to a gradual depletion of Ca in the restricted spaces of transverse tubules (Almers et al., 1981). However, control of I_{Ca} by this phenomenon may be restricted to this particular preparation. Almers et al. (1981) found that I_{Ca} decay could be slowed by buffering the free extracellular Ca concentration with malate. This result was not found with I_{Ca} in frog atrial muscle (Mentrard et al., 1984). In addition, inactivation is accelerated by Ba and Sr substitution for Ca in frog skeletal muscle, presumably because of the increased current size.

A third possible mechanism by which an increase in Ca could indirectly influence the inactivation process is by altering the membrane surface charge. Ca ions are known to be able to bind to anionic sites on the surfaces of cell membranes. This neutralization of negative charges on the membrane alters the

electrical field experienced by membrane proteins, and produces an artifactual shift of the membrane potential (Frankenhauser and Hodgkin, 1957). It may be possible that Ca ions bind to the inside of the cell membrane after entering through the Ca channel, and shift voltage-dependent parameters negative. Although this would only enhance voltage-dependent activation (Eckert and Chad, 1984), it could produce "Ca-dependent" inactivation by enhancing an inherent voltage-dependent inactivation mechanism (Tsien, 1983). This hypothesis has not been rigorously tested, but it has been noted that the shift must be fairly large to account for the difference in the inactivation kinetics of cardiac Ca and Ba currents (Kass and Sanguinetti, 1984).

Several investigators have proposed that Ca_i turns off the Ca channel by means of an intracellular allosteric site (Standen and Standfield, 1982; Chad and Eckert, 1984; Mentrard et al., 1984). There is little or no direct experimental evidence for, or against, this hypothesis. Instead, the success of the hypothesis depends upon the modeling of the Ca transient in the region next to the inner mouth of the pore, the "Ca-domain." It has been demonstrated that such models can account for the inactivation curve and the kinetics of I_{Ca} decay (Plant et al., 1983; Chad et al., 1984).

Finally, an enzymatic mechanism for Ca-dependent inactivation has been recently proposed (Chad and Eckert, 1986). It has long been known that the metabolic state of a cell affects the Ca channel (Sperelakis and Schneider, 1976). The phenomenon of Ca channel rundown was identified in early studies with dialysis techniques (Doroshenko et al., 1982; Byerly and Hagiwara, 1982) and it was found that the rundown was promoted by maneuvers that elevated intracellular Ca, and was slowed by introducing EGTA intracellularly (Fenwick et al., 1982). Rundown was also slowed considerably by introducing agents that promote phosphorylation, such as Mg-ATP or cAMP, intracellularly (Doroshenko et al.,

1982; Byerly and Yazejian, 1986). These data have promoted the idea that Ca channels must be phosphorylated to be functional. Chad and Eckert (1986) have based their hypothesis for Ca-dependent inactivation on this assumption. They have proposed that when Ca enters the cell, it activates a Ca/calmodulin dependent phosphatase, calcineurin, that then dephosphorylates and inactivates the Ca channel. Their evidence for this hypothesis comes from suction pipette experiments on Helix aspersa neurons. They found that placing calcineurin in the pipette solution greatly accelerated Ca channel inactivation, and this effect of calcineurin could be prevented by the addition of intracellular EGTA, or by substituting Ba for external Ca. Also, the inclusion of ATP-γ-S, the slowly hydrolyzable analog of ATP, in the pipette solution greatly slowed inactivation. The authors proposed that this was due to relatively slow dethiophosphorylation of the channels. Thus, calcineurin could be capable of mediating Ca-dependent inactivation. This hypothesis has not been directly tested in other preparations.

C. Drug Interactions with the Inactivated State of the Ca Channel

One of the most interesting aspects of the inactivation process of ion channels is that many drugs and toxins appear to act upon it in some way. In particular, there are several naturally occurring lipid-soluble toxins, such as batrachotoxin, veratridine, and aconitine, that apparently eliminate Na channel inactivation (see Catterall, 1980). This has made these toxins rather valuable tools in studying the basic properties of Na channels. It has also been found that Na channel inactivation can be removed by exposing the inside surface of the membrane to nonspecific proteases (Armstrong et al., 1973). On the other hand, it has been proposed that the local anesthetics, a rather diverse drug group, promote the inactivation of Na channels by preferentially binding to that state of the channel (Hille, 1977; Hondeghem and Katzung, 1977). Thus, the inactivated

state of the Na channel appears to be susceptible to modulation by different agents.

1. Organic Ca channel blockers

The inactivated state of the Ca channel may also have strong interactions with different drugs. A number of different organic Ca channel blockers have been developed, and the mechanism by which they act has been intensely studied. Voltage-clamp studies have indicated that there are many similarities between how these drugs affect the Ca channel and how local anesthetics affect the Na channel. Early studies of verapamil and its methoxy derivative, D600, indicated that they blocked Ca channels in a manner dependent on voltage and time. The blockade of Ca channels with these compounds could be relieved by hyperpolarization, and was enhanced by repetitive depolarization (Ehara and Kaufmann, 1978; McDonald et al., 1980; McDonald et al., 1984). Similar results have also been obtained with another organic Ca channel blocker, diltiazem (Tung and Morad, 1983; Kanyana and Katzung, 1984). Early studies of the dihydropyridines, a more recently developed class of organic Ca channel blockers, indicated that their blockade was neither voltage- or use-dependent (Kass, 1982; Hachisu and Pappano, 1983). However, more careful studies have indicated that dihydropyridine block is voltage- and use-dependent, although not to the extent of the earlier agents (Bean, 1984; Kass and Sanguinetti, 1984; Uehara and Hume, 1985). In particular, Ca channel blockade with these different agents can be divided into tonic block, which is the steady-state inhibition of I_{Ca} that is seen at the holding potential, and use-dependent block, which is the extra block accumulated with repetitive stimulation. It has been found that while verapamil and D600 predominantly manifest use-dependent block, inhibition by the dihydropyridines is usually dominated by tonic block, and block by diltiazem is mixed (Lee and Tsien, 1983; Uehara and Hume, 1985).

These observations have been interpreted in terms of the modulated-receptor hypothesis (Sanguinetti and Kass, 1984; Uehara and Hume, 1985), which was originally developed to explain Na channel block by local anesthetics (Hille, 1977; Hondeghem and Katzung, 1977). This hypothesis is based on three assumptions. First, the receptors for these different drugs is on, or adjacent to, the Ca channel. Second, Ca channels that have bound the drug do not conduct. Finally, the affinity of the receptor for drug is different for the different states (closed, open, and inactivated) of the Ca channel. Therefore, the promotion of Ca channel blockade by depolarization could be interpreted as being due to the channels spending more of their time in high-affinity open or inactivated states. Also, the relief of block by hyperpolarization can be interpreted as being due to the resting state having a low affinity for the drug.

In terms of the modulated-receptor hypothesis, experimental evidence has particularly supported the idea that it is the inactivated state of the Ca channel that has the highest affinity for the organic blockers. Bean (1984) examined this idea by measuring the IC_{50} for Ca channel inhibition by nitrendipine. It was found that nitrendipine was not very potent (IC_{50} = 730 nM) at relatively negative holding potentials, where most of the channels should be in the closed state. However, the potency increased greatly (IC_{50} = 0.36 nM), at more positive holding potentials, where the channels should spend more time in the inactivated state. In addition to this evidence, it has been found that D600 and the dihydropyridines accelerate Ca channel inactivation (Lee and Tsien, 1983; Sanguinetti and Kass, 1984). This latter finding could also be interpreted as block of the open state of the Ca channel (Lee and Tsien, 1983). Binding to the inactivated state has been judged to be especially important, however, as D600, diltiazem, and nifedipine greatly slow recovery from inactivation (Uehara and Hume, 1985).

2. The guarded receptor hypothesis

Recently, an alternative model of local anesthetic blockade of Na channels has been developed which does not require the assumption that the drug preferentially binds to the inactivated state (Starmer et al., 1984; Starmer and Grant, 1985). This model, termed the guarded receptor hypothesis, has simpler assumptions than the modulated receptor hypothesis. It assumes that the receptor has a constant affinity for the drug, but is located in the channel pore. This location of the receptor is what would confer the voltage- and use-dependence of Na channel block, as the receptor is only transiently accessible, since it is guarded by the voltage-dependent gates. This model supposes that the preferred interaction of local anesthetics with the inactivated state of the Na channel is a misinterpretation of some of the data.

It has been found that this model can account for the time course of use-dependent block of Na channels by local anesthetics (Starmer et al., 1985; Moorman et al., 1986). Use-dependent block follows an exponetial time course because of the slow accumulation of drug in the channels. The Na channels would be in an unavailable state at negative potentials, but would become transiently accessible as the activation gate opened with repetitive depolarization. Thus, use-dependent block occurs because with faster stimulation rates, the drug has more time to approach a steady-state level of binding to the drug receptor.

The guarded receptor hypothesis can also explain other experimental results obtained from voltage-clamp studies of local anesthetics. For example, there is supposedly an increased affinity of the local anesthetic receptor (as measured by changes in the IC_{50}) at depolarized potentials (Bean et al., 1983). The guarded receptor hypothesis explains this as being due to the fraction of Na channels that will probably open at each potential (Starmer and Hollett, 1985). At very negative holding potentials, there is little probability of the channels

opening, and thus there will be little block. At more positive holding potentials, the probability of opening increases, and thus more local anesthetic will bind to the receptor. Local anesthetics also induce a hyperpolarizing shift of the Na channel inactivation curve (Weidmann, 1955; Bean, 1983), and the same argument has been applied to this observation (Starmer, 1986). Experimental protocols that determine the inactivation curve measure the steady-state magnitude of the Na current at each potential and compare it to the largest Na current at very negative potentials. These protocols cannot discriminate between channels that are inactivated and those that are blocked. Therefore it has been proposed that the altered inactivation curve is due to the combination of the voltage-dependence of inactivation of drug-free channels, and a changing fraction of drug-complexed channels.

The guarded receptor hypothesis has been extended to explain use-dependent block and unblock by K channel blockers (Starmer, 1986). The type of model has not yet been used to analyze detailed data from experiments with organic Ca channel blockers. Such an analysis would be of interest, as the action of organic Ca channel blockers have many features in common with local anesthetic block of the Na channel.

D. Objectives

The inactivation process plays an important role in the function of the cardiac Ca channel, but is considerably more complex and less understood than its activation process, or the inactivation process of the Na channel. The objectives of this study were therefore as follows. First, the hypothesis of a dual process for Ca channel inactivation will be tested further, and it will be determined if the two processes can be studied separately. Second, the relative roles and importance of each process to the Ca channel will be determined. Third, the

mechanisms of the inactivation processes will be studied in detail. Finally, drugs that are known to affect Ca channel function will be studied for effects on inactivation. The applicability of different models of Ca channel blockade will be examined.

MATERIALS AND METHODS

A. Isolation of single, guinea-pig ventricular cells

The preparation that was used in all of the experiments was single, isolated myocytes from guinea-pig ventricle. The procedure used for isolation of the myocytes was basically that of Powell and Twist (1976), as modified by Hume and Uehara (1985). Guinea-pigs of either sex were injected with heparin (1000 μ, i.p.) and then after 45 minutes were sacrificed by cervical dislocation. The heart was quickly removed and the aorta was cannulated. The heart was then flushed with warm (37°C) Krebs-Henseleit solution to remove the blood. The Krebs-Henseleit solution had a composition of (in mM) NaCl 118, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 2.2, $CaCl_2$ 1.5 and glucose 11, and was gassed with 95% O_2, 5% CO_2. The heart was then transferred to a Langendorff apparatus where the heart was perfused through the aorta with Krebs-Henseleit solution for seven minutes at a rate of approximately 5 ml/min. During this time the heart was completely cleared of blood and was inspected for rhythm or perfusion abnormalities. After this period the perfusate was changed to Krebs-Henseleit solution, with $CaCl_2$ omitted, for five minutes. At the end of this period, collagenase (Sigma, Type I) was added to the low Ca Krebs-Henseleit solution at a concentration of 0.4 mg/ml. Perfusion was continued for an additional fifty minutes under these conditions. The first 25 ml of the effluent was discarded during this period, but thereafter the perfusate was recirculated with a final total volume of 50 ml. The heart was then taken down, the atria were dissected from the ventricles, and the ventricular tissue, which had been softened by the digestion period, was minced into small pieces. The pieces

were then placed in a small beaker containing normal Krebs-Henseleit solution and 0.66 mg/ml collagenase. The tissue was placed in a shaker bath at 35°C for fifteen minutes. After this second period of digestion, the tissue was stored in normal Krebs Henseleit at room temperature until it was used. At that time, a small piece of the tissue was mechanically dispersed into single cells by gentle agitation with a pipet.

The above procedure produced a large number of healthy myocytes, as was demonstrated by their rod-shaped appearance, their clear striations, their quiescence at rest, and their normal resting potential. Examples of the myocytes are shown in Figure 2. The viability of the cell preparation varied widely from day to day, as far as 5-60%. No further effort was made to improve the yield or to separate the live and dead myocytes, as only a small number of myocytes were needed for experiments on individual cells. Experiments were always done within several hours of the isolation. No attempt was made to maintain the cell preparation overnight.

B. Experimental apparatus

The voltage-clamp experiments were performed on the stage of an inverted binocular phase-contrast microscope (Swift Instruments Ltd., San Jose, CA). The myocytes were observed at a magnification of 600X. The microscope itself was mounted on a vibration table (Kinetic Systems, Roslindale, MA), so that the microscope floated above a cushion of compressed air. This greatly reduced unwanted, sudden movements of the microscope stage. The cells were placed in a plastic, 35 x 10 mm tissue culture dish that was mounted on the microscope stage. The dish was superfused by different solutions that were hung in containers above the setup. Solution changes were accomplished by means of a teflon-coated switch placed between the containers and the culture dish. Fluid was removed

Figure 2. Photograph of guinea-pig ventricular myocytes. The photomicrograph on left shows a single guinea-pig ventricular cell, the photomicrograph on right shows a doublet. Hoffman contrast interference optics with total magnification of 600X. Calibration bar is equal to 10 μm.

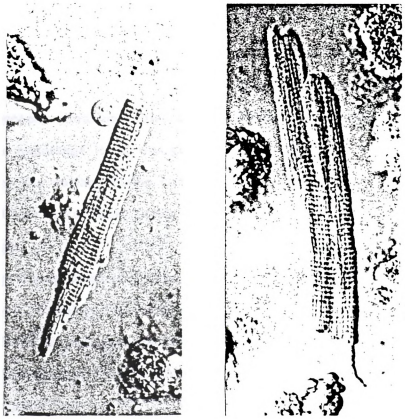


Figure 2

from the culture dish to a waste container by means of gravity. Complete changes of solution took between 15-20 seconds.

The myocytes were voltage-clamped by means of a glass micropipette which was mounted in a plastic pipette holder. (Further details on the voltage-clamp method are explained in the next section.) The pipette holder was connected to the headstage of a patch-clamp amplifer (model 8900, Dagan Instruments, Minneapolis, MN). The headstage was mounted on the microscope stage, and its positioning could be controlled by a hydraulic micromanipulator (model MO-102, Narishige Instruments, Tokyo, Japan).

The entire setup was enclosed in a Faraday cage made of fine copper screen. The pickup of 60 Hz electrical noise was minimized by connecting the Faraday cage, microscope, vibration table, and all electronic instruments to a common ground. The headstage itself was guarded from noise pickup by a driven shield controlled by the voltage-clamp command. The various electronic instruments were housed in a metal rack outside of the Faraday cage and were connected to the headstage.

Voltage-clamp commands were supplied by a digital pulse generator (Galveston Electronic Development, Galveston, TX). The timing of voltage-clamp commands from the pulse generator was controlled by a analog stimulator (model S88, Grass Instruments, Quincy, MA). The command signal was then fed into the patch-clamp amplifier.

Voltage and current outputs from the patch-clamp amplifier were displayed on a storage oscilloscope (Kikisui International, Gardena, CA) for observation during the experiments, and also were recorded on a four-channel FM tape recorder (model 4DS, Racal Recorders Inc., Southampton, England) for later analysis. The current signal was passed through a filter built into the patch-clamp amplifier, which was set at 1 KHz. The signals were recorded on the tape

recorder at a bandwith of DC to 5 kHz. The data were analyzed off-line on a PDP 11-23 computer (Digital Equipment Corporation, Maynard, MA) after the data were digitized at a sampling interval of 0.25-1 ms.

C. Whole-cell patch clamp technique

1. Pipette preparation

The voltage-clamp technique that was used was the whole-cell variant of the patch clamp technique (Hamill et al., 1981). The pipettes were made from Corning 7052 capillary glass. Other types of glass were tried, but this type seemed to produce the best results. The glass was pulled into pipettes of one inch in length by a two-stage vertical pipette puller (model PP-2, Narishije Instruments). Once the pipettes were pulled, the fine-tipped ends were fire-polished by briefly bringing them close to a fine platinum wire through which a small current was passed. The pipettes had a final diameter of 2-3 μm at the tip, and had a resistance of 1-3 megaohms when filled with the standard pipette solution.

The pipettes were filled with internal solution, and then placed in a plastic pipette holder (E.W. Wright, Guilford, CT). The pipette holder had two openings, one for the pipette (1.69 mm), and a smaller suction port. The suction port was connected to a 1 ml gastight glass syringe by a short length of polyethylene tubing. The inside of the holder was filled with the same internal solution that was in the pipette. The electrolyte was connected to the amplifier headstage via a Ag/AgCl pellet that was in contact with both the internal solution and a BNC connector.

2. Voltage-clamp technique

The resistance of a pipette was first measured under voltage-clamp when the pipette was lowered into the bath solution. The resistance was determined by measuring the current deflection during a series of 200 μV

command pulses. The resistance of the pipette was calculated according to Ohm's law. The pipette was then gently pressed against the membrane of the chosen myocyte. Usually the current deflection decreased, indicating an increased resistance of the pipette due to some degree of sealing to the cell membrane. At this point, a small amount of suction was applied to the inside of the pipette by pulling the gastight syringe back about 0.1 ml. This most often resulted in the formation of a gigaohm seal, which was seen as a flattening of the current deflection. An increase in the command pulse, up to 20 mV, allowed the quality of the seal to be measured. Acceptable seals had a resistance of greater than 5 gigaohms. It should be noted that there are some limitations to the use of this method to measure seal resistances quantitatively (Fischmeister et al., 1986), but it is quite satisfactory for the qualitative use employed here. The formation of a gigaohm seal usually occurred fairly quickly after the application of suction. A greater amount of suction or waiting for long periods of time were only moderately helpful in establishing seals. Pipettes were only used once, whether or not a seal was established with them.

After the establishment of a seal on the membrane, the next step was to rupture the patch underneath the pipette. This could be accomplished in one of two ways: 1) the patch could be disrupted by a sudden, large pulse of suction, 2) the patch could be disrupted by a short pulse of current applied through the pipette. It was found that the second method was the more reliable in breaking a large number of patches, but that the application of negative pressure provided better voltage control. It is possible that the burst of current can rupture the patch easily, but often leaves residual membrane material at the tip of the pipette, narrowing its lumen and increasing the series resistance. A hybrid technique was eventually adopted, whereby the patch was first ruptured by a current pulse, and then cleared by suction.

The control of membrane potential by this technique seemed adequate by all accepted indirect measures: a rapid settling of the capacitative transient, a symmetrically shaped current-voltage relationship of I_{Ca}, and a quite rapid activation of I_{Ca}. Voltage-control with this technique (i.e., pipettes with resistances of 1-3 megaohms) has been tested directly with a second intracellular microeletrode, and has been found to be satisfactory (Matsuda and Noma, 1984; Fischmeister and Hartzell, 1986). Series resistance compensation was used in all of these experiments to further improve voltage-control.

The rupture of the patch underneath the pipette allows not only electrical access to the cell interior, but also allows the gradual equilibration of the pipette solution and the cytoplasm of the cell. This allows for the introduction to the cell interior of desired ions and drugs. This was used to isolate I_{Ca} from other membrane currents.

D. Solutions and Drugs

Table 1 shows the composition of the main internal (pipette) and external solutions used in these experiments. A few other solutions were used, but they were not used as often, or were minor modifications of those shown in the Table, and will be defined in the Results section.

There were two basic internal solutions. The one that is called "standard pipette" was used most often. It had Cs as its major constituent cation, as it has been found that replacement of intracellular K with Cs ions greatly reduces outward K currents that may overlap with I_{Ca} (Latorre and Miller, 1983). TEA Cl was also included to further reduce K currents. The disodium salt of ATP was included to reduce Ca channel rundown, and to keep intracellular Na at a physiological concentration. EGTA was kept low to prevent interference with Ca-dependent inactivation. The other internal solution, called "5 mM EGTA"

Table 1

Composition of Solutions

Internal Solution (all concentrations in mM)

	Cs Asp	CsCl	TEA Cl	HEPES	EGTA	ATP Na_2	ATP Mg	$MgCl_2$
1. Standard pipette	110	20	20	5	0.1	4	--	1
2. 5 mM EGTA	110	20	20	5	5	--	1	--

External Solution

	NaCl	$CaCl_2$	$MgCl_2$	KCl	CsCl	glucose	HEPES	EGTA	Tris Cl	$CdCl_2$
1. 2.5 mM Ca	124	2.5	0.5	5.4	20	5.5	5	--	--	--
2. 2 mM EGTA	124	--	0.1	5.4	20	5.5	5	2.0	--	--
3. 20 μM Cd	124	--	0.1	5.4	20	5.5	5	--	--	0.02
4. low Ca	124	1.8	0.1	5.4	20	5.5	5	2.0	--	--
5. 1 mM Mg	124	--	1.0	5.4	20	5.5	5	2.0	—	—
6. 1 mM Ca	124	1.0	--	5.4	20	5.5	5	--	--	--
7. Tris	---	2.5	0.5	5.4	20	5.5	-	--	129	--

All solutions were buffered to pH=7.4, and were gassed with 100% O_2.

solution, was used when the external solution was switched to Na-free solution. It was basically the same as the standard solution, only the EGTA was raised to prevent contracture, and intracellular Na was eliminated as well by switching to the Mg salt of ATP.

It should be noted that both solutions had a significant junction potential relative to the standard external solutions, which was probably attributable to the high proportion of large molecules (aspartate) as current-carriers (cf. Hagiwara and Ohmori, 1982; Matsuda and Noma, 1984). The junction potential between the internal and external solutions was measured with a 3 M KCl-filled microelectrode, and was found to be -14 mV, with the internal solution being more negative. This means that without correction, the "true" membrane potential is 14 mV more negative that that shown by the monitor. This factor was taken into account during the experiments, and the membrane potentials reported here reflect the "true" potential. Finally, the free Ca concentration of the internal solutions was measured by a Ca electrode (courtesy of Dr. Ralph Pax, Michigan State University). The standard pipette solution had a free Ca concentration of approximately 80 nM, and the free Ca of the 5 mM EGTA solution was too low to be estimated.

The most commonly used external solution is labelled "2.5 mM Ca" solution. It contained roughly physiological concentrations of Na, K, Ca, Mg and glucose. CsCl was included to block the inwardly rectifying K current (Isenberg, 1976). Tetrodotoxin (Calbiochem, San Diego, CA), 0.01 mM, was included in all external solutions to reduce currents through Na channels. In addition, the Na current was usually inactivated by using a holding potential of -50 mV. When a more negative holding potential was used, the external solution was switched to a Na-free one, labelled as "Tris," to eliminate current through Na channels. These combinations

of internal and external solutions produced a good isolation of current through Ca channels, as will be shown later.

Table 1 also includes some specialized external solutions. The "2 mM EGTA" solution was used to study Ca channel behavior when permeant divalent cations were absent. The "20 μM Cd" solution was one solution that was used to block all current through Ca channels. The "low Ca" solution was a solution that contained a very low amount of free Ca, approximately 4 μM. The "1 mM Mg" and "1 mM Ca" solutions were used to study different patterns of Ca channel behavior with approximately equal concentrations of total divalent cations.

It should be noted that the external solutions were kept at room temperature (20-22°C). This greatly reduces the magnitude of I_{Ca}, and slows its activation and inactivation (Cavalie et al., 1985), making I_{Ca} easier to study.

Various drugs were used in the course of these experiments. Racemic Bay K 8644 was obtained by courtesy of the Miles Institute, New Haven, CT. Nifedipine was obtained from Pfizer, Inc., New York. D600 was obtained from Knoll Pharmaceutical Co., Whippany, NJ. Trifluoperazine, isoproterenol, and phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co., St. Louis, MO. All drugs were dissolved in distilled water to make stock solutions, except for nifedipine and Bay K 8644, which were dissolved in PEG 400. The final concentration of polyethylene glycol 400 that the myocytes were exposed to was never greater than 0.5%, which does not affect I_{Ca} (Kass, 1982; Uehara and Hume, 1985). However, the same concentration of polyethylene glycol 400 was added to the controls as well. Finally, all light-sensitive drugs were protected from exposure to light. This includes nifedipine, Bay K 8644 and phorbol 12-myristate 13-acetate.

