Interactions of Organic Calcium Channel Antagonists with Calcium Channels in Single Frog Atrial Cells

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ABSTRACT Inhibition of whole-cell calcium currents in enzymatically dispersed frog atrial myocytes by D-600, diltiazem, and nifedipine was studied using a single-micropipette voltage-clamp technique. The objective of these experiments was to test the applicability of a modulated-receptor hypothesis similar to that proposed for local anesthetic interactions with sodium channels to account for the tonic and frequency-dependent interactions of these organic compounds with myocardial calcium channels. Data consistent with such a hypothesis include: (*a*) prominent use-dependent block of i_{Ca} by D-600 and diltiazem, which are predominantly charged at physiological pH; (*b*) i_{Ca} block by an externally applied, permanently charged dihydropyridine derivative is greatly attenuated; (*c*) all three antagonists produce large negative shifts in the voltage dependence of i_{Ca} availability; (*d*) block of i_{Ca} by these compounds is state-dependent; (*e*) reactivation of i_{Ca} in the presence of all three antagonists is biexponential, which suggests that drug-free channels recover with a normal time course and drug-bound channels recover more slowly; and (*f*) the kinetics of the drug-induced slow i_{Ca} recovery process may be determined largely by factors such as size and molecular weight, in addition to lipid solubility of the compounds. Experiments in which the pH was modified, however, reveal some important differences for the interaction of organic calcium antagonists with myocardial calcium channels. Acidification, in addition to changing the proportion of charged and neutral antagonist in solution, was found to selectively antagonize tonic inhibition of i_{Ca} by diltiazem and nifedipine, without changing the kinetics of the drug-induced slow i_{Ca} reactivation process. It is concluded that two distinct receptor sites may be involved in block of i_{Ca} by some of these compounds: a proton-accessible site and a proton-inaccessible site.

INTRODUCTION

Organic Ca channel antagonists are structurally diverse compounds that all have in common the ability to interfere with transmembrane movement of Ca ions

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through voltage-dependent channels in many cells. Clinically, they have proven beneficial as antianginal and antiarrhythmic agents and are believed to be potentially useful for a wide variety of other cardiovascular conditions (Henry, 1980). These compounds are also of basic interest as possible experimental probes of Ca channels.

Of this group of agents, verapamil and its methoxyderivative, D-600, have been most thoroughly investigated. In the heart, these agents inhibit "slow response" action potentials (Cranefield et al., 1974; Bayer et al., 1975), and voltage-clamp experiments have confirmed that these compounds reduce the inward calcium current (Kohlhardt et al., 1972; Kass and Tsien, 1975; Nawrath et al., 1977) and do so in a frequency- or use-dependent fashion (Ehara and Kaufmann, 1978; McDonald et al., 1980). In marked contrast, another organic Ca channel antagonist, nifedipine, has been reported to block the Ca current in cat and guinea pig papillary muscles without altering i_{Ca} kinetics (Kohlhardt and Fleckenstein, 1977), which prompted the suggestion that organic Ca channel antagonists in general might be classified into two separate groups: those that only diminish Ca conductance, and those that, in addition, may modify channel kinetics.

Recent studies, however, have provided new important information regarding the mechanism of Ca channel blockade by these agents which may suggest that such a classification is oversimplified. McDonald et al. (1980) observed that blockade of i_{Ca} in cat trabeculae by D-600 was both use- and voltage-dependent, and suggested that a state-dependent model may explain D-600's inhibitory effects. Use- and voltage-dependent block of i_{Ca} by diltiazem has also recently been reported (Tung and Morad, 1983; Kanaya and Katzung, 1984). Hescheler et al. (1982) studied the effect of D-600 and its quaternary derivative, D-890, on the Ca current in cat ventricular trabeculae. Extracellular application of permanently charged D-890 was found to be ineffective in blocking Ca current, whereas intracellular injection suppressed the action potential plateau in isolated guinea pig myocytes, which implies that the receptor for D-600 and D-890 is located intracellularly. Lee and Tsien (1983) examined Ca channel blockade by a variety of organic antagonists in enzymatically dispersed guinea pig ventricular myocytes. Prominent use-dependent inhibition of i_{Ca} was found for the tertiary amines verapamil, D-600, and diltiazem. However, much less use dependence was observed with nitrendipine, which is uncharged at physiological pH. These results raise the question of whether a modulated-receptor–type hypothesis similar to that proposed for local anesthetic blockade of Na channels (Hille, 1977*a*, *b*; Hondeghem and Katzung, 1977) could account for the inhibitory actions of a variety of organic Ca channel antagonists on Ca channels.

This paper describes the results of a series of experiments carried out to test directly the applicability of a modulated-receptor hypothesis to explain the interaction of three Ca channel antagonists, D-600, diltiazem, and nifedipine, with Ca channels in enzymatically dispersed single frog atrial cells. Our experiments are an extension of those begun by Lee and Tsien (1983). We sought to characterize in greater detail both the kinetic and voltage-dependent interactions of these agents with myocardial Ca channels and the extent to which changes in

external pH may modulate these interactions. To test such a modulated-receptor hypothesis, a number of specific questions were posed: Is frequency-dependent blockade of i_{Ca} more characteristic of the tertiary antagonists D-600 and diltiazem? Does blockade of i_{Ca} by dihydropyridine antagonists involve an intracellular or intramembranous site of action? Do any of these antagonists modify the apparent voltage dependence of inactivation or the kinetics of recovery from inactivation of i_{Ca}? Can blockade by these agents be attributed to binding that is state-dependent (resting, open, or closed)? Are the two different types of channel blockade and drug-induced changes in kinetics of i_{Ca} sensitive to changes in external pH? Finally, do changes in external pH alter the relative proportion of neutral or charged drug forms in solution independently of changes in the drug receptor? Preliminary results of these experiments have been reported in abstract form (Uehara and Hume, 1984*a*).

METHODS

The enzymatic dispersion procedure for obtaining single atrial cells, the experimental chamber, and the optical apparatus that were used in these experiments were identical to those previously described (Hume and Giles, 1981). The voltage-clamp method used, a single suction micropipette technique, is similar to that used by Hume and Giles (1983). The relatively small magnitude of whole-cell i_{Ca} ($3.0–7.0 \times 10^{-10}$ A) recorded made it unnecessary to compensate for the series resistance arising from the micropipette tip. Data were displayed on a storage oscilloscope (model 5103 N, Tektronix, Inc., Beaverton, OR) and simultaneously recorded on an FM instrumentation tape recorder (model 4DS, Racal Recorders, Inc., Corina, CA) at a bandwidth of DC to 5 kHz. Data analysis was done offline by replaying at 0.25 the recording speed data on a chart recorder (model 2400, Gould Instruments, Inc., Cleveland, OH). All current traces used for figures were photographed from the oscilloscope display.

The Ringer's solution contained (mM): 110 NaCl, 2.5 KCl, 2.5 $MgCl_2$, 5.0 $CaCl_2$, 5 HEPES, 10 glucose, and 15 sucrose and was saturated with 100% O_2. pH was titrated to either 6.4 or 7.4 by addition of NaOH. All solutions contained 3×10^{-6} M tetrodotoxin (Sigma Chemical Co., St. Louis, MO). D-600 was kindly provided by Knoll Pharmaceutical Co., Whippany, NJ; diltiazem by Marion Laboratories, Kansas City, MO; and nifedipine by Pfizer Inc., New York. A quaternary dihydropyridine derivative was kindly provided by Dr. D. J. Triggle, State University of New York, Buffalo, NY, and nisoldipine was provided by Miles Institute, New Haven, CT. Dihydropyridine antagonists were dissolved in polyethylene glycol to make 1-mM stock solutions and were continuously protected from exposure to light. Polyethylene glycol in the concentrations used had no observed effect upon Ca currents. D-600 and diltiazem were dissolved directly into Ringer's solution. All experiments were carried out at room temperature. In all voltage-clamp experiments, the holding potential was set to either −90 or −100 mV.

RESULTS

Tonic and Use-dependent Interactions

The chemical structures of the different organic antagonists examined in the present study are shown in Fig. 1. D-600, diltiazem, and nifedipine are quite different structurally and fall along a continuum in terms of both pK_a and molecular weight. Quaternary dihydropyridine (DHP) is a permanently charged

derivative of nifedipine. Since at pH 7.4 these compounds should exhibit markedly different degrees of ionization, a comparison of the types of interactions of these agents with Ca channels was made.

