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ACTIONS OF VERAPAMIL, DILTIAZEM AND OTHER DIVALENT CATIONS ON THE CALCIUM-CURRENT OF *Helix* NEURONES

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1 Effects of organic Ca^{2+}-antagonists, verapamil and diltiazem, and cations, Ni^{2+}, Mn^{2+}, Co^{2+} and La^{3+} on Ca^{2+} current (I_{Ca}) separated from other ionic currents in a *Helix* neurone were studied. A suction pipette technique which allows internal perfusion of the cell body and voltage clamp was used.

2 Verapamil and diltiazem (10^{-6}–10^{-4}M) increased the threshold, and decreased both the amplitude and rate of rise of the soma Ca^{2+}-spike. Both agents inhibited I_{Ca} over the entire range of the current-voltage (I-V) relationship dose-dependently, without shifting the threshold of the I-V relationship. Increases in external Ca^{2+} overcame the inhibitory action of the agents.

3 Divalent cations, Ni^{2+}, Mn^{2+}, Co^{2+} and the trivalent cation, La^{3+} inhibited I_{Ca} dose-dependently, but induced shifts of the I-V relationship to more positive voltages. The order of potency of inhibition of I_{Ca} among these cations was as follows; $Ni^{2+} > La^{3+} > Mn^{2+} > Co^{2+}$.

4 Double reciprocal plots for peak I_{Ca} versus external Ca^{2+} concentrations in the presence or absence of both organic and inorganic Ca^{2+}-antagonists intersect at the ordinate. Results indicate that both organic and inorganic Ca^{2+}-antagonists compete for Ca^{2+} at the common binding site for Ca^{2+}.

5 Internal application of the organic Ca^{2+}-antagonists (10^{-4}M) inhibited I_{Ca} in a time-dependent manner to about 40–60% of the control. Ni^{2+}, when applied internally, also depressed I_{Ca}.

6 The results provide evidence that organic Ca^{2+}-antagonists occupy the binding site for Ca^{2+} in a competitive manner at the surface of the soma membrane of the *Helix* neurone, while divalent and trivalent cations, in addition to inhibiting I_{Ca} in a similar manner to the organic Ca^{2+}-antagonists, change the surface charge of the soma membrane.

Introduction

Since Kohlhardt and his co-workers first showed that verapamil and methoxyverapamil (D-600) competed with Ca^{2+} for a common receptor group or specific carrier in cardiac muscle (Kohlhardt, Bauer, Kraus & Fleckenstein, 1972), these agents have been used as specific Ca^{2+}-antagonists to modify functions of organs or tissues in which Ca^{2+} current plays an important role in electrical or mechanical events. However, their mode of action is still uncertain. Bayer, Kaufman & Mannhold (1975) found that on cat papillary muscle (−)-verapamil and (−)-D-600 acted as allosteric effectors and did not compete with Ca^{2+}. Recently, Payet, Schanne & Ruiz-Ceretti (1980), examining effects of verapamil and D-600 on the slow inward current in the rat ventricular muscle, have drawn the same conclusion. Although it is generally accepted that the slow inward current is mainly carried by Ca^{2+} in cardiac muscle (Reuter & Scholz, 1977 and see French & Adelmann, 1976), participation of Na^+ in the slow inward current cannot be entirely excluded (Rougier, Vassort, Garier, Gargouil & Coraboeuf, 1969; Morad & Maylie, 1980). Therefore, it was felt that analysis of the mode of action of the organic Ca^{2+}-antagonists should be carried out in a preparation in which pure Ca^{2+} current can be separated from other ionic currents in the excitable membrane. In this respect, the snail neurone in which voltage-dependent currents carried by Ca^{2+}, Na^+ and K^+ have been identified, seems to be the most suitable preparation for analysis of drug-action (Kerkut & Gardner, 1967; Geduldig & Gruener, 1970; Standen, 1975; Kostyuk, Krishtal & Pidoplichko, 1975; Akaike, Lee & Brown, 1978), and furthermore, in this preparation pure Ca^{2+} current can be separated for other ionic currents with a suction pipette technique recently developed by our laboratory which allows internal perfusion of the cell body and voltage clamp (Lee, Akaike & Brown, 1978; Akaike *et al.*, 1978). Therefore, the present experiments were designed in an attempt to examine the effects of verapamil, and diltiazem which has been introduced as an organic Ca^{2+}-antagonist (Sato,

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Nagao, Yamaguchi, Nakajima & Kiyomoto, 1971; Nagao, Sato, Nakajima & Kiyomoto, 1972; Nikajima, Hoshiyama, Yamashita & Kiyomoto, 1975) on pure Ca^{2+} current (I_{Ca}) of the snail neurone, using the suction pipette technique. We have also compared effects of these organic Ca^{2+}-antagonists on I_{Ca} with those of divalent and trivalent cations known as inorganic Ca^{2+}-antagonists. The present paper describes results of actions of these agents only on I_{Ca}. Preliminary results have been given elsewhere (Akaike, Lee & Brown, 1979).

