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# INTRACELLULAR CALCIUM IONS MODULATE ACETYLCHOLINE-INDUCED INWARD CURRENT IN GUINEA-PIG ILEUM

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SUMMARY

1. The modulatory effect of internal $Ca^{2+}$ on the current through the ACh-activated non-selective cation channels ($I_{ns,ACh}$) was investigated by the whole-cell patch clamp technique in single isolated cells of guinea-pig ileum.

2. $I_{ns,ACh}$ was isolated with caesium aspartate internal solution of low $Ca^{2+}$-buffering capacity (10 $\mu$M-EGTA). With preceding depolarizations which evoked voltage-operated $Ca^{2+}$ currents ($I_{Ca}$), $I_{ns,ACh}$ increased in amplitude and decayed more rapidly. The extent of this 'facilitating' effect depended on the number and duration of the depolarizations.

3. When depolarizing pulses were applied during the sustained phase of $I_{ns,ACh}$, they were followed by large inward tail currents. These tail currents (tail $I_{ns,ACh}$) resembled the non-facilitated $I_{ns,ACh}$ recorded without the depolarizing pulse, in regard to voltage-dependent gating and dependence on the extracellular $Na^+$ concentration, thus suggesting that the currents are flowing through the same class of channels.

4. The tail $I_{ns,ACh}$ was apparently composed of two components distinguished by the insensitivity to organic $Ca^{2+}$ antagonists. The minor component (about 20% of tail $I_{ns,ACh}$) showed a rapid decay (about 150 ms at $-60$ mV) which could be attributed to voltage-dependent kinetics. The major component decayed slowly within 5 s and appeared to be related to changes in the intracellular $Ca^{2+}$ concentration. The latter component was not recorded when $Ba^{2+}$ or $Sr^{2+}$ were used as a charge carrier for $I_{Ca}$ and was blocked by 10 $\mu$M-D600 or nitrendipine, or $Cd^{2+}$ (0·2–0·5 mM).

5. The tail $I_{ns,ACh}$ increased in proportion to $Ca^{2+}$ influx when the duration of depolarizing pulses were prolonged from 15 to 200 ms, but this 'facilitating' effect was greatly suppressed when the cell was perfused with 40 mM-EGTA.

6. When the pCa in the pipette was varied using 40 mM-Ca-EGTA, the conductance through $I_{ns,ACh}$ increased in a manner dependent on intracellular $Ca^{2+}$ concentration. Half-maximal and submaximal activation occurred at about 200 nM and 1 $\mu$M, respectively.

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7. These results show that the activity of $I_{ns,ACh}$ is very sensitive to the intracellular $Ca^{2+}$ concentration in the physiological range.

## INTRODUCTION

It is now generally accepted that the intracellular $Ca^{2+}$ concentration plays a significant role in controlling the membrane conductance. In excitable tissues like intestine, in response to electrical stimulation or the application of excitatory neurotransmitters like ACh, the intracellular $Ca^{2+}$ concentration can be elevated high enough to cause contraction (Himpens & Somlyo, 1988; Ito, Kuriyama & Parker, 1988; Yagi, Becker & Fay, 1988) and simultaneously induces many sorts of $Ca^{2+}$-dependent conductances ($Ca^{2+}$-dependent $K^+$, $Cl^-$ and non-selective cation channels: Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982; Marty, Tan & Trautman, 1984; Findlay & Petersen, 1985; Singer & Walsh, 1987; Ehara, Noma & Ono, 1988). On the other hand, recent work shows that other channels are also sensitive to changes in intracellular $Ca^{2+}$ concentration, although their activation is initiated by other mechanisms, i.e. de- or hyperpolarization of the membrane, or the activation of receptor channel pathways (voltage-operated $Ca^{2+}$ channels: Ganitkevich, Shuba & Smirnov, 1987; Ohya, Kitamura & Kuriyama, 1988; hyperpolarization-activated inward current: Hagiwara & Irisawa, 1989; GABA-activated $Cl^-$ current: Inoue, Oomura, Yakushiji & Akaike, 1986). Even in guinea-pig ileum, on removal of $Ca^{2+}$ from the PSS, ACh depolarized the membrane less potently and correspondingly produced a smaller inward current than in the control (Inoue, Kitamura & Kuriyama, 1987*a*). Since changes in external $Ca^{2+}$ concentration quickly (30–60 s) modify the intracellular $Ca^{2+}$ concentration in this preparation as measured with $Ca^{2+}$-sensitive dyes (Himpens & Casteels, 1987), a possible effect of intracellular $Ca^{2+}$ on $I_{ns,ACh}$ could be expected. This has also been suggested for other parts of the intestine (Lim & Bolton, 1988). Therefore, in the present study, the sensitivity of $I_{ns,ACh}$ to intracellular $Ca^{2+}$ was examined, especially in relation to $Ca^{2+}$ influx via voltage-operated $Ca^{2+}$ channels, using various pulse protocols and cell dialysis by simple diffusion of various pCa pipette solutions.

## METHODS

The experimental procedures used here are the same as described in the preceding paper (Inoue & Isenberg, 1990). Briefly, the cells were isolated from the longitudinal muscle layer of guinea-pig ileum by enzymic treatment and superfused with a physiological salt solution composed of 130 mM-NaCl, 5 mM-KCl, 2 mM-$CaCl_2$, 2 mM-$MgCl_2$, 10 mM-glucose, adjusted to pH 7·4 with 10 mM-HEPES/Tris. Bath application was usually completed within 5 s. In case of repetitive applications of ACh, an interval of over 5 min was needed for recovery from desensitization. All experiments were performed at room temperature (20–22 °C). For the current and voltage clamp experiments, KCl (20 $\mu$M-EGTA) and caesium aspartate (10 $\mu$M-EGTA) internal solutions were used respectively (see Inoue & Isenberg, 1990). For the control of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), 40 mM-Ca-EGTA mixtures were used and pCa was calculated using the computer program with various inonic strengths introduced by Fabiato (1988). The data were recorded on-line with a Brush pen-recorder and stored on a PDP 23 minicomputer for the later off-line evaluation. All pulse protocols were generated by the computer.