E. Data Analysis

1. Analysis of raw current records

Current traces were usually analyzed after digitization on the PDP 11-23. The magnitude of current through Ca channels was most often measured as the peak inward or outward current deflection, which occured a few milliseconds after depolarization, just after the settling of the capacitative transient. The Ca channel current was measured relative to the time-independent current remaining after complete block of Ca channels by either $CdCl_2$ or nifedipine. Cd (500 μM) was most often used. Both current and voltage traces were displayed with a digital plotter (model 7470A, Hewelett-Packard, San Diego, CA).

2. Statistical analysis

Most numerical data is shown as mean ± SEM. A two-tailed Student's t-test was used to determine significant differences between two experimental means. $P < 0.05$ was deemed significant.

3. Fitting of exponentials

Exponential fits were required on several occasions during these studies. Exponential fits of I_{Ca} current decay was done by regression analysis on the PDP 11-23. All the data points were displayed as the logarithm of current against time, and the exponential fit was selected by eye. Exponential fits that involved a smaller number of data points, such as that for recovery data, were done on an IBM PC-AT. The exponentials were determined by a least-squares fit to the equation $y(t) = A_0 - A_1 \exp(t/tau_1) - A_2 \exp(t/tau_2)$. In this equation, A_0 is a constant, t is time, tau_1 and tau_2 are the time constants of recovery, and A_1 and A_2 are the pre-exponential coefficients for their respective time constants.

4. Analysis of Ca channel blockade

In the studies where use-dependent block was analyzed in terms of the guarded receptor hypothesis, all the successive I_{Ca}s during a train were fit to the

equation $I_n = I_o + (I_o - I_{ss})\, e^{-n\ lambda^*}$ by a least squares method (Starmer et al., 1985). In this equation I_n is the size of I_{Ca} during the nth pulse in the train, I_{ss} is the steady-state level of I_{Ca}, I_o is the control I_{Ca} and lambda* is the use-dependent uptake rate of the drug. This procedure gave values of lambda* for different voltage-clamp protocols. According to the model, the use-dependent uptake rate is the sum of the uptake rates during the activated (or depolarized) period, and the resting (or repolarized) period (Starmer and Grant, 1985). Therefore, lambda* = $lambda_a\ t_a + lambda_r\ t_r$, where $lambda_a$ and $lambda_r$ are the uptake rates during the activated and resting periods, respectively, and t_a and t_r are the duration of the activated and resting periods. Thus, a plot of lambda* versus t_r should give a straight line with a y-intercept of $lambda_a\ t_a$, and a slope of $lamda_r$. Since the uptake rate during the resting period is the converse of the removal of block, $lambda_r = 1/tau_r$, where tau_r is the drug-influenced time constant of recovery.

RESULTS

A. Separation of I_{Ca} from other membrane currents

Figure 3A shows typical current records of I_{Ca} obtained during perfusion with 2.5 mM Ca solution and with the standard pipette solution inside the pipette. The bottom trace shows the current induced by a 500 ms voltage-clamp step to 0 mV from a holding potential of -50 mV. After the settling of the capacitative transient (initial upward deflection), a rapidly activating inward current was seen. The inward current decayed over the course of a few hundred milliseconds. The inward current was completely blocked by the addition of 500 μM Cd, an inorganic Ca channel blocker, to the perfusate (labelled trace). Figure 3B also shows that this inward current (recorded under somewhat different conditions, see legend) was eliminated in the presence of the organic Ca channel blocker nifedipine (10 μM). This inward current was identified as I_{Ca} because of its time course, voltage-dependence, its dependence on the concentration of external Ca (data not shown), and its sensitivity to the Ca channel blockers.

The upper trace of Figure 3A shows a current record obtained during a 500 ms depolarization to +70 mV. In this example, a small outward current that decays with time was seen. Blockade of this current with Cd (labelled trace) indicated that this outward current was probably flowing through Ca channels, and that a reversal of I_{Ca} can be demonstrated in guinea-pig ventricular myocytes. It should also be noted that blockade with Cd revealed a portion of I_{Ca} that did not completely decay, or inactivate, at this potential. These results are in agreement with those of Lee and Tsien (1982; 1984), but are in disagreement with those of

Figure 3. Inhibition of I_{Ca} by Cd and nifedipine. Panel A, top trace shows I_{Ca} evoked by a 500 ms voltage-clamp step to +70 mV from -50 mV in the absence and presence of 500 μM Cd (marked). Bottom trace shows I_{Ca} evoked by a step to 0 mV. The 2.5 mM Ca external solution and the standard pipette solution were used. Panel B, top trace shows a control I_{Ca} evoked by a 500 ms step to 0 mV from a holding potential of -80 mV. The bottom trace shows I_{Ca} after the application of 10 μM nifedipine. The Tris external solution and the 5 mM EGTA pipette solution were used.

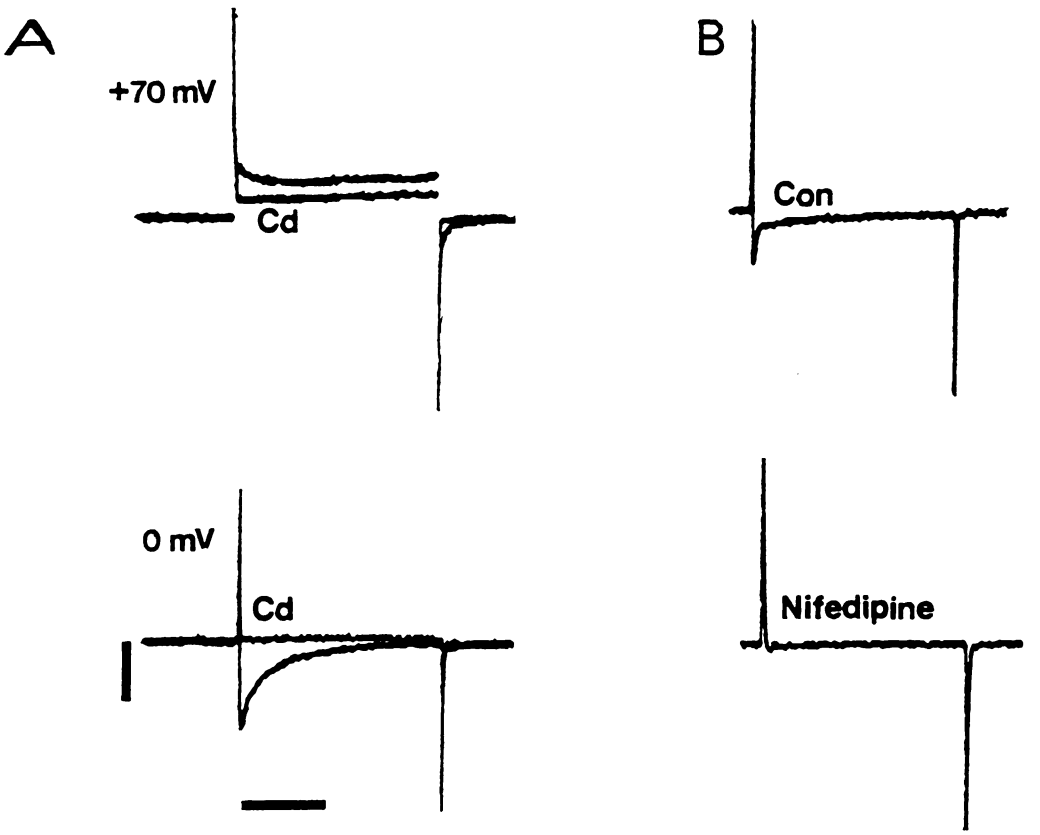


Figure 3

Matsuda and Noma (1984). The latter authors failed to see a clean reversal, since a large, slowly activating outward current contaminated their recordings at very positive potentials. This type of current was also sometimes seen in these experiments. It often did mar recordings of I_{Ca} at very positve potentials, but some cells had little or no component of this current. This current contamination was found to be more troublesome with external solutions that contained no K or Cs, and with external solutions that had Ca as the current-carrier through Ca channels.

It appeared that the precautions taken to reduce current through Na and K channels had produced a satisfactory isolation of current through Ca channels. However, multiple types of Ca channels have recently been characterized (see Introduction). As the Ca channel of interest in these studies is the "L-type," which is believed to be involved in excitation-contraction coupling, and is sensitive to β-adrenergic stimulation, it was necessary to appraise the contribution of other Ca channels to the observed membrane current. This was done by taking advantage of the fact that the second type of Ca channel in heart tissue, the T-channel, is quite insensitive to block by dihydropyridines (Bean, 1985; Nilius et al., 1985; Mitra and Morad, 1986). The top trace in Figure 3B shows I_{Ca} evoked by a depolarization from -80 mV to 0 mV. At this holding potential, the T-type Ca channels should be available. Na-free, Tris-containing external solution was used to eliminate current through Na channels. Application of 10 μM nifedipine eliminated all of I_{Ca}, except for a few pA (bottom trace). It is possible that this residual current may represent Ca influx through T-type Ca channels. However, this was not tested further, as this component would appear to make an insignificant contribution to I_{Ca} under these conditions, as has been suggested by other investigators (Bean, 1985; Nilius et al., 1985). This is also true statistically, as will be shown later (Figure 21). Therefore, when either the term "I_{Ca}" or "Ca

channel" is used in this thesis, it refers solely to the L-type Ca channel, unless otherwise noted.

Figure 4 shows a typical current-voltage relationship of I_{Ca}. The filled symbols show the peak inward or outward current, and the crosses show the magnitude of time-independent current left after Cd was applied. It can be seen that the background conductance of the myocytes under these conditions (Cs on both sides of the membrane) is quite small and has a linear dependence on membrane potential over the range of -100 to 0 mV. This contrasts with the rather large background conductance and its profound nonlinearity under physiological conditions (Hume and Uehara, 1985). The average input resistance of these cells under physiological conditions is 32 megaohms, while the cell shown in Figure 4 had an input resistance of 4 gigaohms. This can almost certainly be attributed to the block by external Cs of the inwardly rectifying K channel, which underlies the resting potential of these cells. The time-independent background conductance seemed to increase, however, at very positive potentials.

I_{Ca}, as measured by the difference between the peak and background currents, had the expected voltage-dependence. It was first activated at about -40 mV, it reached its peak magnitude at about 0 mV, and became a small outward current at potentials greater than +60 mV. The peak magnitude of inward I_{Ca} in this example was about 350 pA at 0 mV. However, the size of I_{Ca} varied between cells, from a few hundred pA to almost 2 nA. Outward currents through Ca channels were almost always fairly small in the 2.5 mM Ca external solution.

Finally, it should be noted with regard to the magnitude of I_{Ca}, that the phenomenon of Ca channel "rundown" (Doroshenko et al., 1982; Fenwick et al., 1982) was seen in these cells. That is, during the course of an experiment, which could be between 10 to 60 minutes, I_{Ca} often slowly decreased in size, although it

Figure 4. Current-voltage relationship of I_{Ca}. Filled symbols show the peak inward or outward I_{Ca}. Crosses show the time-independent current left after application of 500 μM Cd. Currents in this figure were measured with respect to the holding current (V_h = -50 mV). Same cell as in Figure 3A.

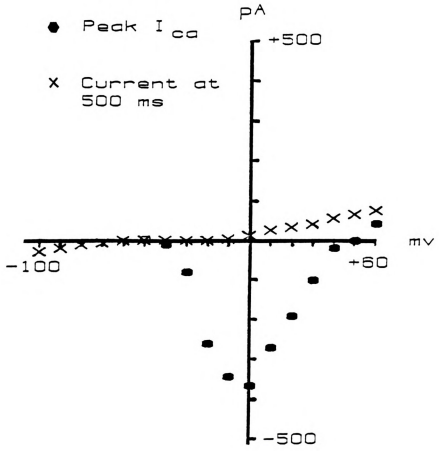


Figure 4

rarely completely disappeared. The rate of this process was quite unpredictable, and seemed to vary more from day to day, rather than between cells on an individual day. As it has been speculated that Ca channel rundown may be due to the loss of some intracellular factor during dialysis (Byerly and Yazejian, 1986), it may be possible that the variation reflects the metabolic state of the cells after enzymatic isolation. The inclusion of ATP in the intracellular solution slowed this process to acceptable levels.

B. Characterization of I_{Ca} inactivation

1. Time course of current decay

As was evident in the previous figures, I_{Ca} inactivated within 500 ms, after activation. All of I_{Ca} appeared to inactivate at a potential of 0 mV, while inactivation appeared to be only partial at more positive potentials (Figure 3A). It has been traditional to describe the time course of channel activation or inactivation as exponential processes, and Figure 5 shows an exponential analysis of a typical I_{Ca} at a test potential of 0 mV. The time course of I_{Ca} decay could be fit by two exponentials. The faster time constant was 12 ms, and the slower time constant was 119 ms. This finding of a biexponential time course of I_{Ca} decay is in agreement with several more recent studies (Isenberg and Klockner, 1982b; Kass and Sanguinetti, 1984; Hume and Uehara, 1985). Figure 6 demonstrates that the time course of I_{Ca} decay represents Ca channel inactivation, and not another process such as development of outward current, by giving an independent measurement of the time course of I_{Ca} inactivation. This was done by using a two-pulse protocol, where the first voltage-clamp step to 0 mV inactivates I_{Ca} to some degree, and the second step to 0 mV elicits a second I_{Ca} that measures the degree of inactivation. The time course for the onset of inactivation was determined by repeating this several times with different

Figure 5. Exponential analysis of the time course of I_{Ca} decay. Panel A shows an example of I_{Ca} evoked by a 500 ms voltage-clamp step to 0 mV from -50 mV. The vertical calibration bar represents 1 nA and the horizontal bar represents 250 ms. Panel B is a semilogarithmic plot of the decay phase of I_{Ca}. The first exponential (tau=119 ms) was subtracted from the trace, and the remaining current was fit by a second exponential (tau=12 ms). The 2.5 mM Ca external solution and the standard pipette solution were used.

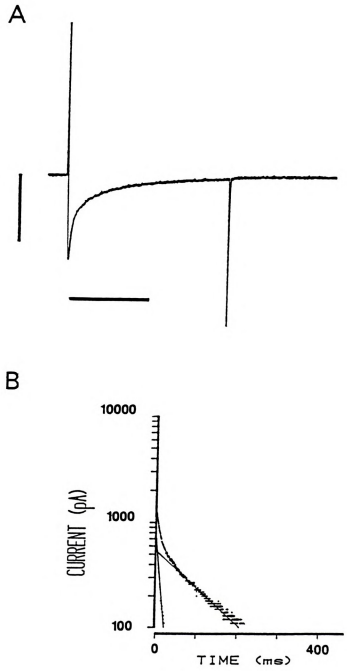


Figure 5

Figure 6. Time course of I_{Ca} inactivation as measured by a two-pulse protocol. The voltage-clamp protocol consisted of a variable duration prepulse, which was separated from a 500 ms test pulse by 10 ms. Both steps were from a holding potential of -50 mV to 0 mV. The graph plots the relative magnitude of the test I_{Ca} (y-axis) against the duration of the prepulse (x-axis). The curve was fit with two exponentials (see Methods) with time constants of 15 and 122 ms. The 2.5 mM Ca external solution and the standard pipette solution were used.

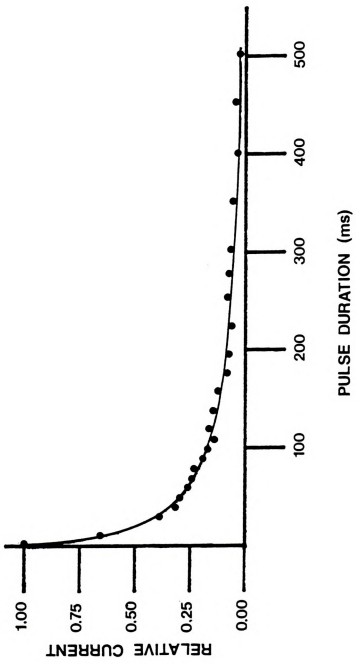

RELATIVE CURRENT
1.00
0.75
0.50
0.25
0.00
100
200
300
400
500
PULSE DURATION (ms)

Figure 6

durations of the first step. In this example, it can again be seen that inactivation is a biexponential process, with time constants of 15 ms and 122 ms at 0 mV.

Despite this information, the characterization of I_{Ca} inactivation as a biexponential process was viewed somewhat skeptically in these studies. This is because further analysis of this kind revealed a number of complexities. The values of the time-constants had a peculiar voltage-dependence that has been noted before (Reuter and Scholz, 1977a, Hume and Uehara, 1985), in that they were fastest at approximately 0 mV, and were slower at either more positive or more negative potentials. This is quite different from the classical example of the Na channel in squid axon (Hodgkin and Huxley, 1952a), or in heart (Brown et al., 1981a). In addition, the relative proportion of two exponentials had a similar voltage-dependence, in that the faster exponential was most prominent at 0 mV, and became less important as the test potential was moved more or less positive. Finally, it was noted the relative proportion of the exponentials also depended on the current magnitude, as an increase in the external Ca concentration enhanced the fast exponential at the cost of the slower, and reduction of I_{Ca} with the inorganic blocker Cd enhanced the slow exponetial. It was concluded that while I_{Ca} inactivation can be described as a biexponential process, it is quite complicated, and functional significance cannot easily be ascribed to the exponentials. It should also be noted that it is difficult to accurately estimate two exponentials, unless their values are widely separated. Further studies used the halftime of decay as a measure of the rate of I_{Ca} inactivation, as exponential analysis gave little additional information, and perhaps was arbitrary. Some of the reasons for the complicated nature of the onset of I_{Ca} inactivation will become clear later.

2. Effect of Ca entry on inactivation

As Ca entry has been postulated to play an important role in the inactivation of Ca channels in many different preparations, it was of interest to determine its influence in this preparation. Figure 7 shows one approach that was taken. It shows current traces of I_{Ca} evoked during 500 ms depolarizations to 0 mV, from a holding potential of -50 mV. This was done in two different external solutions. The current trace next to the triangle shows I_{Ca} in 2.5 mM Ca solution, but with 7.5 mM $MgCl_2$ added. The current trace next to the circle shows I_{Ca} in the same external solution, except that the Ca concentration was increased to 10 mM, and Mg was omitted. Therefore, the total concentration of divalent cations was kept constant, which should help to reduce unwanted surface charge effects. It can be seen that increasing the external Ca concentration increased the size of I_{Ca}, as would be expected. However, the rate of current decay also seemed to be accelerated, as shown by the arrows, which indicate the time it took for I_{Ca} to decline by one-half. The half-time of current decay was 27 ms in the 2.5 mM Ca solution, and diminished to 10 ms in the 10 mM Ca solution. This appeared to be mainly attributable to an enhancement of the fast phase of I_{Ca} inactivation.

If an increase in the amount of Ca entering the cell accelerates inactivation, presumably due to Ca binding at some unknown intracellular site, then the introduction of Ca-chelating agents into the cytoplasm should slow I_{Ca} inactivation. The whole-cell patch clamp technique provides a simple means of undertaking this experiment, as the wide tip-diameter of the pipette allows for dialysis of the cell interior. Figure 8 shows typical results obtained when the pipette was filled with a solution containing (in mM) Cs citrate 65, TEA Cl 20, HEPES 10, Na ATP 4 and $MgCl_2$ 1. I_{Ca} was elicted by 500 ms depolarizations to 0 mV at a frequency of 0.1 Hz. The upper current trace in Figure 8 shows I_{Ca} just after the membrane patch was ruptured, when presumably little dialysis had taken

Figure 7. Time course of I_{Ca} inactivation at two concentrations of external Ca. The top traces show I_{Ca} (voltage-clamp protocol shown at bottom of figure) in the presence of 2.5 mM (triangle) and 10 mM Ca (circle). See text for further details on solutions. Stimulation frequency was 0.1 Hz. The arrows show the half-time for I_{Ca} decay.

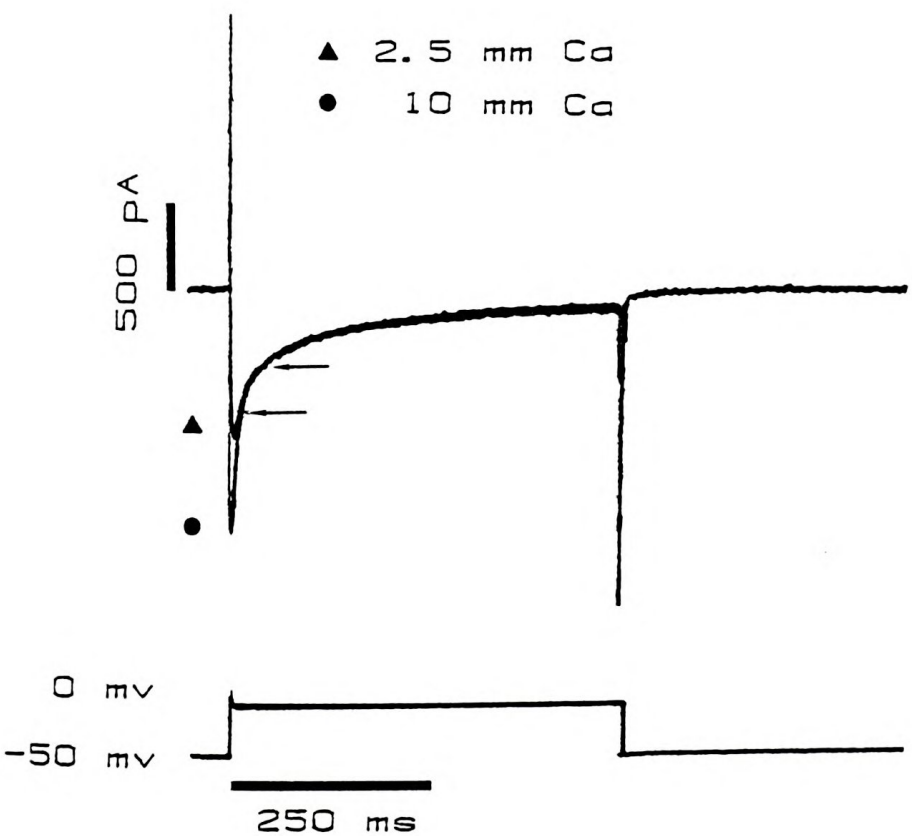


Figure 7

Figure 8. I_{Ca} before and after equilibration with citrate-containing solution. The top traces show I_{Ca} (voltage-clamp protocol shown at bottom of figure) a few seconds after rupture of the membrane patch, and several minutes later, after the citrate-containing pipette solution had equilibrated with the cytoplasm (marked by circle). The 2.5 mM Ca external solution was used. See text for details on pipette solution. The arrows indicate the half-time for I_{Ca} decay.

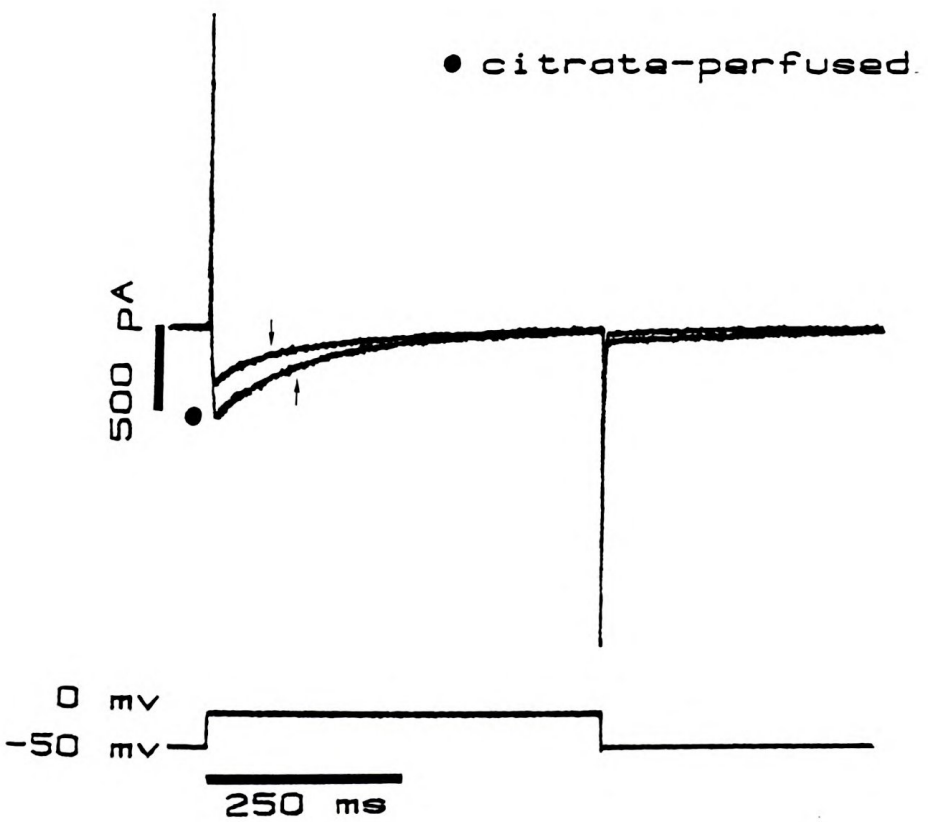


Figure 8

place. The lower trace (marked by a circle) shows I_{Ca} after it had reached a new stable level several minutes later. The introduction of citrate, a Ca buffer, into the cell seems to have increased the size of I_{Ca}. It should be noted that a similar result has been noted with EGTA injection into neurons of Helix aspersa (Plant et al., 1983).