Methods

The experimental method was essentially similar to that previously described (Lee *et al.*, 1978; Akaike, Nishi & Oyama, 1981). In short, the experiments were performed on single neurones isolated from the circumoesophageal ganglia of *Helix aspersa*. The ganglion was removed and connective tissue was stripped off with fine forceps until clusters of neurones floated free in 'normal' snail Ringer. A part of each individual neurone (30–80 μm in diameter) was aspirated under a negative pressure of about −300 mmHg so as to occlude the 10–15 μm diameter tip of a suction pipette, and then the cell body was isolated from residual connective tissue and the axon. Internal perfusion was preceded by disruption of part of the neuronal membrane aspirated into the tip of the suction pipette.

Ca^{2+} current (I_{Ca}) was separated after K^+ current (I_K) and Na^+ current (I_{Na}) were blocked by the substitution of Tris for Na^+, and Cs^+ for K^+, in the internal and external solutions. The compositions of all test solutions are listed in Table 1.

Ionic currents were monitored on a storage oscilloscope (Tektronix 5113), and simultaneously recorded on paper with a fibre optics oscilloscope (Medelec, MS6), or stored on an FM data recorder (Sony, PFM-15) or a digital tape recorder (Kennedy model 9700C). At steps from the usual holding potential (V_H) of −60 or −50 mV for I_{Ca} to voltages of +100 mV, the capacitative current, transient and leakage currents associated with the separated I_{Ca} were subtracted by a signal averager (Medelec, DAV 62), using values obtained from equivalent hyperpolarizing voltage steps.

Organic Ca^{2+}-antagonists employed in the present experiments were: diltiazem[3-acetoxy-2, 3-dihydro-5-2-(dimethylamino)ethyl]-2(4-methoxyphenyl)-1, 5-benzothiazepin-4 (5H) HCl (Tanabe Pharm. Co., Japan) and verapamil[α-isopropyl-α-(N-methyl-N-homoveratryl)γ-aminopropyl]-3,4-dimethoxyphenylacetonitrile HCl (Knoll). They were dissolved in test solution just before use. Unless otherwise stated, solutions of inorganic Ca-antagonists La^{3+}, Ni^{2+}, Mn^{2+} and Co^{2+} were made up from refrigerated stock solutions.

All experiments were carried out at room temperatures of 20–25°C.

Results

Effects of organic Ca^{2+}-antagonists on the soma Ca^{2+}-spike

After the neurone soma was separated from its axon in the 'normal' solution, the preparation was exposed to Na-free solution with 'normal' internal solution. Within a few minutes after exposure to the Na-free solution, somal action potentials with a different amplitude and configuration were produced by stimulus-voltage beyond a threshold level, and the amplitude of the action potential was graded with the voltage applied. This voltage-dependent Ca^{2+}-spike required higher stimulus currents than the action potential produced when the axon remained attached to the soma as described previously (Lee, *et al.*, 1978). Verapamil and diltiazem (10^{-6}–10^{-4}M) did not alter the resting membrane potential but there was a tendency for the membrane input resistance to increase slightly. These agents gradually increased threshold, decreased the amplitude and the rate of rise of the soma Ca^{2+}-spike, and prolonged the spike

Table 1 Ionic composition of snail ringer solutions

	NaCl	*Tris Cl*	*KCl*	*CsCl*	*Cs aspartate*	*K aspartate*	*$CaCl_2$*	*$MgCl_2$*	*Glucose*
External Solution									
Normal	85	5	5	—	—	—	10	15	5.5
Na and K-free Tris	—	85	—	—	5	—	10	15	5.5
Internal Solution									
Normal	—	—	—	—	—	135	—	—	—
Cs	—	—	—	—	135	—	—	—	—

Tris: tris (hydroxymethyl) aminomethane. pH of each solution was 7.4. Internal solution was buffered by adding Trizma base. All values are mM.

duration. The effects appeared within 5 min of application and were almost complete within 10–30 min. When higher concentrations of the agents (10^{-5}–10^{-4}M) were applied, the soma Ca^{2+}-spike was completely abolished within 10 min. After 10 min of exposure at concentrations of 10^{-6} to 10^{-4}M, the depressant action of diltiazem could be reversed by washing, but at a concentration of 3×10^{-4}M recovery was incomplete. On the other hand, in the case of verapamil the inhibitory actions of soma Ca^{2+}-spikes were less readily reversible even at lower concentrations (10^{-5}M).

Effects of organic Ca^{2+}-antagonists on I_{Ca} and its I-V relationship

After Ca^{2+} current (I_{Ca}) was separated by blocking Na^+ current (I_{Na}) and K^+ current (I_K) by external and internal perfusion with the solutions described in Methods (Table 1), under voltage clamp conditions

