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EFFECT OF EXTERNAL Cd^{2+} AND OTHER DIVALENT CATIONS ON CARBACHOL-ACTIVATED NON-SELECTIVE CATION CHANNELS IN GUINEA-PIG ILEUM

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SUMMARY

1. Inhibitory actions of the external divalent cations, Cd^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} on carbachol (CCh)-induced inward current ($I_{ns,ACh}$) were investigated in caesium aspartate-loaded single longitudinal smooth muscle cells of guinea-pig ileum, using a rapid solution-switching device, under voltage-clamped conditions.

2. Cd^{2+} (20–1000 μM), when added to the external solution, reduced $I_{ns,ACh}$ rapidly and reversibly. This effect occurred dose dependently, the relationship being adequately described by a Michaelis–Menten equation with a Hill coefficient (n) of 1·0 and a dissociation constant (K_d) of 98 μM.

3. The inhibitory action of Cd^{2+} was associated neither with agonist concentration (CCh) nor with changes in reversal potential, and was voltage independent.

4. The appearance and removal of the effect of Cd^{2+} were both rapid (a few hundred milliseconds), in sharp contrast with a relatively slow time course of atropine or CCh action (of the order of seconds).

5. Other divalent cations (Ni^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}) applied externally also suppressed $I_{ns,ACh}$, but less potently. The sequence of apparent potency was Cd^{2+} (98 μM) $\geqslant$ Ni^{2+} (131 μM) $\gg$ Co^{2+} (700 μM) $\geqslant$ Mn^{2+} (1000 μM) $\gg$ Mg^{2+} ($\sim$ 10 mM).

6. External Ca^{2+} increased $I_{ns,ACh}$ dose dependently and antagonized the inhibitory effect of Cd^{2+}. However, this effect may not be a simple competition with Cd^{2+}.

7. These results show that external divalent cations strongly modulate $I_{ns,ACh}$ channels, possibly through direct interaction with the channel protein.

INTRODUCTION

Heavy metal ions such as Cd^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+}, mainly owing to their molecular similarity to Ca^{2+}, induce a variety of cellular consequences. In smooth muscle, it is known that such cations can affect voltage-operated Ca^{2+} channels

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(Klöckner & Isenberg, 1985; Ohya, Terada, Kitamura & Kuriyama, 1986), Na^+–Ca^{2+} exchange (Aickin, Brading & Walmsley, 1987), Na^+–K^+–ATPase (Tokushige, Higashino, Searle, Tamura, Kino, Bogden & Aviv, 1984), Ca^{2+}–Mg^{2+}–ATPase (Sumida, Hamada, Takenaka, Hirata, Nishigauchi & Okuda, 1986), other intracellular enzymic activities (Chan, Gallis, Blumenthal, Paller, Wan & Krebs, 1986) and even the contractile system (Nasu & Ishida, 1986). In most previous electrophysiological studies, such divalent cations have been regarded as voltage-operated Ca^{2+} channel (I_{Ca}) blockers and they have been used to separate receptor-operated Ca^{2+} influx and/or neurotransmitter-induced Ca^{2+} release from the internal store sites (sarcoplasmic reticulum; SR), from Ca^{2+} influx via I_{Ca} (Bolton, 1979; Brading & Sneddon, 1980). Little attention has been paid to a possible direct role of external divalent cations on the receptor-operated channels in smooth muscle.

On the other hand, it is now generally accepted in other tissues that external divalent cations Mg^{2+}, Zn^{2+}, Cd^{2+} and even Ca^{2+} strongly modulate neurotransmitter-activated channels (e.g. *N*-methyl-D-aspartate (NMDA) channels, Westbrook & Mayer, 1987; GABA-activated channel, Kaneko & Tachibana, 1986; L-glutamate-activated channels, Hatt, Franke & Dudel, 1988).

In a preliminary stage of the present work, I noticed that external La^{3+} appears to strongly augment the $I_{ns,ACh}$ channel and this effect can be antagonized by Cd^{2+} or Ni^{2+} (Inoue, 1990). The possible role of divalent cations has also been suggested by the observation that Mn^{2+} in mM concentrations greatly suppressed ACh-induced depolarization in single smooth muscle cells dispersed from ileum muscle, associated with a reduction in $I_{ns,ACh}$ (Inoue, Kitamura & Kuriyama, 1987*a*; see also Mironneau, Mironneau & Savineau, 1984).

In this paper, I examine how externally applied divalent cations, Cd^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and physiological ions Ca^{2+} and Mg^{2+}, affect the $I_{ns,ACh}$ channel conductance using a rapid solution-switching device. Furthermore, some mechanistic analysis is attempted in the case of Cd^{2+}, since this ion is the most potent at inhibiting $I_{ns,ACh}$, and is used at low concentrations to eliminate ambiguities arising from non-specific effects of divalent cations, e.g. the membrane-stabilizing effect (Frankenhaeuser & Hodgkin, 1957).

METHODS

Cell isolation. Guinea-pigs of either sex weighing over 600 g were exsanguinated after stunning. The terminal part of the ileum was promptly excised and equilibrated in a Ca^{2+}-free solution which was oxygenated and pre-warmed to 36 °C. Pieces of the longitudinal muscle were prepared according to the method of Paton & Rang (1965). A segment of the ileum 2–3 cm long was slid onto a glass pipette (1 cm in diameter). Two shallow incision lines were made with a sharp blade and a thin sheet of longitudinal muscle was carefully stripped away in the circular direction by gentle strokes using saline-soaked cotton wool. Resultant sheets of muscle were further incubated in Ca^{2+}-free solution to obtain full relaxation, and exposed to two consecutive 'enzyme' solutions (solutions 1 and 2; see below) for 10–15 min at 36 °C. The degree of digestion was judged by the release of spindle-shaped cells on shaking the solution. Finally, digested sheets of muscle were cut into small pieces and then agitated with a blunt-tipped pipette. The cell suspension obtained was diluted 1:1 with the KB medium (see below) and stored in an ice box until used. After settlement of cells on the floor of the experimental chamber, viable cells were identified by their response to CCh ejected from a large pipette. All experiments were carried out at room temperature.