The introduction of citrate into the ventricular cell did not just increase the magnitude of I_{Ca}, it also slowed down the inactivation process. The half-times of current decay (marked by arrows) were 62 ms initially, and 82 ms at steady-state. This effect occurred despite the increase in Ca entry during the pulse. These dialysis effects were also seen when high concentrations of EGTA (5-20 mM) were in the pipette solution, and were never seen during dialysis with the standard pipette solution, which contained 0.1 mM EGTA. Therefore it appears that Ca ions can inactivate Ca channels upon entry, and that this process can be antagonized by internal Ca buffers.

3. Relationship of I_{Ca} inactivation to membrane potential

The dependence of I_{Ca} inactivation on membrane potential was studied using a two-pulse protocol shown in Figure 9A. Cells were held at -50 mV, and then two 500 ms depolarizations were given in succession. The two voltage-steps were separated by a 10 ms interval. The second depolarization is called the test-pulse, and is always to a potential that evokes a large I_{Ca}, usually 0 mV. The first depolarization is called the prepulse, and goes to a wide variety of membrane potentials. This type of protocol was used repeatedly in these studies, and was designed to measure the amount of inactivation remaining after the prepulses. Each prepulse will activate and inactivate I_{Ca} to a different extent. The 10 ms interval between prepulses is long enough for I_{Ca} to deactivate completely (Byerly and Hagiwara, 1982; Isenberg and Klockner, 1982b), and therefore noninactivated channels will have returned to their original resting

Figure 9. Two-pulse protocol for measuring inactivation and representative current traces. Panel A outlines the voltage-clamp protocol used to measure I_{Ca} inactivation. A 500 ms prepulse to a wide variety of potentials is separated by 10 ms from a second, 500 ms step to 0 mV. Panel B shows superimposed current traces evoked by this protocol using prepulses of 0 mV and +110 mV (marked by circle). The 2.5 mM Ca external solution and the standard pipette solution were used.

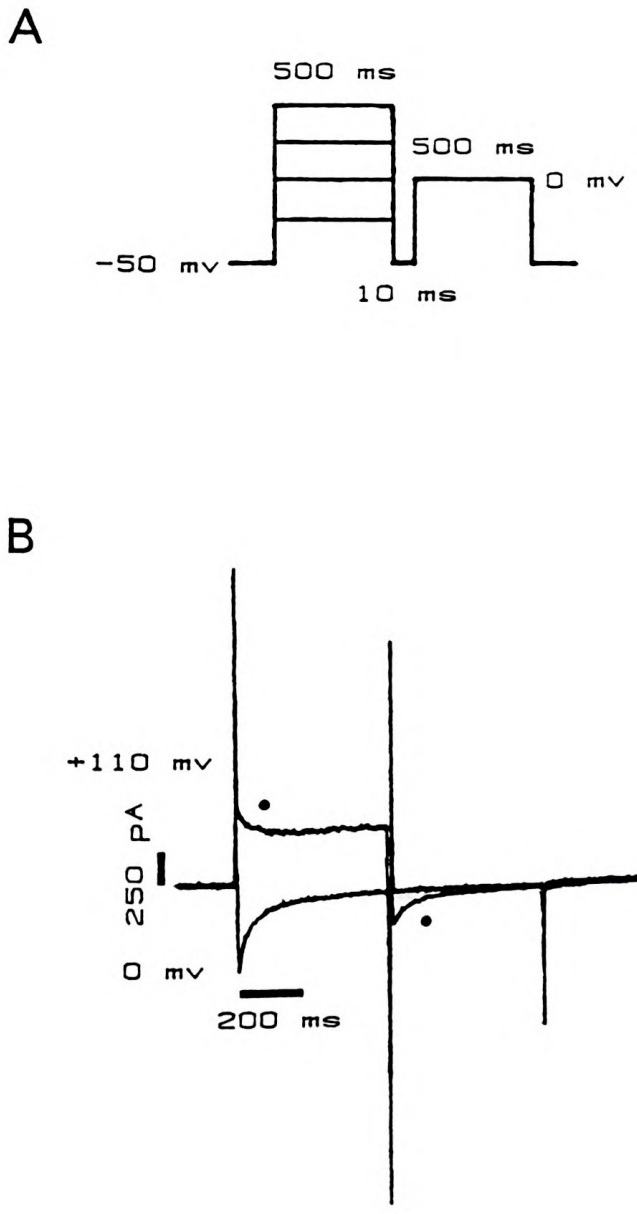


Figure 9

state before the test pulse. However, there will be little or no return of inactivated Ca channels to the resting state during this interval, as recovery from inactivation is relatively slow at -50 mV (see Figure 17 and Table 3). Therefore the I_{Ca} evoked by the test pulse will be reduced in proportion to the extent of inactivation during the prepulse.

Figure 9B shows an example of typical current traces obtained with this protocol in 2.5 mM Ca external solution. A test pulse to 0 mV was preceded by two different prepulses, one to 0 mV, and one to +110 mV. The prepulse to 0 mV evoked a large, inward I_{Ca} that resulted in a large residual inactivation, as judged by the small size of the subsequent test I_{Ca}. If there had been no prepulse at all, the size of the test I_{Ca} would have been the same as that seen during the prepulse. The prepulse to +110 mV, on the other hand, evoked an outward I_{Ca} that seemed to produce less inactivation of Ca channels, as judged by a larger test I_{Ca} (marked by the filled circle). Thus, very strong depolarizations seem to leave behind less Ca channel inactivation. This is the same observation that led to the first proposal of Ca-dependent inactivation for Ca channels in invertebrate preparations (Brehm and Eckert, 1978; Tillotson, 1979), as it is inconsistent with the behavior expected of a classical voltage-dependent inactivation mechanism.

Figure 10 shows the average results from a number of such experiments. The relative size of the test I_{Ca}, when compared to I_{Ca} without a prepulse, is plotted against the prepulse potential. This type of graph was often used in these studies and will be referred to as an inactivation curve. It can be seen that the inactivation curve of I_{Ca} had a partial U-shape. A comparison of the inactivation curve with the current-voltage relationship obtained under similar conditions (Figure 4) reveals some similarities. The amount of inactivation at a given membrane potential seemed to be correlated with the amount of Ca that entered the cell at that potential. Both Ca entry and inactivation were

Figure 10. Relationship of I_{Ca} inactivation to membrane potential. The figure plots the inactivation curve of I_{Ca}, as the relative amplitude of test I_{Ca} against the prepulse potential. The voltage-clamp protocol was that of Figure 9A. Mean values obtained from five cells are shown with the S.E.M. The 2.5 mM Ca external solution and the standard pipette solution were used.

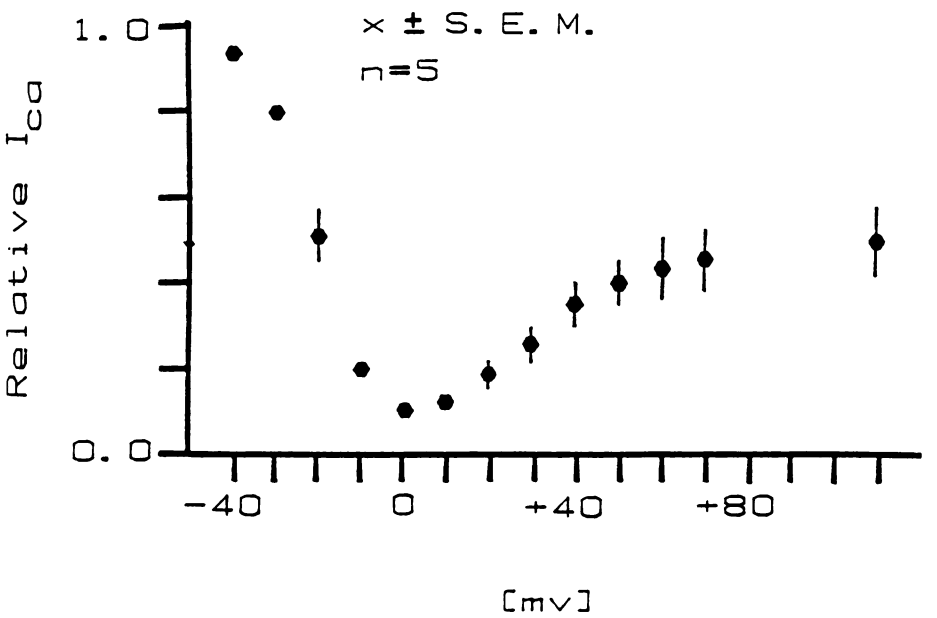


Figure 10

enhanced with more positive depolarizations, until they reached a peak at 0 mV. They both then declined at more positive potentials. However, the correlation did not seem to hold up at very positive potentials. Considerable inactivation of I_{Ca} occurred at +110 mV despite the fact that there should have been little or no Ca entering through the channels at that potential. I_{Ca} inactivation does not seem to show either the simple monotonic dependence on membrane potential that would be predicted of a voltage-dependent mechanism, or the completely U-shaped dependence expected of a mechanism completely dependent on Ca entry. Since the shape of the inactivation curve lay between these extremes, the possibility of a combined mechanism was considered.

C. Ca channel inactivation in the absence of permeant divalent cations

1. General properties of monovalent currents through Ca channels

The previous studies on I_{Ca} inactivation had led to the postulation of a dual mechanism of Ca channel inactivation, where both membrane potential and Ca entry had a direct role in turning off Ca channels. One way of testing this hypothesis is to attempt a separation of the two mechanisms. The approach that was taken to accomplish this, took advantage of the fact that the Ca channel becomes permeable to monovalent cations when the concentration of extracellular divalent cations is greatly reduced (Hess and Tsien, 1984; Almers and McCleskey, 1984).

Figure 11A show representative examples of the membrane currents seen when the external solution was switched from the 2.5 mM Ca solution to the 2 mM EGTA solution. The switch to an external solution low in divalent cations was technically very difficult. The quality of the pipette seal on the membrane quite often deteriorated. In addition, prolonged exposure to Ca-free solutions damages cell membranes so that they become leaky (Winegrad, 1971). Despite

Figure 11. Cd and nifedipine block of I_{ns}. Panel A shows records of I_{ns} evoked by 1 s depolarizations from -50 mV to the potentials shown. Records obtained shortly after switching to the 20 μM Cd solution are marked. Panel B shows inward and outward I_{ns} obtained with 500 ms depolarizations from -50 mV to the potentials shown. Membrane currents after application of 100 nM nifedipine are marked. These records are from two separate cells. The 2 mM EGTA external solution and the standard pipette solution were used.

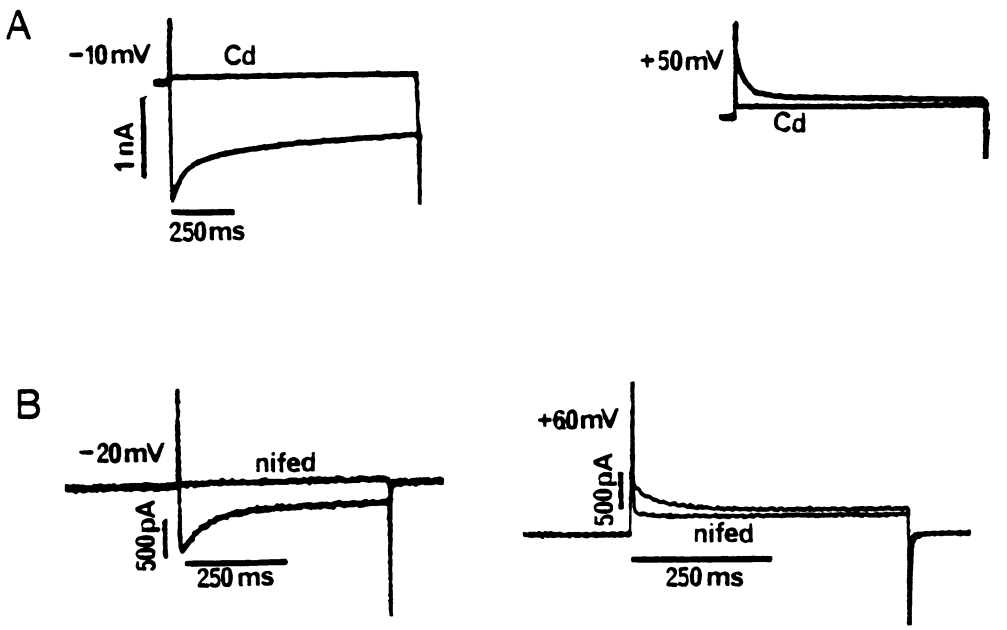

A
-10mV
Cd
1nA
250ms
+50mV
Cd
B
-20mV
nifed
500pA
250 ms
+60mV
500pA
nifed
250 ms

Figure 11

these problems, high-quality recordings were obtained in a number of cells. Experiments were halted if the background current, which was measured by a 50 mV hyperpolarization increased beyond 100 pA.

The left-hand tracing of Figure 11A shows the current evoked during a 1 sec step from -50 mV to -10 mV. A very large inward current is rapidly activated, and then very slowly decays. A stronger depolarization to +50 mV (right-hand trace) produced a large outward current that also slowly decayed. These currents were identified as flowing through Ca channels, as they were completely blocked by switching to the 20 μM Cd external solution (labeled traces in A), or by the addition of 100 nM nifedipine to the superfusate (Figure 11B). These currents were obviously carried by monovalent cations through the channels, as they were only current-carriers available in significant concentrations. As the Ca channel is thought to have only a modest selectivity for different monovalent cations in the absence of Ca (Kostyuk et al., 1983; Fukushima and Hagiwara, 1985), the inward monovalent current is probably carried mostly by external Na, and the outward monovalent current is probably carried principally by internal Cs. For this reason, the precedent of calling this current I_{ns}, for "nonspecific current" (Almers et al., 1984), was followed.

It can be seen in Figure 11 that I_{ns} inactivates with a much slower time course than I_{Ca} did. Also, channel blockade with either Cd or nifedipine reveals that a fraction of current remains uninactivated after long depolarizations. This remained true for depolarizations of up to 10 s (data not shown).

A typical current-voltage relationship for I_{ns} is shown in Figure 12. I_{ns} began to activate at about -40 mV, reached its inward peak at about -20 mV, and became a large outward current at potentials more positive than +20 mV. The reversal potential of I_{ns} was carefully determined in five cells, and was found to be +22±3 mV. It is obvious that the current-voltage relationship had only a small

Figure 12. Current-voltage relationship of I_{ns}. Circles show the peak inward or outward I_{ns} evoked by 500 ms depolarizations from -50 mV. Time-independent, Cd-insensitive current was subtracted off. Same cell as in Figure 11A.

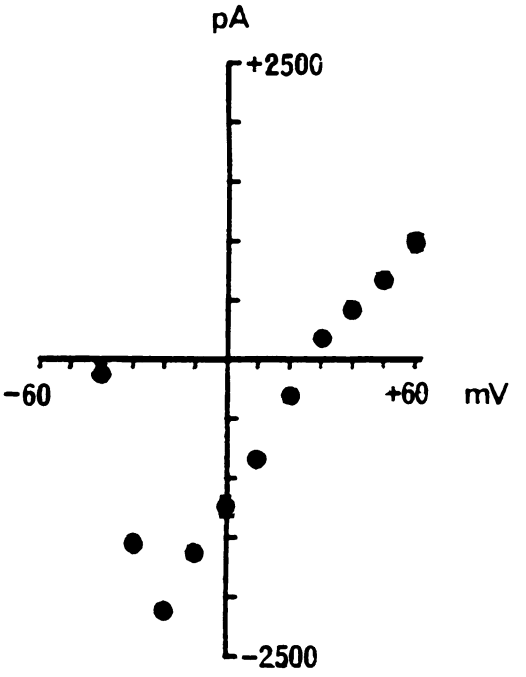


Figure 12

deviation from linearity as the pulse potential was moved more positive. This differs considerably from the strong rectification of I_{Ca} seen around the reversal potential (Figure 4). Finally, it should be noted that the peak inward magnitude of I_{ns} was always much larger than that seen with I_{Ca}.

2. Voltage-dependent inactivation of Ca channels

After the identification and initial description of I_{ns}, the next experiments sought to characterize its slow decay with time. Figure 13A shows a family of I_{ns} traces that were obtained with 1 second depolarizations from -50 mV to a variety of different potentials. Again, I_{ns} was first evoked as a small inward current at -40 mV, and as the pulse potential became more positive, first peaked in an inward direction, and then reversed to become an outward current. Examination of the time course of I_{ns} shows that at quite negative potentials, from -40 to -30 mV, I_{ns} inactivates very slowly or not at all. As the pulse potential was gradually made more positive, the rate of inactivation appeared to become faster. This is the type of behavior expected for a classical voltage-dependent inactivation mechanism.

I_{ns} inactivation, however displayed considerable variability between cells. In the examples shown in Figure 13A, I_{ns} seems to have reached a steady-state by the end of the 1 sec voltage-step. This type of behavior was seen in a number of the cells examined (for example, Figure 11B). However, other cells seemed to have a component of I_{ns} inactivation that was much slower (Figure 11A), and often was not even complete at the end of 10 sec. The reason for this variability is not known.

The time course of I_n inactivation could often be described as a biexponential process, similar to I_{Ca} (see Figure 5). However, because of the variability in the time course of I_{ns}, it was found to be much simpler and more consistent to quantify the rate of I_{ns} inactivation by the time it took to reach half

Figure 13. Kinetics of I_{ns} inactivation. Panel A shows current records obtained by 1 s depolarizations from -50 mV to the potentials shown. Panel B plots the half-time of I_{ns} decay (y-axis) against the pulse potential. The 2 mM EGTA external solution and the standard pipette solution were used.

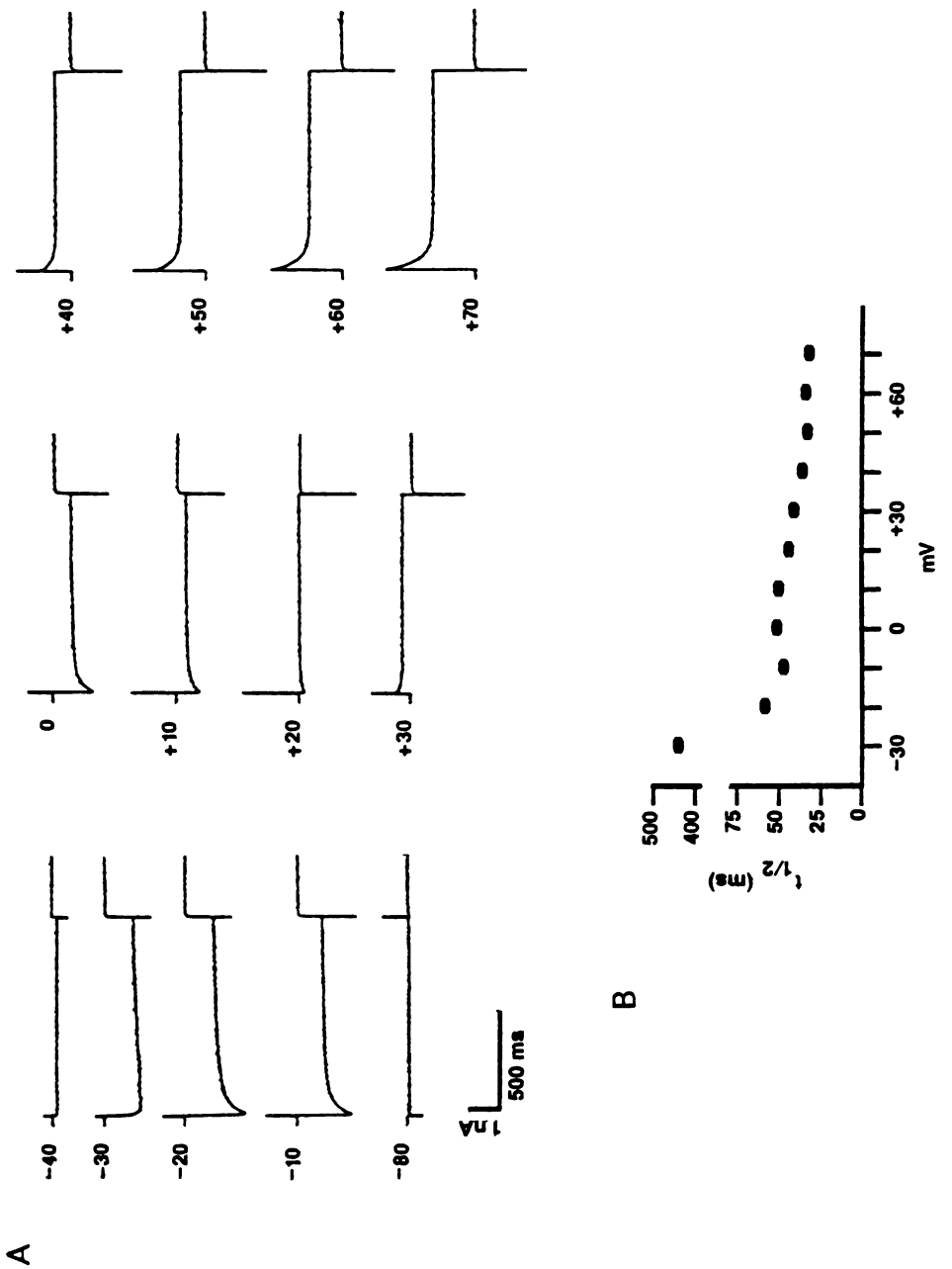

A
-40
-30
-20
-10
-80
0
+10
+20
+30
+40
+50
+60
+70
1 nA
500 ms
B
500
400
75
50
25
0
$t_{1/2}$ (ms)
-30
0
+30
+60
mV

Figure 13

of the steady-state value. Figure 13B shows the half-times of I_{ns} decay for the current traces of Figure 13A. Again, the rate of onset of I inactivation is enhanced in a monotonic fashion by more positive potentials.

The voltage-dependence of I_{ns} inactivation was further studied by examining the inactivation curve. Figure 14A show a typical example of current traces obtained with the two-pulse protocol in the 2 mM EGTA external solution. The 500 ms test pulse was set at -20 mV, as that is where the peak inward I_{ns} occurred. The test pulse was preceded by a 500 ms prepulse to either -20 mV or +50 mV. The prepulse to -20 mV evoked a large, inward I_{ns} that produced a fair amount of inactivation, as judged by the size of the subsequent test pulse. The test pulse would have been the same size as the -20 mV prepulse, if there had been no inactivation. It also appeared that I_{ns} continued to inactivate during the test pulse. The prepulse to +50 mV evoked a large outward I_{ns} that produced even more inactivation, as the initial current level during the subsequent test-pulse (marked by filled circle), was smaller.

There are several differences to be noted with regard to the behavior of I_{Ca} and I_{ns} inactivation during the two-pulse protocol. First, I_{Ca} inactivation decreased with a more positive prepulse, whereas I_{ns} inactivation increased. Second, it can be seen that after the +50 mV prepulse, the subsequent test I_{ns} had an unusual time course, as it actually increased with time. It was smallest at the onset of the -20 mV step, and then increased in size until it approached the same steady-state level as was reached after the -20 mv prepulse. Thus, it appears that with I_{ns}, recovery can occur during the test-pulse. This was never observed with I_{Ca}.

The average inactivation curve obtained during five such experiments is plotted in Figure 14B. The curve is quite different that that obtained with I_{Ca} (Figure 10). The inactivation curve of I_{ns} displays a monotonic relationship to

Figure 14. Relationship of I_{ns} inactivation to membrane potential. Panel A shows examples of I_{ns} traces obtained with the standard two-pulse protocol, except that the test step was to -20 mV. The two prepulses shown were to -20 mV and +50 mV (marked by circle). Panel B shows the average inactivation curve of I_{ns}. The means from a total of five cells are shown, along with the S.E.M. The 2 mM EGTA external solution and the standard pipette solution were used.

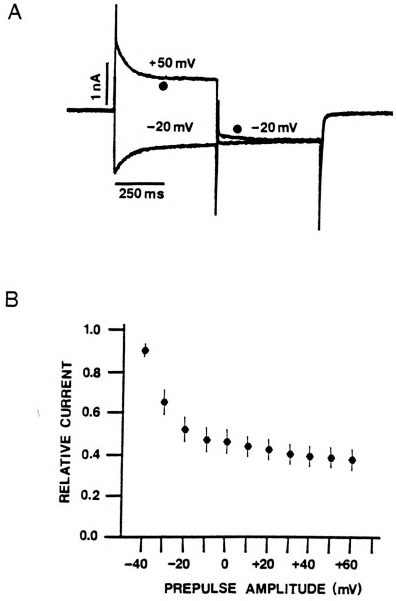

A
+50 mV
1 nA
−20 mV
−20 mV
250 ms
B
RELATIVE CURRENT
1.0
0.8
0.6
0.4
0.2
0.0
−40
−20
0
+20
+40
+60
PREPULSE AMPLITUDE (mV)

Figure 14

membrane potential, rather than a partially U-shaped relationship. There appears to be no correlation of I_{ns} inactivation with current magnitude or direction. Again, it also appears that there is a considerable fraction of I_{ns} left uninactivated after 500 ms, even at very positive potentials.