A standardized protocol was used to assess the relative contribution of tonic and use-dependent blockade of i_{Ca} by the three antagonists (Fig. 2). This protocol consisted of recording a train of i_{Ca}'s elicited by depolarizing voltage-clamp pulses applied at a frequency of 0.33 Hz under control conditions, followed by a rest

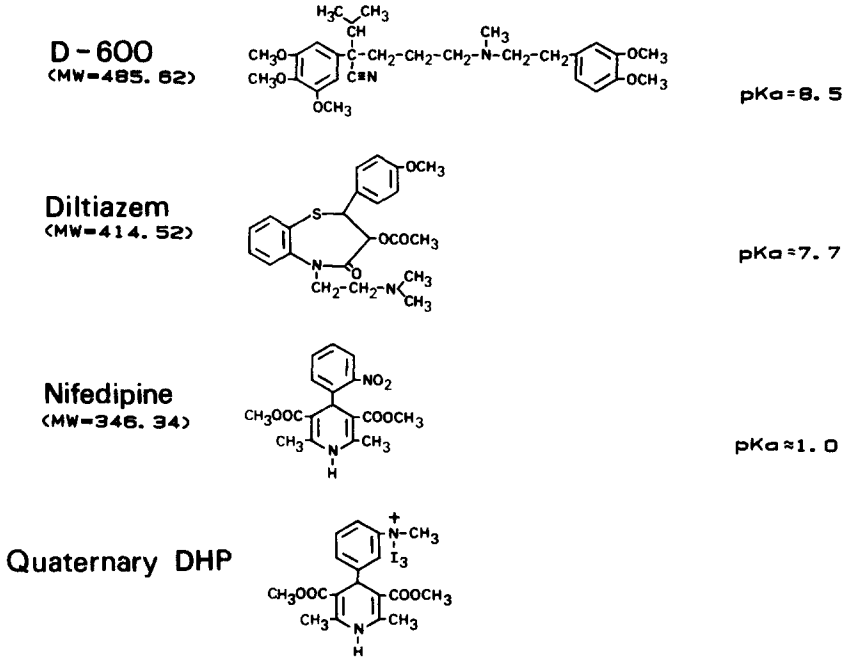

FIGURE 1. Structures of the three organic Ca channel antagonists studied. The molecular weight and pK_a of each compound are also shown. Quaternary DHP is a nifedipine analogue that is permanently charged.

period, during which time the holding potential was set to −90 mV and the organic antagonist was introduced into the experimental chamber. After this 15-min rest period, an identical train of voltage-clamp depolarizations was then applied at a frequency of 0.33 Hz. Total blockade was considered to be the proportional difference in peak i_{Ca} elicited under control conditions and peak i_{Ca} elicited by the 15th pulse after drug exposure (15 pulses at this frequency were enough to produce steady state blockade by each of the antagonists). Tonic blockade was assessed as the difference in peak i_{Ca} in the control and the first pulse after drug exposure. Frequency- or use-dependent blockade was the difference between peak i_{Ca} for the first and 15th pulses after drug exposure.

Fig. 2*A* illustrates a typical result with D-600. Using this protocol, the i_{Ca} elicited under control conditions (C) and the i_{Ca} elicited by the first pulse (1) in

the train after drug exposure were of similar amplitude, which indicates relatively little, if any, tonic block by D-600. There is, however, a progressive decline of i_{Ca} during subsequent voltage-clamp depolarizations in the train, which indicates a large degree of use-dependent inhibition. The same protocol was used to evaluate blockade by diltiazem (*B*) and by nifedipine (*C*). Nifedipine blockade was nearly the opposite of that observed by D-600; blockade was almost completely tonic, with only a small use-dependent component at this frequency. Changes in holding potential to −120 mV for several minutes did not reverse the tonic inhibition produced by nifedipine. Diltiazem, in contrast, appears to be intermediate between D-600 and nifedipine, exhibiting both tonic and frequency-dependent blockade of i_{Ca}. These results are also presented graphically in Fig. 3,

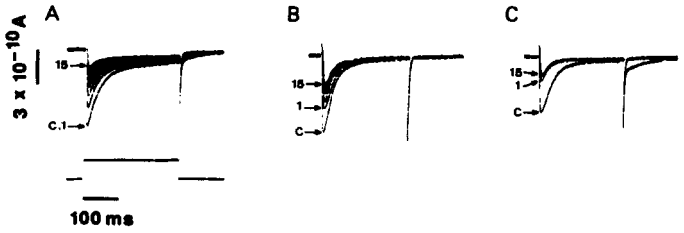

FIGURE 2. Tonic and frequency-dependent inhibition of i_{Ca} by 5×10^{-6} M D-600 (*A*), 5×10^{-5} M diltiazem (*B*), and 3×10^{-7} M nifedipine (*C*). Membrane currents elicited by repetitive 200-ms voltage-clamp pulses (0.33 Hz) to 0 mV from a holding potential of −90 mV are shown. The largest inward current was recorded under control conditions (C) before drug exposure; subsequent currents were recorded in response to a train (0.33 Hz) of 15 identical voltage-clamp pulses applied 15 min after addition of drugs. The numbers 1 and 15 correspond to the inward current elicited by the first and 15th pulses in the train after drug exposure. The bottom trace in panel *A* is membrane voltage. For a further explanation, see text.

with the arrows representing the amount of tonic inhibition. Fig. 3*D* illustrates that the small amount of use-dependent inhibition observed for nifedipine at 0.33 Hz (*C*) can be enhanced if the frequency of depolarizing voltage-clamp pulses is increased.

Although the results presented are from representative experiments, how reproducible are they when examined in a larger number of cells? Experiments such as those shown in Figs. 2 and 3 were repeated in a number of cells and the results are presented in Table I. The three antagonists are compared at doses that produce ~50% total inhibition of i_{Ca} using this experimental protocol. Overall, the compounds that are predominantly charged at physiological pH (D-600 and diltiazem) exhibit prominent frequency-dependent block of i_{Ca}, whereas nifedipine exhibits mostly tonic inhibition of i_{Ca}. An exact correlation between the percentage of use-dependent block and the percentage of protonated drug in solution is not expected since the rate and magnitude of use-dependent binding may be governed by a variety of experimental factors (Schwarz et al., 1977).

These results confirm earlier findings by Lee and Tsien (1983) in isolated guinea pig ventricular myocytes that blockade of i_{Ca} by D-600, diltiazem, and a

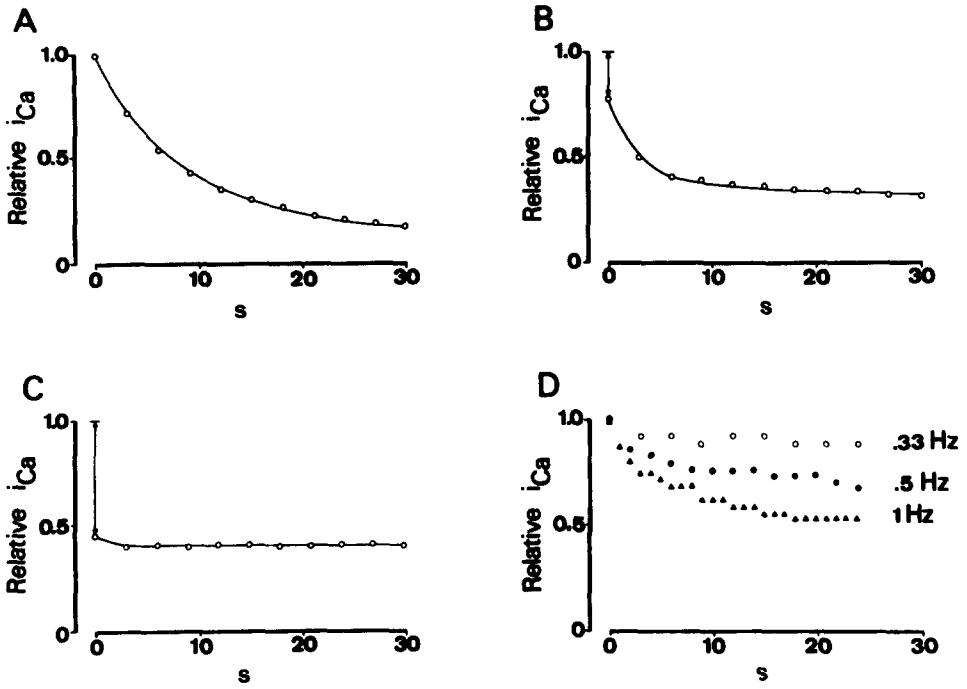

FIGURE 3. Plots of time course of onset of block of i_{Ca} by 5×10^{-6} M D-600 (*A*), 5×10^{-5} M diltiazem (*B*), and 3×10^{-7} M nifedipine (*C*). Data are from same experiments shown in Fig. 1. Arrows indicate the proportion of total block of i_{Ca} attributable to tonic inhibition. The influence of frequency of stimulation on the onset of block by nifedipine (3×10^{-7} M) is shown in *D*. For *A–C*, relative i_{Ca} refers to the amplitude of i_{Ca} normalized by the control i_{Ca} amplitude. For *D*, relative i_{Ca} refers to the amplitude of i_{Ca} normalized to i_{Ca} amplitude in nifedipine after subtraction of tonic inhibition.