Rapid solution-switching device. Prolonged exposure of a single cell to cholinergic agonists caused

slow desensitization, which became most evident at high concentrations. Furthermore, repeated applications of the agonist in most cases resulted in a marked reduction in the response (run-down), particularly when the intracellular Ca^{2+} ion was buffered with relatively high concentrations of EGTA or BAPTA (1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid). This led me to employ a much faster solution-change system than bath application, in order to examine more clearly the effects of various solutions on the same cell. After trial and error, the so-called 'sewer-pipe' system turned out to be very useful for this purpose, as frequently used for the study of other agonist-activated currents (e.g. NMDA-activated channels, Mayer, Westbrook & Vyklický, 1988). Briefly, three tapering polyethylene tubes (tip diameter *ca* 200 μm) were joined in parallel with resin glue and placed on the bottom of the experimental chamber. Test solutions were allowed to flow continuously from the outlets by gravity. A voltage-clamped cell was exposed in turn to the solutions by shifting the electrode swiftly. A change of solution could be achieved within 100–200 ms in successful cases.

Solutions. The current, $I_{ns,ACh}$, was dissected by use of caesium aspartate internal solution as described previously (Inoue & Isenberg, 1990*a*). In addition, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was clamped between 100 and 200 nM by means of intracellular dialysis via the patch pipette containing 10 mM-EGTA or BAPTA and additional Ca^{2+} to give the desired concentration. Under these conditions nearly 50 % of $I_{ns,ACh}$ should be activated and possible changes in the $[Ca^{2+}]_i$ minimized. Any changes in $[Ca^{2+}]_i$ caused by divalent cations might otherwise result in large modifications of $I_{ns,ACh}$ (Inoue & Isenberg, 1990*c*). An approximate value of $[Ca^{2+}]_i$ was determined using Fabiato & Fabiato's formula (1979). However, even using cells with high buffering capacity, occasionally one contracted during the prolonged application of CCh, particularly when the input resistance of the patch pipette exceeded 5 MΩ. Such data were considered invalid and not included in the evaluation.

The composition of the external physiological saline solution (PSS) is given in mM: 140 Na^+, 5 K^+, 2 Ca^{2+}, 1·2 Mg^{2+}, 151·4 Cl^-, 10 HEPES/Tris (pH 7·35–7·4). KB medium consisted of (mM): 85 KCl, 30 K_2HPO_4, 2 Na_2ATP, 5 $MgCl_2$, 5 pyruvic acid, 5 creatine, 20 taurine, 5 succinic acid 10 glucose, 1 EGTA, 10 HEPES and bovine serum albumin (1 mg/ml). Enzyme solution 1 contained pronase (0·5–1·0 mg/ml; type E, Fluka, UK) in Ca^{2+}-free PSS. For enzyme solution 2, two collagenases (1·0 mg/ml, type 1, Sigma; 0·5 mg/ml, Boehringer), trypsin inhibitor (1·0 mg/ml, Sigma) and bovine serum albumin (1·0 mg/ml, Sigma) were dissolved in PSS, free of divalent cations, whose osmolarity was reduced by *ca* 10 % by adding distilled water. This intervention seemed to prevent cells from shrinkage during the course of digestion.

Experimental equipment. The system used in the present experiment was essentially the same as described previously (Inoue & Brading, 1990). A patch-clamp amplifier (EPC7, List Electronics, Darmstadt, Germany) was used to apply voltage signals to cells through a patch electrode made of borosilicate glass (Clark Electromedical Instruments, UK). After filtering at a cut-off frequency of 10 kHz, the signals collected from the cells were digitized using a pulse code modulator (12- or 16-bit resolution, Sony PCM-701, Tokyo, Japan) for later analysis. In some experiments, an A/D, D/A converter (DT2801A, Data translation, UK) was employed, for the generation of voltage signals (ramp and pulses) and data acquisition (digitized at 2 kHz after filtering at 1 kHz) under the control of an IBM compatible computer using programs written in QUICK BASIC 6·0.

Statistics

All data are expressed as means ± standard error of the mean.

RESULTS

ACh- or CCh-induced currents recorded with caesium aspartate internal solution flowed through non-selective cation channels ($I_{ns,ACh}$) as shown previously (Inoue, Kitamura & Kuriyama, 1987*b*; Inoue & Isenberg, 1990*b*). In the continued presence of CCh (100 μM), rapid addition of Cd^{2+} into the bath resulted in a sudden reduction in the amplitude of $I_{ns,ACh}$ (Fig. 1*Aa*).

The inhibitory effect of Cd^{2+} shows at least three features. (1) The onset and offset of the Cd^{2+} effect is rapid and reversible. In satisfactorily rapid solution switching,

the time needed to obtain a steady state did not exceed 100–200 ms, and seemed merely restricted by the speed of manual shifting of the electrode. (2) The effect occurs as a function of Cd^{2+} concentration (compare 20 and 100 μM-Cd^{2+} in Fig. 1*A a*). (3) The reduction in $I_{ns,ACh}$ is associated with a decrease in the noise level (Fig. 1*A b*).