## RESULTS

### *The effect of extracellular $Ca^{2+}$ and $Ca^{2+}$ antagonists on ACh-induced depolarization*

The importance of extracellular $Ca^{2+}$ is demonstrated in Fig. 1. In control conditions, the superfusion of 300 $\mu$M-ACh for 8 s depolarized the membrane very

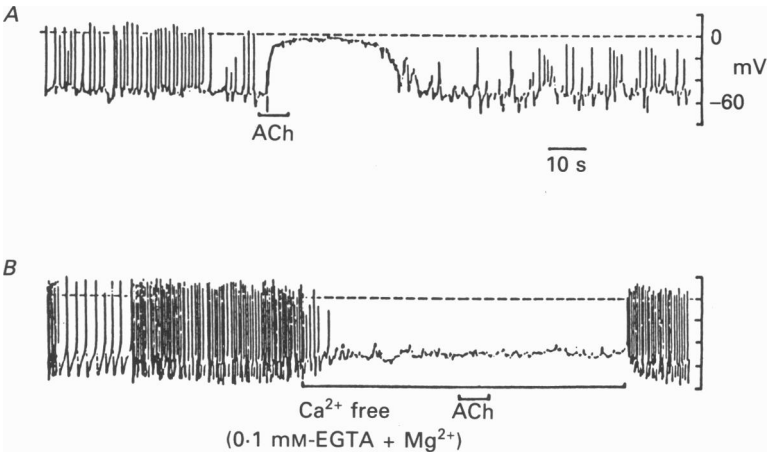


Fig. 1. The influence of $Ca^{2+}$ removal on ACh-induced depolarization. The pipette is filled with KCl solution (20 $\mu$M-EGTA). Superfusing PSS contains 130 mM-NaCl and 2 mM-$CaCl_2$, temperature 22 °C. ACh (300 $\mu$M) was bath applied for 8 s at bars. *A*, ACh-induced depolarizations in normal PSS. *B*, in $Ca^{2+}$-free solution (2 mM-$CaCl_2$ substituted by 2 mM-$MgCl_2$) complemented with 0·1 mM-EGTA.

rapidly to −10 to 0 mV and this depolarization was sustained over tens of seconds (Fig. 1*A*). When spontaneous action potentials are abolished either by replacement of $Ca^{2+}$ with $Mg^{2+}$ or by addition of $Ca^{2+}$ antagonists (0·5–1 mM-$Cd^{2+}$ or $Ni^{2+}$, or 10 $\mu$M-D600 or nitrendipine) in the PSS, ACh produced a less potent depolarization which peaked at more negative potential and repolarized faster than in the control (not shown). Moreover, when the contaminating $Ca^{2+}$ in PSS was chelated by the addition of EGTA (0·1 mM), the membrane depolarized slightly and ACh no longer produced further depolarization (Fig. 1*B*). We interpreted these observations as indicating that changes in $[Ca^{2+}]_i$ might modify the activity of $I_{ns,ACh}$, because all the above-mentioned manoeuvres can decrease $[Ca^{2+}]_i$ (Himpens & Casteels, 1987). To clarify this idea, the following experiments were carried out.

### *Facilitation of $I_{ns,ACh}$ by depolarizations preceding ACh application*

The ACh-induced inward current ($I_{ns,ACh}$) is potentiated by a preceding depolarization as shown in Fig. 2. At a constant holding potential of −60 mV bath application of 300 $\mu$M-ACh for 5 s induced an inward current that peaked within 10 s to −63 pA and slowly faded away afterwards (trace $I_1$). After a recovery period of 5 min, the ACh effect was tested again (trace $I_2$) after a preceding depolarizing clamp step that evoked a voltage-operated $I_{Ca}$. The response to ACh was facilitated, i.e. the inward current peaked within a shorter period of time (3·4 instead of 10 s) to a larger amplitude (−338 instead of −63 pA) from which it decayed more rapidly. Similar

facilitation (4- to 10-fold in current amplitude) was obtained in five other cells. This facilitation fell as a delay between depolarization pulse and application of ACh was increased, and was almost absent if the delay exceeded 10 s. For a given delay, the extent of facilitation increased with the number of preceding depolarizations (data were not shown). Since depolarization activates $I_{Ca}$, these results strongly suggest that $I_{ns,ACh}$ is facilitated by increase in $[Ca^{2+}]_i$.

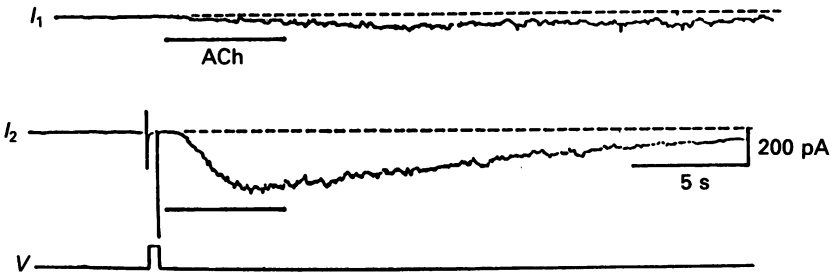


Fig. 2. The facilitatory effect of preceding depolarization on the ACh-induced current. Pipette: caesium aspartate solution and 10 $\mu$M-EGTA. Bath: PSS (2 mM-$Ca^{2+}$). Bars show the bath application of 300 $\mu$M-ACh. The same cell was tested. The ACh-induced inward current was recorded at a constant holding potential of $-60$ mV ($I_1$). It was facilitated in amplitude and speeded up in time course by the preceding clamp step of 500 ms to 0 mV ($I_2$). The delay between the end of the clamp step and the start of ACh application was about 300 ms.