derivative of nifedipine, nitrendipine, fall along a spectrum. D-600 was found to be almost exclusively use-dependent, nitrendipine almost exclusively tonic, and diltiazem intermediate. As pointed out by Lee and Tsien (1983), this pattern of use-dependent blockade by the tertiary amines D-600 and diltiazem and tonic blockade by the neutral amines nifedipine and nitrendipine is reminiscent of the

TABLE I

Tonic and Use-dependent Block of i_{Ca} by Organic Antagonists

	pH	*n*	Dose	Percent total block	Percent use-dependent block	Percent tonic block	Percent charged
D-600	7.4	6	5×10^{-6} M	55±6.0	98.8±0.8	2±1.0	93 ($pK_a = 8.6$)
Diltiazem	7.4	6	5×10^{-5} M	54±7.0	76±7.1	24±7.1	67 ($pK_a = 7.7$)
Nifedipine	7.4	5	3×10^{-7} M	49±7.8	11±2.6	89±2.6	1 ($pK_a \approx 1.0$)
Quaternary DHP	7.4	4	1×10^{-5} M	<1	—	—	100

Data are expressed as means ± SEM.

differences in blockade of i_{Na} exhibited by tertiary and neutral local anesthetics (Hille, 1977*b*; Schwarz et al., 1977; Hondeghem and Katzung, 1977). In the case of the neutral local anesthetic benzocaine, which usually shows little, if any, use-dependent blockade, an increase in stimulation frequency can also enhance the use-dependent effects of this agent (Kendig et al., 1979). A very similar result appears to occur with the neutral Ca antagonist nifedipine.

In a separate series of experiments, we studied a quaternary derivative of nifedpine (see Fig. 1 and Table I) using an identical experimental protocol. In four cells, superfusion of this compound failed to produce any significant inhibition of i_{Ca} at a dose 30 times higher than the dose of nifedipine, which produced ~50% inhibition of i_{Ca}. The failure of this quaternary derivative to produce significant i_{Ca} block implies either: (*a*) that dihydropyridines must cross the cell membrane in order to act at some intracellular or intramembranous site (a conclusion similar to that made previously for D-600 by Hescheler et al., 1982), or (*b*) that only neutral dihydropyridines may bind to an external receptor (see Discussion). The activity of quaternary analogues of nifedipine has also been observed to be greatly reduced in smooth muscle (Rosenberger and Triggle, 1978).

Drug-induced Modification of i_{Ca} Availability

In the modulated-receptor hypothesis for local anesthetic blockade of i_{Na}, a major piece of evidence that there is a single receptor underlying the effects of both charged and neutral forms of these drugs is that both charged and neutral forms modify the inactivation gating process in similar ways: there is a large negative shift in the apparent voltage dependence of i_{Na} inactivation (Weidmann, 1955; Hille, 1977*b*; Schwarz et al., 1977; Kendig et al., 1979). It is therefore important to examine the influence of each of these organic Ca channel antagonists on the voltage dependence of i_{Ca} inactivation.

A recent report (Kass and Scheuer, 1982) has suggested that in cardiac Purkinje fibers, i_{Ca} inactivation may be governed in part by a second ultraslow (seconds) inactivation mechanism. It was important initially to determine whether a similar slow inactivation process contributes to i_{Ca} inactivation in isolated frog atrial cells under control conditions. Fig. 4 illustrates an experiment that was designed to assess the importance of such a slow inactivation process. The voltage dependence of i_{Ca} availability was examined using a two-pulse protocol. A test pulse to 0 mV was preceded by a prepulse of either 200 ms or 10 s duration and of variable amplitude. Both protocols yielded i_{Ca} availability curves with a midpoint at about −20 mV. Thus, no difference in i_{Ca} availability can consistently be observed by using these two protocols, which suggests that if an ultraslow inactivation process for i_{Ca} is present under control conditions, it is either too slow to be measured by these protocols, or it is relatively small in comparison with the faster process and is beyond our resolution of measurement.

A similar two-pulse protocol with 10-s prepulses was used to evaluate possible drug-induced modifications of i_{Ca} availability by each of the three antagonists. As shown in Fig. 5, each of the three antagonists examined produced large hyperpolarizing shifts in the i_{Ca} availability curves. The mean shift in the midpoint of the i_{Ca} availability curve was −20 mV (n = 4) for 5×10^{-6} M D-600, −29.5

mV (n = 4) for 5×10^{-5} M diltiazem, and −18 mV (n = 5) for 3×10^{-7} M nifedipine. No consistent effects on the slopes of the i_{Ca} availability curves (Fig. 5) were observed in these experiments. These drug-induced shifts in i_{Ca} availability are consistent with a modulated-receptor hypothesis in which both neutral and charged drug forms bind to a common single receptor. Thus, blocked channels might be considered to have their voltage dependence of inactivation shifted, whereas unblocked channels may be unaltered. The overall shift in i_{Ca} availability may arise as the result of a shift in equilibrium from resting to inactivated states (Courtney, 1975; Hille, 1978).

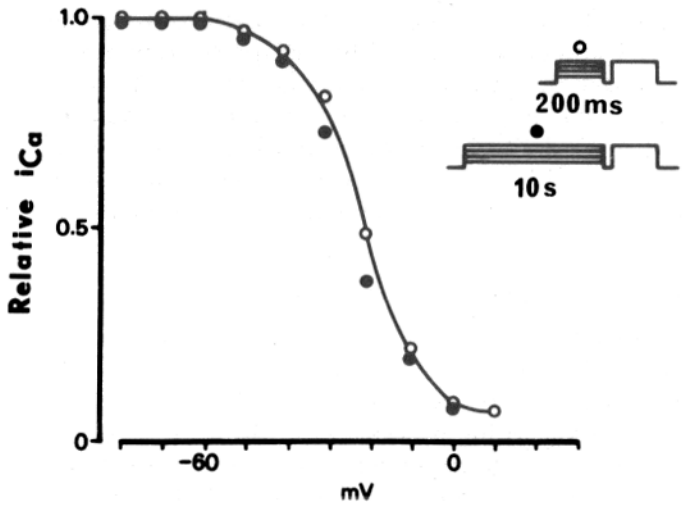

FIGURE 4. Voltage dependence of i_{Ca} availability under control conditions. The protocol consisted of applying a prepulse of either 200 ms (open circles) or 10 s (closed circles) duration to voltages ranging from −80 to +10 mV from a holding potential of −90 mV, followed by a 200-ms test pulse to +10 mV. Peak test pulse current vs. prepulse voltage is plotted, normalized to the peak test pulse current obtained in the absence of a prepulse. Using either protocol, the midpoint of the i_{Ca} availability curve occurs at approximately −23 mV.

The application of 10-s prepulses will produce significant activation of the delayed rectifying K^+ current for voltages positive to −30 mV in single frog atrial cells (Hume and Giles, 1983). The activation of this current might, therefore, distort the i_{Ca} availability curves shown in Figs. 4 and 5. Since we did not attempt to pharmacologically block i_K, contamination by i_K cannot be completely ruled out. Two considerations, however, make a significant contamination by i_K unlikely. First, the data of Fig. 4 show that the i_{Ca} availability curve generated by 200-ms prepulses (which are too short to produce significant activation of i_K) is nearly identical to the curve generated with the longer 10-s prepulses. Second, i_{Ca} availability curves in the presence of the organic antagonists are generated with prepulses to potentials that are negative to the activation range for i_K (because of the −18 to −30 mV shift), and the slopes of these curves

are not significantly different from those obtained under control conditions at a more positive potential range (Fig. 5).