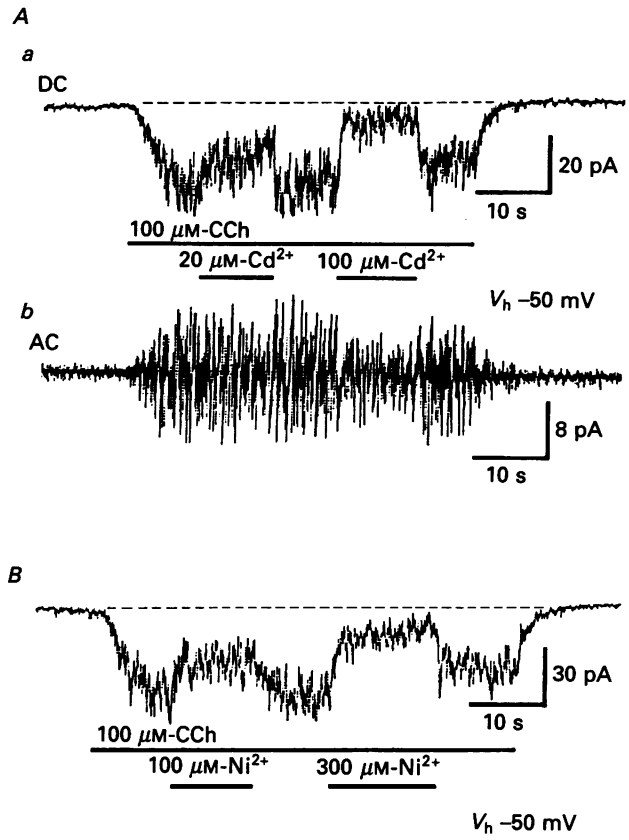

Fig. 1. Cd^{2+} and Ni^{2+} inhibit $I_{ns,ACh}$ channel current. *A*, in the continued presence of 100 μM-carbachol (CCh in PSS), an electrode bearing a voltage-clamped cell was swiftly moved at bars into 20 μM-Cd^{2+} and later into 100 μM-Cd^{2+}. For AC noise, the current was filtered between 0·5 and 100 Hz. *B*, Ni^{2+} (100 μM, 300 μM) was tested in the same way as in *A*.

Other divalent cations Ni^{2+}, Mn^{2+} and Co^{2+}, which resemble Cd^{2+} in nature, also exhibited a similar effect on $I_{ns,ACh}$, but required somewhat higher concentrations to attain the same degree of inhibition as Cd^{2+} (Figs 1*B* and 8).

In the following sections, the mechanisms underlying the inhibitory action of divalent cations are investigated, especially for Cd^{2+}.

Dose–inhibition curve for Cd^{2+} action

The inhibitory effect of Cd^{2+} obeyed the law of mass action, i.e. the dose–inhibition relationship was adequately fitted by the Michaelis–Menten equation (Fig. 2*A*). The best fit of data points at a holding potential of −50 mV (○) gave a Hill coefficient (n) of 1·0 and a dissociation constant (K_d) of 98 μM (continuous curve). These values are

also valid for −25 mV (●), thus suggesting little involvement of the membrane potential in Cd^{2+} action. The K_d of 98 μM is very low with respect to the stabilizing effect of divalent cations, and suggests the interaction of Cd^{2+} with some specific binding sites related to the $I_{ns,ACh}$ channel.

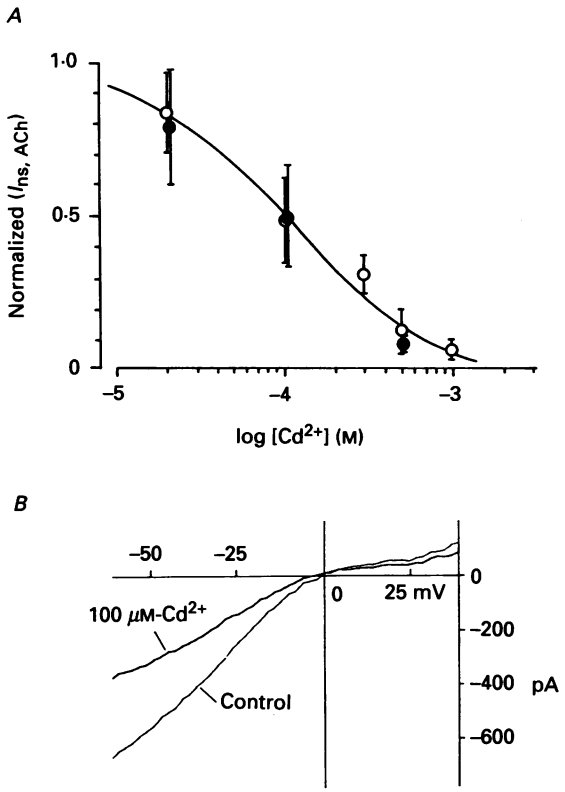

Fig. 2. *A*, dose–inhibition curve evaluated for Cd^{2+} from experiments similar to those shown in Fig. 1. Maintained application of CCh over 10 s caused a slow progressive decay in the amplitude of $I_{ns,ACh}$, probably due to desensitization. Therefore, the change in amplitude of $I_{ns,ACh}$ caused by divalent cations was evaluated from the ratio of mean current levels (this was estimated by eye or by severe low-pass filtering of the current) just before and after the application of divalent cations. ○, holding potential of −50 mV; ●, holding potential of −25 mV. All data points represent the results from five to thirteen experiments. The continuous curve was drawn by non-linear regression with an equation, $1/\{1+(C/K_d)^n\}$, where C denotes the concentration of Cd^{2+} (μM), and K_d and n are 98 μM and 1·0, respectively. *B*, *I–V* curves of CCh-sensitive current evaluated by a fast-falling ramp of 100 ms in the presence of nifedipine (10 μM). The ramp started from +40 and fell to −60 mV after a 600 ms depolarization to 0 mV. CCh-sensitive currents were defined as the difference of net currents in the presence and absence of CCh (100 μM) (see also Inoue & Isenberg, 1990*b*). Both curves derived from the same cell.

Reversal potential

Figure 2*B* shows a quasi-instantaneous *I–V* curve of $I_{ns,ACh}$ evaluated using a 100 ms ramp potential. Two effects can be observed. First, Cd^{2+} (100 μM, close to K_d value) decreased $I_{ns,ACh}$ in a proportional manner over a wide range of membrane