*Depolarization during ACh application facilitates the ACh-induced inward current*

When depolarizations were applied during the response to ACh, large inward-directed tail currents were seen if the depolarization had induced a voltage-operated $Ca^{2+}$ current ($I_{Ca}$). Figure 3*B* demonstrates this observation. Two effects can be seen. First, ACh diminished $I_{Ca}$; the peak was reduced from $-225$ to $-190$ pA. The depression of $I_{Ca}$ by ACh was constantly observed in experiments using a pipette containing 10 $\mu$M-EGTA (see for example Figs 4 or 5), but virtually absent with 40 mM-EGTA in the pipette (R. Inoue & G. Isenberg, unpublished observations), suggesting that $[Ca^{2+}]_i$-mediated inactivation (Ganitkevich *et al.* 1987; Ohya *et al.* 1988) is involved in this observation. Second, the amplitude of ACh-activated current appeared to increase from $-50$ to $-680$ pA following depolarization (facilitated tail current), but decayed time dependently back to the level before the depolarization (non-facilitated). The ACh-sensitive current (Fig. 3*C*: difference between the currents before and during the application of ACh) shows that the extent and time constant of decay of this facilitation effect are 10 times and 1·2 s, respectively.

Experiments on the voltage-dependent gating of $I_{ns,ACh}$ (Inoue & Isenberg, 1990) suggest that the increase in the amplitude and the time constant of relaxation of $I_{ns,ACh}$ are expected to be on average about threefold and 150 ms respectively at $-60$ mV after depolarization to 0 mV. However, the result in Fig. 3*C* shows a much larger increase in amplitude and a much slower decay of the facilitated tail current than expected (about 4 times larger and 10 times slower, respectively). This discrepancy supports the idea that the larger part of the facilitated tail current may result from an additional process, i.e. $Ca^{2+}$ influx induced facilitation of $I_{ns,ACh}$.

Before this hypothesis was tested, the behaviour of the facilitated tail current was studied to confirm whether it possesses the same properties as $I_{ns,ACh}$ or not.

*The properties of facilitated tail current resemble those of non-facilitated* $I_{ns,ACh}$

*Voltage dependence*

To study the instantaneous conductance and the reversal potential of the facilitated tail current, the following pulse protocol was used: the membrane was

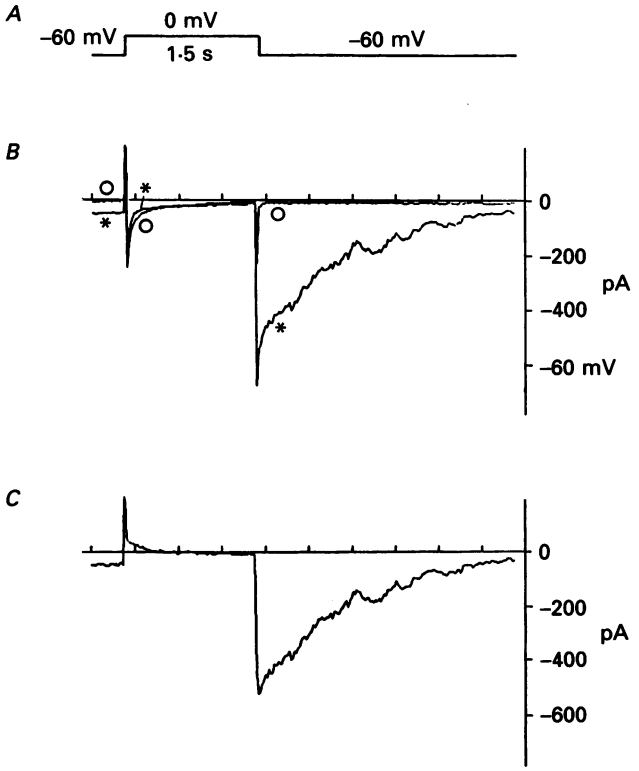


Fig. 3. Tail inward current following depolarization and $I_{Ca}$ (computer print-out). *A*, pulse protocol. *B*, net membrane currents in absence (○) and presence (*) of ACh superimposed. Note the holding current before the pulse is zero with control and inward in the presence of ACh. During the pulse, $I_{Ca}$ was slightly attenuated by ACh. After the pulse, there is a small and short-lasting tail current in the control trace but a huge and long-lasting tail current in the presence of ACh. *C*, difference of the tracings, ACh minus control.

clamped at 0 mV for 0·5 s to obtain the maximal activation of the tail current, then a falling ramp was applied with a rate of 2 V $s^{-1}$ from +80 to −120 mV (Fig. 4*A*). Figure 4*C* compares the ACh-sensitive current at −60 mV. Before the voltage changes, the ACh-sensitive current is small, afterwards it is large. The pulse protocol has facilitated it by a factor of 10. After the ramp pulse, the ACh-sensitive tail current remained constant for 400 ms, indicating that its decay has not proceeded much during the ramp. Figure 4*D* shows the *I–V* curve of the ACh-sensitive tail current. As in the case of non-facilitated $I_{ns,ACh}$, the *I–V* curve shows inward

