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Effects of cadmium on the slow inward current of frog heart muscle in relation to a lowering of pH in external solution

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1 The effect of cadmium (Cd) on the slow inward current (I_{si}) of frog atrial fibres was studied by the double sucrose gap technique.

2 Cd (5 μM) depressed I_{si} in a voltage-dependent manner without alteration of the apparent reversal potential for I_{si}.

3 Dose-response curves indicated an apparent dissociation constant for the Cd blocking effect of 4.5 μM at 0 mV, with a one to one relationship between Cd and the slow channel.

4 Increasing the external concentration of Ca ions ($[Ca]_o$) in the tetrodotoxin (TTX)-containing Ringer solution antagonized the block of I_{si} by Cd. Double reciprocal plots for I_{si} versus $[Ca]_o$ drawn in the presence or in the absence of Cd intersected at the ordinate, indicating that Cd competes with Ca for a common binding site.

5 Lowering the external pH from 7.3 to 6.3 depressed I_{si}. The block caused by H was voltage-dependent. Double reciprocal plots for I_{si} versus $[Ca]_o$ drawn at pH 7.3 and 6.3 intersected at the abscissa, and indicated that H and Ca did not compete for a common site.

6 Lowering the external pH did not change the ability of Cd to inhibit I_{si}.

7 The data suggested the existence of two different sites within the slow channel in frog atrial fibres, one of them being H-sensitive and the other cadmium-sensitive.

Introduction

Diverse accounts of the toxic action of cadmium (Cd) in the cardiovascular (Williams *et al.*, 1978; Kopp *et al.*, 1982; Kopp, 1986) and in the central nervous system (Arvidson, 1981) have been given. Cd ions have been found to be a selective blocker of the slow inward current (I_{si}) in various species (Kostyuk *et al.*, 1977; Lee & Tsien, 1982; Giles *et al.*, 1983; Josephson *et al.*, 1984; Gautier *et al.*, 1987). In addition, H ions, known to depress I_{si} in heart muscle (Chesnais *et al.*, 1975), have also been found to cause a dramatic enhancement of the negative inotropic effect of Cd in frog cardiac muscle mechanical activity (Horiuchi & Hayashi, 1979). These findings prompted us to investigate the combinative effect of H and Cd ions on I_{si} in frog cardiac tissue. The study of concentration- and voltage-dependence of Cd and H blockade as well as the determination of the nature of I_{si} inhibition by H and Cd ions, provide information for characterizing and localizing ionic binding sites within the slow channel and hence, for understanding the mechanism of the Cd-induced cardiotoxicity.

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Methods

Current clamp and voltage clamp experiments were performed at 5–8°C on fine atrial trabeculae (75–150 μm in diameter, 2–4 mm in length) isolated from heart muscles of *Rana esculenta*. The double sucrose gap technique with vaseline seals was used (Rougier *et al.*, 1968). The experimental set up was similar to that described by Sauviat & Suchaud (1981).

The composition of the Ringer solution was (mM): NaCl 110.5, KCl 2.5, $CaCl_2$ 2; HEPES buffer (5 mM), pH 7.3. In pH experiments, HEPES was replaced by MOPS buffer (5 mM). In the present study, the effect of Cd (used as $CdCl_2$) on the slow conductance was

studied. Thus, the Ringer control solution contained tetrodotoxin (TTX, 0.57 μM) at a concentration that inhibits Na conductance (Sauviat, 1981) and 1 mM Cd was added to the control solution to block completely I_{si} (Gautier *et al.*, 1987). In all experiments, measurements were made when the preparations reached an apparent steady-state, that is after 1 min in test solution.