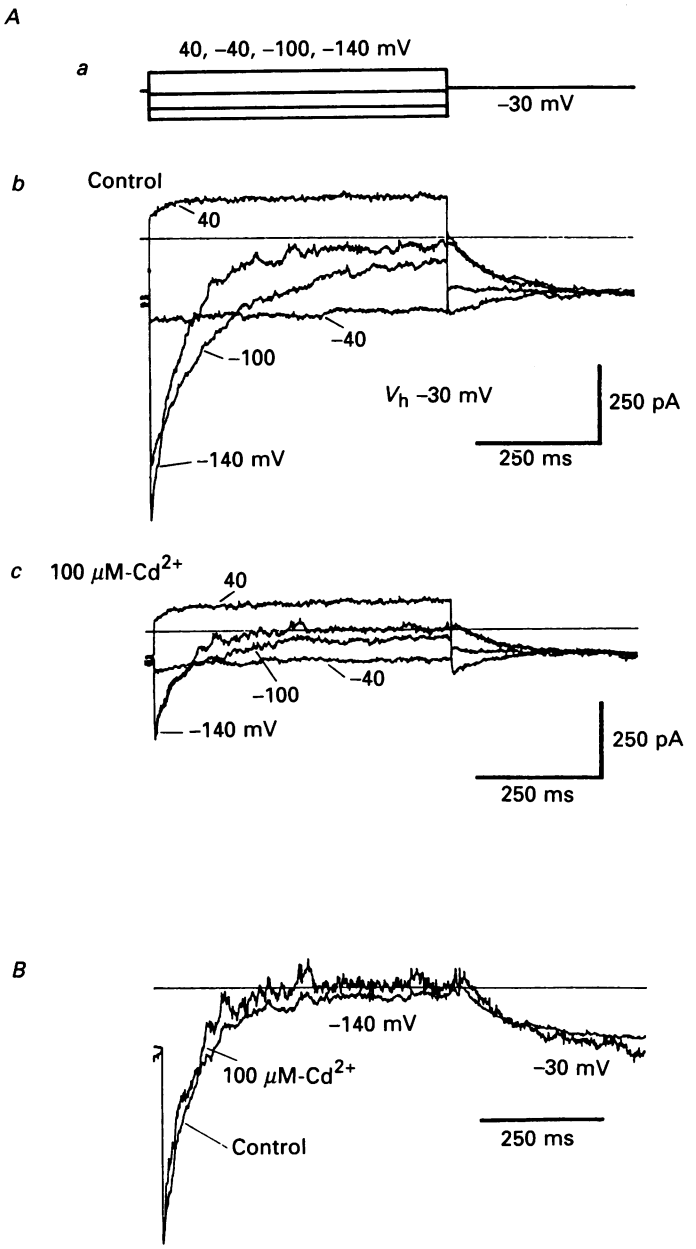

Fig. 3. $I_{ns,ACh}$ relaxations during voltage jumps. *A*, from a holding potential of −30 mV, 600 ms voltage pulses to various membrane potentials (*a*) were applied in the absence and presence of CCh (100 μM). I_{Ca} was blocked by 10 μM-nifedipine. Examples of CCh-sensitive currents (the difference of net currents in the presence and absence of CCh) are displayed at −140, −100, −40 and 40 mV (*b*). Addition of 100 μM-Cd^{2+} reduced $I_{ns,ACh}$ (*c*). All traces are recorded from the same cell. Horizontal line indicates zero current level. Note that tail currents at −30 mV converge to the same level. *B*, normalized $I_{ns,ACh}$ at −140 mV from *A* (control and 100 μM-Cd^{2+}). The currents are normalized to their peaks and superimposed.

potential (but see Fig. 4*B*). Second, the presence of Cd^{2+} caused only a small, but not significant, negative shift in the reversal potential of $I_{ns,ACh}$ ($+2{\cdot}3 \pm 3{\cdot}0$ mV in PSS *vs.* $+0{\cdot}2 \pm 3{\cdot}7$ mV in 100 μM-Cd^{2+}-containing PSS, $n = 8$, respectively). These results suggest that the Cd^{2+} effect cannot be caused by alteration of ionic selectivity of $I_{ns,ACh}$.

Voltage-dependency

In previous work, I investigated voltage-dependent gating of $I_{ns,ACh}$ by observing relaxation of the current during an abrupt perturbation of the membrane potential (Inoue & Isenberg, 1990*b*), as performed for nicotinic receptor-operated channels (Adams, 1974). The relevance of the 'minimal reaction' model proposed is still uncertain since there is considerable shortage of information on the link between muscarinic receptors and the $I_{ns,ACh}$ channel. Nevertheless, the protocol used in the experiments is useful to assess whether Cd^{2+} can influence the voltage dependence of $I_{ns,ACh}$, because parameters obtained with such an approach should reflect its voltage-dependent behaviour. These properties were not significantly affected by 100 μM-Cd^{2+}. As demonstrated in Fig. 3*Ac*, Cd^{2+} decreased both instantaneous peak and steady-state level (end of the pulse) of $I_{ns,ACh}$, the extent of inhibition being almost independent of the membrane potential. The time course of relaxation was only modestly accelerated by Cd^{2+}, as indicated by normalized currents in both the absence and presence of Cd^{2+} (Fig. 3*B*).

Figure 4*A* shows instantaneous and steady-state *I–V* relationships averaged from six different experiments. In order to facilitate comparison between currents of different size, the current amplitude was normalized to that measured at a holding potential of -30 mV. As predicted by inspecting the actual records in Fig. 3, *I–V* curves in the absence and presence of Cd^{2+} superimpose both for instantaneous and steady state *I–V* relationships (○ *vs.* ●, △ *vs.* ▲). Furthermore, the steady-state activation curves of $I_{ns,ACh}$ hardly shifted in the presence of Cd^{2+}. Thus, the Boltzmann parameters, half-activation voltage ($V_{\frac{1}{2}}$) and slope factor (k) were not significantly different (Table 1).

In order to inspect more accurately the effect of Cd^{2+}, the percentage of $I_{ns,ACh}$ remaining after Cd^{2+} inhibition was plotted against the membrane potential (Fig. 4*B*). A slight relief of Cd^{2+} inhibition was observed at more positive potentials for steady-state current, but not for instantaneous current. This suggests that the site of Cd^{2+} binding may be close to the external surface of the sarcolemmal membrane.

In conclusion, proportional inhibition by Cd^{2+} is not associated with changes in its voltage-dependent gating parameters. This is largely different from an ion permeation blockade mechanism observed for various types of channels (e.g. Mg^{2+} block of NMDA channels; Nowak, Bregestovski, Asher, Herbert & Prochiantz, 1984), but cannot exclude the possibility that Cd^{2+} may occlude the outer mouth of $I_{ns,ACh}$ channel, thereby blocking ion permeation through the channel.