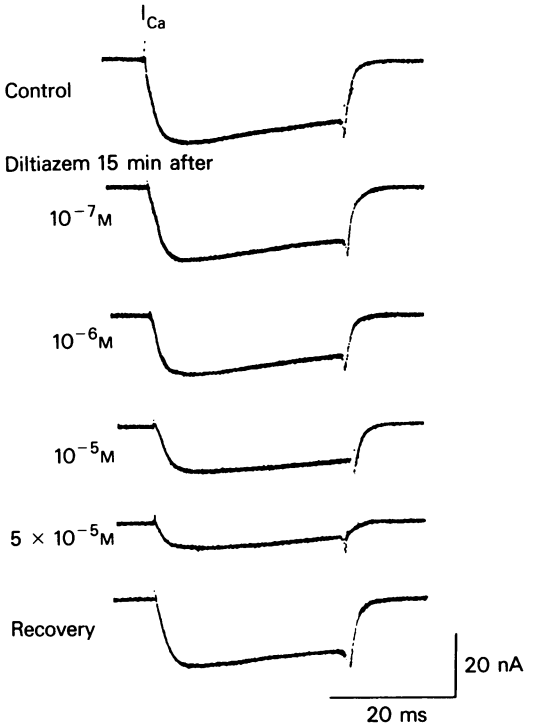

Figure 1 Effects of diltiazem on the calcium current (I_{Ca}) in a *Helix* neurone. Effects of diltiazem at various concentrations on peak I_{Ca} elicited by voltage steps of +20 mV from the holding potential of −50 mV. Sample records of I_{Ca} were obtained after subtraction of leakage, shunt and linear capacitative currents. From the top to bottom: control, 15 min after application of diltiazem of 10^{-7}M, 10^{-6}M, 10^{-5}M and 5×10^{-5}M, respectively, and 30 min after washing the preparation with normal solution.

depolarizing voltage steps of 10 mV were applied to the preparation. A slowly rising inward current of I_{Ca} appeared at depolarizing voltage steps of 10–15 mV from the holding potential (V_H) of −50 mV, rose smoothly, reached its peak within 5 ms and persisted over the subsequent 15–100 ms. At larger depolarizing voltages a transient peak current appeared at 1–3 ms, declined and merged with a slowly inactivating current (Akaike *et al.*, 1978). After ensuring that there was no change in the I_{Ca} current-voltage (I-V) relationship during the control period of 30 min, the preparation was superfused with the external solution containing various concentrations of verapamil or diltiazem at a flow rate of 5 ml/min in a 1 ml perfusion chamber. Figure 1 shows the effects of diltiazem at various concentrations on I_{Ca} produced by a voltage step to +20 mV from V_H (−50 mV). Each record was taken 15 min after adding the agent. Diltiazem at a concentration as low as 3×10^{-6}M started to inhibit I_{Ca}, and a further increase in the concentration of diltiazem reduced I_{Ca} dose-dependently. When the concentration increased, the time for I_{Ca} to reach its peak was slightly prolonged and the time course of the inactivating current was slowed. I-V curves of I_{Ca} in the presence of verapamil and diltiazem at various concentrations at 15 min after the start of superfusion are shown in Figure 2. Both agents produced dose-dependent depression of I_{Ca} and did not shift the I-V relationship. Dose-response curves for depressant effects of the agents were obtained by calculating the ratios of values of the peak I_{Ca} to the control 15 min after exposure to various concentrations of the agents (Figure 3). At a concentration lower than 10^{-7}M, both agents exerted no effect on I_{Ca} but diltiazem started to depress the I_{Ca} at 3×10^{-6}M by 3–17% of the control (mean = 10 ± 6 (s.e.mean)%, $n = 5$), while the threshold dose of verapamil to depress the I_{Ca} was 10^{-6}M (mean = 5 ± 4 (s.e.mean)%, $n = 5$). A further increase in the concentration of the agents depressed the peak I_{Ca} dose-dependently. Half inhibition doses for diltiazem and verapamil were 2×10^{-5}M and 4×10^{-5}M, respectively. At a high concentration of 3×10^{-4}M, both agents completely abolished I_{Ca}.

Effects of inorganic Ca^{2+}-antagonists on I_{Ca}

In the presence of Ca^{2+} 10 mM in the external solution, the effects of Ni^{2+}, La^{3+}, Mn^{2+} and Co^{2+} at various concentrations on I_{Ca} were examined. When preparations were exposed to solutions containing these inorganic Ca^{2+}-antagonists, I_{Ca} was depressed within 5 min of exposure. As a typical example, effects of Co^{2+} on I_{Ca} are presented in Figure 4. It was found that there were shifts of threshold and the peak I_{Ca} of the I-V relationship to more positive potentials in the presence of these cations. These shifts of