One question that might be raised about the decay of I_{ns} is whether it is due to true inactivation. It might be considered possible that accumulation or depletion phenomena could be contributing to the time course of I_{ns}. For example, during the time course of a smaller depolarization, a large amount of Na ions would enter the cell due to the large size and prolonged time course of the inward I_{ns}. It is possible that these entering Na ions would accumulate intracellularly and diminish the normally large gradient for Na ions to enter the cell. If this were to occur over the time course of a depolarization, I_{ns} would gradually decay because of the slowly diminishing gradient, and this might be mistaken for true channel inactivation.

Figure 15 shows an experiment designed to test this hypothesis. A 500 ms voltage-clamp step was given to the reversal potential of I_{ns}. In this cell, the reversal potential was +23 mV. It can be seen that the current trace was essentially flat during this step. This was done with and without a 1 sec prepulse to -20 mV. This potential was chosen as it produces the largest I_{ns}, and thus would be most likely to result in Na accumulation. The idea was that if I_{ns} decayed due to a diminished Na gradient, the reversal potential of I_{ns} would shift in the negative direction, as it is partly dependent on the Na gradient. As can be seen in the current trace, the presence of the prepulse did evoke a large inward I_{ns} of 1.3 nA. However, despite this large entry of Na ions, the reversal potential of I_{ns} did not shift, as the current during the subsequent test pulse to +23 mV was flat, and exactly overlays the current without a prepulse. This was seen with three other myocytes examined in the same way. If the inward I_{ns} had declined

Figure 15. Effect of Na entry on the I_{ns} reversal potential. The top of the figure shows superimposed current records obtained when a 500 ms test pulse to +23 mV, the reversal potentia for I_{ns} in this cell, was given with and without a 1 s prepulse to -20 mV. The interval between the two steps was less than 3 ms. The voltage records are shown at the bottom of the figure. The 2 mM EGTA external solution and the standard pipette solution were used.

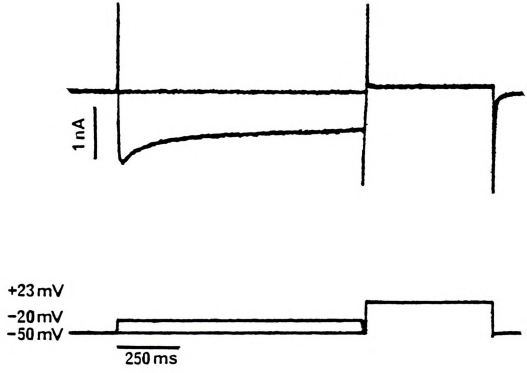


Figure 15

because of Na accumulation, the magnitude of the shift of the reversal potential could be predicted, assuming reasonably ohmic behavior of the open channel. The application of Hodgkin and Huxley's (1952b) adaptation of Ohm's law to the Ca channel gives: $I_{ns} = g_{ns} (E-E_{ns})$, where I_{ns} is the current in pA, g_{ns} is the channel conductance, E is the membrane potential, and E_{ns} is the reversal potential of I_{ns}. If the 45% reduction in current amplitude seen during the -20 mV prepulse were to be accounted for by a reduction in driving force alone, the factor $E-E_{ns}$ must decline by 45%, from 43 mV (-20 mV -(23 mV)) to 24 mV. Since a 19 mV negative shift was predicted, and the reversal potential of I_{ns} could be determined very precisely (within 1 mV), this experiment rules out a change in driving force as the reason for I_{ns} decay. Therefore, I_{ns} decay is due to a true drop in g_{ns}.

Figure 16 shows the final test made of the mechanism of I_{ns} inactivation. It involved an examination of the I_{ns} inactivation curve under somewhat different conditions. First, the external solution was changed to the low Ca solution, which had a Ca/EGTA mixture designed to buffer free Ca in the micromolar range. Second, the two-pulse protocol is somewhat different, in that the test-pulse was set at +80 mV. Figure 16A shows an example of the current traces seen with and without a prepulse to -10 mV. In the absence of the prepulse, the step to +80 mV evoked a large outward I_{ns} that decayed with time. The currents seen with the prepulse to -10 mV (next to the filled circles), however, were quite remarkable. First, instead of the usual large inward I_{ns} seen at -10 mV, the current during the prepulse was very small and almost flat. This indicates that I_{ns} is induced by the absence of Ca, and not by a pharmacological effect of EGTA (cf. Almers et al., 1984; Fukushima and Hagiwara, 1985). The current level during the pulse did not change when Ca channels were blocked by switching to the 20 μM Cd external solution, which meant that the only current seen during the prepulse was a small, outward leakage current. As outward I_{ns}

Figure 16. I_{ns} inactivation in the absence of ion permeation. Panel A shows superimposed current records obtained with the standard two-pulse protocol, using a test-pulse of +80 mV. The test pulse was given in the absence and presence (marked by circles) of a prepulse to -10 mV. The low Ca external solution and the standard pipette solution were used. Panel B shows the average inactivation curve under these conditions (n=4).

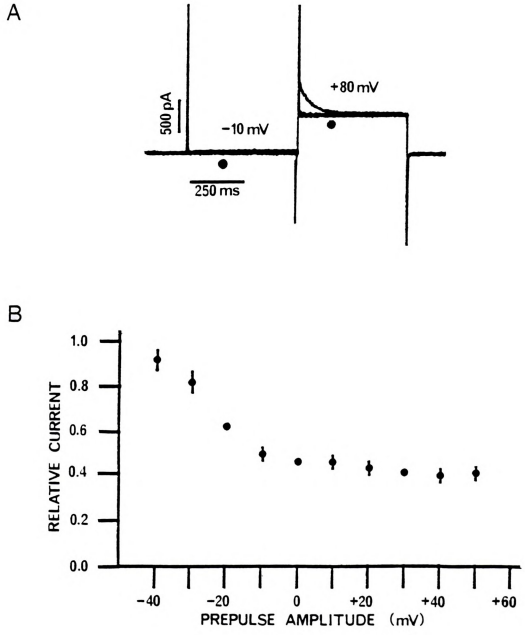

A
500 pA
−10 mV
+80 mV
250 ms
B
RELATIVE CURRENT
1.0
0.8
0.6
0.4
0.2
0.0
−40
−20
0
+20
+40
+60
PREPULSE AMPLITUDE (mV)

Figure 16

was still present, the Ca channel was apparently converted to an outwardly rectifying channel by the presence of Ca in micromolar concentrations. Second, despite the complete absence of current movement through the Ca channel, the prepulse could still inactivate the outward test I_{ns} to a great extent. Therefore, Ca channel inactivation can occur in the complete absence of ion permeation through the pore.

The average inactivation curve seen under these conditions is plotted in Figure 16B. Inward currents were small or completely absent in these cells, while the outward test currents were large. The degree of inactivation of outward I_{ns} shows a monotonic relationship to the membrane potential, and in general quite closely resembles the inactivation curve obtained in the 2 mM EGTA external solution, although it may be somewhat smoother. This leaves little doubt that I_{ns} inactivation occurs through a voltage-dependent mechanism. The only remaining alternative, that I_{ns} inactivation occurred in response to voltage-dependent release of Ca from the sarcoplasmic reticulum, was rejected as I_{ns} inactivation was unaffected by long periods of exposure to Ca-free solutions, by the application of 10 μM ryanodine, or by the inclusion of 20 mM EGTA in the pipette solution.

D. Comparison of voltage- and Ca-dependent inactivation

Now that it appeared that a successful separation of voltage- and Ca-dependent inactivation could be obtained in EGTA-containing external solutions, it was important to compare some of their properties. One property that was of interest was the recovery from channel inactivation, as there has been contradictory reports of the effects of varying the concentration and species of external divalent cations on the time course of recovery. Recovery from inactivation was studied by using a two-pulse protocol, where two 500 ms depolarizations to a

potential that produced large currents, were separated by an interval that varied in duration. The size of the current during the second, or test pulse, was measured relative to that during the prepulse. A series of these measurements with different interpulse intervals allowed the time course of the removal of inactivation to be studied.

The time course of I_{Ca} recovery was studied in a number of cells using two 500 ms depolarizations from -50 mV to 0 mV. The time course was analyzed, and it was found that it could be described as a single exponential process. The exponentials were fit according to the procedure described in Methods. The average time constant for I_{Ca} at -50 mV was 221±19 ms (n=5). When I_{ns} was studied in an analogous manner, its recovery was found to be much slower. The average time constant for I_{ns} recovery was found to be 1060±63 ms (n=6). However, it may be argued that this difference is artifactual, as it may arise from changes in the surface charge of the membranes. The total concentration of divalent cations is much lower in the 2 mM EGTA external solution than it is in the 2.5 mM Ca solution. Divalent cations are known to bind to sites on the membrane surface, and this can strongly influence membrane properties. In particular, a reduction in external divalent cations mimics depolarization of the membrane. Since the recovery of Ca channels is strongly voltage-dependent, and in particular, is slowed by more positive holding potentials, it is possible that the slowed I_{ns} recovery is just a secondary effect of the reduction of divalent cations.

Figure 17 shows an examination of I_{Ca} and I_{ns} recovery under conditions where surface charge effects should be greatly reduced. Current traces of I_{Ca} recovery while the cell was in the 1 mM Ca solution are superimposed in Figure 17A. It takes approximately 800-900 ms for the removal of inactivation to be complete. Figure 17B shows I_{ns} recovery in the same cell, when the external solution was switched to the 1 mM Mg solution. The time course of recovery

Figure 17. Comparison of I_{Ca} and I_{ns} recovery from inactivation. Panel A shows superimposed I_{Ca} traces evoked by a two-pulse protocol, where two 500 ms depolarizations from -50 mV to -10 mV were separated by a variable interval. The 1 mM Ca external solution and the standard pipette solution were used. Panel B shows I_{ns} recovery elicited by the same voltage-clamp protocol in the same cell. The 1 mM Mg external solution and the standard pipette solution were used.

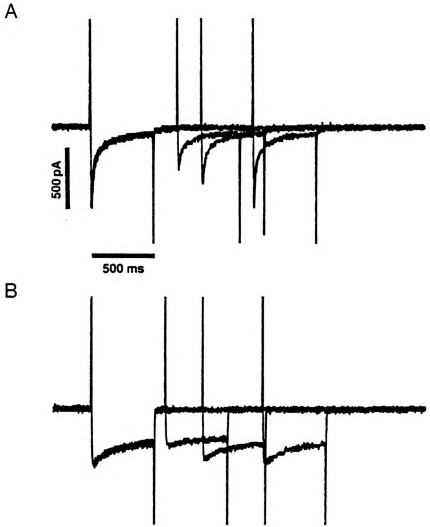


Figure 17

appeared to be quite similar to that of I_{Ca}. The 1 mM Mg solution contained 2 mM EGTA, so that the free Ca concentration was low enough to permit monovalent ions to pass through the Ca channel. The 1 mM Mg it also contained served to minimize surface charge differences from the 1 mM Ca solution. The size of I_{ns} was much smaller than usual under these conditions. It was apparent that Mg ions could block I_{ns}, but much less effectively than Ca or Cd ions. I_{Ca} and I_{ns} time constants of recovery were determined in the same cell a total of four times, with the average time-constants being 275±26 ms for I_{Ca}, and 313±35 ms for I_{ns}. The difference between the two values was not significant, which means that the slowed I_{ns} recovery seen in the 2 mM EGTA solution is probably completely attributable to the reduction in extracellular divalent cations.

The results of the previous study were intriguing, as it had proven possible to compare the properties of Ca-dependent and voltage-independent inactivation in the same cell. Therefore, it should be possible, with these techniques, to evaluate the role and relative importance of both types of Ca channel inactivation in one cell. In principle, this could be achieved by a comparison of the I_{Ca} and I_{ns} inactivation curves shown in Figures 10 and 14, but the solutions differed greatly in total divalent cation concentration, and the protocols were somewhat different as well. Figure 18 shows both types of inactivation curves obtained in a single cell. The curve shown with the filled circles is the I_{Ca} inactivation curve obtained with the 1 mM Ca external solution, and the curve indicated with the open circles is that obtained for I_{ns} in the 1 mM Mg solution. The protocol used for both curves was the standard two-pulse protocol with the test-pulse set at -10 mV, in order to obtain large inward currents under both conditions.

The I_{Ca} inactivation curve has its typical partial U-shape, and can be assumed to represent total Ca channel inactivation. The I_{ns} inactivation curve has a smooth dependence on the membrane potential, and can be assumed to

Figure 18. Direct comparison of I_{Ca} and I_{ns} inactivation curves. The figure plots inactivation curves for I_{Ca} (filled circles), and I_{ns} (open circles) obtained from the same cell. The voltage-clamp protocol was the standard two-pulse protocol with the test-pulse set at -10 mV. I_{Ca} was studied in the 1 mM Ca external solution, while I_{ns} was studied in the 1 mM Mg external solution. The standard pipette solution was used.

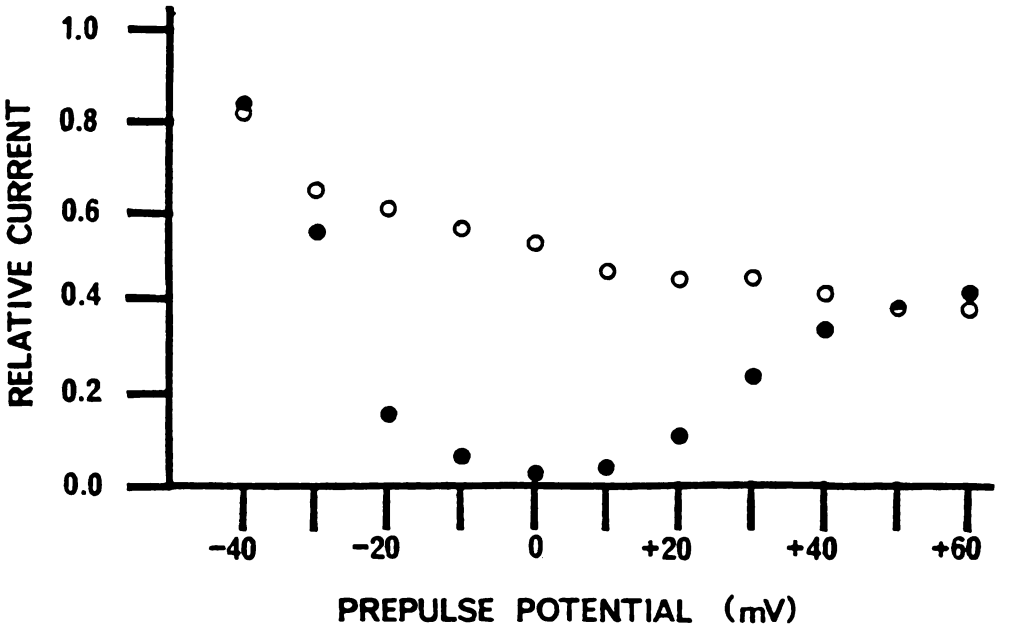

1.0
0.8
0.6
0.4
0.2
0.0
RELATIVE CURRENT
-40
-20
0
+20
+40
+60
PREPULSE POTENTIAL (mV)

Figure 18

represent the role of voltage-dependent inactivation. The role of Ca-dependent inactivation in turning off I_{Ca} would then be reflected by the difference between the two curves. It can be seen that voltage-dependent inactivation accounts for much of the total inactivation of Ca channels, with Ca-dependent inactivation making a strong contribution over the important range of potentials from -30 to +40 mV. The inactivation curves approach each other again at +50 mV, which indicates that voltage-dependent inactivation is the dominant mechanism at very positive potentials. Quite similar results were seen with three other cells.

E. Drug Interactions with the inactivated state of the Ca channel

A quite important aspect of Ca channel function is how it is influenced by various organic Ca channel blockers, such as verapamil, diltiazem and nifedipine. It has been proposed that the modulated receptor hypothesis, which was originally developed to explain local anesthetic interactions with the Na channel, may explain blockade of Ca channels as well. The basic premise of the modulated receptor hypothesis is that the site for drug binding on the channel is profoundly affected by the state of the channel. In particular, it has been proposed that in the inactivated state, the channel may have a thousand-fold higher affinity for these drugs. It was therefore of interest to more closely examine the interaction of certain drugs with the inactivated state of the channel.

1. Voltage-dependent effects of Bay K 8644 on I_{Ca}

In the last few years, a new dihydropyridine, Bay K 8644, has been introduced that can increase Ca influx through Ca channels (Schramm et al., 1983). However, it has also been found to have some inhibitory effects on I_{Ca} (Thomas et al., 1984; Sanguinetti and Kass, 1984b).

The effects of Bay K 8644 on I_{Ca} were examined under somewhat different conditions than were used in the previous studies. Holding potentials

more negative than -50 mV were required for many of the experiments, and so more extreme measures had to be taken to eliminate current through Na channels. The cells were kept in the 2.5 mM Ca solution, but after impalement the external solution was switched to the Na-free Tris solution. The pipette solution contained 5 mM EGTA in order to prevent contracture of the cell during the removal of Na.

Figure 19 shows an experiment designed to examine the concentration-dependence of Bay K 8644's effects. The cell was held at either -80 or -40 mV, and stimulated at 0.1 Hz by 500 ms depolarizations to 0 mV. Various concentrations of Bay K 8644 were then added to the superfusate, and measurements were remade after I_{Ca} had reached its new steady-state value. It can be seen that at a holding potential of -80 mV, Bay K 8644 produces a concentration-dependent enhancement of I_{Ca}. However, if the cell was held more positive, at -40 mV, a concentration-dependent blockade of I_{Ca} was seen. The difference between the two holding potentials is most striking at a concentration of 5 μM. The average results obtained with Bay K 8644 are shown in Table 2.

Since it seemed clear that Bay K 8644 could block Ca channels at positive holding potentials, the voltage-dependence of Bay K 8644's effects was examined more quantitatively. I_{Ca} was evoked by 200 ms voltage-clamp steps to 0 mV from holding potentials between -30 and -90 mV. The cells were held at the new holding potential for 10-20 s, long enough for I_{Ca} to reach steady-state, before being stimulated. This was done under control conditions, and after the application of 250 nM Bay K 8644, a concentration that produced both strong stimulatory and inhibitory effects. The average percent change in the peak magnitude of I_{Ca} that was induced by Bay K 8644 is plotted against the holding potential in Figure 20. Bay K 8644 produced stimulatory effects at negative holding potentials, as the size of I_{Ca} more than doubled. Little difference in Bay K 8644's effects were seen between holding potentials of -90 and -60 mV. The

Figure 19. Concentration-dependent enhancement and inhibition of I_{Ca} by Bay K 8644. The top of each panel shows the voltage-clamp protocol, where a 500 ms step to 0 mV was given from -80 mV (left panel) or -40 mV (right panel). The bottom of each panel shows I_{Ca} under control conditions and after exposure to different concentrations of Bay K 8644. The cell was stimulated at 0.1 Hz. The Tris external solution and the 5 mM EGTA pipette solution were used.

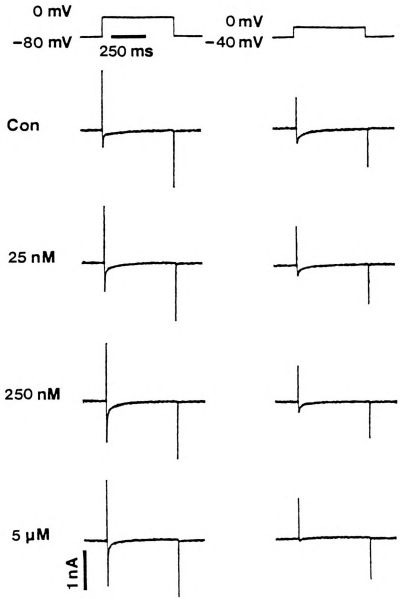


Figure 19

Table 2

Dose-Dependence of Bay K 8644's Effects on I_{Ca}

V_h	Concentration of Bay K 8644		
	25 nM	250 nM	5 μM
-40 mV	-12.0±9.8	-44.4± 7.9	-75.9±3.5
-80 mV	58.6±6.6	118.7±22.0	126.7±15.8

The mean percent change in peak I_{Ca} is shown with the SEM. N=6.

Figure 20. Voltage-dependence of Bay K 8644's effects on I_{Ca}. The average percent change (with S.E.M.) in peak I_{Ca} magnitude induced by 250 nM Bay K 8644 is plotted against the holding potential. The holding potential was allowed to remain at the new value for 10-20 s before I_{Ca} was evoked by a 200 ms step to 0 mV. The Tris external solution and the 5 mM EGTA pipette solution were used.

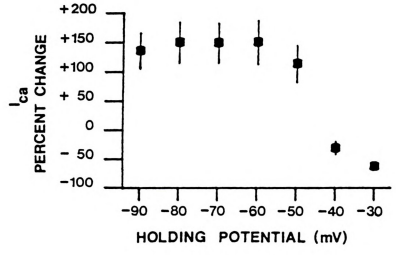

+200
+150
+100
+ 50
0
- 50
-100
I_{Ca}
PERCENT CHANGE
-90 -80 -70 -60 -50 -40 -30
HOLDING POTENTIAL (mV)

Figure 20

stimulatory effect, however, become somewhat smaller at -50 mV, and abruptly changed into an inhibitory effect at more positive potentials.

A second way of examining the voltage-dependence of Bay K 8644's actions is to study its effect on the inactivation curve. The inactivation curves were obtained with the usual two-pulse protocol, with a holding-potential of -50 mV. The curves are plotted in Figure 21. The open symbols plot the control inactivation curve, which has the typical partial U-shape. It is notable that hyperpolarizing prepulses as negative as -100 mV do not increase the size of I_{Ca} very much under control conditions. This must mean that the T-type Ca channel, which is mostly inactivated at -50 mV, contributes little current even at more negative potentials. The inactivation curve after application of 250 nM Bay K 8644 is marked by the filled symbols. It is apparent that Bay K 8644 induced several changes in the curve. First, the potential where I_{Ca} is apparently half-inactivated was -19 mV under control conditions, but was -36 mV in the presence of Bay K 8644. Second, the slope of the inactivation curve seemed to be less steep in the presence of Bay K 8644. Finally, Bay K 8644 seemed to partly suppress the upturn in the inactivation curve at positive potentials. Such changes in the inactivation curve have been noted with other Ca channel blockers, and have been suggested to be due to preferential interactions of the drug with the inactivated state of the channel (Kass and Sanguinetti, 1984; Uehara and Hume, 1985).

Obviously, relatively modest alterations in the holding potential can greatly alter the effect of Bay K 8644 on I_{Ca}. In particular, there is a reduction in I_{Ca} amplitude associated with more positive holding potentials. This reduction in I_{Ca} was termed "tonic block," although at this point it had not been demonstrated whether the effect was due completely to active block of Ca channels, or whether a voltage-dependent loss of the stimulatory effect could be

Figure 21. Effect of Bay K 8644 on I_{Ca} inactivation curve. The inactivation curves of I_{Ca} before (open symbols), and after application of 250 nM Bay K 8644 (filled symbols) are plotted. The voltage-clamp protocol was a standard two-pulse protocol with a test-pulse of 0 mV, and with both depolarizing and hyperpolarizing prepulses from the holding potential of -50 mV. The Tris external solution and the 5 mM EGTA pipette solution were used.

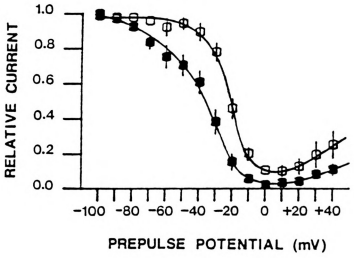

RELATIVE CURRENT
1.0
0.8
0.6
0.4
0.2
0.0
−100 −80 −60 −40 −20 0 +20 +40
PREPULSE POTENTIAL (mV)

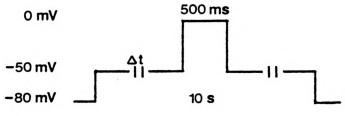
Figure 21

involved as well. The kinetics of these changes in Bay K 8644's effects were then studied. This was, of course, necessary for the adequate design of later voltage-clamp protocols, but such data can also sometimes provide information as to the underlying mechanism of drug action.

The top half of Figure 22 shows the voltage-clamp protocol used to examine the time-dependence of the onset of tonic block by Bay K 8644. Cells were first exposed to 250 nM Bay K 8644 for several minutes. The magnitude of I_{Ca} was then determined at a holding potential of -80 mV. The steady-state value of I_{Ca}, after switching to a holding potential of -50 mV, can be expected to be somewhat smaller. The kinetics of this reduction in current magnitude were analyzed by means of a voltage-clamp step from -80 mV to -50 mV for 10 sec. This step was interrupted after a variable interval of time by a 500 ms test step to 0 mV. This was repeated for a number of different intervals, and the peak magnitude of I_{Ca} obtained with the test pulses were compared to the steady-state values obtained at -80 and -50 mV. The bottom half of the figure plots the results obtained with a representative experiment. The current size is plotted on the y-axis, and the length of the interval is plotted on the x-axis. I_{Ca} started out at 720 pA at a holding potential of -80 mV, and reached a value of about 500 pA after 10 s at -50 mV. The time course of the onset of the tonic block could be fit by a single time constant of 3132 ms. A total of four experiments gave an average value of 3038 ± 443 ms for the onset of tonic block at -50 mV.