Effect of Cd^{2+} on CCh concentration–response relationship

The voltage-independent reduction in $I_{ns,ACh}$ might result from a decreased sensitivity or activation of muscarinic receptors. However, since $I_{ns,ACh}$ recorded with high $[Ca^{2+}]_i$ buffer usually shows 'run-down', it was difficult to evaluate the CCh

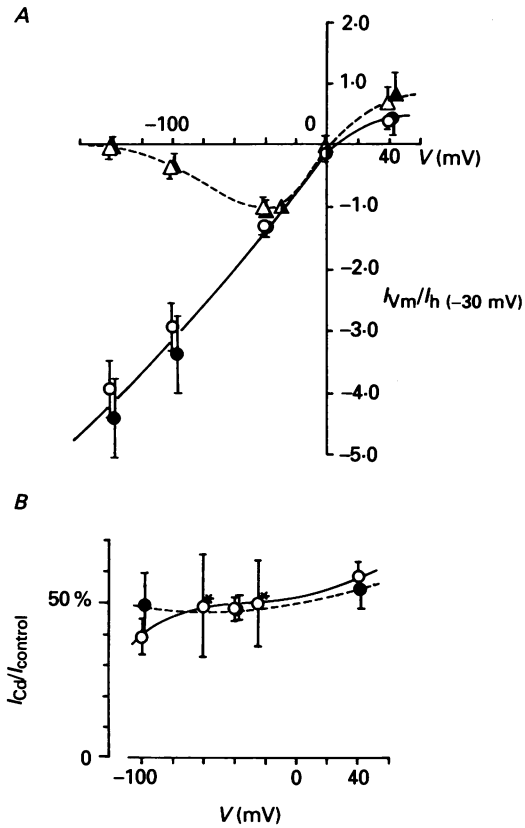

Fig. 4. *A*, instantaneous and steady-state *I–V* relationships of 100 μM-CCh-sensitive currents evaluated from six paired experiments (absence *vs.* presence of 100 μM-Cd^{2+} in PSS) in the presence of nifedipine (10 μM). The values are normalized to that at −30 mV (holding potential) and averaged. ○, △, in PSS; ●, ▲, 100 μM-Cd^{2+} in PSS. Circles and triangles represent instantaneous and steady-state (at the end of 600 ms pulse) values of $I_{ns,ACh}$, respectively. *B*, the $I_{ns,ACh}$ in the presence of 100 μM-Cd^{2+} (I_{Cd}) expressed as a percentage of the control at different holding potentials. ● and ○ represent the values for instantaneous peak and steady-state level, respectively (obtained from the voltage jump experiments of *A*). Asterisks indicate data from Fig. 2*A*.

TABLE 1. The parameters of steady-state activation curves in the absence and presence of 100 μM-Cd^{2+}

	Control (PSS)	PSS + 100 μM-Cd^{2+}
k (mV)	−21·4 ± 2·1 (n = 6)	−18·4 ± 3·2 (n = 6)
$V_{\frac{1}{2}}$ (mV)	−45·9 ± 4·6 (n = 6)	−44·6 ± 5·3 (n = 6)

Steady-state activation curves were fitted by a non-linear least-squares method using an equation, $p = 1/\{1+\exp[(V-V_{\frac{1}{2}})/k]\}$, where p, V, $V_{\frac{1}{2}}$ and k denote normalized current to that at 0 mV, the membrane potential, half-maximum activation potential and slope factor (see Inoue & Isenberg, 1990*b*)

concentration–$I_{ns,ACh}$ relationship from the same cell using repetitive applications of CCh. Therefore, a different approach was employed, i.e. the degree of inhibition caused by one concentration of Cd^{2+} (either 300 μM or 1 mM) was compared among

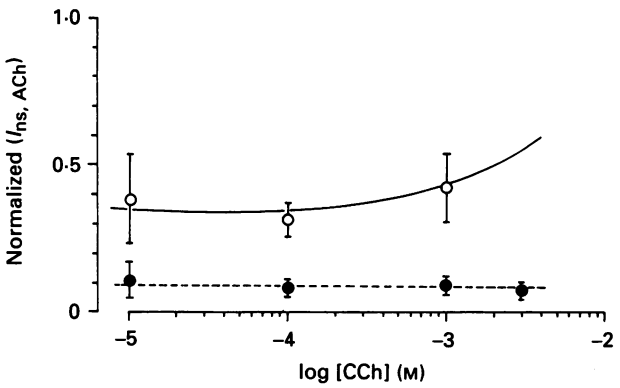

Fig. 5. CCh concentration does not affect Cd^{2+}-induced inhibition of $I_{ns, ACh}$. At a given concentration of CCh (10 μM–3 mM), the effects of 300 μM- and 1 mM-Cd^{2+} were tested on the same cell in a way similar to Fig. 1. In order to avoid the influence of run-down, only one concentration of CCh was tried in each cell. Each data point in the figure represents an average from more than six cells (n = 6–14). ○, the data with 300 μM-Cd^{2+}; ●, with 1000 μM-Cd^{2+}. At 1 μM-CCh, the amplitude of $I_{ns, ACh}$ was usually too small to evaluate correctly, and was not included. V_h was −50 mV. The curve was drawn by eye.

TABLE 2. Time constants (t) for activation and deactivation of $I_{ns, ACh}$ by 100 μM-CCh and those for 1 μM-atropine block of 100 μM-CCh-activated $I_{ns, ACh}$

	CCh (100 μM)	
$t_{on(50\%)}$	−25 mV	2·8 ± 1·3 s (n = 13)
$t_{off(50\%)}$	−25 mV	2·1 ± 0·7 s (n = 13)
$t_{on(50\%)}$	−50 mV	3·6 ± 1·1 s (n = 12)
$t_{off(50\%)}$	−50 mV	2·3 ± 0·9 s (n = 12)
	Atropine (1 μM) + CCh (100 μM)	
$t_{50\% block}$	−50 mV	4·2 ± 1·2 s (n = 4)
$t_{100\% block}$	−50 mV	13·7 ± 2·5 s (n = 4)

different concentrations of CCh (Fig. 5). This approach does not demand any assumption about the response ($I_{ns, ACh}$) as a function of CCh concentration, and is convenient for detecting changes in receptor activation. At CCh concentrations between 10 μM and 3 mM, there is no obvious change in the degree of inhibition by Cd^{2+}. This implies that the degree of receptor activation is not strongly affected by Cd^{2+}.