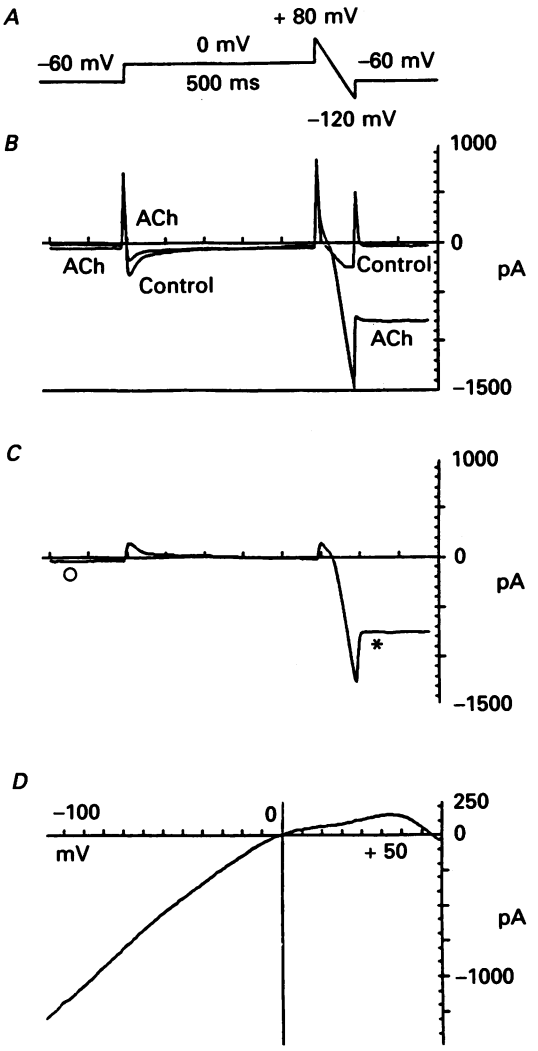


Fig. 4. Voltage dependence of ACh-sensitive tail current as evaluated with ramp clamps. *A*, the protocol of voltage changes starts from the holding potential of −60 mV. A 500 ms long step pulse to 0 mV is followed by a ramp signal which repolarizes the membrane from +80 to -120 mV at a constant rate of 2 V $s^{-1}$. Finally, the potential is set back to −60 mV. *B*, computer print-outs of net membrane currents measured in the absence (control) and presence of ACh. *C*, ACh-sensitive current as difference current. Note: the ACh-sensitive current before the pulse (○) is small in comparison to the ACh-sensitive tail current after the pulse (*). *D*, the instantaneous *I–V* curve evaluated from the ramp as the difference of tracings (ACh minus control in panel *B*). For clarity the more positive potentials are displayed at the right side in the figure.

rectification. From eight experiments we evaluated a chord conductance of $6{\cdot}4 \pm 1{\cdot}5$ nS close to 0 mV per cell. This value is fourfold larger than the conductance evaluated without facilitation by $I_{Ca}$ (1·5 nS in the presence of $Ca^{2+}$ antagonist).

*Reversal potential*

From a total of eight experiments, we estimated the reversal potential of ACh-sensitive tail current as $0{\cdot}9 \pm 2{\cdot}4$ mV. When extracellular $Na^+$ concentration was reduced from 130 to 30 mM, the reversal potentials of the tail current shifted to $-31{\cdot}3 \pm 2{\cdot}9$ mV ($n = 3$). These values are nearly identical to those obtained for non-facilitated $I_{ns,ACh}$ (+1·6 mV and −32·6 mV, Inoue & Isenberg, 1990).

The dependence of the facilitated tail current on ACh-concentration is not identical to that of the non-facilitated $I_{ns,ACh}$ (not shown). When the concentration–response relationships were normalized, a small difference was observed. Namely, the Michaelis constant of 10 μM for non-facilitated $I_{ns,ACh}$ shifted to 3–5 μM for the facilitated tail current, but the Hill coefficient of 0·8–1·0 was not significantly changed. This means that a moderate increase in sensitivity to ACh can partly account for the fourfold increase of conductance of the facilitated tail current.

Voltage-dependent gating of the facilitated tail current was analysed with square pulses that followed the facilitating depolarization (Fig. 5*A*). At positive potentials, the ACh-sensitive tail currents were nearly time independent. At negative potentials, they appeared to decay with a two exponential process (Fig. 5*C*). The fast exponential may be due to voltage-dependent gating. The time constant of the relaxation was faster the more negative the clamp step, being approximately 35 ms for −140 mV, 55 ms for −120 mV, 60 ms for −100 mV, and 90 ms for −80 mV. At −60 mV and more positive potentials, the contribution of the fast exponential was too small for time constant analysis.

*Steady-state activation curve*

The peak of the ACh-sensitive tail current was taken as a measure of the instantaneous *I–V* relationship. It is nearly ohmic at negative potentials and reverses at 0 mV but bends toward the voltage axis at positive potentials (○, Fig. 5*D*). This resembles the *I–V* relationship obtained by ramp currents in Fig. 4 and is similar for non-facilitated $I_{ns,ACh}$. The steady-state values of the ACh-sensitive tail current were measured 450 ms after the end of the conditioning depolarization pulse, long enough for the voltage-dependent relaxation but too short for the decay of facilitation. The *I–V* curve of steady-state activation (● Fig. 5*D*) has the shape of an inverted bell as it is for non-facilitated $I_{ns,ACh}$ (Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987*b*; Inoue & Isenberg, 1990). The ratio of steady-state to peak tail current was calculated from four cells and plotted in Fig. 5*E*. The data points are fitted by a Boltzmann sigmoid curve with half-maximal activation of −65 mV which is about 15 mV more negative than that obtained for non-facilitated $I_{ns,ACh}$.

*Conclusion*

The facilitated tail current resembles non-facilitated $I_{ns,ACh}$ in reversal potential, dependence on extracellular $Na^+$ concentration and voltage-dependent gating. The

resemblance suggests that the non-facilitated $I_{ns,ACh}$ and facilitated tail current flow through the same population of non-selective cation channels. In this sense, these tail currents can be called facilitated 'tail $I_{ns,ACh}$'. The moderate changes on the ACh sensitivity and the voltage-dependent gating will be discussed in the context of an elevated $[Ca^{2+}]_i$.