Membrane currents were measured as net inward or outward currents if not otherwise specified. Starting from a holding potential (HP), the potential of the test node was displaced in rectangular steps at a rate of 0.2 Hz; positive potentials correspond to depolarization. In current-voltage relationships (I–V curves), outward current corresponds to positive current and depolarizations to positive potentials applied from HP. The apparent reversal potentials (E_{rev}) for I_{si} were determined as the intersection of the positive slope of I–V curves drawn in the absence and in the presence of the studied cation in the control solution and after total blockade of the current by the appropriate inhibitor (Gautier *et al.*, 1987). According to Horackova & Vassort (1979), the double sucrose gap technique does not seriously affect the measurement of either slow inward current or tension, or the accuracy of voltage-tension-current relationships since only a very small deviation (a few mV) of the intracellularly recorded potential from the applied clamp potential occurred during the initial 5 to 10 ms of the imposed clamp. Nevertheless, we have determined the series resistance (R_s) values at the beginning and at the end of each experiment to check that the correction of the I–V curves, by taking into account the voltage drop across R_s, did not modify results described below and particularly shifts of I–V curves such as those shown in Figures 1 and 3. We have considered only experiments in which R_s did not change. Transmembrane potentials and currents were either displayed on an oscilloscope (Tektronix 5110) or recorded on a magnetic tape recorder (3M) by means of an 8 bit data acquisition system (Datalab) and plotted on an X–Y plotter (Ifelec) or photographed for further analysis. Data analysis was performed by means of a Hewlett Packard desk computer 9826. Calculations are expressed as mean values ± s.e. mean; (*n*) corresponds to the number of experiments.

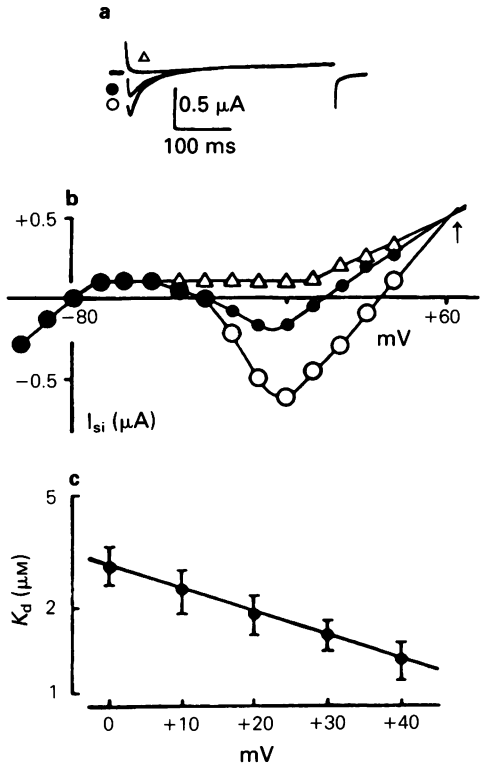

Figure 1 (a) Superimposed records of I_{si} recorded for a 80 mV depolarizing pulse applied from HP = −80 mV in tetrodotoxin (TTX)-containing solution before (○), after 1 min Cd (5 μM) treatment (●) and a further Cd (1 mM) addition to the Cd-containing solution (△). (b) Current-voltage relationships of I_{si} in (○) TTX-containing Ringer solution; (●) Cd (5 μM)-containing control solution. The curve indicated by (△) was recorded after addition of Cd (1 mM) to the control solution. The arrow indicates the point at which E_{rev} was measured. (c) Voltage-dependence of the apparent dissociation constant K_d for Cd blockade. K_d values obtained from equation (1) are plotted against membrane potential on semi logarithmic scale. Straight line was drawn according to the following regression line: $y = -0.01x + 5.7$ ($r = 0.99$). Vertical bars represent s.e. mean for $n = 6$.

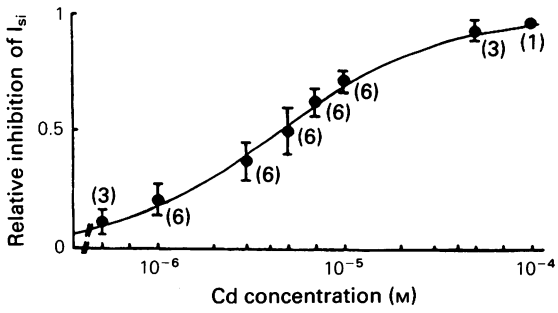

Figure 2 Log concentration-response relationships of the effect of Cd (abscissa scale) on I_{si} amplitude (ordinate scale). The theoretical concentration-response curve which fits the experimental data was drawn according to Langmuir equation with $m = 1$ and $K_d = 4.5$ μM. The number beside each point represents the number of experiments performed; vertical lines represent s.e. mean.