The top-half of Figure 23 shows the protocol used to determine the rate of removal of tonic block. Cells that were exposed to Bay K 8644 were first held at -50 mV, and then were given a 10 s voltage-clamp step to -80 mV. This hyperpolarizing step was then interrupted at different points by a 500 ms test-pulse to 0 mV. The bottom-half of the figure shows a typical time course of the removal of tonic blockade. The recovery process could be fit as a single

Figure 22. Kinetics of Bay K 8644 tonic block. The top half of the figure shows a diagram of the voltage-clamp protocol. The cell was held at -80 mV for 15 s, and then was stepped to -50 mV for 10 s. At varying times during this voltage-clamp step, the cell was depolarized further to 0 mV for 500 ms in order to evoke I_{Ca}. The bottom half of the figure plots the magnitude of the test I_{Ca} during a typical experiment, against the duration of the prepulse to -50 mV. The time course of the onset of the tonic block was fit with a single exponential with a time constant of 3132 ms. The Tris external solution and the 5 mM EGTA pipette solution were used.



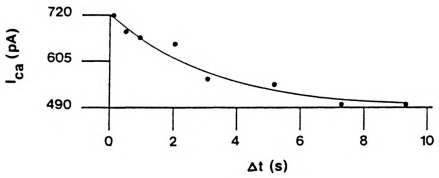


Figure 22

Figure 23. Kinetics of removal of Bay K 8644 tonic block. The top half of the figure shows the voltage-clamp protocol. The cell was held at -50 mV for 15 s, and then hyperpolarized to -80 mV for 10 s. The hyperpolarization was interrupted after a variable interval with a 500 ms test pulse to 0 mV. The bottom half of the figure shows a plot of the test I_{Ca} against the prepulse duration. The data was fit by an exponential with a time constant of 356 ms. The Tris external solution and the 5 mM EGTA pipette solution were used.

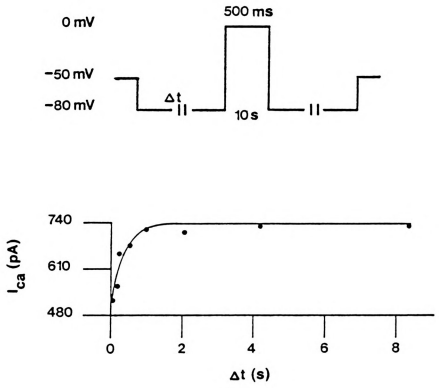


Figure 23

exponential process as well, as the example shown was fit with a time-constant of 356 ms. The average time-constant in four such experiments was 533±108 ms.

2. Use-dependent block of I_{Ca} by Bay K 8644

Since Bay K 8644 produces an inhibition of I_{Ca} at more positive membrane potentials, it would be expected that the more time the cell is depolarized, the more inhibition will be seen. In other words, Bay K 8644 may be expected to produce more Ca channel block the faster the cell is stimulated. This has been seen with other Na and Ca channel blockers, and is referred to as frequency-dependent, or use-dependent blockade. Bay K 8644 was examined for this effect by repetitively stimulating the cell at different frequencies with 200 ms depolarizations to 0 mV. This was done under control conditions, and after the cell was exposed to 250 nM Bay K 8644. Figure 24 shows the results from a typical experiment. The percentage change in the steady-state I_{Ca} magnitude that was seen with Bay K 8644 is plotted against the stimulation rate. The filled symbols show the effect of different stimulation rates when the cell was held at -80 mV. At a low frequency, Bay K 8644 caused an approximately 70% increase in the size of I_{Ca}. This cell, therefore, had a somewhat less robust response to Bay K 8644 than the average (see Table 2). An increase in the stimulation rate reduced Bay K 8644's stimulatory effect. If the cell was instead held at -50 mV (open symbols), the same result was seen; more rapid stimulation reduced peak I_{Ca}. Peak I_{Ca}, however, was smaller for each frequency when the cell was held at -50 mV. In addition, an increase in the stimulation rate had a more profound effect when the cell was held at -50 mV. The percentage change seen when the rate was switched from 0.1 to 1 Hz was greater at -50 mV, and actual net inhibition of I_{Ca} could be seen with high stimulation rates at -50 mV. In general, the same results were obtained with ten other cells, that is, faster stimulation

Figure 24. Frequency-dependence of Bay K 8644's effects on I_{Ca}. The percent change in I_{Ca} magnitude induced by 250 nM Bay K 8644 is plotted against the frequency of stimulation. The cell was held at either -50 mV (open symbols) or -80 mV (filled symbols), and stimulated at the given frequency with 200 ms depolarizations to 0 mV. The data points show the steady-state change in I_{Ca} magnitude, as compared to the control I_{Ca} for that holding potential and frequency. The Tris external solution and the 5 mM EGTA pipette solution were used.

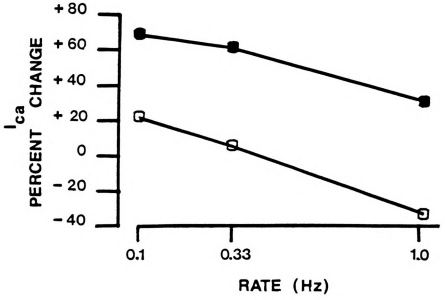


Figure 24

rates and more positive holding potentials accentuated inhibition of I_{Ca}. However, the cells displayed some variability, in that the exact frequency at which the net effect of Bay K 8644 changed from being stimulatory to inhibitory was different.

As the inhibitory effect of Bay K 8644 did appear to have a use-dependent component, it would be useful to study the onset of the use-dependent block, and to determine what factors influenced it. The onset of the use-dependent block was analyzed by repetitively stimulating the cell from a certain holding potential and at a given frequency, after a 15 sec rest at that holding potential. The magnitude of I_{Ca} was then measured for each pulse in the train. Figure 25A plots the results from a typical cell, when the cell was stimulated by 20 ms depolarizations to 0 mV from a holding potential of -80 mV. This was done at two different frequencies, and under control conditions (open symbols) and after the application of 250 nM Bay K 8644 (filled symbols). The size of I_{Ca} during each pulse in the train was normalized relative to the first pulse of that train. It can be seen that under control conditions, repetitive stimulation after a rest at -80 mV resulted in a gradual, modest increase in the size of I_{Ca} during the train. The increase was more prominent with a faster rate. This result has been seen before in cardiac tissue (Noble and Shimoni, 1981a,b; Lee, 1987). The addition of Bay K 8644 caused a reversal of this trend, as I_{Ca} then decreased with repetitive stimulation, and the decrease was larger with a more rapid rate.

Figure 25B demonstrates the influence of pulse duration on the onset of use-dependent block by Bay K 8644. The conditions were the same as in Figure 25A, except that the depolarizing pulse was widened from 20 to 200 ms. The results were basically the same as before, except that the extent of use-dependent block was enhanced, and it appeared to take longer for the block to approach a steady-state level.

Figure 25. Onset of use-dependent block by Bay K 8644. All of the panels show time-dependent changes in the size of I_{Ca} due to repetitive voltage-clamp steps to 0 mV after a 15 s rest at the holding potential. The curves in Panels A, B and C differ as labelled with regard to holding potential, pulse duration and frequency of stimulation. The currents are all scaled relative to the first I_{Ca} of each train. Trains were done under control conditions (open symbols) and in the presence of 250 nM Bay K 8644 (filled symbols). The Tris external solution and the 5 mM EGTA pipette solution were used.

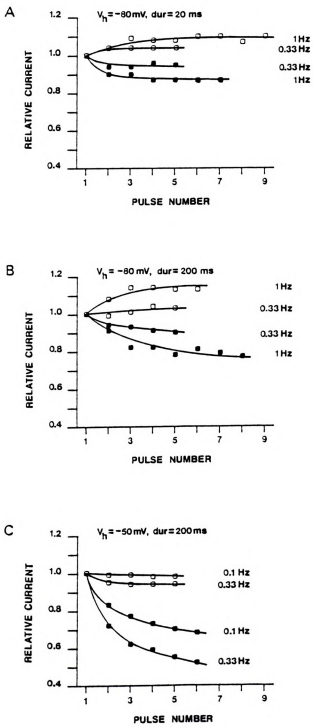

A
V_h = −80 mV, dur = 20 ms
1 Hz
0.33 Hz
0.33 Hz
1 Hz
RELATIVE CURRENT
1.2
1.0
0.8
0.6
0.4
1
3
5
7
9
PULSE NUMBER
B
V_h = −80 mV, dur = 200 ms
1 Hz
0.33 Hz
0.33 Hz
1 Hz
RELATIVE CURRENT
1.2
1.0
0.8
0.6
0.4
1
3
5
7
9
PULSE NUMBER
C
V_h = −50 mV, dur = 200 ms
0.1 Hz
0.33 Hz
0.1 Hz
0.33 Hz
RELATIVE CURRENT
1.2
1.0
0.8
0.6
0.4
1
3
5
7
9
PULSE NUMBER

Figure 25

The influence of the holding-potential in the acquisition of use-dependent block is demonstrated in Figure 25C. Trains were run at 0.1 and 0.33 Hz with 200 ms depolarizations from -50 mV. First, it can be seen that the positive-staircase effect was not present under control conditions, thereby implying that the membrane potential strongly influences it. The more positive holding potential also seems to have greatly enhanced use-dependent block by Bay K 8644. It also apparently takes longer to approach steady-state conditions. Thus, use-dependent blockade is favored by more positive holding potentials, more rapid stimulation, and longer duration of depolarization.

3. Effects of Bay K 8644 on I_{Ca} kinetics

Inspection of I_{Ca} traces before and after the application of Bay K 8644 usually showed that the rate of I_{Ca} decay was faster in the presence of the drug (see Figure 19 for an example). This effect has been noted with some other Ca channel blockers (Lee and Tsien, 1983; Sanguinetti and Kass, 1984a). In the instance of Bay K 8644, however, the observation is complicated by the concomitant increase in Ca current size. The increased Ca influx by itself could accelerate Ca channel inactivation. This effect of Bay K 8644 on the inactivation kinetics was examined more quantitatively, as shown in Figure 26. The current trace in the upper left hand corner shows I_{Ca} evoked by a 500 ms step to 0 mV from -80 mV under control conditions. The half-time for I_{Ca} to inactivate is marked by the arrow, and was 9 ms. The current trace in the lower left hand corner shows I_{Ca} after the application of 250 nM Bay K 8644. I_{Ca} had become bigger and decayed faster, as the half-time for inactivation was 4 ms. Again, the acceleration of I_{Ca} decay seems to be mainly attributable to an enhanced fast phase of inactivation. The question of whether this effect could be attributed to the enhanced entry of Ca, was tested by adding 25 μM Cd to the Bay K solution (upper right hand trace). I_{Ca} was much smaller under these conditions, but still

Figure 26. Effect of Bay K 8644 on I_{Ca} decay. The labelled panels show a representative experiment in one cell, where the half-time of I_{Ca} decay was determined under control conditions (upper left), with 250 nM Bay K 8644 present (lower left), and with Bay K 8644 and 25 μM $CdCl_2$ present (upper right). I_{Ca} was evoked by 500 ms depolarizations to 0 mV from a holding potential of -80 mV. Half-times for inactivation are marked by arrows, and are given in the text. Complete block of I_{Ca} by 500 μM Cd is also shown (lower right). The Tris external solution and the 5 mM EGTA pipette solution were used.

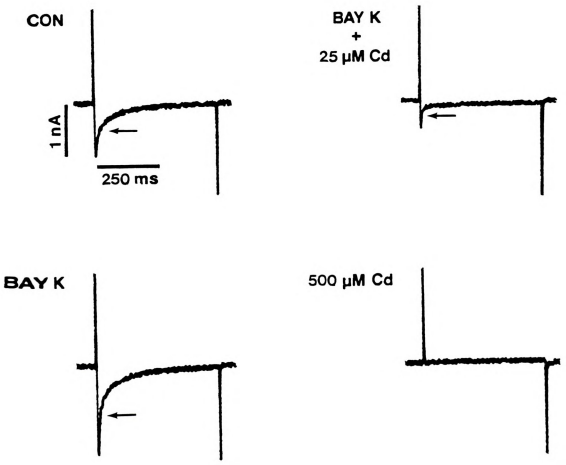


Figure 26

turned off very fast, as the half-time was 4 ms. The effect of 25 μm Cd alone was not tested in this cell but prior experiments established that it slows I_{Ca} decay in proportion to the reduction of Ca entry. Complete block of I_{Ca} by Cd is shown in the lower right hand trace. The average half-times obtained from a total of five cells was 17.2±2.9 ms for control, 6.2±1.6 ms with 250 nM Bay K 8644 present, and 6.0±1.1 ms for Bay K 8644 and 25 μM Cd present. Therefore, the acceleration of inactivation by Bay K 8644 is a direct effect of the drug.

Since Bay K 8644 accelerated inactivation, it was of interest to examine its effects on the recovery from inactivation. There has been one report that Bay K 8644 accelerated the recovery of Ca channels (Sada et al., 1986). The recovery process was studied with the same protocol described before, two 500 ms depolarizations to 0 mV separated by a variable interval. The time course of Ca channel recovery was then plotted as the relative I_{Ca} (the second I_{Ca} relative to the first) against the duration of the interval. Figure 27A shows an example of I_{Ca} recovery under control conditions, when the cell was held at -50 mV. The recovery process can be described well as a single exponential process with a time constant of 422 ms. The recovery process was voltage-dependent in that it was faster at more negative potentials. Figure 27B shows I_{Ca} recovery in the same cell at a holding potential of -80 mV. The data could be fit with a time constant of 93 ms.

The application of 250 nM Bay K 8644 strongly affected the recovery process. Figure 27C shows I_{Ca} recovery in the same cell as before, at a holding potential of -50 mV, after exposure to the drug. The recovery process takes much longer to be complete than before, and can no longer be described satisfactorily by a single exponential. The data could instead be fit well by the sum of two exponentials. The fast exponential had a time constant of 400 ms, and there was also a slower one with a time constant of 3924 ms. Both exponentials became

Figure 27. Effect of Bay K 8644 on I_{Ca} recovery from inactivation. I_{Ca} recovery was studied with a two-pulse protocol where two 500 ms depolarizations to 0 mV were separated by a variable interval. The panels plot the magnitude of I_{Ca} during the second, test pulse (measured relative to I_{Ca} with no prepulse) against the interval between the two pulses. Panel A shows an example of control recovery at a holding potential of -50 mV, and Panel B shows the control recovery at -80 mV. Panels C and D show the effect of 250 nM Bay K 8644 on I_{Ca} recovery, at holding potentials of -50 mV, and -80 mV, respectively. Parameters used to fit the curve are given in the text. The Tris external solution and the 5 mM EGTA pipette solution were used.

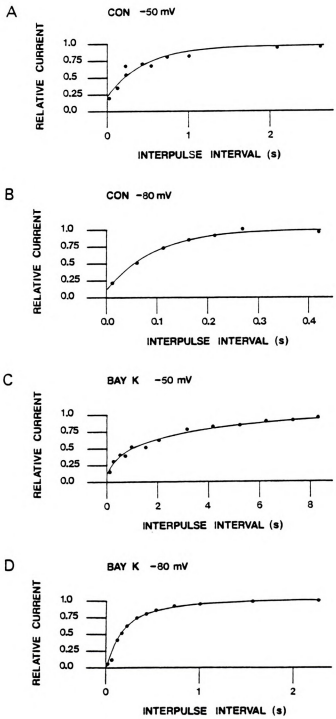

A
CON −50 mV
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0
1
2
INTERPULSE INTERVAL (s)
B
CON −80 mV
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0.0
0.1
0.2
0.3
0.4
INTERPULSE INTERVAL (s)
C
BAY K −50 mV
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0
2
4
6
8
INTERPULSE INTERVAL (s)
D
BAY K −80 mV
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0
1
2
INTERPULSE INTERVAL (s)

Figure 27

faster when the cell was held at -80 mV. The time constants for the recovery data shown in Figure 27D were 130 and 697 ms. The fast exponential seemed to account for more of the recovery at -80 mV than it did at -50 mV.

The average results obtained with a number of different recovery experiments are shown in Table 3. It can be seen that in the presence of Bay K 8644, the fast recovery process has time constants that are nearly identical to that of the control recovery. There was no significant difference between these time constants at either holding potential. The effect of Bay K 8644 to slow the recovery of I_{Ca} from inactivation seems to be completely attributable to the induction of a second, very slow component of recovery. It is interesting to note that this drug-induced component of recovery has almost the same voltage-dependence as the normal recovery process. Both processes are about 3-3.5 times faster at -80 mV than at -50 mV. However, the slowing of recovery by Bay K 8644 does not get its voltage-dependence just from changes in the values of the time constants. The relative importance of the two time constants also changes with membrane potential. A_1, the relative amplitude of the fast component of recovery, is significantly larger at -80 mV than at -50 mV, while A_2, the relative amplitude of the slow component, is more prominent at -50 mV.

Although it has been demonstrated that a moderate amount of Ca entering the guinea-pig ventricular myocyte does not affect recovery (Figure 17), there are reports of Ca entry slowing recovery in other preparations (Mentrard et al., 1984). This again raises the problem of discriminating between direct and indirect effects of Bay K 8644. In this instance, it is with regard to the possibility of an enhanced Ca entry slowing the subsequent recovery. Figure 28 shows an experiment that tested this possibility. A typical biexponential recovery time course seen at -80 mV in the presence of 250 nM Bay K 8644 is shown in Figure 28A. The time constants had values of 117 and 1012 ms. Figure 28B shows the

Table 3

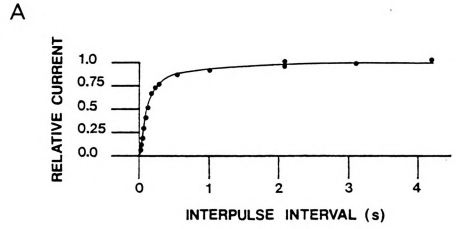
Voltage-Dependence of Fast and Slow Phases of I_{Ca} Recovery

Condition	V_h	N	tau_1	tau_2	A_1	A_2
Control	-50 mV	6	337±56 ms			
Control	-80 mV	6	104± 9 ms			
Bay K 8644	-50 mV	5	368±94 ms	3077±266 ms	.31±.05*	.64±.07*
Bay K 8644	-80 mV	5	123±10 ms	866± 85 ms	.81±.01*	.19±.03*

The concentration of Bay K 8644 that was used was 250 nM. Tau_1 and tau_2 are the fast and slow time constants of recovery. A_1 and A_2 are the relative amplitudes of the fast and slow phases of recovery, respectively.

*, indicates a significant difference between values in a column when compared with a Student's t-test ($p < 0.05$).

Figure 28. Effect of Bay K 8644 and Cd on I_{Ca} recovery. The same voltage-clamp protocol and solutions were used as in Figure 27. Panel A plots the recovery of I_{Ca} at a holding potential of -80 mV, when 250 nM Bay K 8644 was present. Panel B shows I_{Ca} recovery when 25 µM $CdCl_2$ was added along with the Bay K 8644. The Tris external solution and the 5 mM EGTA pipette solution were used.


A
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0
1
2
3
4
INTERPULSE INTERVAL (s)

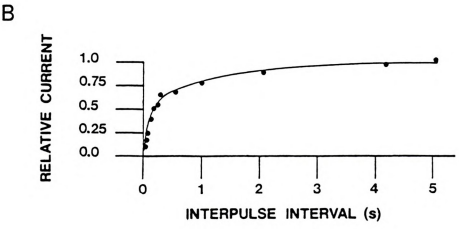

B
RELATIVE CURRENT
1.0
0.75
0.5
0.25
0.0
0
1
2
3
4
5
INTERPULSE INTERVAL (s)

Figure 28

recovery data obtained under the same conditions, except for the addition of 25 μM Cd to the superfusate. The addition of Cd reduced the size of peak I_{Ca} from 1770 to 540 pA. I_{Ca} was 920 pA under control conditions. The reduction in Ca entry had only a small effect on the time constants of recovery, as the data was fit using time constants of 102 and 1280 ms. Therefore, the slowed recovery process was a direct effect of the drug, and was not Ca-dependent. However, the addition of the Cd did have one effect. The relative amplitude of the slow component was increased from .196 to .448. These effects were seen in the four other cells that were examined.

4. An alternative model of Ca channel blockade: the guarded receptor hypothesis

The Bay K 8644 data that has been presented supports the hypothesis of strong interactions of certain drugs with the inactivated state of the Ca channel. As discussed in the Introduction, similar results have been previously obtained with local anesthetics and traditional Ca channel blockers. However, an alternative model has been developed that explains this data in terms of "gate-trapping" (Starmer and Grant, 1985). Therefore, it was decided to test the applicability of this model to Ca channel blockade, in order to see if it can be determined whether or not there are indeed preferential interactions of drugs with the inactivated state of the Ca channel.

The drug that was chosen for these experiments was D600, the methoxy deriviative of verapamil. The reason for this was that there is an extensive literature on D600 blockade of Ca channels, and that the D600 molecule is predominantly charged (> 90%) at physiological pH. This means that this drug will, for the most part, be confined to enter the channel through the hydrophilic pore. This makes D600 one of the more likely Ca channel blockers to be heavily influenced by the gating properties of the channel.

Figure 29 shows the experimental results from a study of the block of I_{Ca} by D600. An I_{Ca} current trace resulting from a 500 ms depolarization from -110 to 0 mV is shown in Figure 29A. This was obtained with the Na-free Tris external solution, and the 5 mM EGTA-containing internal solution, as very negative holding potentials were used. After obtaining a control record, the cell was exposed to 5 µM D600 for several minutes. The cell was then given a train of 500 ms depolarizations to 0 mV at a frequency of 0.2 Hz. Several examples of I_{Ca} obtained at differents pulses in this train are shown in Figure 29B. I_{Ca} during the first depolarization is quite similar in size to the control, indicating that little tonic block is seen with D600. Use-dependent block, however, is prominent with D600, as can be seen by the diminishing size of the subsequent I_{Ca}. After holding the cell at -110 mV for 30 sec, a length of time sufficient to remove virtually all of the D600 block (McDonald et al., 1984b), another train of depolarizations was given, this time at 1.0 Hz. Figure 29C shows examples of I_{Ca} obtained at different points during this train. It can be seen that at a higher frequency, D600 block is considerably more extensive.

The use-dependence data from the same cell is shown again in Figure 30, this time in numerical form. The peak magnitude of I_{Ca} is plotted against the pulse number for three different trains. The open squares show the data resulting from a train at 0.2 Hz, the filled squares represent a train at 0.5 Hz, and the filled diamonds represent the train at 1 Hz. It is obvious from the data that increasing the rate of stimulation greatly increases the level of steady-state blockade of I_{Ca}, and also increases the number of depolarizations it takes to reach a steady-state level. The time-course of use-dependent block, or frequency-dependent uptake of D600, has been fitted for all three trains by an exponential function, $I_n = I_o + (I_o - I_{ss})\ e^{-n\ lambda^*}$, derived from the assumptions of the guarded-receptor hypothesis (see Methods). The theoretical

Figure 29. Use-dependent block of I_{Ca} by D600. Panel A shows the control current and voltage records. I_{Ca} was evoked by a 500 ms voltage-clamp step to 0 mV from -110 mV. Panel B shows I_{Ca} during a train of similar depolarizations at 0.2 Hz, after the cell had been exposed to 5 μM D600. I_{Ca} during the first, second, fourth and eighth stimuli are shown. Panel C shows I_{Ca} during a similar train at 1.0 Hz. The cell was held at -110 mV for 30 s in between trains. The Tris external solution and the 5 mM EGTA pipette solution were used.

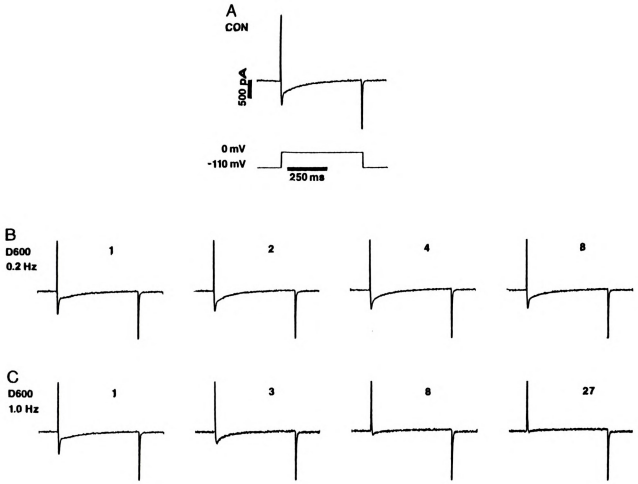


Figure 29

Figure 30. Analysis of frequency-dependent uptake of D600. The peak magnitude of successive I_{Ca}s during different trains are plotted against the pulse number. These trains were done in the presence of 5 μM D600, and represent data from the same cell and experimental protocol as in Figure 29. Data are shown for trains of 0.2 Hz (open squares), 0.5 Hz (filled squares), and 1.0 Hz (diamonds). A nonlinear least squares fit was used to estimate use-dependent uptake rates, lambda*, for each frequency. The fitted curves and lambda*s are shown.