The ability of each of the three organic Ca antagonists tested to produce large negative shifts in i_{Ca} availability may also give some insight into the mechanism

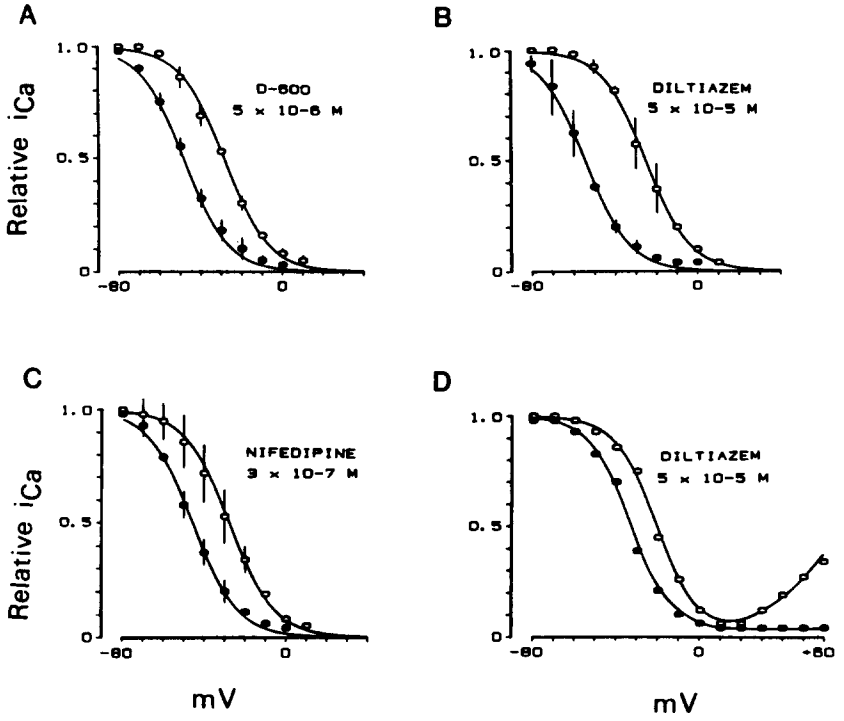

FIGURE 5. Influence of organic Ca channel antagonists on i_{Ca} availability. Voltage dependence of i_{Ca} availability determined as in Fig. 4 using 10-s prepulses. The data in *A* represent mean normalized values from four complete experiments with D-600; the data in *B* are from four experiments with diltiazem; the data in *C* are from five experiments with nifedipine (open circles, control; closed circles, drug; vertical bars, standard error). In *D*, i_{Ca} availability was determined using 200-ms prepulses over a wider voltage range in the absence (open circles) and in the presence (closed circles) of diltiazem. A holding potential of −100 mV was used throughout. The solid lines in *A*, *B*, and *C* are drawn according to the function $\{1 + \exp[(V - V_h)/k]\}^{-1}$ with parameters chosen for the best visual fit. In *A*, $V_h = -28.2$ mV and $k = 10.8$ for control; and $V_h = -48.2$ mV and $k = 10.8$ for D-600; in *B*, $V_h = -24.8$ mV and $k = 10.8$ for control, and $V_h = -54.3$ mV and $k = 10.8$ for diltiazem; in *C*, $V_h = -27.1$ mV and $k = 11.0$ for control, and $V_h = -45.1$ mV and $k = 11.0$ for nifedipine.

of inactivation of i_{Ca} in single frog atrial cells. Similar effects are produced by local anesthetics on i_{Na} availability in nerve (Weidmann, 1955; Hille, 1977*b*), and the mechanism of inactivation of i_{Na} is believed to be controlled by a predominantly voltage-dependent process (Hodgkin and Huxley, 1952; Meves, 1978).

By analogy, the similarity in action of the organic Ca antagonists on i_{Ca} may suggest some similarity in the process governing inactivation of i_{Ca}. A number of reports in heart have described a Ca entry-dependent process underlying i_{Ca} inactivation (Brown et al., 1981; Marban and Tsien, 1981; Hume and Giles, 1982; Mentrard et al., 1984), which is based primarily upon the observation that the voltage dependence of i_{Ca} availability is U-shaped when examined at positive membrane potentials. Some decrease in i_{Ca} inactivation is also observed with prepulses to very positive membrane potentials in isolated frog atrial cells (Fig. 5*D*); however, considerable inactivation still persists even at +70 mV. Therefore, inactivation of i_{Ca} in single frog atrial cells may be controlled by both Ca_i-dependent and voltage-dependent processes, as in other heart cells (Tsien, 1983). Fig. 5*D* also shows that in the presence of 5×10^{-5} M diltiazem, there is an approximately −15-mV shift of the voltage dependence of i_{Ca} availability and a decline in the upturn of i_{Ca} availability at positive potentials (e.g., i_{Ca} inactivation appears to be purely voltage-dependent in the presence of diltiazem). The absence of a Ca_i-dependent inactivation mechanism in the presence of organic antagonists may be indirect and may be caused by an overall decline in the magnitude of i_{Ca}, since similar results are observed after other experimental manipulations that reduce the magnitude of i_{Ca} without shifting the voltage dependence of i_{Ca} availability (e.g., inorganic Ca channel antagonists; Uehara, A., and J. R. Hume, unpublished observation).

Drug-induced Changes in i_{Ca} Reactivation

It is well established that in the presence of local anesthetics, i_{Na} recovers with two distinct time courses: a normal phase of recovery, and a second, much slower phase of recovery (Khodorov et al., 1974, 1976; Courtney, 1975). Within the context of a modulated-receptor hypothesis, slow recovery is believed to result from restricted diffusion of drug molecules from the receptor via a hydrophobic pathway. Hence, the kinetics of recovery for drugs that are predominantly charged are slower than for drugs that are predominantly neutral (Hille, 1977*b*).

Earlier voltage-clamp experiments in the heart have shown that verapamil and D-600 slow the rate of recovery of i_{Ca} from inactivation (Kohlhardt and Mnich, 1978; McDonald et al., 1980), whereas nifedipine has been reported not to influence the rate of recovery of i_{Ca} (Kohlhardt and Fleckenstein, 1977). In contrast, recent experiments by Lee and Tsien (1983) as well as Woods and West (1983) have suggested that nifedipine or its derivative, nitrendipine, might also slow i_{Ca} recovery. The influence of D-600, diltiazem, and nifedipine on the reactivation kinetics of i_{Ca} in frog atrial cells was examined to evaluate whether the drugs fall along a continuum, as would be expected if drug charge was an important determinant of channel-drug dissociation rates.

Fig. 6 shows results from paired-pulse experiments in which i_{Ca} reactivation was studied before and after addition of 5×10^{-6} M D-600 (*A* and *B*), 5×10^{-5} M diltiazem (*C*), and 3×10^{-7} M nifedipine (*D*). In all three cells, i_{Ca} reactivation is a relatively rapid, single-exponential process under control conditions (holding potential = −90 mV) and is complete within 500 ms. In the presence of each of the three antagonists, an additional, slower process of recovery is observed. In

each case, a proportion of channels appears to recover with the same time course observed under control conditions, and another proportion of channels appears to recover by a much slower exponential process. The second drug-induced slow phase of i_{Ca} recovery is faster in the case of nifedipine (*D*) compared with either D-600 (*B*) or diltiazem (*C*). Table II summarizes the results obtained from a

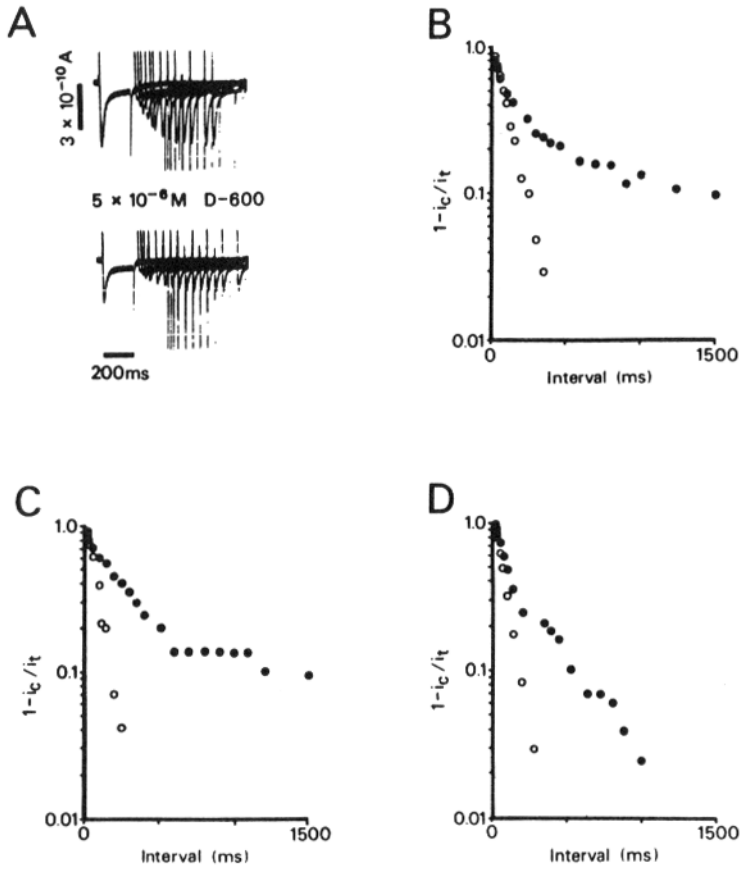

FIGURE 6. Effect of organic antagonists on kinetics of i_{Ca} reactivation. Two voltage-clamp pulses to +10 mV for 200 ms duration were applied at a frequency of 0.05 Hz and the interval between the pulses was varied between 10 ms and 10 s. The superimposed current traces shown in *A* illustrate i_{Ca} reactivation under control conditions (top) and in the presence of 5×10^{-6} M D-600 (bottom). In the control, recovery is complete when the interval between i_c and i_t approaches 500 ms. However, after exposure to D-600, recovery is clearly incomplete over the time interval shown. i_c is the peak inward current elicited by the first of the pulse pair; i_t is the peak inward current elicited by the second pulse following a variable interval. Semilog plots of $1 - i_t/i_c$ vs. the interpulse interval are shown for controls (open circles) and after exposure (closed circles) to 5×10^{-6} M D-600 (*B*), 5×10^{-5} M diltiazem (*C*), and 3×10^{-7} M nifedipine (*D*). The holding potential was −90 mV throughout.