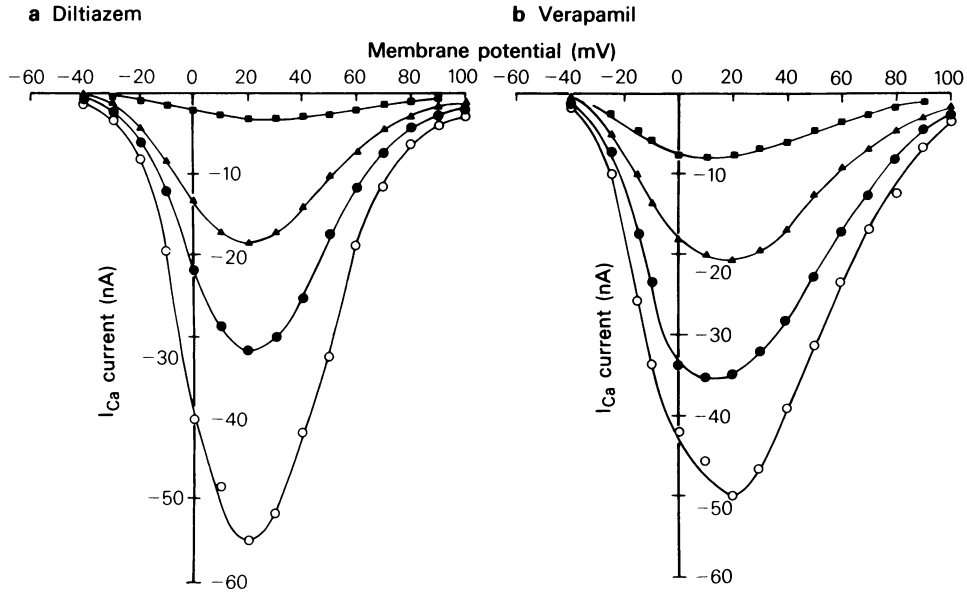

Figure 2 Effects of changes in concentrations of diltiazem (a) and verapamil (b) on current-voltage relationship of I_{Ca}: (a and b) (○) control; (●) 15 min after application of agents at 10^{-5}M, (▲) 3×10^{-5}M, and (■) 10^{-4}M. (a) and (b) were obtained from different experiments. Holding voltage = −50 mV.

threshold and peak I_{Ca} were more pronounced when the concentration of the cation was increased. Thus, at smaller depolarizations the depressant effect of Co^{2+} on I_{Ca} was more marked than at larger depolarizations, and furthermore, in the presence of 1 mM Co^{2+}, the peak I_{Ca} occurred at a voltage step 10 mV more positive than the voltage to induce peak I_{Ca} in the control. There was no qualitative difference in the depressant actions of these inorganic Ca^{2+}-antagonists. Figure 5 shows dose-response curves for depressant effects of Ni^{2+}, La^{3+}, Mn^{2+} and Co^{2+} on the peak I_{Ca} in the order: $Ni^{2+} > La^{3+} > Mn^{2+} > Co^{2+}$, and half-inhibition concentrations for these inorganic Ca^{2+}-antagonists were 0.057 mM (Ni^{2+}), 0.18 mM (La^{3+}), 1.9 mM (Mn^{2+}) and 3 mM (Co^{2+}).

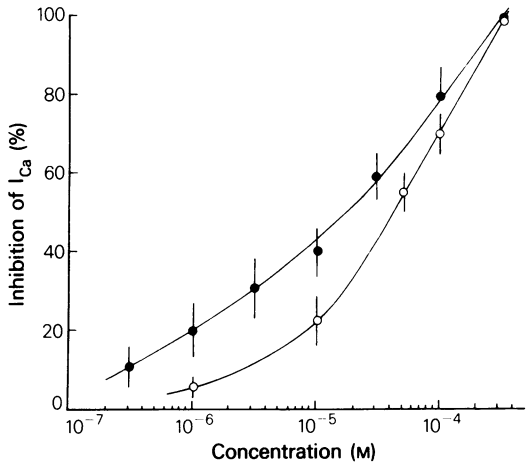

Figure 3 Dose-response curves for % inhibition of peak I_{Ca} after application of diltiazem (●) and verapamil (○). The % inhibition was calculated from peak current before and after the drug application. Each point indicates the average value of 5–6 experiments, and vertical bars show s.e.mean.

Effects of various $[Ca^{2+}]_o$ upon the actions of Ca^{2+}-antagonists

I_{Ca} disappears in Ca-free external solution and increases in a hyperbolic manner when the external $[Ca^{2+}]_o$ is augmented (Akaike *et al.*, 1978). Effects of various concentrations of Ca^{2+} ($[Ca^{2+}]_o$) in the external solution on the depressant actions of both organic and inorganic Ca^{2+}-antagonists were examined. $[Ca^{2+}]_o$ was varied between 2.5 and 20 mM, and Lineweaver-Burk plots were used to characterize the nature of inhibition. Measurements were first performed in 5 mM $[Ca^{2+}]_o$ for 10 min and then the solution was changed to one containing a different $[Ca^{2+}]_o$ concentration. The preparation was exposed to the test solution for 10 min and at the end of the exposure the peak I_{Ca} was recorded. In the present series of experiments, as examples of organic and inorganic Ca^{2+}-antagonists, diltiazem (10^{-5}M) and Co^{2+} (3×10^{-4}M, 10^{-3}M) were used. In each experi-