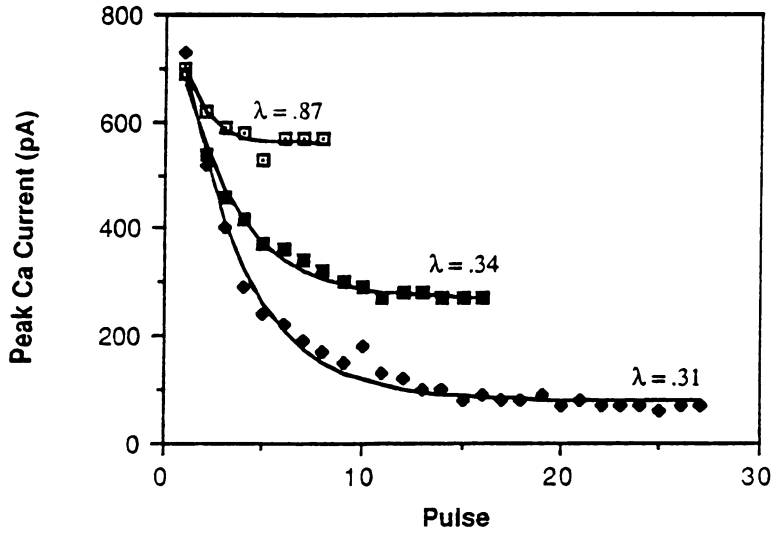


Figure 30

equation fitted the data quite well. The apparent uptake rate, lambda*, was calculated for each of the trains from the fit. They were 0.87 at 0.2 Hz, 0.34 at 0.5 Hz, and 0.31 at 1.0 Hz.

Such experiments were done with a variety of different holding potentials, different rates of stimulation, and different pulse durations. The idea was to test the applicability of the guarded-receptor hypothesis under greatly varying conditions. Theoretical fits to the data were, in general, quite good, and the apparent uptake rates were calculated. One prediction of the model comes from the definition of the apparent uptake rate, where $\text{lambda}^* = \text{lambda}_a\, t_a + \text{lambda}_r\, t_r$. Lambda_a and lambda_r are the uptake rates during the depolarized and resting interval respectively, and t_a and t_r are the durations of the depolarized and resting intervals. Therefore, a plot of lambda* against the resting, or recovery interval should be a straight line with an intercept of $\text{lambda}_a\, t_a$, and a slope of t_r.

Figure 31 plots the lambda*s from a number of different experiments against the stimulus, or recovery interval. The data are grouped according to the holding potential. The open squares represent different lambda*s obtained at -60 mV, the diamonds are data at -80 mV, and the filled squares are data from -110 mV. It can be seen that the prediction of the model is reasonably well followed, as lambda* appears to be a linear function of the stimulus, or recovery interval.

Another test of the model can be derived from the data shown in Figure 31. The slope of the lines, lambda_r, is inversely related to the recovery time constant, tau_r; $\text{lambda}_r = 1/\text{tau}_r$. Therefore, the slopes of the theoretical lines shown in Figure 31 predict the value of the time constant of I_{Ca} recovery after the application of D600. This prediction was tested by determing the time constants experimentally. The effect of D600 on I_{Ca} recovery could not be determined by the same two-pulse protocol used for the Bay K 8644 studies for

Figure 31. Uptake rate of D600 at different stimulus intervals. The uptake rate, lambda*, is plotted against the interval between stimuli, for three different holding potentials, -60 mV (open squares), -80 mV (diamonds) and -110 mV (filled squares). A nonlinear least squares method was used to fit the data points at each holding potential (see Methods). Data were pooled from several cells. The Tris external solution and the 5 mM EGTA pipette solution were used.

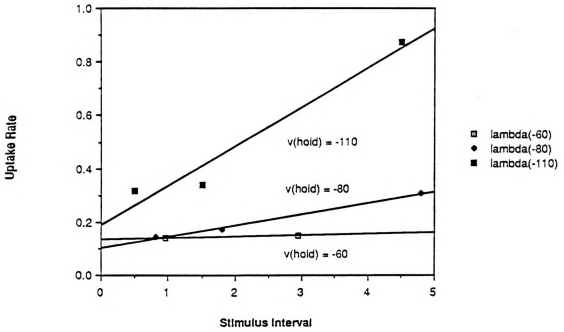

1.0
0.8
0.6
0.4
0.2
0.0
Uptake Rate
0
1
2
3
4
5
Stimulus Interval
v(hold) = -110
v(hold) = -80
v(hold) = -60
lambda(-60)
lambda(-80)
lambda(-110)

Figure 31

two reasons. First, the effect of D600 on Ca channels is highly use-dependent; the channels must open before D600 has much effect. As can be seen by examining Figures 29 and 30, this means that a single prepulse would do very little. Second, D600 recovery is extraordinarily slow, which can produce problems if there are even small instabilities of the cell. The protocol used to study recovery is shown in Figure 32A. A train of 100 ms depolarizations to 0 mV was given for a total of 2 sec. This repetitive stimulation accumulated a fair amount of I_{Ca} block. The cell was then restimulated with a 500 ms test-pulse after a variable interval. The degree of inactivation was measured by dividing the peak magnitude of each test I_{Ca} by the peak magnitude of the first I_{Ca} during the train. Figure 32B shows an example of the currents observed during such a train, when the holding potential was -110 mV. Examples of test I_{Ca}s observed after different intervals are shown in Figure 32C. The raw data were then fit by an exponential as before. The average time constants in the presence of D600 were found to be 7,360±550 ms at -110 mV (n=6), and 25,440±4,850 ms at -80 mV (n=4). This compares with 38.6±3.1 ms at -110 mV (n=5), and 90.0±3.3 ms (n=5), under control conditions.

Table 4 compares the theoretical recovery time constants obtained from Figure 31, with the experimental time constants. However, experimental data could not be obtained at very positive potentials, as the time constant (>>5 minutes) was longer than could be accurately measured in the single cells. For this reason, additional data from an earlier report (McDonald et al., 1984b) are shown. This data was obtained using the single sucrose gap method with cat ventricular muscle. This preparation has the advantages of being more stable, and being fairly comparable to the guinea-pig ventricular myocytes. All of the theoretical and experimental data seem to be in good agreement, a result that lends support to the supposition that the guarded-receptor hypothesis can ade-

Figure 32. I_{Ca} recovery from inactivation in the presence of D600. Panel A is a diagram of the voltage-clamp protocol that was used. A 2 s long train of 100 ms duration depolarizations from -110 mV to 0 mV at 4 Hz, was first given to accumulate D600 block, and then after a variable duration interval, I_{Ca} was remeasured by a 500 ms test pulse to 0 mV. Panel B shows a series of I_{Ca}s elicited by such a train, showing progressive block by D600. Panel C shows I_{Ca} elicited by test pulses after different rest periods after the train. The Tris external solution and the 5 mM EGTA pipette solution were used.

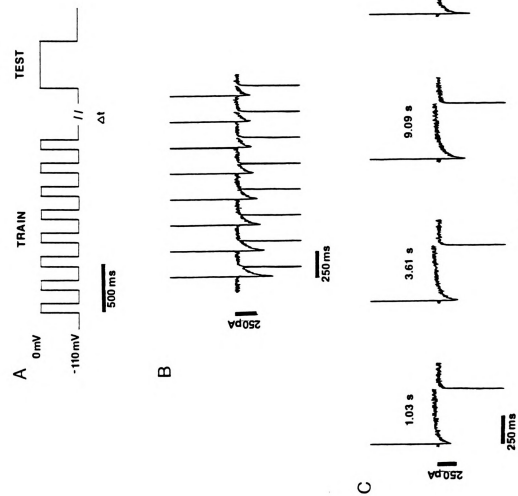

A
0mV
-110mV
TRAIN
TEST
Δt
500 ms
B
250pA
250ms
C
1.03 s
3.61 s
9.09 s
18.55 s
250pA
250ms

Figure 32

Table 4

Comparison of Theoretical and Experimental Rates of D600-influenced I_{Ca} Recovery

Method	-110 mV	-90 mV	-80 mV	-70 mV	-60 mV	-50 mV
Uptake	6.77	---	24.01	---	333	---
Recovery	7.36	---	25.44	---	---	---
Recovery (McDonald)	5	16	---	144	---	528

quately describe Ca channel blockade, at least that of D600. In addition, it is obvious that I_{Ca} recovery has a steep voltage-dependence in the presence of D600.

F. Possible Mechanisms for Ca-dependent inactivation

By this point in the experimental studies, several things about the complicated inactivation process of the Ca channel had become more clear. The Ca channel was clearly inactivated by two different processes which could be separated by studying monovalent ion currents through the channels. The first inactivation process was defined as a classical voltage-dependent inactivation mechanism. The fact that the second process was dependent on the entry of divalent cations into the cell had also been clearly established. The main question that remains at this point is the exact mechanism by which Ca ions act to turn off the Ca channel. Several possible mechanisms have been proposed in recent years, and some experiments have been done to test some of these hypotheses.

1. Effect of Ca entry on the surface charge of the membrane

Since changes in the concentration of external divalent cations are known to have profound surface charge effects on the voltage-dependence of ion channels (Frankenhauser and Hodgkin, 1957), the possibility of entering Ca ions causing intracellular surface charge effects has drawn attention. In particular, one possible mechanism that has been considered for Ca-dependent inactivation is that intracellular Ca ions might bind to, and neutralize, negatively charged groups on the internal side of the membrane (Tsien, 1983). This loss of negative charges on the intracellular surface would cause an additional, artifical depolarization of the membrane. A voltage-dependent inactivation mechanism would be influenced by this additional depolarization, and more channel inactivation than expected would occur at each potential where Ca entered the cell.

One way to test this hypothesis is to examine the effect Ca entry has on the activation of the channel. The activation process is a less complex process than inactivation, but still depends on the membrane potential. Thus, if prior Ca entry altered Ca channel inactivation via surface charge effects, it should also alter the activation process. Figure 33 shows a typical current-voltage relationship of I_{Ca} obtained with 500 ms depolarizations from -50 mV to a variety of different potentials (open symbols). After obtaining this curve, the current-voltage relationship was then repeated, but with a 50 ms prepulse to 0 mV added before each pulse (filled symbols). This prepulse allowed a large amount of Ca to enter the cell just prior to the examination of the current-voltage relationship. If the Ca entry caused an artifical depolarization of the membrane, the current-voltage relationship should have shifted in the negative direction. As can be seen, the current-voltage relationship displays no shift at all. The only effect of the prepulse was to reduce the available I_{Ca} equally at all potentials.

2. Effects on inactivation of drugs that alter Ca channel phosphorylation

A second hypothesis that has been recently proposed is that the entry of Ca into the cell activates a Ca/calmodulin-dependent phosphatase, calcineurin, that then dephosphorylates the Ca channel (Chad and Eckert, 1986). This hypothesis assumes that the Ca channel is nonconducting in the dephosphorylated state.

There are a number of ways to test this hypothesis, as there are several drugs that can influence various facets of the phosphorylation process. One approach is to try to interfere with an early step in the dephosphorylation scheme, the activation of the phosphatase by Ca/calmodulin. There are a number of drugs that are calmodulin inhibitors. Few are particularly potent, and most of them have only somewhat specific effects. Nevertheless, a high concentration of a calmodulin inhibitor, such as trifluoperazine, should be able to prevent the

Figure 33. Effect of prior Ca entry on current-voltage relationship of I_{Ca}. I_{Ca} was elicited by 500 ms voltage-clamp steps from -50 mV to the potentials indicated. The magnitude of the peak inward or outward I_{Ca} is plotted for the test step alone (open symbols), and when the test step was preceded by a partially inactivating, 50 ms prepulse to 0 mV (filled symbols). I_{Ca} was measured relative to values obtained in a parallel run with 500 μM $CdCl_2$ present. The 2.5 mM Ca external solution and the standard pipette solution were used.

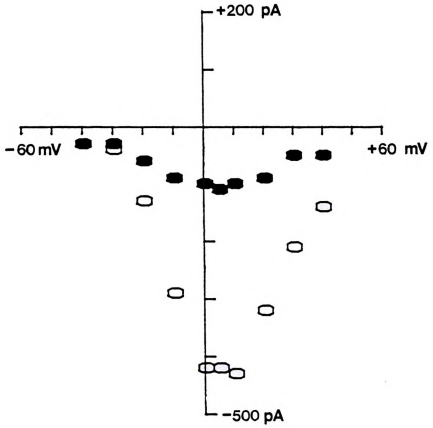


Figure 33

activation of the phosphatase, and reduce Ca-dependent inactivation. The upper trace in Figure 34 shows an example of I_{Ca} evoked by a 500 ms voltage-clamp step to 0 mV under control conditions. The lower trace shows I_{Ca} in the same cell after 10 minutes of exposure to 10 μM trifluoperazine. Trifluoperazine seemed to reduced the peak amplitude of I_{Ca} in this example by about 50%. This effect of trifluoperazine was seen in several other cells, but was not seen in others. However, it is obvious that trifluoperazine had little or no effect on the time course of current decay. The half-times of inactivation were 11 ms (control) and 14 ms (trifluoperazine). Preliminary observations also indicated that trifluoperazine had little effect on the U-shaped inactivation curve, and that external application of calmidazolium, a more potent calmodulin antagonist (Mazzei et al., 1984), had little effect on Ca channel inactivation either.

Ca channel inactivation appeared to occur normally in the presence of calmodulin antagonists, but a more direct means of testing the dephosphorylation hypothesis may be to see if agents that promote phosphorylation antagonize channel inactivation. Phorbol esters are one group of compounds that promote phosphorylation by stimulating protein kinase C (Nishizuka, 1984). The effect of phorbol esters on I_{Ca} is shown in Figure 35. I_{Ca} under control conditions, and after the application of 100 nM phorbol 12-myristate 13-acetate are superimposed. Little or no change was seen. The time course of current decay was almost the same as control, as was the peak magnitude. The half-time of decay for I_{Ca} was 16 ms in both control and drug-containing solutions. The average change in peak I_{Ca} induced by 100 nM phorbol 12-myristate, 13-acetate was $+1.8 \pm 3.0\%$.

One agent that is known to promote the phosphorylation of the Ca channel is isoproterenol (Reuter, 1983). Isoproterenol, like the other agents tried, did not greatly affect the time-course of I_{Ca}. Figure 36 shows an example of

Figure 34. I_{Ca} inactivation in the presence of trifluoperazine. Panel A shows an example of I_{Ca} obtained under control conditions by a 500 ms voltage-clamp step from -50 mV to 0 mV. Panel B shows I_{Ca} in the same cell several minutes after exposure to 10 μM trifluoperazine. The vertical calibration bar represents 1 nA, and the horizontal calibration bar represents 250 ms. The half-times of I_{Ca} decay are marked by arrows. The 2.5 mM Ca external solution and the standard pipette solution were used.

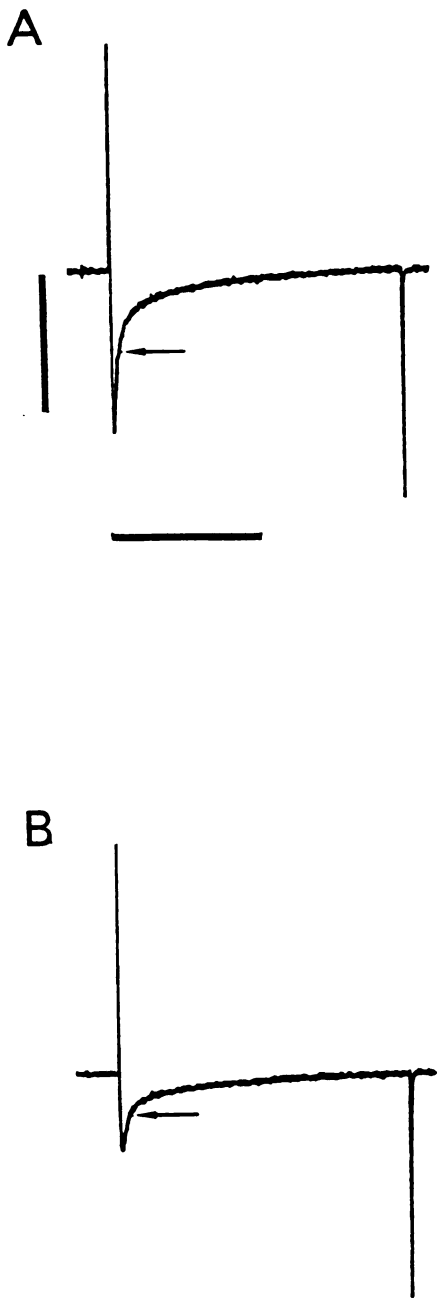


Figure 34

Figure 35. Effect of a phorbol ester on I_{Ca}. I_{Ca} traces are superimposed for control conditions, and after exposure to 100 nM phorbol 12-myristate, 13-acetate. The vertical calibration bar represents 500 pA, and the horizontal calibration bar represents 500 ms. Half-times of inactivation are marked by arrows. The 2.5 mM Ca external solution and the standard pipette solution were used.

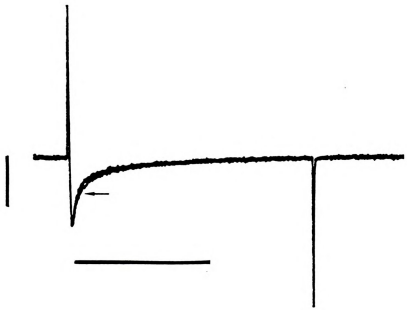


Figure 35

Figure 36. Time course of I_{Ca} inactivation in the absence and presence of isoproterenol. Current traces of I_{Ca} under control conditions (top), and after the application of 50 nM isoproterenol (bottom) are superimposed. The vertical calibration bar represents 500 pA, and the horizontal calibration bar represents 250 ms. Half-times of inactivation are marked by arrows. The 2.5 mM Ca external solution and the standard pipette were used.

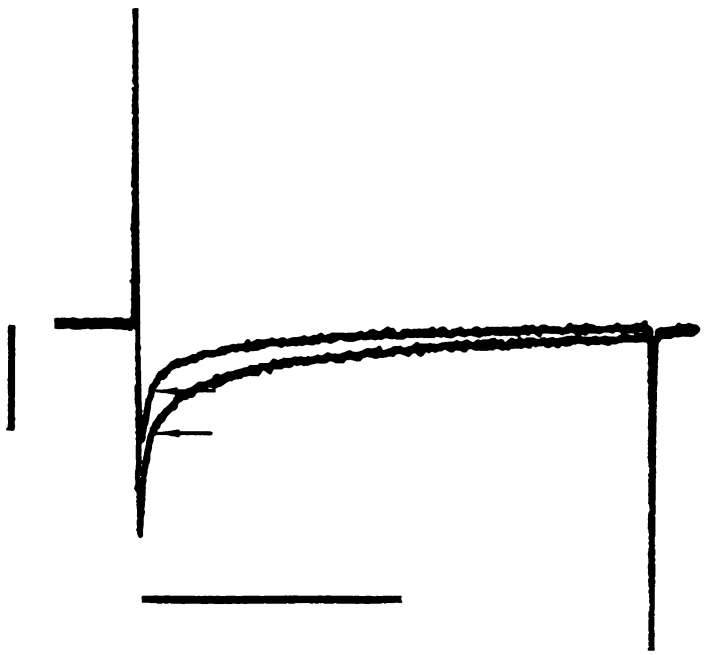


Figure 36

this. The figure shows superimposed traces of the control I_{Ca} and I_{Ca} after application of 50 nM isoproterenol. Isoproterenol greatly increased the size of I_{Ca}, but did not affect the half-time of inactivation. The half-time of inactivation was 14 ms in both control and isoproterenol-containing solutions.

Isoproterenol was also examined for any effects on I_{Ca} recovery. If inactivation of the channel was partly due to dephosphorylation, it would be expected that agents that enhanced channel phosphorylation would accelerate recovery. Figure 37A shows a typical example of I_{Ca} recovery under control conditions. The time constant of recovery was 329 ms. Recovery in the same cell, after exposure to 50 nM isoproterenol, is shown in Figure 37B. Recovery was only slightly faster, with a time constant of 279 ms. The average time constants of recovery were 353 ± 48 ms and 313 ± 29 ms under control conditions, and in the presence of 50 nM isoproterenol, respectively (n=5). The difference was not significant.

Figure 37. Effect of isoproterenol on I_{Ca} recovery from inactivation. Panel A shows I_{Ca} recovery under control conditions. Panel B shows I_{Ca} recovery several minutes after application of 50 nM isoproterenol. The 2.5 mM Ca external solution and the standard pipette solution were used.

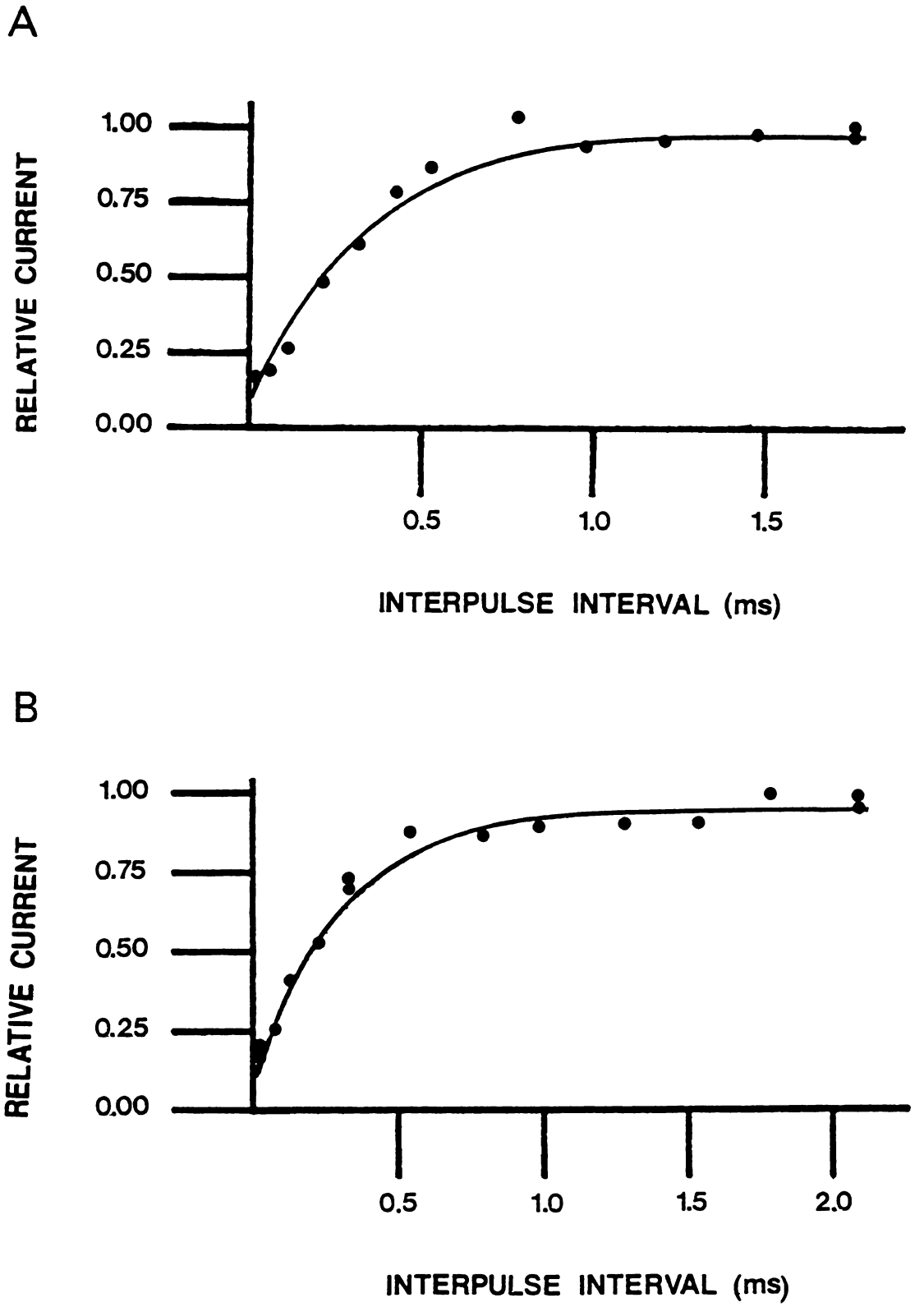

A
1.00
0.75
0.50
0.25
0.00
RELATIVE CURRENT
0.5
1.0
1.5
INTERPULSE INTERVAL (ms)
B
1.00
0.75
0.50
0.25
0.00
RELATIVE CURRENT
0.5
1.0
1.5
2.0
INTERPULSE INTERVAL (ms)

Figure 37

DISCUSSION

A. Characterization of I_{Ca} in Guinea-pig Ventricular Myocytes

A prerequisite for the study of any properties of the Ca channel is a satisfactory isolation of the Ca current from other components of membrane current. The introduction of voltage-clamp techniques that allow for the dialysis of the cell interior was an important advance in the achievement of this goal. These techniques have been used in this study to produce a satisfactory isolation of I_{Ca} in guinea-pig ventricular cells. K currents have been effectively suppressed by the introduction of the K channel blockers Cs and TEA intracellularly, and the inclusion of Cs in the superfusate as well. The inwardly rectifying K current, I_{K1}, was very effectively blocked, as judged by the slope conductance of the cell at negative potentials, and by its linearity. The time-dependent current was also suppressed to a great extent. This was evident from the many flat current records obtained at very strong depolarizations. However, it should be noted that a small, slowly activating outward current, such as was noted by Matsuda and Noma (1984), was seen with very strong depolarizations in many of the experiments. This current was more troublesome in Ca-containing solutions, but this may have been because of the much smaller conductance of the outward current through Ca channels with Ca present. The identity of this current was not put to any rigorous tests, but two possibilities include some type of voltage-dependent nonspecific conductance (Matsuda and Noma, 1984), or residual time-dependent K current. This latter possibility was suggested by the observation that

the addition of 1 mM Ba, a potent K channel blocker (Osterrieder et al., 1982), to the Ca-containing solution strongly suppressed the current (data not shown).