TABLE II

Effects of Organic Antagonists on i_{Ca} Reactivation Kinetics

	pH	*n*	Dose	τ Control*	τ Drug*
				ms	*ms*
D-600	7.4	4	5×10^{-6} M	98±1.7	14,800±3677
Nifedipine	7.4	5	3×10^{-7} M	97±3.2	493±97
Diltiazem	7.4	5	5×10^{-5} M	90±15	2183±878
Diltiazem	6.4	4	5×10^{-5} M	200±32	2372±705

* For these experiments, i_{Ca} reactivation was studied using a two-pulse protocol similar to that described in Fig. 6 from a holding potential of −90 mV.

number of cells for each of the antagonists studied. The mean time constant for the slow phase of recovery for D-600 was 14,800 (±3,677) ms, for diltiazem 2,183 (±878) ms, and for nifedipine 493 (±97) ms. These results provide evidence that the slower i_{Ca} recovery phases observed in the presence of the three antagonists do indeed fall along a spectrum. The slowest rate is observed for D-600, which is predominantly charged in solution at pH 7.4; the fastest rate occurs with nifedipine, which is expected to be completely neutral at pH 7.4. An intermediate rate is found for diltiazem.

Factors such as molecular weight and size, in addition to drug charge, may be important determinants of drug-channel dissociation rates. Although drug-channel dissociation rates for the three organic antagonists tested appear to fall along a continuum in terms of pK_a, the molecular weight of the antagonists also appears to be an important factor. Fig. 7 shows a nearly linear relationship between the

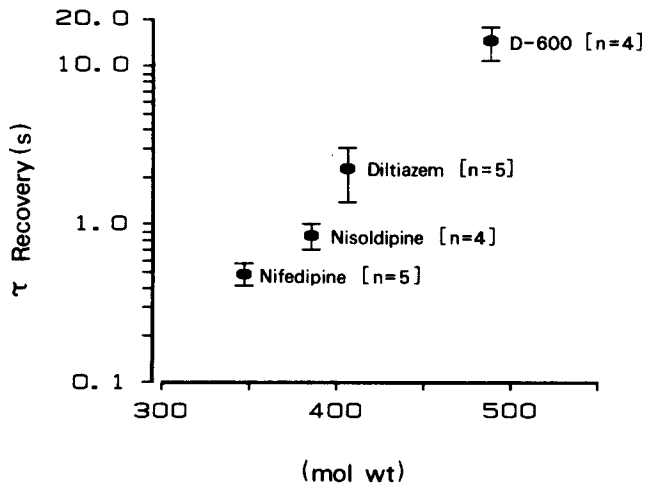

FIGURE 7. Relationship between log i_{Ca} recovery time constant induced by four organic Ca channel antagonists and the molecular weight of the compounds. All experiments were carried out using the same experimental protocol described in Fig. 6 from a holding potential of −90 mV. Drug concentrations were: D-600, 5×10^{-6} M; diltiazem, 5×10^{-5} M; nifedipine, 3×10^{-7} M; nisoldipine, 3×10^{-8} M (from Uehara and Hume, 1984*b*). Data are expressed as mean ± SEM and *n* is the number of cells tested for each antagonist.

log i_{Ca} recovery time constant induced by four organic antagonists and the molecular weight of the compounds. Courtney et al. (1978) and Kendig et al. (1979) have found that factors such as molecular weight and size, in addition to lipid solubility, are important determinants of drug dissociation rates of anesthetics from Na channels.

Channel State-dependent Block by Organic Antagonists

Rate and equilibrium constants for binding of local anesthetics to the Na channel have been shown to depend on whether the channel is resting, open, or inactivated (Strichartz, 1973; Courtney, 1975; Hille, 1977*b*). Tonic block by neutral anesthetics is believed to be due primarily to drug binding to resting and inactivated channels, whereas the use-dependent block observed with quaternary and tertiary anesthetics is believed to result primarily from preferential binding to inactivated channels. Use dependence arises because the accessibility of the drug to the receptor is restricted to a hydrophilic pathway (Hille, 1977*a*, *b*; 1978; Hondeghem and Katzung, 1977).

In the case of organic Ca antagonists, tonic block may also be the result of preferential binding to resting and inactivated states of the Ca channel. However, is use-dependent block due to preferential binding to open or inactivated channels, and is it similar for each of the antagonists? In a recent study by Bean et al. (1983) of use-dependent block of cardiac Na channels by lidocaine, a two-pulse protocol was used to distinguish between open channel and inactivated channel blockade by lidocaine. A similar pulse protocol has been used to distinguish between open channel and inactivated channel blockade by D-600, diltiazem, and nifedipine of i_{Ca} in atrial cells.

Fig. 8 shows results from three experiments in which channel state-dependent block by D-600, diltiazem, and nifedipine was examined in three different cells. A conditioning depolarizing pulse was applied for durations ranging from 10 ms to 2.5 s, followed by a return to a holding potential of −90 mV for 500 ms, followed by a test pulse to 0 mV. The 500-ms interval at the holding potential is long enough to permit drug-free channels to recover completely (see Fig. 6). A decline in i_{Ca} elicited by the test pulse is a measure of the development of drug-induced block occurring during the conditioning pulse. In response to the conditioning pulse to 0 mV, channels are activated quickly and then as a function of time undergo transitions from the open state to the inactivated state. With enough time, most channels would be expected to become inactivated. For a drug that binds preferentially to the inactivated state, a progressive decline in the test current amplitude as a function of the conditioning pulse duration would be expected. A very sudden drop in the test current amplitude would be expected for a drug that binds rapidly to the open state of the channel. Using this criterion, it is evident from Fig. 8 (*A* and *B*) that both D-600 and diltiazem preferentially block inactivated Ca channels. Nifedipine (*C*), in contrast, produces an immediate decline in the test current amplitude with the shortest-duration conditioning pulse tested (10 ms), followed by a later slow decline. This may indicate binding of nifedipine to both open and inactivated channels. These results are consistent with the data of Lee and Tsien (1983), who concluded that D-600 and the nifedipine derivative nitrendipine bind to both open and inactivated Ca channels,

whereas diltiazem block of i_{Ca} is primarily the result of binding to inactivated channels.

Influence of pH on Tonic and Use-dependent Interactions with Ca Channels

The effects of changes in external pH on both the type of i_{Ca} blockade and on the kinetics of the drug-induced slow recovery process were examined. These

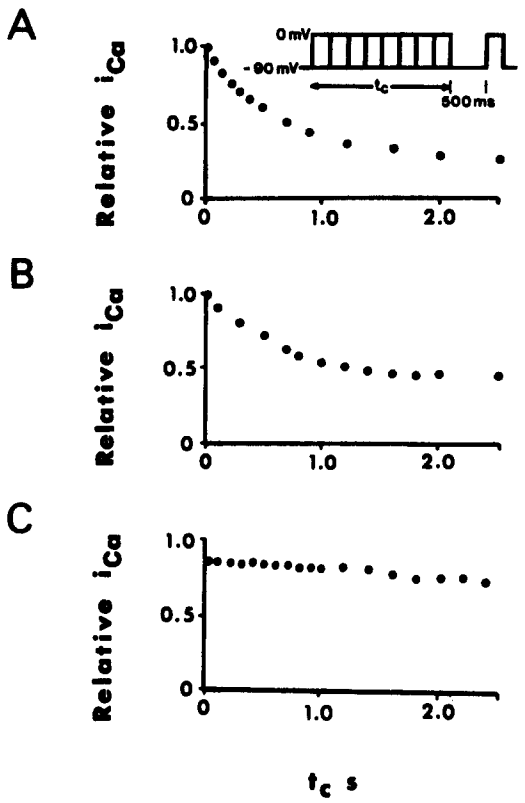

FIGURE 8. Time course of development of blockade of i_{Ca} by 5×10^{-6} M D-600 (*A*), 5×10^{-5} M diltiazem (*B*), and 3×10^{-7} M nifedipine (*C*). A conditioning depolarization to 0 mV was applied for durations ranging from 10 ms to 2.5 s, followed by a return to a holding potential of −90 mV for 500 ms, followed by a 200-ms test pulse to 0 mV (see inset). The amplitude of i_{Ca} was normalized with respect to the amplitude obtained in the presence of antagonist with no conditioning prepulse. Therefore, these results do not reflect any tonic component of inhibition.