Time course of CCh and atropine actions

In another series of experiments, the time constants for activation and deactivation of $I_{ns, ACh}$ by CCh, and the time needed to diminish $I_{ns, ACh}$ by atropine, were measured. These parameters would reflect all of the processes participating in the coupling of muscarinic receptor to the channel, which may involve biochemical processes and G-proteins (Inoue & Isenberg, 1990*a*; Komori & Bolton, 1990). The results are summarized in Table 2. As can also be seen in Fig. 1, activation and deactivation of $I_{ns, ACh}$ occurred in the order of seconds, but atropine needed more

time to exert its antagonistic action. Considering that the onset and offset of Cd^{2+} inhibition occurs within a few hundred milliseconds, the target of Cd^{2+} action must be very close to the final effector, presumably the $I_{ns,ACh}$ channel itself.

Ca^{2+} antagonizes the inhibitory effect of Cd^{2+}

In contrast with the inability of membrane potential or CCh concentration to affect the Cd^{2+} inhibition, a physiological divalent cation, Ca^{2+}, appeared to

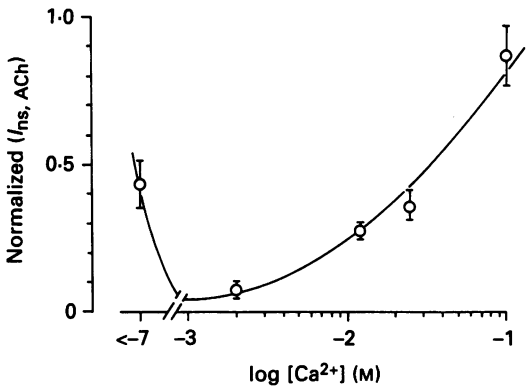

Fig. 6. Antagonism by external Ca^{2+} on the Cd^{2+} effect on $I_{ns,ACh}$. The degree of inhibition of CCh (100 μM)-activated $I_{ns,ACh}$ caused by 1 mM-Cd^{2+} is plotted against $[Ca^{2+}]_o$. The degree of inhibition was measured as a fraction of $I_{ns,ACh}$ in the presence of 1 mM-Cd^{2+} of the control ($I_{ns,ACh}$ activated by 100 μM-CCh in the absence of Cd^{2+} at a given value of $[Ca^{2+}]_o$). Each data point represents a mean calculated from several experiments (n = 7–13). External Na^+ concentration was kept to 140 mM, except for at 100 mM-Ca^{2+}, where no other cations were included. Below 2 mM-Ca^{2+}, Ca^{2+} was replaced by Mg^{2+}. In Ca^{2+}-free solution (i.e. pCa < −7), 0·1–0·3 mM-EGTA was added. The curve was drawn by eye.

antagonize it (Fig. 6). Raising the external concentration of Ca^{2+} ($[Ca^{2+}]_o$) relieved Cd^{2+} (1 mM)-induced inhibition of $I_{ns,ACh}$ in a dose-dependent manner, although omission of Ca^{2+} also resulted in significant attenuation of the Cd^{2+} effect. This observation need not necessarily be due to competitive antagonism between Cd^{2+} and Ca^{2+}. Using high $[Ca^{2+}]_o$ (over 10 mM), the stabilizing effect on the membrane and alterations in the permeability of $I_{ns,ACh}$ must be taken into account. In order to get more insight into the nature of this Ca^{2+} antagonism, the following experiments were attempted.

External Ca^{2+} augments $I_{ns,ACh}$

In this series of experiments, the possible influence of the membrane-stabilizing effect was minimized by keeping the total divalent cation concentration constant (i.e. $[Ca^{2+}]_o + [Mg^{2+}]_o$ = constant). Under this condition, increase in $[Ca^{2+}]_o$ clearly augmented the amplitude of $I_{ns,ACh}$. An example is shown in Fig. 7*A*, and is summarized graphically in Fig. 7*B*. The effect of lowering $[Ca^{2+}]_o$ resembled that for the Cd^{2+} effect. It is quick and reversible, and dose dependent. The shift of reversal potential of $I_{ns,ACh}$ was not pronounced, i.e. it was not larger than a few millivolts in all paired experiments where two different $[Ca^{2+}]_o$ (pCa = < −7·0 to −2·7) were tested

on the same cell. These observations suggest that external Ca^{2+} might regulate the $I_{ns,ACh}$ channel acting on the same site as Cd^{2+} does and therefore could antagonize the inhibitory effect of Cd^{2+}. However, in two particular experiments where voltage dependency was compared, in 2 mM-Ca^{2+}-containing and Ca^{2+}-free (replaced with