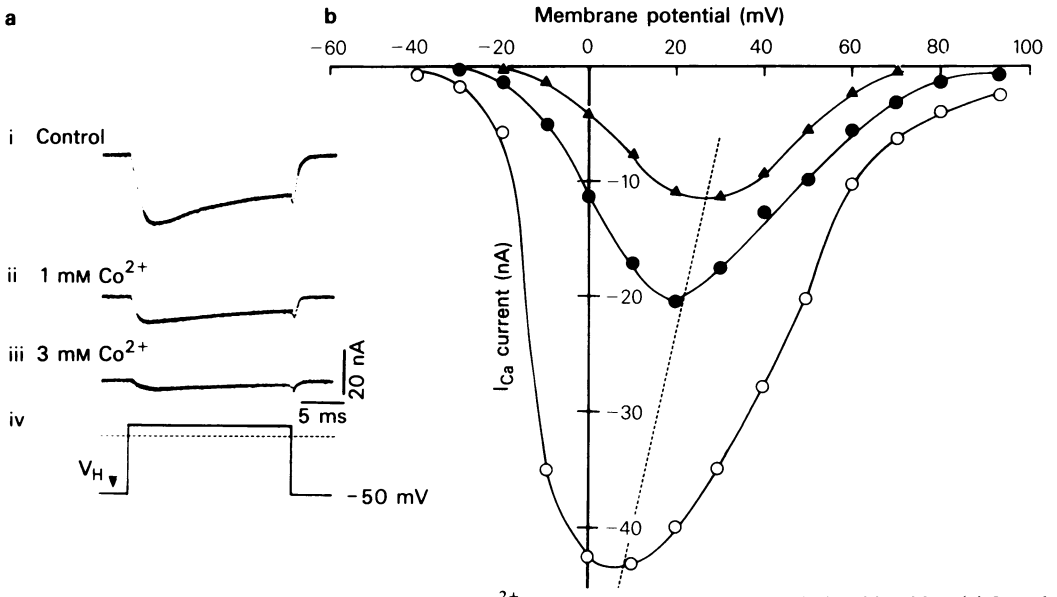

Figure 4 Effects of changes in concentrations of Co^{2+} on I_{Ca}, and current-voltage relationship of I_{Ca}. (a) Sample records of I_{Ca} elicited by a voltage step to +10 mV from the holding potential of −50 mV. From the top to bottom: (i) control; (ii) 15 min after application of Co^{2+} 1 mM; (iii) Co^{2+} 3 mM, and (iv) the voltage step applied. (b) I-V relationship of I_{Ca} in the absence (○: control) and presence of Co^{2+} (●) 10^{-3}M, and (▲) 3×10^{-3}M. A dotted line indicates a shift of the peak current in the presence of Co^{2+}. Results obtained from 2 different experiments.

ment, at least three to four different $[Ca^{2+}]_o$ concentrations were tested per preparation. Figure 6 shows an example of actual records taken from an experiment in which effects of various $[Ca^{2+}]_o$ concentrations on the inhibitory action of diltiazem (10^{-5}M) were examined. It is evident from this figure that the depressant effects of diltiazem are dependent on $[Ca^{2+}]_o$. The effect of the agent observed in 10 mM $[Ca^{2+}]_o$ could be overcome in 20 mM $[Ca^{2+}]_o$. while the depression was enhanced in 2.5 mM $[Ca^{2+}]_o$ compared to 10 mM $[Ca^{2+}]_o$. In the case of Co^{2+}, qualitatively similar results to those obtained with diltiazem were obtained. Lineweaver-Burk plots intersect at the ordinate (Figure 7). Therefore, it can be assumed that diltiazem and Co^{2+} compete with Ca^{2+} for the same receptor sites in inhibiting I_{Ca} in the snail neurone.

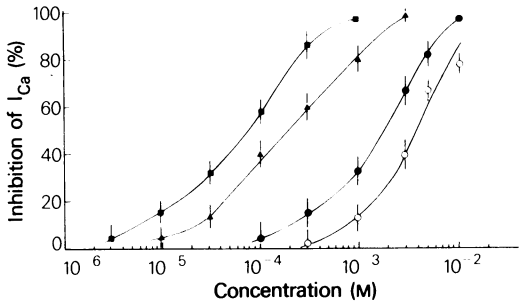

Figure 5 Dose-response curves for inhibitory effect on peak I_{Ca} after application of Ni^{2+} (■), La^{3+} (▲), Mn^{2+} (●), and Co^{2+} (○). The % inhibition was calculated from peak current before and after the application of each cation. Each point indicates the average value of 5–6 experiments, and vertical bars show s.e.mean.

Increases in $[Ca^{2+}]_o$ cause saturation of I_{Ca} and the relationship between peak I_{Ca} and $[Ca^{2+}]_o$ is hyperbolic (Akaike *et al.*, 1978). The relationship is described by Langmuir or Michaelis-Menten type relationships of the sort used by Hagiwara (1975). By using his argument that the relationship is due to a calcium binding action, we have:

$$\frac{1}{I_{Ca}} = \frac{1}{I_{Camax}} + \frac{K_{Ca}}{I_{Camax}} \cdot \frac{1}{[Ca]_o}\left(1 + \frac{M}{K_m}\right) \quad (1)$$

and the blocking effect of Ca^{2+}-antagonists upon peak I_{Ca} in the presence of 10 mM $[Ca^{2+}]_o$ can be expressed as the K_m value using the above equation (1), where I_{Camax} = 24 nA (peak I_{Ca} at +20 mV), I_{Ca} = 12 nA (half of I_{Camax}), K_{Ca} = 3.8 mM (the dissociation constant of the membrane Ca^{2+}-binding site with external solution), M = concentration (mM) of Ca^{2}+-antagonist required to reduce I_{Ca} by one half, and K_m = the dissociation constant for Ca^{2+}-antagonist (mM). The relative dissociation constants thus calculated from the half inhibition doses of both organic and inorganic Ca^{2+}-antagonists are listed in Table 2.