The goal of studying Ca-dependent inactivation required that the internal solution have a minimal capability of buffering Ca. This meant that the physiological studies had to be done in Na-containing external solution, as intracellular EGTA is customarily used to prevent the contracture due to Na-free solutions. Therefore, the Na current was usually eliminated by holding the membrane potential relatively positive, and by applying TTX. In the pharmacological studies, Ca-buffering was of less concern, and Na-free solutions were used. However, there is one concern regarding the use of normal Na-containing solutions. There has been some theoretical and experimental evidence that the Na/Ca exchanger may contribute significant inward current during moderate depolarizations (Noble, 1984; Hume, 1987; Fedida et al., 1987). This possibility does not lead to many complications regarding the interpretation of the present results. The contribution of the Na/Ca exchange current would appear to be small under these conditions, as the characteristic large, inward tail currents seen upon repolarization (Hume and Uehara, 1986a,b) are absent in the records. The Na/Ca exchanger would also not be expected to contribute to the Ca-dependent inactivation of I_{Ca}, as the current is expected to be inwardly directed, and thus would be moving Na ions into the cell, not Ca. However, there is a possibility that the Na/Ca exchange current could complicate the exponential analysis of the inactivation kinetics.

Finally, the contribution of other types of Ca channels to I_{Ca}, besides L-channels, needs to be evaluated. The N-type Ca channel (Nowycky et al., 1985) has not yet been found in any cardiac preparation. This channel does not appear to contribute significantly to I_{Ca} in this study, as there was little inactivation of I_{Ca} up to -50 mV, where the N-channels would be completely unavailable.

Furthermore, I_{Ca} was completely inhibitable by dihydropyridines, to which N-channels are insensitive. The same arguments also apply to the possibile contribution of the t-type Ca channels (Nilius et al., 1985; Nowycky et al., 1985).

It was found in these studies that a clear reversal of I_{Ca} could be demonstrated, such as has been reported by Lee and Tsien (1982, 1984). Matsuda and Noma could not observe such a reversal in this preparation, due to the time-dependent outward current discussed earlier. However, the outward current through Ca channels, which was presumably carried by Cs ions, was always fairly small in Ca-containing solutions.

I_{Ca} was found to inactivate with a biexponential time course. This differs from earlier studies of I_{Ca} inactivation, where only a single time constant was used to fit the data (New and Trautwein, 1972; Kohlhardt et al., 1975; Isenberg and Klockner, 1980; Mentrard et al., 1984), but is in agreement with several recent studies (Isenberg and Klockner, 1982b; Mitchell et al., 1983; Josephson et al., 1984; Hume and Uehara, 1985). It was also found that the relative importance of the slow component was enhanced whenever the magnitude of I_{Ca} was reduced. However, this traditional type of analysis was not pursued too earnestly, as it was felt that there were many complications involved. The first, of course, was the possibility of the Na/Ca exchange current marring the records. However, this did not seem to be too important, as the biexponential time course of inactivation was also found by measuring the progressive reduction of peak I_{Ca} with time, and by examination of I_{Ca} records in Na-free solution (data not shown). It is doubtful that further analysis of the inactivation kinetics would have much significance, as it appears that I_{Ca} inactivation is not a simple voltage-dependent process, as is assumed in traditional analyzes, but is dependent on two separate processes. However, some studies have indicated that Ba currents, which should be considerably less affected by current-dependent inactivation, still have two time

constants of inactivation (Kass and Sanguinetti, 1984; McDonald et al., 1986). Furthermore, these observations do not fit well with the finding in these studies that I_{Ca} recovery can be described by a single exponential. However, it should noted that there has been one report of biexponential I_{Ca} recovery in cardiac Purkinje fibers (Kass and Sanguinetti, 1984). It seems that the kinetics of the onset of I_{Ca} inactivation are quite complicated, and analysis in terms of exponentials may not be appropriate, and may be misleading.

It was found that I_{Ca} inactivation was strongly associated with entry of Ca through the channels. Evidence for this included the similarity of the I_{Ca} I-V and inactivation curves, and the effects of altering external Ca or introducing Ca chelators intracellularly. However, it would appear that not even very high concentrations of citrate or EGTA can completely prevent Ca-dependent inactivation. This may be due to either to the limited buffering capacity, or to an overwhelming size of the Ca transient in the microscopic domain around each channel. If the latter reason was true, it would indicate that the inactivating effect of Ca occurred in the immediate vicinity of the channel. Evidence for the inability of intracellular EGTA to control intracellular Ca transients has been obtained in Aplysia neurons (Eckert and Ewald, 1983).

B. General Properties of I_{ns}

The properties of Ca channels have also been studied under conditions when they are permeant to monovalent cations. The membrane potential where I_{ns} was first activated, and where the inward current peaked, were both shifted negative relative to the values for I_{Ca}. This is in agreement with previous studies of this current (Kostyuk and Krishtal, 1977; Imoto et al., 1985). This is most likely due to the removal of external Ca, which should induce a negative shift in voltage-dependent parameters, because of the increase in the surface charge of the

membrane. The reversal potential of I_{ns} is also shifted negative relative to that of I_{Ca}, but to an even greater extent. This is no doubt due both to the surface charge effect, and to a true shift in the reversal potential, as the selectivity of the Ca channel is considerably diminished in the absence of Ca. The fact that the reversal potential was still +23 mV indicates that the Ca channel under these conditions is somewhat more permeant to the dominant extracellular cation Na, than to the dominant intracellular cation Cs. This has been confimed recently in rather detailed voltage-clamp and single channel experiments in this same prepartion (Hess et al., 1986). However, the moderately positive reversal potential, the large magnitude of both inward and outward I_{ns}, and the near linearity of the I-V relations at positive potentials, indicates that the selectivity of the channel among these monovalent cations is only moderate.

The observation that raising extracellular Ca into the micromolar range converts the Ca channel into an outwardly rectifying channel is of great interest. This result can be interpreted in terms of the two-binding site model of ion permation (Almers and McCleskey, 1984; Hess and Tsien, 1984). Micromolar concentrations of extracellular Ca would only give a low probability of double occupancy of the pore, and thus little Ca influx would occur because of the lack of ionic repulsion within the pore. However, there would be a high probability of Ca binding to a single site, which would in most cases be the external site. This bound Ca ion would then repel entering monovalent cations, and thus block the pore. However, since the Ca ion itself is charged, its blocking effect could be modulated by the membrane potential, if the binding site was within the electrical field. Thus, the outward rectification of I_{ns} under these conditions is likely to be due to the relief of the block, as strong positive potentials forces the Ca back out of the pore. A similar argument has been made to explain voltage-dependent block of Ca channels in mouse lymphocytes by Mg and Ca (Fukushima and

Hagiwara, 1985). However, this interpretation differs from that of Lansman et al. (1986), who studied divalent cation block of monovalent currents through cardiac Ca channels at the single-channel level. The entry and exit of Ca from the pore was determined by observing "flickering" block during prolonged channel openings induced by Bay K 8644. These investigators found that the entry of Ca into the channel was not affected by voltage, but the exit of Ca from the channel was hastened by hyperpolarization. They interpreted the data to indicate that the blocking Ca ions could be pulled into the cell by hyperpolarization, but the first Ca binding site probably lay outside the electrical field, as the onset of block was not voltage-dependent. There are a number of questions about this data that need to be addressed. First, it is not inherently obvious why the the removal of the blocking Ca ion can be influenced by the membrane potential but the onset can not, unless there is some movement of the Ca ion deeper into the pore during the blockade. Second, Lansman et al. (1986) could only investigate the voltage-dependence of block over a small range of potentials (-60 to -20 mV), where the the single channel openings were fairly large. It can be argued that this range of potentials is not broad enough to fully test the voltage-dependence of the entry of Ca into the channel. These negative potentials certainly do not test the hypothesis that postive membrane potentials can cause the ejection of blocking ions from the pore. Thus, there appears to be considerable doubt left about the precise location of the Ca binding sites.

C. Separation of Two Types of Ca Channel Inactivation

Dual mechanisms for inactivation have been suggested for Ca channels in Helix neurons (Brown et al., 1981b), and more recently, for Ca channels in heart tissue (Kass and Sanguinetti, 1984; Lee et al., 1985). Others have argued that the inactivation of cardiac Ca channels is strictly Ca-dependent (Mentrard et al.,

1984; Bechem and Pott, 1985), or is dominated by a voltage-dependent mechanism (Trautwein and Pelzer, 1985). Some of the evidence that was found in these studies for Ca-dependent inactivation has already been summarized. The strongest evidence for Ca-dependent inactivation, however, were the studies of the inactivation curves. I_{Ca} had a partially U-shaped dependence on the membrane potential, which has already been offered as evidence for a dual mechanism (Lee et al., 1985). Certainly, the relationship of I_{Ca} inactivation to the degree of Ca entry during a prepulse is obvious by comparing the I_{Ca} I-V and inactivation curves. Furthermore, Ca channel inactivation was found to have a monotonic relationship to the membrane potential when Ca was absent. Therefore, the correlation of the I_{Ca} I-V and inactivation curves was not coincidental, and it appears that Ca-dependent inactivation does occur in this preparation. The demonstration of I_{ns} inactivation clearly eliminates the possibility that the inactivation of I_{Ca} at positive potentials is due to the presence of Ca tail currents between the prepulse and test pulse (Standen and Stanfield, 1982).

The residual Ca channel inactivation that occurred with I_{ns} appeared to be due to a voltage-dependent mechanism, as suggested by the monotonic inactivation curve. This conclusion was further supported by examination of I_{ns} kinetics, and by the demonstration that accumulation or depletion of permeant ions played no role in the process. Furthermore, as it was shown that inactivation could take place in the absence of ion permeation, there is little doubt that it is voltage alone that inactivates the channel. The most likely mechanistic explanation would seem to be an intrinsically voltage-dependent inactivation "gate" on the channel protein itself, similar to certain models of Na channel inactivation (see Armstrong, 1981). A more detailed hypothetical mechanism would only be speculative in the absence of any detailed structural information about the channel protein. Nevertheless, preliminary experiments that introduced nonspeci-

fic proteases into the cell interior indicated that the inactivation gate was not as labile as that of the Na channel (Armstrong and Bezanilla, 1973). However, these experiments were severely limited by a rapid rundown of I_{Ca}, presumably due to enzymatic degradation. A fundamental dissimilarity between the voltage-dependent inactivation gates of the Na and Ca channels is further suggested by the vast kinetic differences between the two processes.

An important question that arises from the existence of a dual mechanism for Ca channel inactivation concerns the relative importance of each mechanism, with regard to both experimental studies of I_{Ca}, and to the normal functioning of the Ca channel during an action potential. This issue has been addressed by the experiment shown in Figure 18, where the inactivation curves of I_{Ca} and I_{ns} were compared in a single cell. It is obvious that, with respect to 500 ms depolarizations, Ca-dependent inactivation accounts for much of the Ca channel inactivation that occurs between -30 and +30 mV. Voltage-dependent inactivation can also account for much of the inactivation over this range, and is the dominant mechanism at even more positive potentials. This analysis would appear to be appropriate under most conditions. Most voltage-clamp protocols use depolarizations of 500 ms or shorter duration, and the duration of the action potential of this preparation is also about 500 ms at 22°C (Hume and Uehara, 1985). However, the kinetics of voltage-dependent inactivation are often extremely slow, and thus voltage-dependent inactivation would become more prominent with experimental protocols that used longer depolarizations. This may help explain differences between the inactivation curves obtained with whole-cell measurements and the monotonic steady-state inactivation curves obtained in single-channel studies (Reuter et al., 1982; Cavalie et al., 1983).

At this point, it should be recognized that an outward current generated by a Na/Ca exchanger (Kimura et al., 1986; Hume and Uehara, 1986a,b), may produce

Ca influx at very positive potentials under some conditions. This would inactivate additional Ca channels in proportion to the extent of Ca entry.

It was often observed in these studies that there was a fraction of I_{ns} that had not inactivated by the end of the voltage step. This was found to be true even for depolarizations of up to 10 s. It appears that there is a noninactivating fraction of Ca channel current, or window current, at most of the potentials studied. This may even be true at very positive potentials, but this is complicated by the often exceedingly slow kinetics. It would appear that it is this noninactivating fraction of I_{ns} that supports the very long (> 10 s) cardiac action potentials that are found in the presence of external EDTA or EGTA (Rougier et al., 1969; Linden and Brooker, 1982).

D. Possible Mechanisms of Ca-dependent Inactivation

The most important issue about the inactivation of cardiac Ca channels that remain unresolved is the precise mechanism by which Ca inactivates the channel. Unfortunately, the data that has been accumulated in this and previous studies does not allow a definitive conclusion to be reached. Nevertheless, the data does allow for the evaluation of a number of possible mechanisms, and for the exclusion of some.

One possibility that has repeatedly been discussed is that during the time course of I_{Ca}, the driving force for Ca may change due to accumulation of Ca in a restricted intracellular space, or due to the depletion of extracellular Ca. As discussed in the Introduction, there is theoretical evidence against intracellular Ca influencing the driving force for Ca to any great extent (Hagiwara and Byerly, 1981; Chad and Eckert, 1984). However, depletion of extracellular divalent charge-carriers would seem to be plausible in cardiac tissue. Ion depletion in the transverse tubules of frog skeletal muscle fibers has been found to account for the

decay of I_{Ca} in that preparation (Almers et al., 1981). The presence of transverse tubules raises this possibility in cardiac muscle as well. However, buffering of extracellular Ca did not slow I_{Ca} inactivation in frog atrial fibers (Mentrard et al., 1984), and the reversal potential of Sr current through Ca channels was not altered by prior channel inactivation (Kass and Sanguinetti, 1984). Furthermore, the inactivation of cardiac Ca channels is slower when Ba and Sr are substituted for Ca (Kass and Sanguinetti, 1984; Lee et al., 1985), even though they have a greater flux through the channels, and would be expected to deplete or accumulate faster. Nevertheless, the use of the Ca-sensitive dye tetraethylmurexide has shown that a small depletion of extracellular Ca does occur in guinea-pig atrial muscle upon stimulation (Hilgemann et al., 1983). Also, in the present studies it was often observed that size of I_{Ca} was usually a little larger when the experimental chamber was constantly being perfused with fresh solution, than when it was not. It would appear that the depletion of external charge-carriers may play a small role in determining peak I_{Ca}, but probably has little influence over its time course.

A second possible mechanism for Ca-dependent inactivation is that there are anionic sites on the inner surface of the cell membrane, and that entering Ca ions can bind to them. This neutralization of the negative intracellular charges could cause an artifical depolarization of the cell membrane, and thus promote voltage-dependent inactivation to a greater extent than the same depolarization without Ca entry. This possibility has been examined before in Purkinje fibers, and it was suggested the shift would have to be extremely large to account for Ca-dependent inactivation (Kass and Sanguinetti, 1984). These studies have extended this observation, as it has been shown that with a 500 ms depolarization, Ca-dependent inactivation can cause complete inactivation at 0 mV, while voltage-dependent inactivation was still incomplete at potentials as positive as

+110 mV. In addition, it was found that Ca entry produced little or no shift in the voltage-dependence of Ca channel activation. These results strongly suggest that surface charge effects are of little importance in cardiac Ca channel inactivation.

The possibility that the dual mechanism for I_{Ca} inactivation reflects the properties of separate populations of Ca channels should be briefly considered. This does not appear to be a significant possibility, as I_{Ca} and I_{ns} appear to be quite similar physiologically, except for inactivation properties, and also to be quite similar with respect to pharmacology. Also, the absence of a slowly decaying component of I_{Ca} indicates that the presence of Ca accelerates the inactivation of the channel that carries I_{ns}. Thus, both inactivation processes appear to take place on the same Ca channel.

Another mechanism that has often been envisioned for Ca-dependent inactivation is that Ca might bind to the channel, or to an associated adjacent protein, and directly regulate Ca channel function in an allosteric manner. There are a number of possible alternative models that can be hypothesized. One is that voltage- and Ca-dependent inactivation act through two completely independent mechanisms on the channel protein. Physically, this would probably be represented by a binding site on the channel protein, which would close the channel pore when it bound a Ca ion, and would open the pore when the Ca unbound. This model has been used by several investigators, but leads to some untenable predictions. As noted by Lee et al. (1985), there should be a secondary increase in I_{Ca} as intracellular Ca diminishes late in the depolarization. This plainly does not occur. However, it is arguable that a positive intracellular potential might prevent Ca from diffusing away from the membrane, and thus the Ca concentration in the domain around the channel might still be high.

A specialized model of the above hypothesis would be involved if the binding site for Ca was in the pore itself, and in particular, if it was the inner binding site of the two that are supposed to control ion permation (Almers and McCleskey, 1984; Hess and Tsien, 1984). If this were the case, Ca-dependent inactivation would actually consist of Ca block of the channel once Ca_i became high enough. The K_m of the inner site is not known, but if it were similar to the outer site, it would be in the micromolar range. This is approximately the Ca concentration at which I_{Ca} is completely blocked (Plant et al., 1983; Byerly and Moody, 1986). However, this version of the hypothesis again has the difficulty of inactivation not following the time-course of the Ca transient. Furthermore, it would seem unlikely that block of the channel at the internal site could be maintained, as entering Ca ions should clear the pore by ionic repulsion. There is some evidence for a similar intracellular block by Ni, however (Akaike et al., 1981).

A second form that an allosteric effect of Ca might take is that intracellular Ca could modulate voltage-dependent inactivation. That is, bound Ca might promote and accelerate the inherent, but somewhat ineffective, inactivation process. This hypothesis thus bestows some voltage-dependence to Ca-dependent inactivation, and would explain much of the data in this and previous studies. In particular, this would give a satisfactory explanation of why I_{Ca} does not recover from Ca-dependent inactivation at positive potentials (Eckert and Tillotson, 1981; Mentrard et al., 1984). However, injection of Ca can by itself inactivate Ca channels in snail neurons (Plant et al., 1983), even at a negative holding potential. An exactly analogous experiment has not been done in cardiac tissue, probably because of the difficulties involved in injecting Ca into muscle cells. However, the consistent effect of intracellular citrate or EGTA to increase I_{Ca} in these studies suggests that some degree of resting inactivation could be relieved. A direct test of this hypothesis was attempted by observing what effect altering the

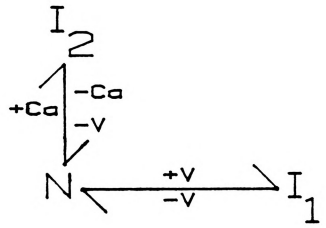
concentration of Ca in the pipette had on the I_{ns} inactivation curve. No consistent enhancement of voltage-dependent inactivation was observed, but the experiment was severely limited by the technical difficulties associated with the muscle cell, and by the uncertainity of the actual intracellular Ca concentration in such experiments (Byerly and Moody, 1984).

Two possible allosteric models for Ca-dependent inactivation are shown as a state diagram in Figure 38. The closed states and the open state of the Ca channel are represented collectively as N, for noninactivated channels, as voltage-dependent inactivation can occur with both closed and open channels (Cavalie et al., 1986), and the same is apparently true of Ca-dependent inactivation (Plant et al., 1983). Ca channels can spontaneously enter an inactivated state (I_1) with a probability and rate that depends on the membrane potential. They also can leave this state with a probability dependent on the membrane potential. Another inactivated state (I_2) can be reached through an independent pathway involving the binding of a Ca ion. However, the removal of Ca-dependent inactivation appears to be more complicated than recovery from voltage-dependent inactivation. A reduction of Ca_i is necessary, but repolarization appears to be an additional requirement. This voltage-dependence of Ca-dependent inactivation can either be a separate property of the I_2 state (Figure 38A), or be due to a coupling of the I_1 and I_2 states. Figure 38B represents this possibility as a conversion of I_2 to I_1. Additionally, it is possible that I_1 and I_2 are an identical state of the Ca channel, with two different mechanisms of induction.

These models can explain why positive potentials, which ordinarily would cause little Ca-dependent inactivation, can nevertheless maintain Ca-dependent inactivation. It can also explain why I_{Ca} and I_{ns} recovery kinetics were found to be almost identical, after taking surface charge effects into consideration. In agreement with this finding is the report that I_{Ca} and I_{Ba} have identical recovery

Figure 38. Model of Ca- and voltage-dependent inactivation. N represents non-inactivated Ca channels, while I_1 and I_2 represent voltage- and Ca-inactivated channels, respectively. Panel A shows a model of inactivation with two independent mechanisms. Panel B shows one model where the two mechanisms are coupled. See text for details.

A



B

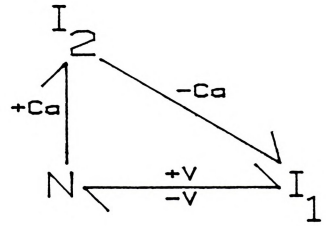


Figure 38

kinetics over a broad range of potentials in the calf Purkinje fiber (Kass and Sanguinetti, 1984). However, a survey of the literature indicates that there have been diverse results when cardiac Ca channel recovery was examined after altering the concentration or type of divalent cation present. An increase in extracellular Ca has been shown to speed (Kohlhardt et al., 1975; Shimoni, 1981) or to slow recovery (Mentrard et al., 1984). Substitution of Sr or Ba for Ca has also been shown to slow recovery (Noble and Shimoni, 1981), or to speed recovery (Mentrard et al., 1984). These diverse results may indicate that the recovery process is quite complicated, and that factors such as surface charge effects, the "positive staircase" of I_{Ca} (Noble and Shimoni, 1981a,b; Lee, 1987), and the possible effect of intracellular Ca to increase I_{Ca} (Marban and Tsien, 1982), should be considered in analyzing recovery. The results presented in this report indicate that Ca entry under these conditions has little effect on recovery. This must mean either that the removal of Ca-dependent inactivation is not the limiting factor in recovery, or that it is removed by the same voltage-dependent process that underlies I_{ns} recovery. Obviously, the availability of new techniques for measuring Ca_i in single cells (Williams et al., 1985) will make it possible to more closely examine allosteric models of Ca-dependent inactivation.

A final hypothesis for the mechanism of Ca-dependent inactivation involves metabolic control of the Ca channel. There have been repeated suggestions over the years, that Ca channels must be phosphorylated in order to open (Sperelakis and Schneider, 1976; Reuter and Scholz, 1977b), although the point is quite controversial. The debate, for the most part, has been centered on the issue of whether β-adrenergic stimulation increases cardiac I_{Ca} by increasing the number of functional Ca channels (Bean et al., 1984), or by increasing the probability of opening of already functional channels (Cachelin et al., 1983; Brum et al., 1984). This controversy has recently been extended into the area of Ca channel

inactivation, as it has been proposed that Ca-dependent inactivation of L-type Ca channels is due to dephosphorylation (Chad and Eckert, 1986). This has been described as being due to the actions of calcineurin, a Ca/calmodulin-dependent phosphatase. After activation by Ca/calmodulin, calcineurin would then dephosphorylate the Ca channel, rendering it nonfunctional. Recovery from inactivation would result from diminished calcineurin activity, and from channel rephosphorylation, probably due to cAMP-dependent protein kinase.

The applicability of this hypothesis to cardiac I_{Ca} was tested in these studies by examining the effects on I_{Ca} inactivation of drugs that could alter the phosphorylation of the Ca channel. Little support was found for this postulate, as the application of the calmodulin inhibitor trifluoperazine did not seem to alter the time-course of I_{Ca} decay, although it did appear to produce a small amount of block. Block of Ca channels by calmodulin inhibitors have been noted before (Bkaily et al., 1984; Bkaily and Sperelakis, 1986). Also, activation of protein kinase C by a phorbol ester did not appear to slow I_{Ca} inactivation. In addition, the phorbol ester appeared to have little effect on the size of I_{Ca}, suggesting that it did not promote channel phosphorylation (cf. Rinaldi et al., 1982). It should be noted that the activation of protein kinase C has been found to have both stimulatory (Wakade et al., 1985; DeRiemer et al., 1985; Strong et al., 1987) and inhibitory effects (Hammond et al., 1987) on Ca influx in other preparations. Finally, contrary to the prediction made by the dephosphorylation hypothesis, and to two previous reports in older preparations (Shimoni et al., 1984; Tsuji et al., 1985), stimulation of cAMP-dependent protein kinase with isoproterenol did not significantly accelerate recovery. In addition, it has been recently reported that isoproterenol slows I_{Ca} recovery in isolated frog atrial cells (Fischmeister and Hartzell, 1986). Isoproterenol was also found to have little effect on the time course of I_{Ca} decay.