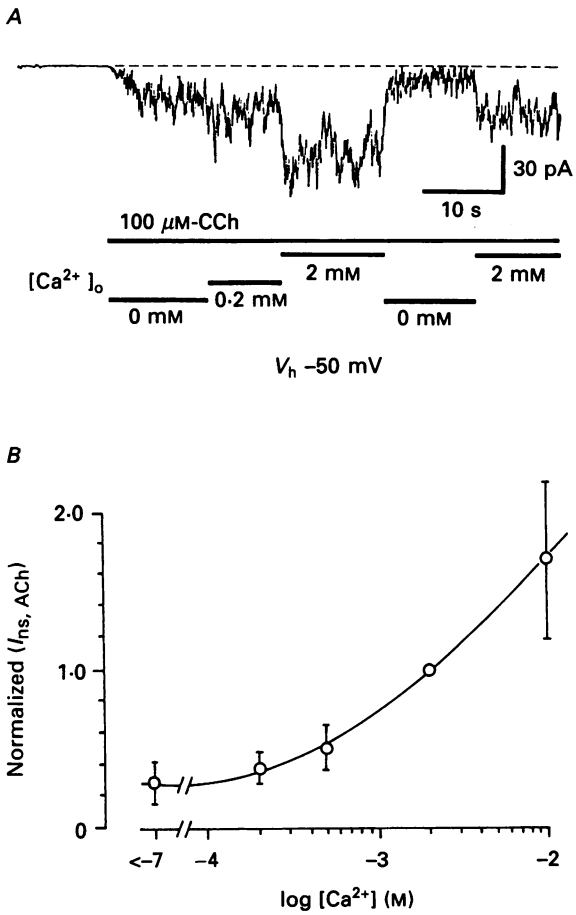

Fig. 7. Augmentation of $I_{ns,ACh}$ by external Ca^{2+}. *A*, actual record showing the effects on $I_{ns,ACh}$ of changing $[Ca^{2+}]_o$ to three different concentrations (0, 0·2, 2 mM). The sum of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ was kept to 3·2 mM. EGTA (0·1 mM) was used for the case of 0 mM-Ca^{2+}. *B*, the degree of activation of $I_{ns,ACh}$ is plotted against $[Ca^{2+}]_o$. The data were averaged from five to thirty-five experiments for each data point in the figure.

2 mM-Mg^{2+}) external solutions, the steady-state activation curve was shifted in a parallel fashion toward positive potentials by *ca* 15 mV in Ca^{2+}-free solution (i.e. negative shift on increasing $[Ca^{2+}]_o$). This shift could produce a decrease of $I_{ns,ACh}$ to 60–70% of the control (2 mM-Ca^{2+}), but can only partly account for the strong reduction observed in Ca^{2+}-free solution (29 ± 14% of the control). Taken together with the antagonism to Cd^{2+}, these results may imply that external Ca^{2+} can modify the gating properties of $I_{ns,ACh}$ channel as well as influence the channel in a similar way to Cd^{2+}.

Dose–inhibition curves for other divalent cations

Finally, dose–inhibition relationships were evaluated for other divalent cations (Ni^{2+}, Co^{2+}, Mn^{2+}) (Fig. 8). Fitting with a Michaelis–Menten equation gave K_d values of 131, 700 and 1000 μM for Ni^{2+}, Co^{2+} and Mn^{2+}, respectively. Mg^{2+} appeared

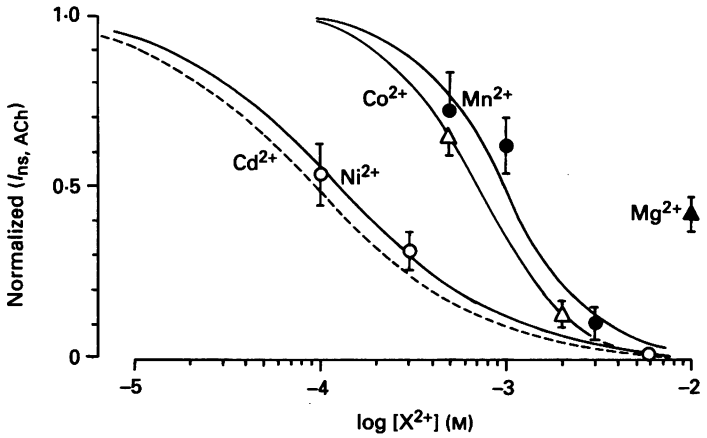

Fig. 8. Dose–inhibition curves of $I_{ns, ACh}$ for various divalent cations. Data were evaluated at −50 mV (n = 3–8 for each point). ○, Ni^{2+}; ●, Mn^{2+}; △, Co^{2+}; ▲, Mg^{2+}. Curves were drawn using the equation in Fig. 2*A*. Apparent values of K_d and n were 131 μM and 1·0, 700 μM and 1·8, and 1000 μM and 1·8 for Ni^{2+}, Co^{2+} and Mn^{2+}, respectively.

to require a fairly high concentration to produce half-reduction of $I_{ns, ACh}$ (only one concentration was tested; ▲). On the other hand, an n value of 1·0 was not seen with Co^{2+} and Ni^{2+}, namely their dose–inhibition curves were steeper (n = 1·8) than that for Cd^{2+} or Ni^{2+}. This might be a consequence of two effects superimposed, a Cd^{2+}-like effect and the membrane-stabilizing effect, the latter being more apparent at concentrations over 1 mM (see also Discussion). However, the inhibitory effects of Co^{2+} and Mn^{2+} were still clearly observed even when the total divalent cation concentration was kept constant.

DISCUSSION

The present work has clearly demonstrated that divalent cations in the extracellular space can modulate the non-selective cation channels of muscarinic receptor ($I_{ns, ACh}$) in guinea-pig ileal smooth muscle, and a detailed study of the actions of Cd^{2+} has allowed some explanations of the mechanism involved to be proposed.

Divalent cations act on the extracellular site of $I_{ns, ACh}$

Any possible contribution of changes in $[Ca^{2+}]_i$ to the external divalent cation effect can be largely eliminated by use of a high concentration (10 mM) of either EGTA or the more favourable Ca^{2+} chelator, BAPTA (Tsien, 1980) in the pipette.

This is further supported by the fact that the inhibitory effect of divalent cations is not appreciably affected either by membrane potentials, where Ca^{2+} influx via non-inactivated Ca^{2+} channels is unlikely i.e. < -50 mV (Ganitkevich & Isenberg, 1990), or by use of nifedipine, and that the time course of this action seems too rapid to involve any 'indirect effect' mediated by the facilitatory effect of $[Ca^{2+}]_i$ (Inoue & Isenberg, 1990*c*). It is therefore very likely that divalent cations exert their action on $I_{ns,ACh}$ by acting on a site close to the extracellular side of the sarcolemma.

The major mechanism of divalent cation action on $I_{ns,ACh}$

Substances affecting neurotransmitter-operated channels, in general, could interact at any of the steps that exist between receptor binding and channel opening. The important steps include the effective concentration of agonist in the bulk solution, the binding process of neurotransmitter to its receptor, the effector channel consisting of ion-conducting pores and regulatory proteins, and in some cases intervening biochemical elements (e.g. G-proteins). Each of these should be considered in order to elucidate the action of external divalent cations on $I_{ns,ACh}$.