experiments were limited to small increases in $[H^+]_o$, since strong external acidification is known to block transmembrane Ca current (Chenais et al., 1975; Vogel and Sperelakis, 1977) and external alkalization will reverse TTX blockade of i_{Ca} (Ulbricht and Wagner, 1975), making the separation of i_{Ca} from i_{Na} difficult. In the absence of the organic Ca channel antagonists, changing external pH from 7.4 to 6.4 produced a small attenuation of peak i_{Ca} amplitude in single frog

atrial cells (Fig. 9) without any significant voltage shift of peak i_{Ca}. This change in external pH would be expected to produce a small change in the membrane surface charge that may not be detected in our experiments since voltage-clamp steps were applied in 10-mV increments. For instance, Hille et al. (1975) found

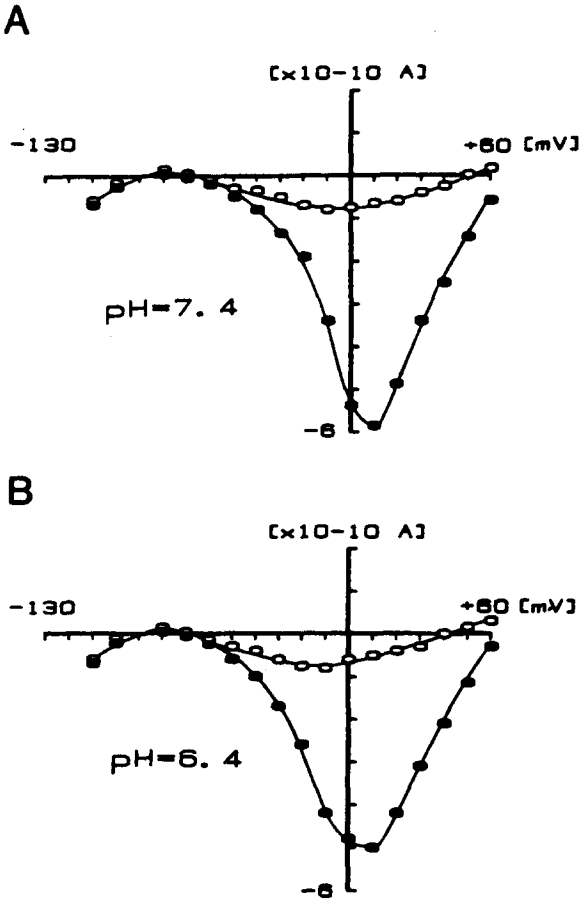

FIGURE 9. Effect of external acidification on the peak inward (closed circles) and late (open circles, measured at the end of 250 ms) current-voltage relationship. The data in *A* were obtained from a cell bathed in HEPES-Ringer's solution at pH 7.4. The data in *B* were obtained from the same cell after 15 min exposure to HEPES-Ringer's solution at pH 6.4. TTX (3×10^{-6} M) was present throughout.

that a change in external pH from 7.4 to 6.4 (with $[Ca^{++}]_o$ = 2 mM) produced an approximately +5-mV shift in the membrane surface charge in myelinated nerve fibers. Also, in agreement with the results of Kohlhardt et al. (1976), we observed no significant shift of the voltage dependence of i_{Ca} availability at pH 6.4 compared with external pH 7.4 in the absence of any drug.

Because diltiazem's pK_a is intermediate (pK_a = 7.4) compared with the other two antagonists, small changes in extracellular pH would be expected to produce greater changes in the relative proportion of neutral and charged drug forms. Therefore, to test whether drug-channel dissociation rates are dependent upon drug charge, i_{Ca} recovery was examined in the presence of 5×10^{-5} M diltiazem

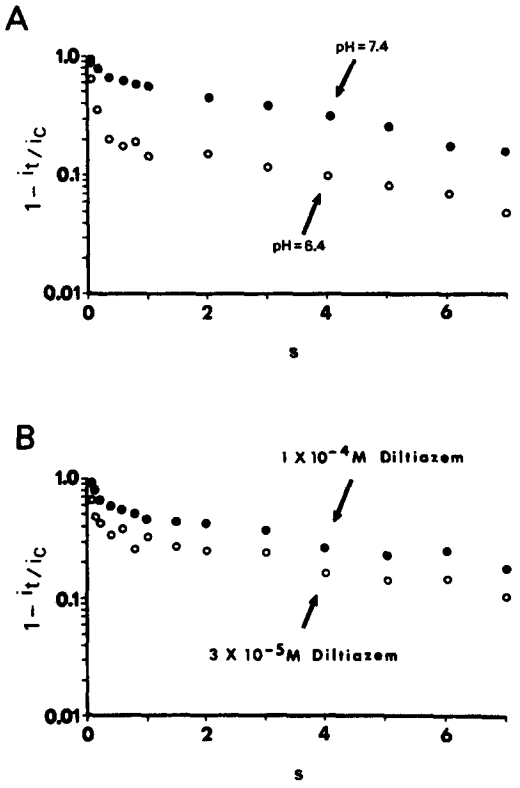

FIGURE 10. Effect of pH and drug concentration on the kinetics of slow i_{Ca} reactivation. In *A*, the kinetics of i_{Ca} reactivation were examined in the presence of 5×10^{-5} M diltiazem at external pH 7.4 (open circles) and pH 6.4 (closed circles). In *B*, the kinetics of i_{Ca} reactivation were examined in the presence of 3×10^{-5} M diltiazem (open circles) and in 1×10^{-4} M diltiazem (closed circles) at a constant external pH of 7.4. The experimental protocol is the same as in Fig. 5. The holding potential was −80 mV throughout.

at external pH 7.4 and 6.4. Fig. 10*A* shows a surprising result: extracellular acidification does not appear to modify the time course of the slow, drug-induced recovery phase. The decrease in pH appears only to decrease the amplitude of the slow recovery phase, which suggests that a smaller proportion of channels is drug-associated at the lower pH. Such a result might be expected if the primary effect of external acidification is to reduce the effective intracellular drug concentration. Fig. 10*B* verifies that changes in the concentration of diltiazem

change the relative amplitude of the slow recovery process without changing the kinetics of the slow process.

Table II summarizes additional data for diltiazem block at pH 6.4. In these experiments, external pH was 6.4 before the introduction of an antagonist. Again, no substantial change in the kinetics of the slow phase of recovery of i_{Ca} was produced by reductions in extracellular pH. There was, however, a small prolongation of the time constant of normal recovery of i_{Ca} in the absence of drug, an effect similar to that produced by extracellular acidification on i_{Na} in myelinated nerve (Courtney, 1979). It is not completely unexpected that a decrease in extracellular pH is equivalent to reducing the diltiazem concentration, because an increase in the proportion of charged extracellular drug would be expected to result in less net intracellular accumulation of drug. However, analogous experiments on i_{Na} block by local anesthetics in nerve (Khodorov et al., 1976; Schwarz et al., 1977) and in heart (Bean et al., 1983) have demonstrated a slowing of the drug-induced phase of recovery at lower external pH. In terms of the modulated-receptor model, it is hypothesized that external protons are capable of interacting directly with channel-bound neutral drug species to increase the concentration of charged-bound drug, which can only slowly dissociate from the receptor (Schwarz et al., 1977). The absence of an effect of external pH change on the kinetics of i_{Ca} recovery in the presence of organic Ca channel antagonists is a clear deviation from the modulated-receptor hypothesis as formulated to account for the interactions of local anesthetic agents with i_{Na}.

In a final series of experiments, we examined whether external acidification would modify tonic and use-dependent block of i_{Ca} by diltiazem and by nifedipine. External acidification will increase the proportion of the cationic drug form of diltiazem in solution. In the case of nifedipine, which has a very low pK_a, a relatively minor change in external pH from 7.4 to 6.4 should have negligible effects on the proportion of neutral and charged drug species in solution. These experiments are important in order to verify that the changes in block produced by external acidification are in fact attributable to changes in drug ionization and are not due to protonation of the drug receptor.

To test whether changes in extracellular pH modify use-dependent block by diltiazem, an experimental protocol identical to that used in Figs. 2 and 3 and Table I was carried out at pH 6.4 using the same concentration of diltiazem (5×10^{-5} M). These results are summarized in Table III. In a total of 10 cells, ~97% of the total inhibition of i_{Ca} by diltiazem at pH 6.4 was use-dependent, much like that observed with D-600 at pH 7.4. However, at pH 6.4, the total percent inhibition of i_{Ca} was reduced from 54 to 28. The apparent increase in frequency-dependent inhibition at pH 6.4 actually reflects an overall reduction in the absolute amount of tonic block. Since extracellular acidification increases the amount of protonated diltiazem in solution, it may be expected that less drug would accumulate intracellularly and would account for a decrease in the observed overall potency, but a reduction in tonic block at lower pH is unexpected and is not consistent with a modulated-receptor model as formulated for local anesthetic block of Na channels (Schwarz et al., 1977).