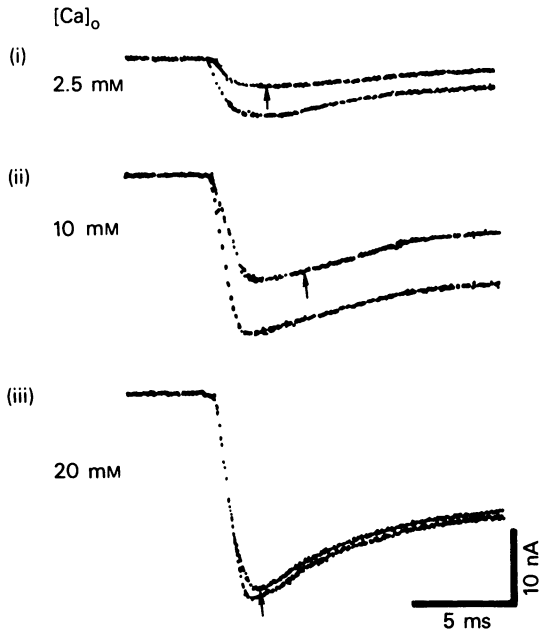

Figure 6 Effects of changes in concentrations of $[Ca^{2+}]_o$ on I_{Ca} in the presence of diltiazem 3×10^{-3} M: (i) 2.5 mM, (ii) 10 mM and (iii) 20 mM $[Ca^{2+}]_o$. The direction of an arrow in each record shows I_{Ca} elicited by a voltage step to +20 mV from the holding potential of −50 mV before and 10 min after the drug application.

Table 2 Dissociation constants for various Ca^{2+} antagonists

Ca^{2+} antagonist	*Half inhibition dose* (mM)	K_m (mM)
Diltiazem	0.02	0.034
Verapamil*	0.04	0.028
Ni^{2+}	0.057	0.035
La^{3+}	0.18	0.111
Mn^{2+}	1.9	1.165
Co^{2+}	3	1.839

*Incomplete recovery

Internal application of Ca^{2+}-antagonists

The experiments so far described indicate that both organic and inorganic Ca^{2+}-antagonists act on the surface membrane of the neurone soma, and exert their depressant effects on I_{Ca}. The question arises as to whether the organic Ca^{2+}-antagonists, verapamil or diltiazem, can also induce their effects from the inside of the plasma membrane. To answer this question, the organic Ca^{2+}-antagonists were applied intracellularly. Internal application of verapamil and diltiazem at concentrations lower than 10^{-5} M did not produce any appreciable changes in I_{Ca}. However, with increases in doses of the organic Ca^{2+}-antagonists, depression of I_{Ca} was observed. Results are shown in Figure 8. Internal application of dil-

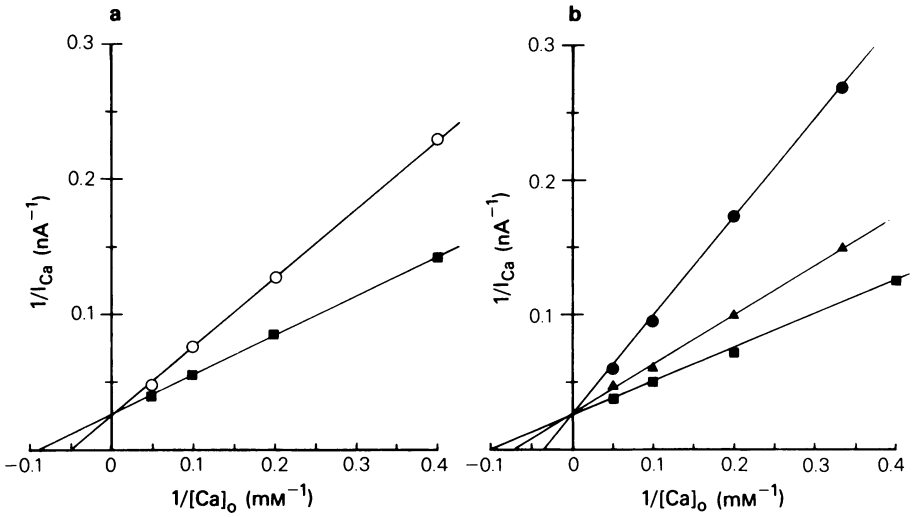

Figure 7 Lineweaver-Burk plot for diltiazem (a) and Co^{2+} (b). Line obtained by varying $[Ca^{2+}]_o$ at a fixed concentration of diltiazem or Co^{2+}. (a) (■) Control; (○) diltiazem 10^{-5} M. (b) (■) Control; (▲) Co^{2+} 0.3 mM; (●) Co^{2+} 1 mM. Straight lines were drawn by eye. (a) and (b) were obtained from different experiments. In (a) peak I_{Ca} elicited by a fixed voltage step to +20 mV from the holding potential of −50 mV, was measured, while in (b), peak I_{Ca} elicited by different voltage steps of 10–30 mV from the holding potential of −50 mV was measured.