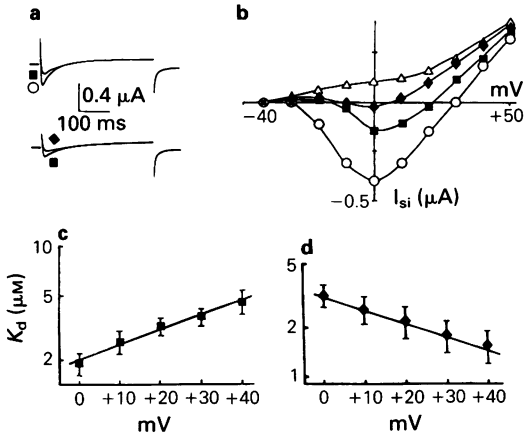

Figure 3 (a) Superimposed records of I_{si} recorded in the tetrodotoxin (TTX)-containing Ringer solution for a 70 mV depolarizing pulse applied from HP = −80 mV. Upper traces (○) pH 7.3; (■) pH 6.3; lower traces (■) pH 6.3; (◆) pH 6.3 Cd (5 μM)-containing solution. (b) Effects of change in external pH from 7.3 (○) to 6.3 (■) on the current-voltage relationships of I_{si}. The other I–V curves drawn for I_{si} were obtained in the presence of 5 μM (◆) and 1 mM (△) Cd respectively in the TTX-containing solution at pH 6.3. (c and d) Voltage-dependence of K_d for H block (c) and Cd block in pH 6.3 medium (d). The method for obtaining these plots is identical to that described in Figure 1c. Straight lines were drawn according to the following regression line equations: (c) $y = 0.01x + 5.5$ ($r = 0.98$); (d) $y = 0.01x + 5.7$ ($r = 0.99$). Vertical bars represent s.e. mean for $n = 6$.

Results

Voltage- and concentration-dependence of Cd block of I_{si}

Figure 1a shows that Cd (5 μM) reduced the magnitude of I_{si} by 53% within 1 min while the addition of Cd (1 mM) to the cation-containing solution entirely suppressed the remaining current without a marked influence on the later outward current that is presumably carried in a large part by K ions. A typical current-voltage relationship for the effect of Cd on I_{si} is presented in Figure 1b. Cd (5 μM) reduced the amplitude of the current and did not modify significantly E_{rev} (+60 ± 1 mV, $n = 6$). Moreover, I_{si} (measured by subtracting the current recorded at the peak time before and after total blockade by Cd (1 mM)) was inhibited by 39%, 64% and 73% at membrane potential values of −20 mV, 0 mV and +10 mV respectively. Indeed, the I–V curves plotted in Figure 1b indicate that the inhibition of I_{si} induced by Cd developed in an apparent potential-dependent manner compared to the I–V curve drawn in the control solution. Without making any specific assumptions about the molecular events, the potential-dependent block of I_{si} by Cd can formally be described by assuming that the equilibrium dissociation constant $K_d = k_2/k_1$ of the reaction between the ion (X) and receptor (R) changes with the depolarization.

$$X + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \cdot R$$

From the voltage-dependence of the inhibitory effect of Cd, we calculated the voltage-dependence of the equilibrium dissociation constant:

$$K_d = k_2/k_1 = (a/(1 - a)) \cdot [X] \qquad (1)$$

a being the current ratio ($I_X/I_{control}$) between the current recorded in the presence (I_X) and in the absence of Cd in the control solution ($I_{control}$) and ([X]) the cation concentration. The voltage-dependence of Cd has been described more fully in Figure 1c by plotting in semilogarithmic scale K_d values against membrane potentials in the range 0 to +40 mV where the current is fully activated (Lenfant *et al.*, 1972). The data are best fitted by a straight line with a correlation coefficient ($r = 0.99$) which indicates the existence of a dependence of K_d upon membrane potentials; K_d determined at zero membrane potential is 2.88 μM in Figure 1c.

The dose-response curve for the inhibitory effect of Cd on I_{si} is shown in Figure 2. The experimental points were best fitted by use of the Langmuir equation $Y = Y_{max}[X]^m/(K_d + [X]^m)$ where Y is the percentage of I_{si} inhibition, [X] the concentration of Cd, K_d the apparent dissociation constant (the average value of K_d was: $K_d = 4.5 \pm 0.9$ μM, $n = 5$), Y_{max} being taken as 100% and m the stoichiometric parameter (m = 1 in Figure 2).