An examination of i_{Ca} block by nifedipine at an external pH of 6.4 also yielded

an unexpected result. In comparison with the inhibition observed at pH 7.4, there was also an apparent marked decrease in overall potency and an apparent increase in the relative proportion of use-dependent block. This change also reflects a decrease in the absolute amount of tonic block at the lower pH as well. Since such a small decrease in external pH would not be expected to produce any significant change in the concentration of neutral nifedipine, it would appear that small changes in external pH are capable of modulating tonic block of i_{Ca} by nifedipine independently of changes in the proportion of neutral vs. ionized drug concentration.

TABLE III

Effect of Changes in Extracellular pH on Tonic and Use-dependent Block of i_{Ca} by Organic Antagonists

	pH	*n*	Dose	Percent total block	Percent use-dependent block	Percent tonic block	Percent charged
Diltiazem	7.4	6	5×10^{-5} M	54±7.0	76±7.1	24±7.1	67 ($pK_a = 7.7$)
Diltiazem	6.4	10	5×10^{-5} M	28±4.2	97±2.9	3±2.9	95 ($pK_a = 7.7$)
Nifedipine	7.4	5	3×10^{-7} M	49±7.8	11±2.6	89±2.6	1 ($pK_a \approx 1.0$)
Nifedipine	6.4	10	3×10^{-7} M	20±3.5	45±9.9	55±3.2	1 ($pK_a \approx 1.0$)

Data are expressed as means ± SEM.

DISCUSSION

Although some earlier voltage-clamp studies have concluded that a modulated-receptor model (Hille, 1977*a*, *b*; Hondeghem and Katzung, 1977) may explain the interactions of some organic Ca channel antagonists with myocardial Ca channels (McDonald et al., 1980; Hescheler et al., 1982; Lee and Tsien, 1983), such a hypothesis has not previously been tested directly. The experiments described in this paper were carried out to test directly the applicability of such a model to i_{Ca} inhibition by three structurally diverse organic compounds: D-600, diltiazem, and nifedipine.

Our experiments demonstrate many remarkable similarities between the interactions of these compounds with myocardial Ca channels and previously characterized interactions of quaternary, tertiary, and neutral forms of local anesthetics with Na channels in nerve and cardiac muscle. Frequency-dependent inhibition of i_{Ca} by these compounds appears to be associated with the charged drug form. All three antagonists at physiological pH produce large shifts of i_{Ca} availability in the hyperpolarizing direction. Since at this pH, D-600 can be considered to be predominantly charged and nifedipine completely neutral, it can be concluded that both charged and neutral organic antagonists produce a shift in equilibrium of i_{Ca} from resting to inactivated states.

Voltage-dependent block of i_{Ca} has previously been observed with verapamil and its methoxy derivative D-600 (Pelzer et al., 1982; Trautwein et al., 1983) and also recently with diltiazem (Tung and Morad, 1983; Kanaya and Katzung, 1984). A new and interesting result of our experiments is the demonstration of

voltage-dependent block of i_{Ca} by the dihydropyridine nifedipine. A similar voltage-dependent interaction has also been observed with the nifedipine derivative nisoldipine (Uehara and Hume, 1984*b*). These results are in marked contrast to earlier reports (Bayer et al., 1982; Kass, 1982), which failed to detect a voltage-dependent effect of dihydropyridines on i_{Ca}. This discrepancy may be due to the shorter prepulses used to assess i_{Ca} availability and the higher drug concentrations used in those studies. A re-examination of dihydropyridine blockade in calf cardiac Purkinje fibers has recently confirmed that i_{Ca} blockade by these compounds is voltage-dependent (Sanguinetti and Kass, 1984).

In the presence of all three organic antagonists, the i_{Ca} reactivation time course is bi-exponential: Ca^{++} channels that are free of drug appear to recover with a normal time course; drug-bound channels appear to recover much more slowly. The kinetics of the drug-induced reactivation process appear to fall along a spectrum. The slowest phase (~15 s) is produced by D-600 (which is predominantly charged at pH 7.4); the fastest phase (~500 ms) is produced by nifedipine (which is neutral at pH 7.4). This effect explains why use-dependent block by D-600 can be observed at slow frequencies of stimulation, whereas rates of stimulation in excess of 1 Hz are required to demonstrate appreciable use-dependent inhibition by nifedipine. The kinetics of the slow phase of recovery produced by the organic antagonists also appears to be independent of drug concentration. Changes in concentration appear to change only the amplitude of the slow recovery process, which indicates that higher drug doses increase the proportion of drug-bound channels that recover with the same kinetics.

The similarities in action between organic Ca antagonists and previously characterized actions of local anesthetic agents on nerve and cardiac Na channels suggest that a similar modulated-receptor hypothesis is essential for understanding the interactions of these agents with myocardial Ca channels. By analogy, organic Ca channel antagonists may interact with resting channels (R), open channels (O), and inactivated channels (I) (see Fig. 11), and each antagonist may have characteristic association and dissociation rate constants for each of the channel states. Our experiments provide evidence that blockade of myocardial Ca channels by D-600, diltiazem, and nifedipine is state-dependent. Nifedipine and the neutral form of diltiazem exhibit tonic inhibition, which may be associated with binding to resting Ca channels, since holding potentials were used that were negative enough to ensure that inactivation was absent (even in the presence of large hyperpolarizing drug-induced shifts in the voltage dependence of i_{Ca} inactivation) and changes in holding potential to more negative levels for minutes failed to reverse the tonic block. Thus, the tonic block observed in the present experiments is not due to binding to inactivated channels (cf. Bean et al., 1983). Each of the three antagonists, in addition, appears to bind to some extent to inactivated Ca channels, as assessed by examining the time course of the development of the drug-induced slow i_{Ca} reactivation phase (Fig. 8). For D-600 and diltiazem, long-duration depolarizing prepulses are required to allow the proportion of Ca channels exhibiting slow reactivation to reach equilibrium. This is consistent with strong binding to inactivated Ca channels (I-I^x pathway), in agreement with earlier findings with verapamil and diltiazem in ferret papillary

muscles (Kanaya et al., 1983). Hydrophilic drug forms of diltiazem and D-600 may, however, have the access to their receptor site restricted to a hydrophilic pathway (O-O* pathway).

Nifedipine, in contrast, is neutral at physiological pH and can gain access to the receptor site via any of the three possible pathways. The time course of development of block by nifedipine, which is associated with slow i_{Ca} reactivation, is composed of a very fast component, attributable to open channel block, and an extremely slow component, associated with block of inactivated channels. Both components together appear to represent only a small additional interaction of nifedipine with Ca channels when compared with the interaction of nifedipine with resting i_{Ca} channels (tonic inhibition) studied at relatively negative holding

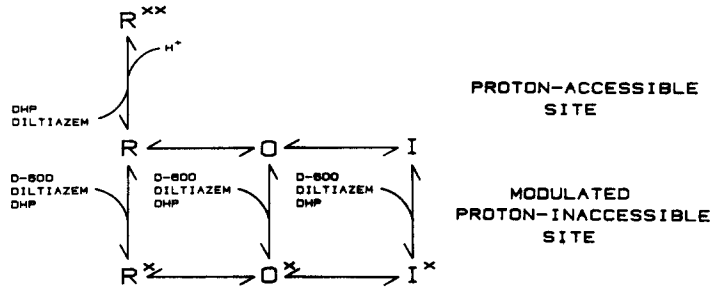

FIGURE 11. Modified modulated-receptor scheme to account for the inhibitory actions of organic Ca channel antagonists with myocardial Ca channels. The Ca channel is shown in three conformational states: resting, open, and inactivated. Hydrophilic and hydrophobic forms of D-600 and diltiazem and the hydrophobic form of nifedipine may bind to a proton-inaccessible site, the affinity of which is dependent upon the state of the channel. In addition, a separate proton-accessible site is postulated to account for the tonic inhibition of i_{Ca} by hydrophobic forms of nifedipine and diltiazem. Drug-blocked states are denoted by *x*.