The effective concentration of CCh, or ACh in the bulk solution is not likely to be affected by divalent cations, since these agonists are highly positively charged molecules, which repulse any ions of the same polarity and would not form inactive complexes with divalent cations (compare with NMDA-activated channels, Westbrook & Mayer, 1987 and ATP-activated channels, Honoré, Martin, Mironneau & Mironneau, 1989).

Changes in receptor binding or subsequent signal transducing processes (Inoue & Isenberg, 1990*a*; Komori & Bolton, 1990) are also unlikely. The action of Cd^{2+} and other divalent cations (Ni^{2+}, Mn^{2+}, Co^{2+}) is rapid and reversible. The time required to reach a steady state (100–200 ms) is more than ten times shorter than required for CCh or atropine to exhibit their effects (>2 s). Furthermore, the degree of Cd^{2+}-induced inhibition is almost independent of the CCh concentration. These facts are consistent with the hypothesis that Cd^{2+} short-cuts the receptor and subsequent biochemical processes, and acts directly on the $I_{ns,ACh}$ channel protein itself. However, this scheme is probably true only for the short-term effect of divalent cations (in the order of hundreds of milliseconds), since muscarinic receptors are thought to exist in several conformations with different agonist affinities, with interconversions sensitive to divalent cations (Sokolovsky, 1988). Indeed, when a cell was exposed for a long time to Ca^{2+}-free or Cd^{2+}-containing solutions, the response to ACh or CCh was often greatly reduced (R. Inoue, unpublished observations).

The parameters determining voltage-dependent gating of $I_{ns,ACh}$ also seem to be almost insensitive to Cd^{2+}. Similar properties have been reported for other neurotransmitter-operated channels (NMDA-activated channel, Westbrook & Mayer, 1987; GABA-activated Cl^- channels, Kaneko & Tachibana, 1986) which are affected by some divalent cations in a way different from that expected for open channel blockade (Neher & Steinbach, 1978; Nowak *et al.* 1984; Lansmann & Hess & Tsien, 1986). All such cases share common properties, i.e. rapid, reversible and voltage-independent effect of divalent cations in the order of 10–100 μM. In a recent detailed study using single-channel recording, Zn^{2+}- or Cd^{2+}-induced inhibition of NMDA channels was shown to be complex. Zn^{2+} reduced large conductance events

and shortened the mean open time of NMDA channels in a voltage-independent manner (Legendre & Westbrook, 1990). It also caused a reduction both in the number of bursts and burst duration. This implies that any alteration of the parameters characterizing the channel kinetics could be responsible for the effect of divalent cations on the $I_{ns,ACh}$ channel. In preliminary experiments, the single-channel conductance of $I_{ns,ACh}$ was not markedly affected by the presence of Cd^{2+}, but the characteristic frequency (half-power frequency) of fluctuation analysis, which reflects the relaxation time constant (e.g. Fig. 3*B*), was increased in the presence of Cd^{2+}, suggesting that the mean open lifetime of the $I_{ns,ACh}$ channel (in the model proposed in Inoue & Isenberg, 1990*b*) may become shorter. This could partly explain the effect of Cd^{2+} on the $I_{ns,ACh}$ channel as suggested by fluctuation analysis for Zn^{2+} action on NMDA channel (Mayer *et al.* 1988). Further information should be obtained on the basis of single-channel recording.

Is a surface charge effect involved?

Divalent cations are known to screen or bind to negatively charged sites on the sarcolemmal membrane and reduce the surface potential (Hille, 1984). This is thought to produce a positive shift of voltage-dependent variables in various kinds of ionic channels (Frankenhaeuser & Hodgkin, 1957; Ohmori & Yoshii, 1977; Cohen & van der Kloot, 1978). Also, in smooth muscle, Ganitkevich, Shuba & Smirnov (1988) reported that Ba^{2+}, Sr^{2+}, Mg^{2+} and Ca^{2+} ions all caused a positive shift of $V_{\frac{1}{2}}$ in the Ca^{2+} channel. The shift was larger than the values predicted by a simple screening effect assuming uniformly distributed non-specific binding sites (Gouy-Chapman's theory, Hille, 1984), and could be explained by considering specific binding sites on the sarcolemma. The case of $I_{ns,ACh}$ channel seems to be complicated. The shift of the steady-state activation curve in the presence of Cd^{2+} (100 μM) was negligibly small. However, Mn^{2+}, Co^{2+} or Mg^{2+} were only effective at concentrations of 1 mM or more which might produce screening of surface charge; Mn^{2+} (3 mM) did cause a strong positive shift of the steady-state activation curve by *ca* 20 mV in one experiment (this sort of experiment was technically difficult to perform). In contrast, the physiological ion, Ca^{2+} caused a 'negative' shift, and a similar effect is seen for La^{3+} which strongly shifts the steady-state activation curve leftward as well as prolonging relaxation time constants (Inoue, 1990). These effects are not consistent with a screening effect and therefore suggest that Ca^{2+} and La^{3+} might be unique multivalent cations capable of modifying the voltage-dependent gating of $I_{ns,ACh}$ channel in some direct way.

Taken together, this multiplicity of observations suggest that several distinct non-specific and specific sites are involved in the action of external divalent cations on the $I_{ns,ACh}$ channel. More careful evaluation is required of the effects of external divalent cations on receptor-operated channels in smooth muscle, since they may greatly alter the excitatory effects of neurotransmitters on the muscle.

Physiological implications of the divalent cation effect

The inhibitory effect of Cd^{2+} and related divalent cations may be unique to the $I_{ns,ACh}$ channel amongst the family of neurotransmitter-activated non-selective cation channels of smooth muscle. For instance, ATP-activated (Benham & Tsien,

1987) and noradrenaline-activated non-selective cation channels (Amédée, Benham, Bolton, Byrne & Large, 1990) have been reported to be resistant to external Cd^{2+}. In those channels, Cd^{2+} in hundred micromolar concentrations, failed to block the agonist-activated current. Nevertheless, the results of the present work show that great caution is needed in interpreting the effects of divalent cations on receptor-operated response. This is particularly important, since divalent cations described in this paper have frequently been considered as an important pharmacological tool to block voltage-operated Ca^{2+} channels and thereby distinguish between receptor-operated and voltage-dependent Ca^{2+} influx.

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