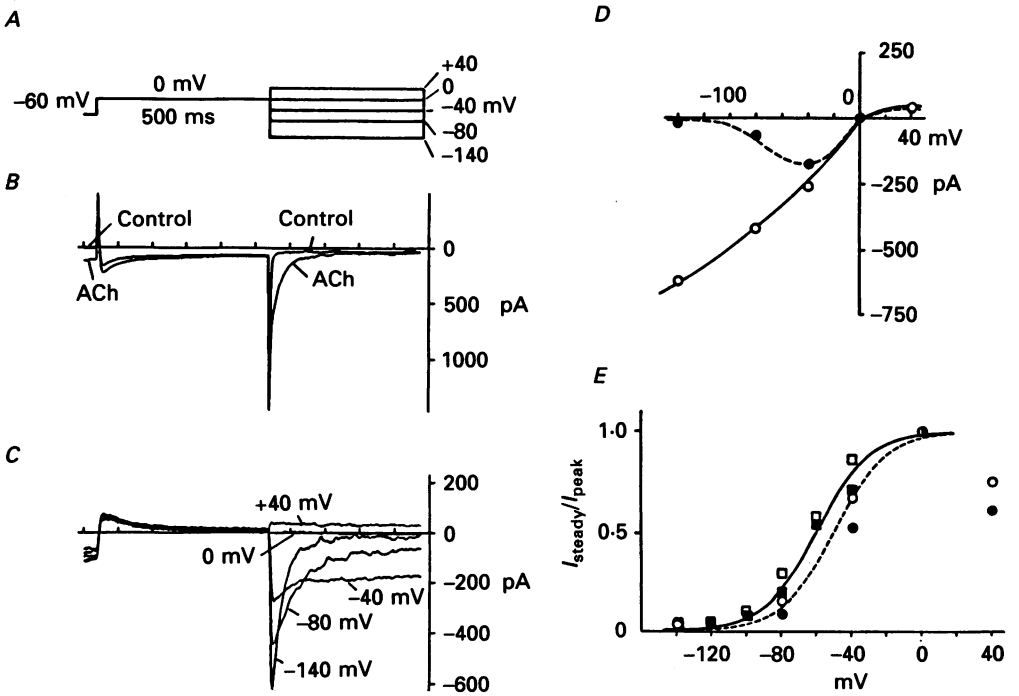


Fig. 5. Voltage dependence of the ACh-sensitive tail inward current evaluated with square pulses. Pulse protocol applied immediately before (control) and 5–30 s after ACh application. *A*, pulse protocol. *B*, example of net currents (test step to 0 mV, tail step to −140 mV). Note: during −60 mV (pre-step) and −140 mV (tail step) ACh-induced inward currents are due to the activation of $I_{ns,ACh}$. At 0 mV ACh-reduced $I_{Ca}$ ($I_{ns,ACh}$ can be excluded because 0 mV is the reversal potential of $I_{ns,ACh}$). *C*, $I_{ns,ACh}$ as ACh-sensitive currents (ACh minus control). Potentials during the tail steps are labelled. Note the crossing of the tracings, i.e. at −40 mV the steady-state current is more inward than at −140 mV. *D*, instantaneous *I–V* curve (○; measured at the peak) and steady-state current (●; measured at the end of the test pulse, i.e. at 450 ms) of ACh-sensitive tail currents. *E*, steady-state activation curve of ACh-sensitive tail currents evaluated from the ratio of the steady-state current to the instantaneous current. The points at 0 mV were estimated to be 1·0 from the *I–V* curves in panel *D* (continuous and dashed lines). Data from four different cells are shown by the different symbols. Line drawn by eye. The dashed line shows the activation curve of non-facilitated $I_{ns,ACh}$ for comparison (see Fig. 6 in Inoue & Isenberg, 1990).

### *The role of $Ca^{2+}$ influx on facilitation of $I_{ns,ACh}$*

In the following paragraphs, we attempted to clarify the causal relationship between facilitation of tail $I_{ns,ACh}$ and preceding $Ca^{2+}$ influx. Figure 6 shows the effects of $Ca^{2+}$ antagonist nitrendipine on facilitation tail $I_{ns,ACh}$. As expected,

nitrendipine blocked the voltage-operated $I_{Ca}$ during the pulse. At −60 mV before the pulse, nitrendipine has no effect. After the pulse, upon repolarization to −60 mV, tail $I_{ns,ACh}$ peaked to a smaller amplitude (about 50 % of control (○) in this case) and decayed more rapidly (time constant of decay shortened from 1·5 s to 150 ms).

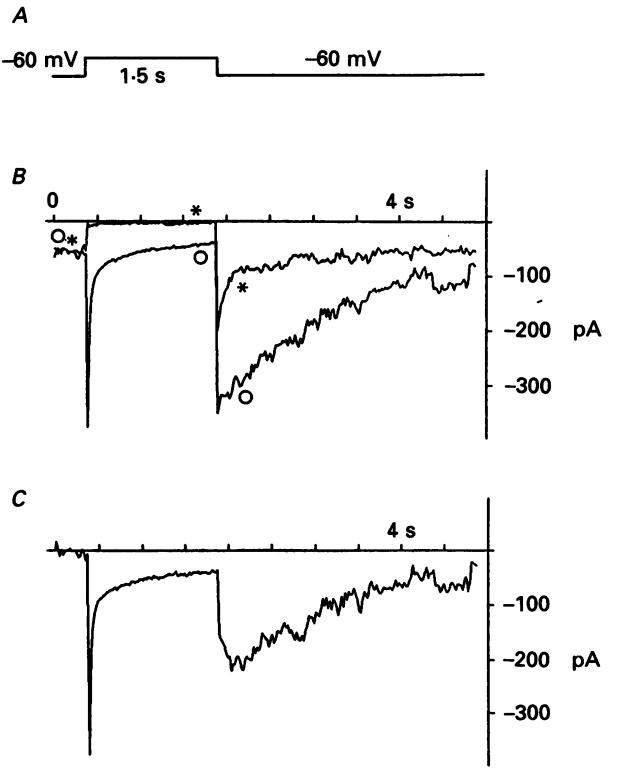


Fig. 6. Tail $I_{ns,ACh}$ is depressed by the $Ca^{2+}$ antagonist nitrendipine. *A*, pulse protocol. *B*, net currents in the continuous presence of 300 μM-ACh, either in the absence (○) or in the presence of 10 μM-nitrendipine (*). *C*, nitrendipine-sensitive current as the difference. Note: $I_{ns,ACh}$ before the pulse is insensitive to nitrendipine. During the pulse, at 0 mV, $I_{ns,ACh}$ does not contribute to the record because it is set at the reversal potential, thus the nitrendipine-sensitive current is $I_{Ca}$. Tail $I_{ns,ACh}$ after the pulse is strongly diminished by nitrendipine.