potentials. However, the interaction of nifedipine with resting Ca channels cannot account for the large hyperpolarizing shifts of Ca channel availability produced by this compound (Fig. 5*C*). These shifts presumably reflect an overall change in equilibrium from the resting to the inactivated state once the drug is bound (Hille, 1978; Bean et al., 1983). This observed shift of i_{Ca} availability suggests an even greater interaction of nifedipine with inactivated Ca channels than implied by the experiment shown in Fig. 7*C*. In fact, this particular experimental protocol does not readily distinguish between open channel block and very fast block of inactivated channels (with a sudden drop in the current amplitude). From Table I, we can estimate the dissociation constant for nifedipine binding to resting Ca channels to be 3×10^{-7} M, since these experiments were carried out at negative holding potentials and at stimulation frequencies that minimized frequency-dependent block of i_{Ca}. The following equation from Bean et al. (1983) can be used to estimate the dissociation constant for nifedipine binding to inactivated Ca channels:

$$\Delta V_h = k \ln[(1 + [N]/K_R)/(1 + [N]/K_I)], \quad (1)$$

where ΔV_h is the shift in midpoint of i_{Ca} availability curve, k is the slope factor of the inactivation curve, [N] is the nifedipine concentration, and K_R and K_I are the apparent dissociation constants for resting and inactivated channels. If $V_h = -18$ mV (mean of 5 experiments) and k is taken as 10.9 mV (mean of 13 experiments), K_R as 3×10^{-7} M, and [N] as 3×10^{-7} M, then $K_I = 3 \times 10^{-8}$ M. This calculation and the experiment in Fig. 7*C* predict that nifedipine may interact slowly with inactivated Ca channels with an affinity approximately one order of magnitude greater than the interaction with resting Ca channels. Although we have not directly tested this prediction (by examining nifedipine block at depolarized holding potentials, where h would be small), recent data by Bean (1984) in canine isolated myocytes with the dihydropyridine derivative nitrendipine indicate a higher K_d for interactions with inactivated Ca channels compared with resting Ca channels. Our data, obtained primarily at negative holding potentials, are therefore consistent with a higher-affinity interaction of dihydropyridine antagonists with inactivated Ca channels and may account for the substantial changes in i_{Ca} availability produced by nifedipine in our experiments.

Important differences between the interactions of organic Ca channel antagonists with myocardial Ca channels and local anesthetic interactions with Na channels were observed. These differences may require some modifications of the modulated-receptor hypothesis as proposed for local anesthetic interactions with Na channels (Hille, 1977*a*, *b*; Hondeghem and Katzung, 1977). For Na channels, external acidification increases the time constant of the local anesthetic-induced slow recovery phase (Khodorov et al., 1976; Schwarz et al., 1977; Bean et al., 1983). Protons are considered to be able to enter the external mouth of the channel and equilibrate with neutral-bound drug, thereby increasing the concentration of charged-bound drug, which slowly dissociates from the receptor. Our experiments indicate that the three antagonists examined appear to fall along a continuum: D-600, the most charged at pH 7.4, produces the longest time constant for the slow i_{Ca} recovery process; nifedipine (neutral) produces the fastest; and diltiazem produces an intermediate time constant. However, external acidification, which is expected to increase the amount of charged diltiazem, does not appear to modify the kinetics of the drug-channel dissociation rate. This result indicates that for myocardial Ca channels, protons may not be able to equilibrate with neutral-bound antagonist. More experiments are needed for a full understanding of the significance of why lowering external pH fails to modify the kinetics of i_{Ca} reactivation induced by the organic Ca antagonists.

It was possible to verify in nerve (Schwarz et al., 1977) that a change in external pH, independent of changes in the receptor or indirect changes in intracellular pH, was the important variable influencing use-dependent block of i_{Na} by local anesthetics. Thus, changes in external pH did not influence block of i_{Na} by the neutral anesthetic benzocaine. Analogous experiments on i_{Ca} block by the predominantly neutral antagonist nifedipine gave quite different results. Acidification attenuates the tonic inhibition of i_{Ca} by nifedipine (see Table III). Therefore, in the experiments in which diltiazem block was examined at different pH values, two primary effects could have occurred as a result of acidification: (*a*) a modification of the degree of ionization of diltiazem, and (*b*) some additional effect of protons either on a drug receptor site or on the surface membrane.

Several plausible mechanisms may account for the attenuation of tonic i_{Ca} block of diltiazem and nifedipine at low pH. Ca bound to anionic sites on the cell surface membrane has been hypothesized to play a crucial role in both transmembrane Ca influx and excitation-contraction coupling in the heart (Langer, 1978; Bers et al., 1981). One possible mechanism by which external acidification may attenuate tonic block of i_{Ca} by nifedipine is the displacement of bound Ca ions from external sarcolemmal binding sites by external protons, which would produce a net increase in the availabiity of Ca as a charge source. However, such an effect of external protons would be expected to produce an increase in the magnitude of i_{Ca} in the absence of nifedipine, and this effect was not observed. In fact, decreases in external pH of a magnitude larger than those used here are known to inhibit transmembrane Ca current (Chenais et al., 1975; Vogel and Sperelakis, 1977). Attenuation of tonic i_{Ca} block by diltiazem and nifedipine at low pH may be the result of a shift in membrane surface charge (Hille, 1968; Hille et al., 1975). For instance, a depolarizing shift in i_{Ca} availability at lower pH may antagonize the voltage-dependent block of i_{Ca} by diltiazem and nifedipine. However, in our experiments, we failed to find a shift in the membrane surface charge or i_{Ca} availability at pH 6.4 that was large enough to account for the attenuation of tonic block by diltiazem and nifedipine. Another possibility is that the pK_a of the drug may actually change once it is bound to the channel.

Alternatively, protons may be interacting directly with a drug receptor site. The rather selective effect of protons on tonic inhibition of i_{Ca} by organic antagonists contrasts markedly with the inability of protons to modify drug receptor dissociation rates (Table II and Fig. 8). This apparent paradox may be resolved if separate receptors mediate the tonic and frequency-dependent inhibition of i_{Ca} by these agents at negative holding potentials: one site that is accessible to protons, and another site that is inaccessible. A separate external receptor for neutral local anesthetic interactions with Na channels in nerve was originally proposed by Khodorov et al. (1976). It is possible that the receptor site for tonic block is either intracellular or is at least within the sarcolemma, since even tonic block by the quaternary dihydropyridine derivative is greatly attenuated upon external application of the compound. However, an external receptor for tonic block that only binds neutral drug forms cannot be excluded.

The tonic block of i_{Ca} by the organic antagonists observed in the present experiments was not reversed by hyperpolarization, which is similar to the tonic block of i_{Na} observed after internal application of QX-314, a permanently charged derivative of lidocaine, in squid axon (Cahalan and Almers, 1979). In these experiments, nonspecific adsorption of local anesthetic to the lipid bilayer, rather than a separate receptor site for tonic block, was considered as a possible mechanism. A similar nonspecific adsorption of the organic Ca antagonists to the sarcolemma might mediate the tonic block of i_{Ca} observed in our experiments. However, the considerably higher potency of the organic Ca antagonists for tonic inhibition of i_{Ca} (see Table I), compared with the millimolar concentrations of local anesthetics usually required to produce tonic inhibition of i_{Na}, makes it unlikely that organic Ca antagonist intramembrane concentrations ever approach those achieved with high doses of local anesthetics (Almers, 1976).

Both the tonic and frequency-dependent interactions of the organic Ca channel antagonists and the influence of pH changes on those interactions might be explained by a modified version of the modulated-receptor hypothesis originally proposed for local anesthetic interactions with Na channels. The new hypothesis (Fig. 11) incorporates most of the features of the local anesthetic model with the following modifications: (*a*) binding by both hydrophilic and hydrophobic forms of D-600 and diltiazem and the hydrophobic form of nifedipine to a proton-inaccessible site, the affinity of which is state-dependent and characteristic for each compound, and (*b*) additional binding by the hydrophobic form of dihydropyridine antagonists and also diltiazem to a proton-accessible site that may be associated primarily with resting channels. Binding to this site may be voltage-independent, whereupon channels remain resting but blocked and thus electrically silent. The channels that remain resting but blocked (R^{**}) are distinguishable from channels that have bound drug at the proton-inaccessible site and become blocked and inactivated (I^{*}) by the fact that in the continued presence of drug the latter pool of channels can eventually be made to recover from block as a function of both time and voltage, whereas the former cannot. Although our data suggest that binding to this proton-accessible site is associated primarily with resting Ca channels when examined at relatively negative holding potentials, they do not exclude the possibility that binding to this site may actually be state-independent.

There is evidence that suggests important differences between the dihydropyridine family of Ca channel antagonists and other organic antagonists. It has recently been proposed that a nifedipine derivative (BAY K8644) may act as a Ca channel agonist (Schramm et al., 1983). The positive inotropic effect of this compound was competitively antagonized by nifedipine, but not by verapamil or diltiazem. Receptor binding studies indicate that the nifedipine family of organic antagonists may bind to a common receptor site that differs from the sites at which verapamil and diltiazem bind (Murphy et al., 1983; Holck et al., 1983; DePover et al., 1982; Ferry and Glossman, 1982), and two recent reports describe separate high-affinity and low-affinity binding sites for dihydropyridine antagonists (Bellemann et al., 1981; Marsh et al., 1983). These sites may be similar to the proton-accessible and proton-inaccessible sites described for nifedipine in this paper, and allosteric interactions between these sites may be possible (Ferry and Glossman, 1982; DePover et al., 1982).

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