Despite this contrary evidence, the importance of phosphorylation to Ca channel function is clearly undeniable, and a relationship of inactivation to the phosphorylation state of the channel has been suggested by several investigators. There are two basic questions that have to be addressed to resolve this problem. First, is the Ca channel nonfunctional when it is not phosphorylated? Second, does the entry of Ca into the cell promptly lead to the dephosphorylation of the channel?

The hypothesis that the dephosphorylated Ca channel is nonfunctional has arisen from two observations. First, it is known that exposure of heart cells to dinitrophenol, cyanide, or anoxia, which decrease the level of intracellular ATP, diminish Ca influx through the channels (Sperelakis and Schneider, 1976). Second, it has been a common finding that the rundown of I_{Ca} during cell dialysis can be slowed considerably by the inclusion of phosphorylating agents, such as ATP or cAMP, in the pipette (Doroshenko et al., 1982; Irisawa and Kokubun, 1983). However, in most cases, phosphorylating agents by themselves cannot completely prevent rundown, and it has been suggested that other processes, such as proteolysis, may have some role (Eckert and Chad, 1986). This may be quite important, since in many cases, Ca channel rundown is irreversible. In addition, it has been suggested that phosphorylating agents may not prevent rundown through a direct effect on the Ca channel, but may instead help to maintain a low Ca_i through activation of the Ca ATPase (Byerly and Yazejian, 1986).

The view that the availability of Ca channels is controlled by phosphorylation, has also been supported by fluctuation analysis of the effect of isoproterenol on I_{Ca} in frog ventricular cells (Bean et al., 1984). These investigators found that isoproterenol increased the functional number of Ca channels in a cell. The opposite view has been taken by Brum et al. (1984), who found in patch-clamp studies on isolated mammalian myocytes, that β-adrenergic stimulation never

could induce the appearance of Ca channels when they were absent under control conditions. β-adrenergic stimulation instead seemed to increase the probability of opening of channels that were already functioning. In addition, Bean et al. (1984) demonstrated that isoproterenol has little effect on the size of outward currents through Ca channels, which suggests a strong enough depolarization can recruit as many channels as β-adrenergic stimulation.

Evidence on this subject has also been obtained by introducing catalytic enzymes or their inhibitors into myocytes. The dialysis of myocytes with the regulatory subunit of cAMP-dependent protein kinase or protein kinase inhibitor (Kameyama et al., 1986a) or phosphatase 1 (Kameyama et al., 1986b), can strongly counteract the stimulatory effects of isoproternol, but only diminish basal I_{Ca} slightly. This contrasts sharply with recent results obtained with outside-out patches on GH3 clonal pituitary cells (Armstrong and Eckert, 1987). These investigators found that they could only maintain the function of L-type Ca channels in these patches in the presence of ATP, cAMP, and the catalytic subunit of cAMP-dependent protein kinase.

Direct measurements of the phosphorylation state of the Ca channel would clearly be very useful in approaching this question. However, few of these studies have been done, and none has yet correlated the degree of phosphorylation of the channel with its functionality. The dihydropyridine receptor from skeletal muscle t-tubules has recently been purified, and found to consist of three subunits: α, β and γ (Curtis and Catterall, 1984). This protein has recently been reconstituted into planar lipid bilayers, and forms a functional Ca channel (Flockerzi et al., 1986). The dihydropyridine receptor from skeletal muscle is thought to be phosphorylated on only one subunit in vivo, although it is controversial over whether it is the α or β subunit that is phosphorylated (Curtis and Catterall, 1985; Hosey et al., 1986). It also appears from these reports that phosphorylation

of the receptor by cAMP-dependent protein kinase only yields one mole of phosphate per mole receptor. This is intriguing, in light of the fact that β-adrenergic stimulation has been shown in patch clamp studies to alter the kinetics of the opening of cardiac Ca channels (Cachelin et al., 1983; Brum et al., 1983). If this occured as a result of cAMP-dependent phosphorylation, and there is only one phosphorylation site on the channel, it implies that cardiac Ca channels can open in the dephosphorylated state.

Aside from the question of whether the dephosphorylated Ca channel is nonfunctional, it is necessary to ask whether such a dephosphorylation can account for Ca-dependent inactivation of I_{Ca}. It has been established that calcineurin can dephosphorylate the dihydropyridine receptor from skeletal muscle (Hosey et al., 1986). However, it does not appear that calcineurin, which is also known as phosphatase 2B, is present in as great an amount in cardiac muscle as it is in neural tissue or skeletal muscle (Pallen and Wang, 1985). However, this has not been studied to a great extent, and there is always the possibility of other Ca-dependent phosphatases. Another important question is whether such an enzymatic process could occur quickly enough to account for the often rapid time course of I_{Ca} decay. The "inactivation" that was induced after the injection of calmodulin and calcineurin into Helix neurons (Chad and Eckert, 1986) was somewhat sluggish, and apparently had a time constant of hundreds of milliseconds. Perhaps this could occur faster if some of the enzyme was membrane-bound, instead of floating in the cytoplasm. It also should be recalled that in these studies, isoproterenol had no consistent effect to delay inactivation or speed recovery. This agrees with the observation that intracellular ATP or cAMP, if anything, accelerated I_{Ca} inactivation in guinea-pig ventricular cells (Irisawa and Kokubun, 1983).

It is apparent that there is not yet a clearly defined mechanism for Ca-dependent inactivation of Ca channels. However, it has proven possible to definitely rule out some possibilities, and put constraints on others. The two general mechanisms that need to be tested further are Ca-dependent phosphorylation, and the possible existence of an allosteric site on the intracellular side of the Ca channel. The issue of whether the Ca channel is functional when it is dephosphorylated remains controversial, but it should be emphasized that the possibility of dephosphorylation accounting for Ca-dependent inactivation is a separate question. For example, the Ca channel might be nonfunctional when it is nonphosphorylated, but might not be dephosphorylated significantly during the time course of I_{Ca}. The allosteric model, on the other hand, suffers from a lack of any direct demonstration of its presence. However, preliminary observations with reconstituted cardiac Ca channels have indicated that openings cannot be seen when the Ca concentration on the "intracellular" side of the bilayer is raised into the micromolar range (Ehrlich et al., 1985; Rosenberg et al., 1986). It would seem that further experiments with reconstituted channels could directly test these postulated mechanisms by adding Ca or different kinases and phosphatases to the "intracellular" side of the bilayer. Finally, it should be noted that the possibility of there being more than one mechanism for Ca-dependent inactivation should not be discounted.

E. Ca Channel Antagonist Properties of Bay K 8644

One of the principal aims of these studies was to ascertain whether certain drugs that affected cardiac Ca channels had preferential interactions with the channel's inactivated state. One drug that was examined for this effect was the new dihydropyridine, Bay K 8644, which has been shown to enhance Ca influx through Ca channels (Schramm et al., 1983). However, the action of Bay K 8644

on Ca channels is quite complicated, as the drug has been found to have inhibitory effects, along with its stimulatory effects, when very high concentrations of the drug are used (Thomas et al., 194), or when the cell is depolarized (Sanguinetti and Kass, 1984b; Sanguinetti et al., 1986). On the other hand, it has been reported that the net effect of Bay K 8644 on cardiac I_{Ca} is chiefly stimulatory, with little indication of voltage-dependent inhibitory effects (Brown et al., 1984b; Brown et al., 1986). The present studies clearly show that Bay K 8644 has Ca channel antagonist properties that are dependent on both the drug concentration and the membrane potential, and provide considerable quantitative information about both the stimulatory and inhibitory effects of the drug.

It has been shown that the inhibitory effects of Bay K 8644 consist of a component of block that occurs at rest (tonic block), and a second component that appears upon stimulation (use-dependent block). Tonic block of I_{Ca} by Bay K 8644 only becomes prominent at holding potentials positive to -50 mV; that is, at potentials positive to the threshold of Ca channel activation and inactivation. However, I_{Ca} in the presence of the drug was somewhat smaller at a holding potential of -50 mV than at more negative holding potentials, although it was still larger than the control. The time course of the onset of this "tonic block" had a time constant inthe order of seconds at -50 mV, while the relief of tonic block at -80 mV was faster, with a time constant of several hundred ms. Kinetically, the process closely resembles the Bay K 8644-induced slow phase of recovery at both potentials. There was no significant difference between the time constants of recovery and the onset or relief of tonic block at either potential. This suggests that the processes are similar, and that tonic block is due to active inhibition of channels, rather than due to some unknown voltage-dependence of the stimulatory effect of the drug.

If the decrease in the size of I_{Ca} at depolarized potentials can be attributed to active block of Ca channels, then it appears that the stimulatory effect of the drug was not affected to any great extent by the holding potential. It was apparent in Figure 20 that Bay K 8644 had a constant effect on I_{Ca} over the voltage-range of -90 to -60 mV, where the stimulatory effect of the drug is dominant. Some voltage-dependence of the stimulatory effect has been noted in other studies (Hess et al., 1984, Sanguinetti and Kass, 1984b), but this was simply a negative shift of the IV relations, so that a larger increase in I_{Ca} was seen with a test-potential of -20 mV than at +10 mV. This may simply be due to the inherent activation properties of the channel. A smaller increase in the size of I_{Ca} would be seen at positive potentials, because the probability for Ca channel opening is already quite high. Such an effect has been noted with isoproterenol as well (Bean et al., 1984). This type of voltage-dependence may have no particular significance with regard to the interaction of the drug and its receptor.

The accumulation of use-dependent block by Bay K 8644 was found to be accentuated by faster rates of stimulation. This has been noted in previous studies of both Bay K 8644 (Sanguinetti et al., 1986), and similar dihydropyrdines such as CGP 28392 (Kamp et al., 1985) and the Sandoz compound 202-791 (Kongasmut et al., 1985). Use-dependent block by Bay K 8644 was also accentuated by increased duration of depolarization or by more positive holding potentials. This is suggestive of block by Bay K 8644 of Ca channels once they are in the open or inactivated state.

Other effects of Bay K 8644 on I_{Ca} closely resembled that of classical organic Ca channel blockers. Bay K 8644 accelerated the rate of current decay, an effect that can again be interpreted as block of open (Lee and Tsien, 1983) or inactivated channels (Sanguinetti and Kass, 1984a; Hess et al., 1984). The fact that Bay K 8644 also shifted the inactivation curve and slowed I_{Ca} recovery would

seem to suggest that block of the inactivated state would be more important. This will be discussed in greater detail below.

The similarity of the inhibitory effects of Bay K 8644 to that of other, more typical, dihydropyridines strongly suggests that it acts through a similar mechanism. This casts doubt upon the suggestion that the inhibitory effects of Bay K 8644 arise as a special consequence of its promotion of Ca channel opening (Sanguinetti et al., 1986).

It is important to ask why the inhibitory effects of Bay K 8644 were such a prominent feature in these studies, and were conspicuously absent in other studies that used the same technique and the same preparation (Brown et al., 1984b; Brown et al., 1986). This discrepancy is quite difficult to explain. It should be noted, however, that the degree of drug-induced shift of the inactivation curve that is seen is probably dependent on at least two factors. First, it seems likely, after examination of Figure 19, that the degree of shift will be proportional to the drug concentration. The size of I_{Ca} at the holding potentials of -80 mV and -40 mV are only moderately different under control conditions, but grow more widely disparate as the Bay K 8644 concentration increases. Second, the duration of the prepulse would appear to affect the inactivation curve. The inactivation curve shown in Figure 21, which was obtained with 500 ms prepulses, has a gradual slope at negative potentials. Figure 20, which shows absolute current changes induced by Bay K 8644, has a more abrupt slope, and was obtained using longer intervals. The reason for this may be that short prepulses do not allow enough time for I_{Ca} to reach steady-state levels (compare Figures 22 and 23).

A question that needs to be addressed at this point is the relative importance of the two enatiomers of Bay K 8644. It has been reported that the activity of the two enatiomers is differentiable, with (-) Bay K 8644 having chiefly stimulatory effects, and (+) Bay K 8644 having principally inhibitory

effects (Franckowiak et al., 1985). Similar observations have also been seen with the stimulatory dihydropyridine 202-791 (Kongasmut et al., 1985). Racemic Bay K 8644 was used in all of the present experiments, but it is necessary to evaluate the importance of the two enatiomers to the inhibitory effects described here. It would seem likely that (+) Bay K 8644 would make a strong contribution to the inhibitory effects, and examination of the dose-response data presented in Table 2 suggests that this may be the case. It can be seen in Table 2 that the stimulatory effects seen at -80 mV seem to rise and peak at lesser concentrations than the inhibitory effects seen at a holding potential of -40 mV. This is consistent with the possible importance of (+) Bay K 8644 to the inhibitory effects, as this enatiomer has a somewhat lower affinity for the dihydropyridine receptor than (-) Bay K 8644 (Bellemann and Franckowiak, 1985; Wei et al., 1986). However, the stimulatory enatiomer of 202-791, (+) 202-791, has been recently shown to have antagonist-like effects of its own (Kokubun et al., 1986; Kamp et al., 1987). Also, clear evidence of concentration-dependent inhibitory effects of (-) Bay K 8644 has been reported in smooth muscle from rat tail artery and guinea-pig ileum (Wei et al., 1986). Therefore, it seems likely that (-) Bay K 8644 also contributes to the inhibitory effects of the racemate.

F. Comparison of different models of Ca channel blockade

It has been shown in these studies that the new dihydropyridine, Bay K 8644, has many properties in common with classical Ca channel blockers. These properties include the hyperpolarizing shift of the inactivation curve, the acceleration of I_{Ca} inactivation, the importance of use-dependent block to its antagonistic effects, and the slowing of recovery from inactivation. Such effects have been noted before in many studies with both local anesthetics and Ca channel blockers, and have usually been interpreted in terms of the modulated receptor

hypothesis; that is, they were attributed to high-affinity drug binding to the inactivated state of the channel (Hondeghem and Katzung, 1984).

It has also been shown in these studies that the guarded receptor hypothesis can adequately describe the use-dependent block of Ca channels produced by D600 with a variety of protocols. Additional analysis demonstrated further agreement between the theoretical predictions and the observed data. These are important findings, as they constitute the first evaluation of the applicability of the guarded-receptor hypothesis to the action of a Ca channel blocker.

The modulated-receptor hypothesis has also been used to successfully describe block of Ca channels by D600 (Uehara and Hume, 1985). As of yet there has been no complimentary appraisal of the adequacy of the guarded receptor hypothesis in describing dihydropyridine block. Consideration of all of these studies makes it clear that while a model of Ca channel blockade must first be able to predict the observed voltage- and time-dependence of block, this alone does not allow discrimination between different models. In particular, more direct tests of the basic hypotheses underlying the models are needed.

An additional technique that has been used to investigate the mechanism of Ca channel blockade is receptor-binding studies. These studies have used a variety of different Ca channel blockers, but the most detailed and informative of them have concentrated on the dihydropyridines. Early comparisons of dihydropyridine binding and functional studies in cardiac tissues revealed a significant discrepancy; the K_D was found to occur at concentrations up to a thousand-fold less than the IC_{50} (Schwartz and Triggle, 1984). The discovery of the voltage-dependence of dihydropyridine block led to the suggestion that this could be accounted for by the modulated receptor hypothesis, as the IC_{50} at very positive holding potentials was much more closely correlated with the K_D (Bean, 1984; Cognard et al., 1986). In support of this idea, there has been one report of

interconvertible high and low-affinity dihydropyridine receptors in rat brain synaptosomal membranes (Weiland and Oswald, 1985).

This proposal has caused considerable interest in the development of preparations where the voltage-dependence of receptor binding could be directly measured. One study of nitrendipine binding to isolated rat ventricular myocytes only found high-affinity sites, and depolarization of the cells with high external K increased the maximum receptor density, but did not affect the K_D (Green et al., 1985). However, this report has been sharply criticized by other investigators (Kokubun et al., 1986), who have pointed out that since isolated myocytes contain a mixture of live and dead cells, Green et al. may have been studying high-affinity binding to the dead cells, and the K-induced depolarization may have increased the apparent maximal receptor density by adding the live cells to this pool. These investigators own studies were done with cultured rat ventricular cells, and similar experiments indicated that depolarization did decrease the K_D, with no change in maximal receptor density. Thus, there does appear to be some direct evidence that supports the modulated-receptor hypothesis, although the data is limited and still controversial.

One intriguing possibility that has not drawn much attention is that neither theory can explain all of the available data, and that both mechanisms may be involved to a greater or lesser extent with each Ca channel blocker. Such a possibility is suggested by the recovery experiments in these studies. The Bay K 8644-induced slow component of I_{Ca} recovery had a voltage-dependence that was quite close to that of normal recovery. This is apparently not true with D600, as the slow component of recovery has a much steeper voltage-dependence. This is particularly noticeable at more positive potentials. This indicates that for some Ca channel blockers, the removal of block resembles the removal of normal inactivation, while the unblocking process may be more complicated for other

drugs. This is supported by the observation that factors such as molecular weight and lipophilicity can strongly influence the removal of Ca channel blockade (Uehara and Hume, 1985).

The above suggestion does find some additional support in preliminary studies of the effect of D600 on single Ca channels (Trautwein and Pelzer, 1985). It was found that D600 had two effects on Ca channel activity. D600 both shortened the average open time of the channel, and promoted long lasting silent periods. It is possible to interpret the shortened mean open time as being due to the entry of D600 into the pore, once the activation gate opens. Likewise, the long silent periods can be interpreted as being due to D600 stabilizing the inactivated state of the channel. This last effect has also been noted with nitrendipine (Hess et al., 1984). Further experiments on this subject are obviously needed.

The question of the applicability of the guarded-receptor hypothesis to Ca channel block is closely tied to the question of where the drug receptors are located on the channel. The guarded receptor hypothesis assumes that channel block is due to "plugging" of the pore by the drug, and that the receptor is within the pore, behind the channel gates. It is known from radioligand binding studies that the receptor for D600 is separate from the dihydropyridine receptor, although it appears to be allosterically linked (Murphy et al., 1983). Both types of Ca channel blockers apparently must pass the cell membrane before they can reach their receptors, as Ca channels are relatively insensitive to block by external D890, the quaternary derivative of D600 (Hescheler et al., 1982; Affolter and Coronado, 1986), or to an externally-applied, quaternary dihydropyridine derivative (Uehara and Hume, 1985). The available evidence seems to indicate that D600 does bind to its receptor in the pore itself. The application of D600 for as long as 30 minutes produced no block of I_{Ca} in cat ventricular muscle, as long

as the activation gates remained closed (Pelzer et al., 1982). Thus the drug, which is predominantly charged at a physiological pH, can be shielded from its receptor by the activation gate. This is apparently true of the inactivation gate as well (McDonald et al., 1984a).

The precise site of action of the dihydropyridines seems to be less clear. The existence of stimulatory dihydropyridines, such as Bay K 8644, has prompted the proposal that all dihydropyridines act through subtle effects on the gating processes of the Ca channel (Hess et al., 1984). It is certainly difficult to imagine dihydropyridine block as being due to plugging of the channel, if both stimulatory and inhibitory dihydropyridines act at the same site (Schramm et al., 1984; Wei et al., 1986). Nevertheless, block of I_{Ca} by nicardipine, a dihydropyridine that is partially charged at physiological pH, appears to be influenced by the activation gate of the channel (Sanguinetti and Kass, 1984a). It is possible that this may be explained by separate stimulatory and inhibitory dihydropyridine binding sites (Dube et al., 1985). Evidence for this includes the existence of positive cooperativity between stimulatory and inhibitory dihydropyridines with regard to both electrophysiological effects and binding properties (Kokubun et al., 1986), and different time courses for the onset of the stimulatory and inhibitory effects (Brown et al., 1986). Other receptor hypotheses have been developed as well (Brown et al., 1986). At this point, it would appear that some of the properties and the site of the inhibitory dihydropyridine receptor are ill-defined.

It is obvious that at this point, what is needed is a more direct way of testing these alternative hypotheses. Perhaps the clearest results could be obtained if Ca channel inactivation could be removed, as can be accomplished with the Na channel by using proteolytic enzymes (Armstrong and Bezanilla, 1973), various group-specific protein reagents (Oxford et al., 1978), or toxins (Caterall, 1980). Such experiments have been useful in evaluating the importance

of inactivation to the block of Na channels by various compounds (Cahalan, 1978; Yeh and Ten Eick, 1987). The possible removal of Ca channel inactivation by similar agents has not drawn much attention, as simple methods of introducing intracellular agents have only recently become available, and the existence of two mechanisms for Ca channel inactivation have caused additional complications. That it may be feasible to eliminate Ca channel inactivation has been inadvertently demonstrated recently, during planar lipid bilayer experiments with purified sarcolemmal vesicles (Ehrlich et al., 1986), and with purified dihydropyridine receptors (Flockerzi et al., 1986). In both cases, Ca channel activity was observed (with Ba as the permeant cation) that was completely non-inactivating. Furthermore, this activity could be inhibited with D600 (Ehrlich et al., 1986; Flockerzi et al., 1986), or with nitrendipine (Ehrlich et al., 1986). However, the interpretation of these results is complicated by additional alterations in channel properties, including channel activation. Nevertheless, it is clear that some unknown step in the purification process was capable of eliminating Ca channel inactivation. Further studies need to be undertaken to ascertain whether this can be taken advantage of in cellular preparations.

SUMMARY AND CONCLUSIONS

The properties of Ca channel inactivation were studied in isolated guinea-pig ventricular cells with the whole-cell patch clamp technique. This technique made detailed studies of Ca channel behavior possible, as I_{Ca} could be satisfactorily isolated from other current through other membrane channels.

I_{Ca} was found to completely inactivate within 500 ms at a test potential of 0 mV, but inactivation was incomplete at more positive potentials. Inactivation was found to follow a biexponential time course at 0 mV, with the two time constants having values of appoximately 15 and 120 ms. No functional interpretation was placed on this finding, as the behavior of the time constants was complex.

I_{Ca} inactivation appeared to be correlated with the entry of Ca into the cell, as inactivation occured faster at higher extracellular Ca concentrations, and was slower if certain Ca chelators were introduced into the cell interior. In addition, the inactivation curve of I_{Ca} was partially U-shaped, as the degree of I_{Ca} inactivation at different test potentials was related to the amount of Ca entering the cell at that potential.

That I_{Ca} inactivation was partially due to a Ca-dependent process was confirmed by examining Ca channel behavior when Ca was absent, and monovalent cations carried the current (I_{ns}). The U-shaped portion of the inactivation curve was eliminated, and the remaining inactivation that occured had no correlation with current magnitude. That I_{ns} still inactivated to a great extent indicated that there was a second mechanism for Ca channel inactivation. This mechanism was

identified as a classical voltage-gated inactivation mechanism, as the degree and rate of I_{ns} inactivation had a monotonic relationship to the membrane potential, and it could be demonstrated that this inactivation could occur in the complete absence of ion permeation through the Ca channel.

The respective roles and physiological importance of voltage- and Ca-dependent inactivation were determined by examination of the I_{Ca} and I_{ns} inactivation curves. Voltage-dependent inactivation accounts for much of the total Ca channel inactivation, and is the dominant mechanism at very positive potentials. Ca-dependent inactivation is very important over the plateau range of potentials, and appears to be essential for complete inactivation.

The mechanism for Ca-dependent inactivation could not be identified precisely, but some possibilites could be eliminated, and additional information was obtained on other possibilites. The possibility that surface charge effects were involved in Ca-dependent inactivation was ruled out, as it could be demonstrated that the Ca that entered the cell during the time course of I_{Ca} did not alter the voltage-dependence of I_{Ca} activation. The hypothesis that the entry of Ca may lead to dephosphorylation and closure of the Ca channel was tested with several drugs that may alter Ca channel phosphorylation: trifluoperazine, phorbol esters and isoproterenol. No evidence was found that phosphorylation strongly influenced the onset or removal of I_{Ca} inactivation. Nevertheless, this hypothesis, along with the hypothesis of allosteric control of the channel by Ca ions, is still under consideration.

Finally, the hypothesis that the organic Ca channel blockers preferentially bind to the inactivated state of the Ca channel was examined by studying Ca channel blockade by D600, and the stimulatory dihydropyridine Bay K 8644. Bay K 8644 was found to have strong inhibitory effects on I_{Ca}. These effects resembled those of classical Ca channel blockers, in that Bay K 8644 blocked Ca

channels in both a tonic- and use-dependent fashion, shifted the inactivation curve in a negative direction, accelerated I_{Ca} decay and induced a slow component of recovery. All of these results are consistent with the modulated receptor hypothesis, which assumes preferential drug-binding to the inactivated state of the channel.

The possibility of applying the guarded receptor hypothesis to Ca channel blockers was examined using D600. It was found that theoretical predictions of this model matched well with experimental observations of use-dependent block and the removal of block. Therefore, it appears that both models can be used to explain the action of Ca channel blockers. There is some suggestion from the different voltage-dependencies of the removal of Bay K 8644 and D600 block, that both binding to inactivated channels and gate trapping may be involved in block of Ca channels.

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