Nearly identical results were obtained using 10 μM-D600 or 0·2–0·5 mM-$Cd^{2+}$. These observations allow two components of the tail $I_{ns,ACh}$ to be distinguished. The first part is insensitive to $Ca^{2+}$ channel antagonists, and would be voltage-dependent gating, the second part of tail $I_{ns,ACh}$ can be attributed to a nitrendipine-sensitive $Ca^{2+}$ influx and its consequences.

### *Facilitation in tail* $I_{ns,ACh}$ *parallels the amount of* $Ca^{2+}$ *influx*

Facilitation of tail $I_{ns,ACh}$ was highly correlated to the amount of preceding $Ca^{2+}$ influx. When the duration of depolarization was varied between 15 and 60, 150, 300 and 600 ms, tail $I_{ns,ACh}$ increased up to a duration of 300 ms, but then saturated (Fig. 7*D*). Figure 8*A* shows a plot of facilitation of tail $I_{ns,ACh}$ *versus* amount of charge

transported by $I_{Ca}$ obtained from such experiments. In spite of the large scatter in the absolute values, the half-maximal and maximal facilitation are always observed at 2–4 and 10 pC for each case. The extent of maximal facilitation is 2–5 times larger for each symbol in the figure (six different experiments) than that obtained in the presence of $Ca^{2+}$ antagonists (arrow).

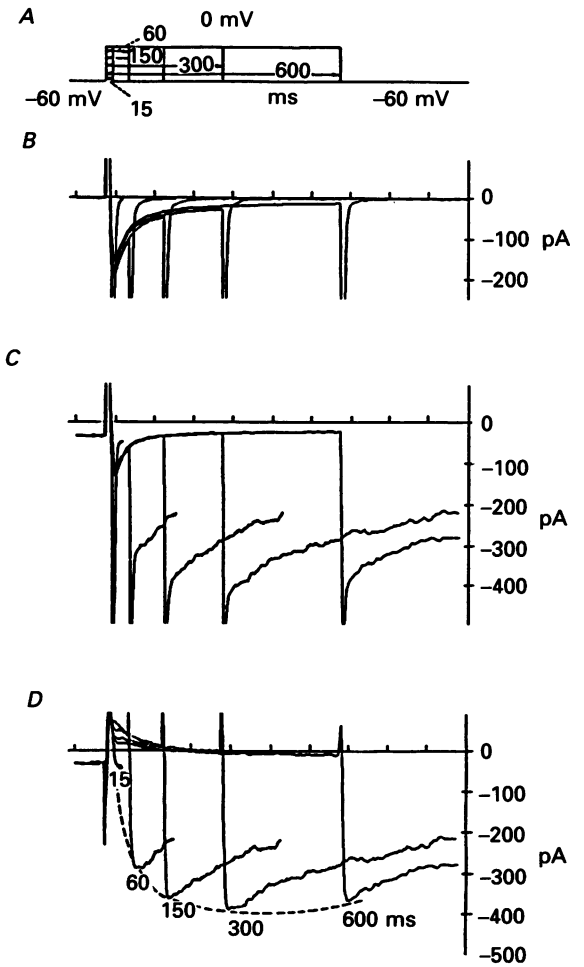


Fig. 7. Facilitation of tail $I_{ns,ACh}$ depends on the duration of the test step. *A*, pulse protocol; the pulse duration is set to 15, 60, 150, 300 and 600 ms. *B* and *C*, net membrane currents before (*B*) and in the presence (*C*) of 300 $\mu$M-ACh. *D*, tail $I_{ns,ACh}$ (*C*−*B*). The duration of each test step is labelled.

Figure 8*B* shows the time course of the facilitation in tail $I_{ns,ACh}$ in relation to $Ca^{2+}$ influx normalizing the data of Fig. 8*A*. Within the first 100 ms of the pulse, the extent of facilitation (×) increases in parallel with the $Ca^{2+}$ influx (dashed line) and then saturates within 200 ms. In the absence of $Ca^{2+}$ influx (i.e. in the presence of $Ca^{2+}$ antagonist), the extent and time course of the facilitation are much smaller and slower (○; $n = 6$). The maximal facilitation observed after nearly 1 s long depolarization (time constant about 200 ms at 0 mV) was reduced to only 24% of