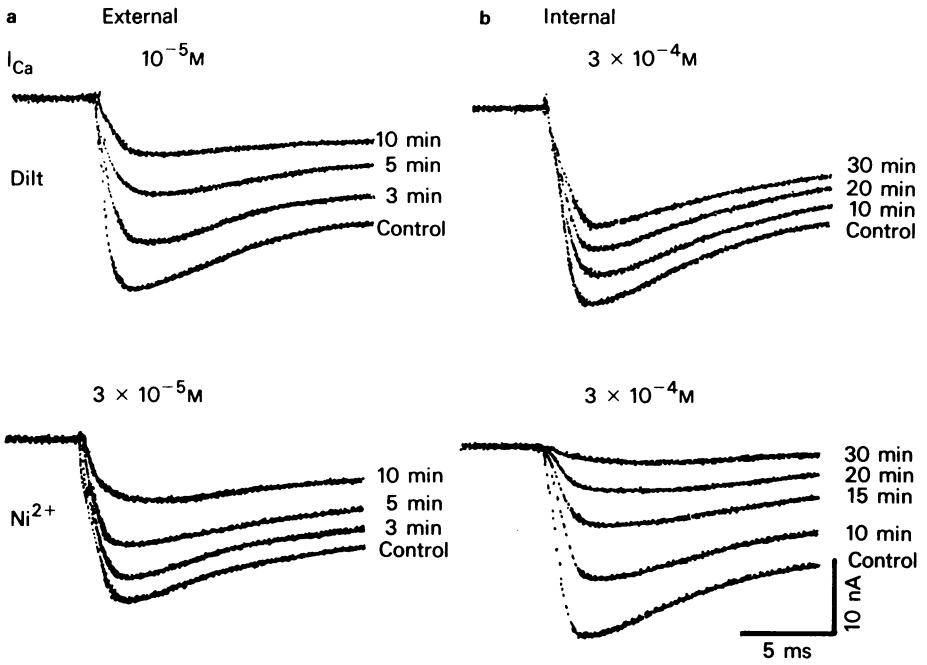

Figure 8 Effects of internal perfusion of diltiazem and Ni^{2+} at a constant external Ca^{2+} concentration (10 mM) on I_{Ca}. Data were obtained from 4 different experiments. (a) External application of diltiazem (Dilt) 10^{-5}M, or Ni^{2+} 3×10^{-5}M. (b) Internal application of diltiazem 3×10^{-4}M, or Ni^{2+} 3×10^{-4}M. I_{Ca} was elicited by a voltage step to +20 mV from the holding potential of −50 mV.

tiazem at a concentration of 3×10^{-4}M reduced peak I_{Ca} in a time-dependent manner, and decreased the I_{Ca} by 30–40% of the control within 30 min in 4 cases examined. Depression occurred over the entire voltage range and no shifts of the threshold and the voltage to induce peak I_{Ca} were observed. Similar effects were observed during internal application of verapamil (3×10^{-4}M). The inhibitory effects of diltiazem applied internally were reversible following a period of inhibition (20–30 min) after the start of perfusion with Cs-aspartate internal solution, but in the case of verapamil recovery was only partial.

It has been reported that an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Cs-aspartate internal solution reduced peak I_{Ca} (Akaike *et al.*, 1978). Another divalent ion, Ni^{2+} which was found to be the most potent inorganic Ca^{2+}-antagonist examined, was applied intracellularly in 5 cases. In the present experiments, to compare the effect of internal application of Ni^{2+} with that of the organic Ca^{2+}-antagonist, the same concentration (3×10^{-4}M) of Ni^{2+} was applied internally. Ni^{2+} (3×10^{-4}M) also depressed I_{Ca} in a time-dependent manner and at the end of 30 min, I_{Ca} evoked by a given depolarizing pulse was markedly decreased. The threshold for I_{Ca} was shifted in the hyperpolarizing direction in the presence of $[Ni]_i$ and the voltage to induce peak I_{Ca} was also shifted to a less positive potential. At a constant voltage step from V_H, marked depression of I_{Ca} was observed as shown in Figure 8, while peak I_{Ca} was reduced by 40–60% of the control after 30 min of internal application of Ni^{2+}. The effects were partially reversible after perfusing the preparation with Cs-aspartate internal solution, but in some instances I_{Ca} remained depressed for a long period of time (1–2 h) without showing any recovery.

Discussion

Characteristics of I_{Ca} separated from other ionic currents in the *Helix* neurone are: (1) I_{Ca} has single activation and single inactivation components, (2) the Ca^{2+} conductance (G_{Ca}) transports Ca^{2+} and other divalent cations but not Na^+, and (3) G_{Ca} has a binding site for divalent cations (Akaike *et al.*, 1978). Using this preparation, present experiments have demonstrated that the organic Ca^{2+}-antagonists, verapamil and diltiazem, inhibited I_{Ca} dose-dependently. It is most likely that the organic Ca^{2+}-antagonists exert their action on I_{Ca} by occupying the specific binding site for Ca^{2+} and hence, would have resulted in depression or blockade of G_{Ca}. Evidence