Effect of Cd at low extracellular pH

In order to examine the dependence of the Cd inhibitory effect upon acidic pH, we have first studied the effect of lowering the external pH of the TTX-containing Ringer solution on I_{si}. The upper trace of Figure 3a shows that a reduction in the extracellular pH in the control solution from 7.3 to 6.3 inhibited I_{si} by about 50%. Current-voltage relationships plotted for I_{si} in Figure 3b show that lowering the pH of the control solution resulted in a decrease in I_{si} associated with a shift in voltage range of I_{si} activation toward more positive membrane potentials. Current ratios ($I_{pH\,6.3}/I_{pH\,7.3}$) varied with the amplitude of the depolarization; the current being more inhibited in the range −30 to −10 mV than at more

positive membrane potentials. Moreover, E_{rev} ($+64 \pm 0.8$ mV, $n = 6$) was not significantly changed by acidic pH. Afterwards, the addition of Cd (5 μM) to the pH 6.3 solution reduced the remaining I_{si} by 50% as shown in the lower traces of Figure 3a. The I–V curves in Figure 3b indicate that the inhibition induced by Cd (5 μM) at pH 6.3 developed in an apparent potential-dependent manner compared to I–V curve drawn at pH 6.3. To characterize the potential-dependence of both the H binding reaction and Cd binding reaction at low pH, we have followed the same procedure as the one used in Figure 1c. Figures 3c and 3d illustrate the dependence of K_d upon membrane potential at pH 6.3 in the absence and in the presence of Cd respectively. In both figures, values of logarithm of K_d plotted versus voltage are best fitted by a straight line with correlation coefficients of 0.98 and 0.99 respectively, indicating the existence of a dependence of K_d upon membrane potential. At zero mV, K_d determined from theoretical straight lines is 2 μM and 3.1 μM in the absence and in the presence of Cd respectively.

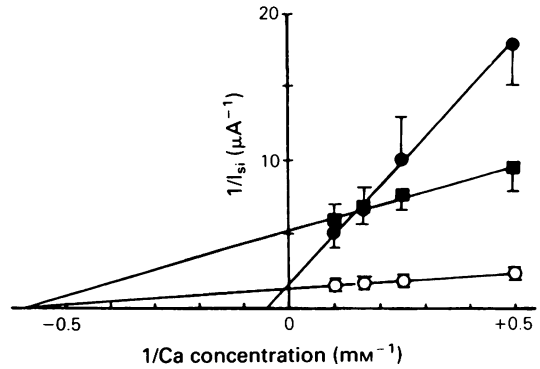

Figure 4 Lineweaver-Burk plot for Cd and H. Points were obtained by varying $[Ca]_0$ in the tetrodotoxin (TTX)-containing Ringer solution: (○) pH 7.3; (●) pH 7.3, Cd (10 μM)-containing medium; (■) pH 6.3. Straight lines were drawn according to the following regression line equations: (○) $y = 0.13x + 1.28$; (●) $y = 0.001x + 1.437$; (■) $y = 0.47x + 5.17$. I_{si} was elicited by a fixed voltage step 80 mV applied from the HP = −80 mV. $K_m = 1.67$ mM; $K_{m'} = 23.6$ mM; $K_{m''} = 1.72$ mM. Vertical bars represent s.e.mean for $n = 6$.

Nature of the inhibitory effect of Cd and H on I_{si}

In order to test the nature of the inhibition of the slow channel by Cd and H we used the following procedure. For a given depolarizing pulse, external Ca concentration ($[Ca]_0$) was increased in the TTX-containing Ringer solution from 2 to 10 mM before and after Cd (10 μM) exposure and also after changing the external pH from 7.3 to 6.3. As the Ca influx through the slow channel exhibits saturation kinetics, we have used the Lineweaver-Burk relationship to characterize the nature of the inhibition of I_{si} by Cd and H ions (Hagiwara, 1975). Figure 4 shows that Lineweaver-Burk plots drawn for I_{si} in the absence and in the presence of Cd intersected at the ordinate. Therefore, it can be assumed that Cd competed with Ca for the same receptor site by inhibiting I_{si}. According to Michaelis-Menten relationships, the half-saturation constants for Ca in the absence of Cd (K_m) and in presence of Cd ($K_{m'}$) are the negative reciprocal of the intercept of the straight line drawn for I_{si}^{-1} with the abscissa. The average values for K_m and $K_{m'}$ were 1.8 ± 0.4 mM ($n = 6$) and 23.6 ± 4.0 mM ($n = 6$) respectively. Figure 4 also shows that Lineweaver-Burk plots, drawn at pH 6.3, intersected at the abscissa with the plots drawn at pH 7.3. The average value of the half saturation constant for Ca at pH 6.3 $K_{m''}$ (2.0 ± 0.3 mM, $n = 6$) was of the same order of magnitude as the value obtained at pH 7.3. Therefore, it can be assumed that H did not compete with Ca for the same receptor site.