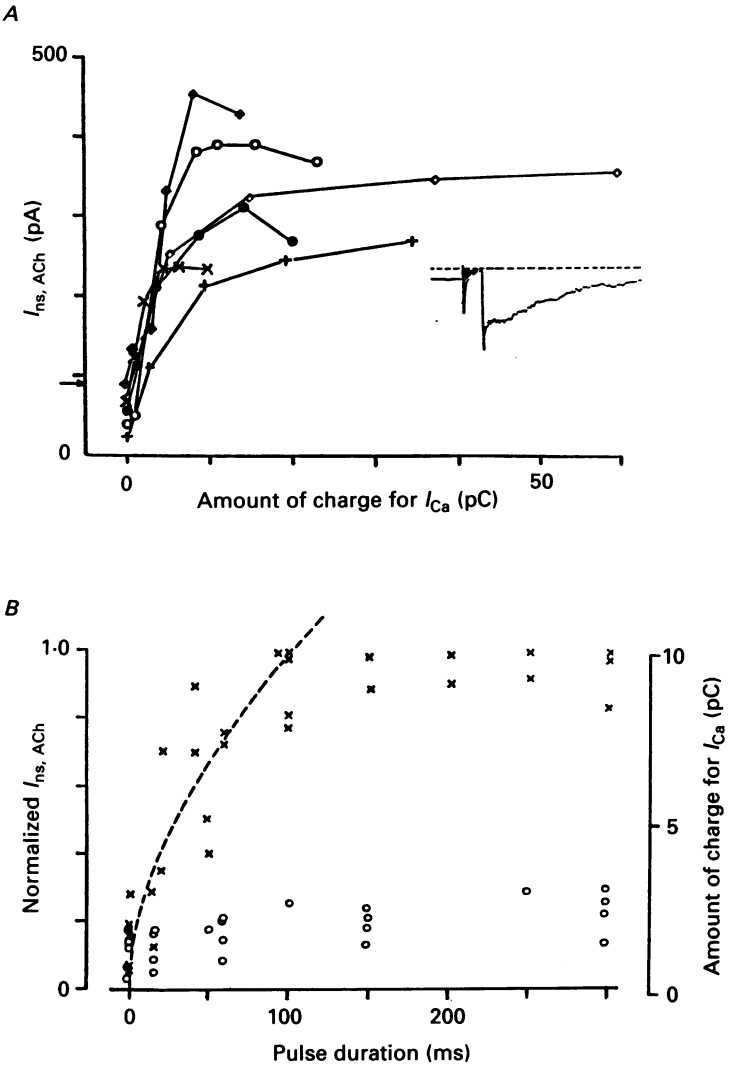


Fig. 8. Peak amplitude of tail $I_{ns, ACh}$ as a function of $Ca^{2+}$ influx through voltage-operated $Ca^{2+}$ channels ($I_{Ca}$). *A*, $Ca^{2+}$ influx is indicated by the charge (pC) calculated from the time integral of $I_{Ca}$ (see shaded area in inset). Different symbols indicate results from six experiments. The mean of the voltage-dependent maximal activation of $I_{ns, ACh}$, i.e. the amplitude of $I_{ns, ACh}$ after depolarization to 0 mV in the presence of inorganic $Ca^{2+}$ antagonists, is indicated by an arrow. *B*, influence of test pulse duration on normalized tail $I_{ns, ACh}$ in the absence ( × : data from panel *A* was divided by the maximal facilitated tail $I_{ns, ACh}$ which was usually seen with 200 ms long pulses) and that in the presence of $Ca^{2+}$ antagonists (○: for better comparison data in the presence of $Ca^{2+}$ antagonists were normalized by the mean facilitated tail $I_{ns, ACh}$ in the absence of $Ca^{2+}$ antagonists, i.e. 375 pA from panel *A*). The dashed line shows the charge which is typically transported by $I_{Ca}$ within 100 ms.

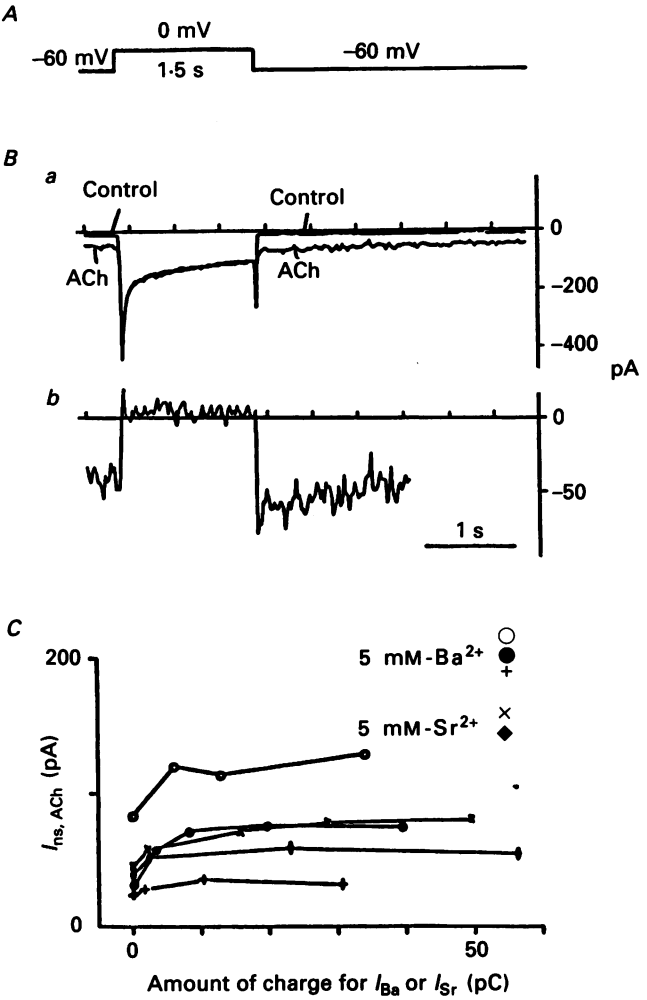


Fig. 9. Facilitation of tail $I_{ns,ACh}$ is abolished by substitution of extracellular $Ca^{2+}$ with $Ba^{2+}$. *A*, pulse protocol. *B*, net membrane currents in the absence (control) and presence of ACh (*a*), and the ACh-sensitive current as the difference (*b*), under substitution of $Ca^{2+}$ by $Ba^{2+}$ in the bath. *C*, peak of tail $I_{ns,ACh}$ as a function of charge transported by $Ba^{2+}$ or $Sr^{2+}$ through $Ca^{2+}$ channels.

that in the presence of $Ca^{2+}$ influx. These results further support the idea that $Ca^{2+}$ influx and the consequent increment in $[Ca^{2+}]_i$ cause the large facilitation of $I_{ns,ACh}$.