for blockade at a common site for Ca^{2+} comes from the observation that the inhibitory action of verapamil and diltiazem could be overcome by an increase in $[Ca^{2+}]_o$, or enhanced by a decrease in $[Ca^{2+}]_o$. The fact that double reciprocal plots of peak I_{Ca} versus $[Ca^{2+}]_o$ in the presence or absence of the organic Ca^{2+}-antagonists intercept at the ordinate, further supports the idea, and suggests that the organic Ca^{2+}-antagonists compete with Ca^{2+} for the same binding site in inhibiting I_{Ca}. In addition, the blockade curves for the agents are fitted quite well by the single site model used in equation (1), originally proposed by Hagiwara (1975). Thus, present results agree with the results obtained by Kohlhardt & Mnich (1978), but not with the recent conclusion of Payet *et al.* (1980). The latter authors have reported that Lineweaver-Burk plots for a slow inward current (G_{Si}) of rat ventricular muscle intercepted at the abscissa, and hence concluded that verapamil and D-600 do not compete with Ca^{2+} for the same receptor membrane. Although it is generally accepted that G_{Si} is produced mainly by Ca^{2+}, the possibility that other ionic currents, such as I_{Na} and I_K, may also participate in eliciting G_{Si} in the cardiac cell, cannot be entirely ruled out (Rougier *et al.*, 1969; Morad & Maylie, 1980). Therefore, there is the possibility that verapamil and D-600 might have exerted their actions on other ionic currents in the component of G_{Si}. In fact, verapamil has a non-specific inhibitory action on I_K of the *Helix* neurone (Akaike *et al.*, 1979; Nishi & Oyama, unpublished observations), and Kass & Tsien (1975) have shown inhibitory effects of verapamil and D-600 on other cardiac ionic currents. Thus, the validity of the conclusion drawn by Payet *et al.* is questionable. The question whether the Ca^{2+}-antagonist causes inhibitory actions on the pure Ca^{2+}-current of mammalian cardiac muscle in a similar manner to that found in *Helix* neurones, deserves further study.

An increase in concentrations of verapamil and diltiazem prolonged the time for I_{Ca} to reach its peak and at the same time, slowed the time course of the inactivation current at a given depolarizing voltage pulse. In the present experiments, we have not done quantitative analysis of effects of the organic Ca^{2+}-antagonists on the activation and inactivation processes of I_{Ca}, but the results indicate that both the activation and inactivation processes are equally affected by the agents.

The I-V relationship was shifted to more positive potentials in the presence of divalent and trivalent cations. The observation is similar to the effects of $[Ca^{2+}]_o$ on the relationship between the I_{Ca} and voltage in *Helix* neurones (Akaike *et al.*, 1978) and also to that observed on the threshold potential for Ca^{2+}-spike in barnacle muscle fibre membrane (Hagiwara & Takahashi, 1967). The most satisfactory explanation for these results is the binding model proposed by Hagiwara (1975). The surface of the membrane probably has negative fixed charges (Ling & Ochsenfeld, 1965), and with increasing external divalent cation concentrations, the cations are absorbed at this surface and form a compact electric double layer. The surface charge of the membrane, thus neutralized by the occupying divalent cations, requires more positive potential to activate I_{Ca}, and at the same time, these cations occupy the binding site for Ca^{2+}. The null potential seemed to be less positive, but the difference is uncertain because of relatively large leakage currents at large depolarizing potentials. The inhibitory effectiveness had the order $Ni^{2+} > La^{3+} > Mn^{2+} > Co^{2+}$ in the presence of Ca^{2+} in *Helix* neurones. This order is slightly different from that found in a lobster giant axon (Blaustein & Goldman, 1966) and a barnacle muscle fibre (Hagiwara & Takahashi, 1967).

According to previous work, internal perfusion of Ca^{2+} and Mg^{2+} blocks the Ca^{2+}-spike in a barnacle muscle fibre (Hagiwara & Nakajima, 1966) and I_{Ca} in a *Helix* neurone (Akaike *et al.*, 1978). The present experiments have shown that both the organic and inorganic Ca^{2+}-antagonists also inhibit I_{Ca} upon internal application. The membrane sensitivity to internal application of the organic Ca^{2+}-antagonists was less than that to external application and it seemed that it took more time to inhibit I_{Ca}, when applied internally. It is difficult to explain the results. However, possible explanations for this can be offered: (1) if it is assumed that the membrane is permeable to internally applied verapamil or diltiazem because of the high lipophilicity of these agents (Bayer, Rodenkirchen, Kaufmann, Lee & Henekers, 1977; Rosenberger & Triggle, 1978), then a small amount of the agent having passed through the membrane from the intracellular phase to the external space would act on the site for Ca^{2+} on the surface of the membrane and thus the inhibition of I_{Ca} would have been resulted, or (2) the agent could have occupied the Ca^{2+}-channel somewhere between the outer surface and inner phase of the membrane or from the inside of the membrane. Internal application of Ni^{2+} inhibited I_{Ca} more markedly than organic Ca^{2+}-antagonist. The mode of action of internally applied Ni^{2+} on I_{Ca} is similar to that of an increase in $[Ca^{2+}]_i$; the null potentials became less positive at higher $[Ca^{2+}]_i$, and the threshold for I_{Ca} shifted in a hyperpolarizing direction. It is unlikely that internally applied Ni^{2+} acts at the external Ca^{2+} binding site. Therefore, it is assumed that the presence of divalent cations *per se* would act as a modifier of I_{Ca}. This assumption can be supported by previous observations that an increase in $[Ca^{2+}]_i$, in addition to shifting the null potential of I_{Ca} according to a Nernst-type relation, further sup-

pressed peak I_{Ca} in a way not predicted from either constant field or absorption isotherm theory (Akaike *et al.*, 1978). In this respect, further characterization of the effects of internal application of inorganic Ca^{2+}-antagonists is required.

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