Discussion

In the present study, cadmium ions are shown to be selective blockers of I_{si} in frog heart muscle. The block of I_{si} by Cd developed without any change in the current time course and presented five main properties: (1) the inhibition of I_{si} by the cation was found to be potential-dependent; (2) the apparent reversal potential for I_{si} was unchanged suggesting that Cd did not alter the selectivity of the slow channel; (3) the stoichiometry of the relation between Cd and the slow channel is one to one; (4) the block of the slow channel by Cd is independent of the external pH; (5) the inhibitory effect of Cd on I_{si} was reversed by an excess of Ca ions. The block of I_{si} by Cd affected only the inward directed component of the membrane current, which developed during the first 100–150 ms, whereas the outward current which developed later, remained unaltered whatever the membrane potential investigated. Thus we did not find any Cd-resistant slow inward current in our experiments as previously reported by Hume & Giles (1983) in frog atrial cells and by Lee *et al.* (1984) and Nilius *et al.* (1985) in mammalian cardiac cells. However, in I–V curves, we observed that the current recorded between membrane potentials −40 to −10 mV, seems to be more resistant to Cd ions than the current recorded at more positive membrane potentials. This can account for the potential-dependent block of I_{si} by Cd.

The present results concerning the pharmacological properties of the slow channel give rise to several points. One of them concerns the existence of an antagonism between Cd and Ca within the slow channel. Evidence for blockade at a common site for Ca comes from the observation that the inhibitory action of Cd on I_{si} could be overcome by increasing $[Ca]_o$. One of the simplest interpretations of Lineweaver-Burk plots is that Cd ions do not alter the maximal Ca entry, but increase the apparent half-saturation constant by competing with Ca. The antagonism between Ca and Cd suggests the existence of a divalent cation-sensitive site within the channel. A similar Cd-Ca competition has already been described in synaptosomes (Nachshen, 1984) and appeared to be one of the main properties of divalent cations such as Co and Ni (Akaike *et al.*, 1981).

The behaviour of the slow channel at acidic pH is the second point of interest. Lowering the external pH was found to block I_{si} (Chesnais *et al.*, 1975; Uehara & Hume, 1985). The shift observed in voltage range of I_{si} activation may be due to neutralization of negative surface charges located at the mouth of the slow channel as the result of the protonation of 'non specific anionic groups', as has been proposed for the fast Na channel (Hille, 1975; Mozhayeva *et al.*, 1982) and the slow channel (Vogel & Sperelakis, 1977). Moreover, Lineweaver-Burk plots show that lowering the pH did not modify the half-saturation constant for Ca but reduced the maximal Ca entry; in other words, H did not compete with Ca ion for a common site. This last finding implies, at least, a modification of the interpretation of pH effects, in assuming that the protonation caused by an increase of the extracellular H ion concentration diminishes the Ca concentration at the mouth of the slow channel and, thereby, reduces or blocks the Ca inward current. Indeed, proton ions do not bind at specific Ca sites since inhibition of the sites with Ca ions is non-competitive. Moreover, we observed that lowering the external pH altered neither the efficiency of Cd inhibition on I_{si} nor the affinity of Cd for the site. The present results might account for the increase in the negative inotropic effect of Cd in low pH solution reported by Horiuchi & Hayashi (1979), since low pH media is shown to reduce markedly the maximal Ca entry while the ability of Cd to block I_{si} is unchanged. This might also suggest that the blocking effect of H and Cd ions on I_{si} was additive. Both inhibitory effects led to a consequent reduction of I_{si} and of the tension since, in frog heart, the quantity of Ca ions entering the cell upon depolarization was linearly related to peak phasic tension (Horackova & Vassort, 1976).

All the results lead us to suggest the existence of two kinds of sites involved in the cation permeation process within the slow channel: one is proton-sensitive and located at the external mouth of the channel; the other is inside the channel and Cd-sensitive.

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