### *Influx of $Ba^{2+}$ or $Sr^{2+}$ does not facilitate $I_{ns,ACh}$*

Whereas influx of $Ca^{2+}$ facilitates tail $I_{ns,ACh}$, that of $Ba^{2+}$ or $Sr^{2+}$ does not. When $Ca^{2+}$ was substituted by equimolar $Ba^{2+}$ or $Sr^{2+}$ which can induce nearly the same amplitude of $I_{Ca}$ (Ganitkevich, Shuba & Smirnov, 1988), tail $I_{ns,ACh}$ was brief and small (Fig. 9*Bb*). The relationship between $Ba^{2+}$ and $Sr^{2+}$ influx and the peak amplitude of tail $I_{ns,ACh}$ suggests that $Ba^{2+}$ and $Sr^{2+}$ have little facilitating effect on

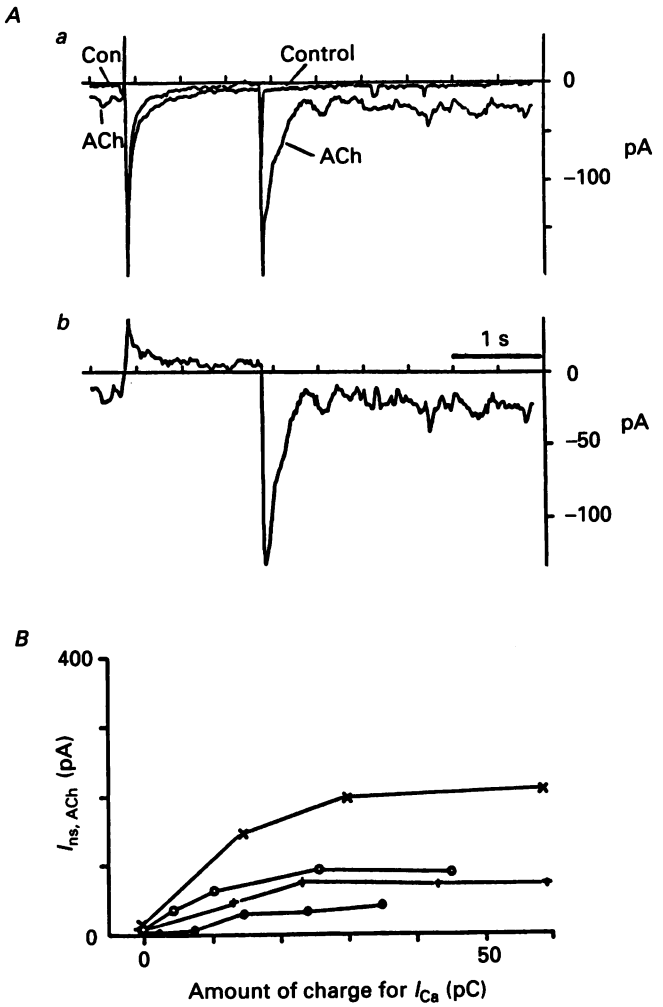


Fig. 10. Facilitation of $I_{ns, ACh}$ is diminished with 40 mM-intracellular EGTA. The pipette contained 40 mM-EGTA. The same pulse protocol as in Fig. 9 was used. *A*, net membrane currents before (control) and after ACh application (*a*), and the ACh-sensitive current as the difference (*b*). *B*, peak of tail $I_{ns, ACh}$ as a function of charge transported by $I_{Ca}$ from *B* ($n = 4$).

$I_{ns, ACh}$ (Fig. 9*C*). These results suggest that facilitation of tail $I_{ns, ACh}$ specifically requires $Ca^{2+}$.

*Facilitation is reduced by chelation of* $[Ca^{2+}]_i$ *with high EGTA*

When the buffering capacity for $[Ca^{2+}]_i$ was enhanced using 40 mM-EGTA instead of 10 $\mu$M, the facilitation of tail $I_{ns, ACh}$ was greatly attenuated. Under this condition, ACh(300 $\mu$M) produced a small inward current (−20 pA) and facilitating depolarization for $I_{ns, ACh}$ resulted in a tail $I_{ns, ACh}$ of rather low amplitude and fast time course of decay (time constant, *ca* 200 ms, Fig. 10*Ab*). To some extent, the depressing

effect of 40 mM-EGTA could be overcome by increasing the $Ca^{2+}$ influx with longer depolarizations. The plot of peak tail $I_{ns,ACh}$ *versus* the amount of charge transported during the preceding $I_{Ca}$ shows that maximal facilitation is about threefold lower with 40 mM-EGTA than with 10 $\mu$M-EGTA (Fig. 10*B versus* Fig. 8*A*). Furthermore, half-maximal facilitation required a greater amount of charge (15–25 pC instead of 2–4 pC). These results suggest that a large fraction of the $Ca^{2+}$ entering via $I_{Ca}$ binds to EGTA and, therefore, is not available for interaction with the ACh-activated channel.

*Ca-EGTA mixtures with high* $[Ca^{2+}]_i$ *mimic depolarization* ($I_{Ca}$)*-induced facilitation*

According to our hypothesis $Ca^{2+}$ influx caused by depolarization leads to facilitation of $I_{ns,ACh}$, probably via an elevation in $[Ca^{2+}]_i$ which might interact with the channel protein. If that is true, a similar facilitation of $I_{ns,ACh}$ would be directly obtained by raising $[Ca^{2+}]_i$ using Ca-EGTA mixtures in the pipette. Figure 11*A* shows three representative results where the free $Ca^{2+}$ concentration in the pipette was adjusted to $< 1$ nM, 158 nM or 1·4 $\mu$M using 40 mM-Ca-EGTA (values based on the computer program of Fabiato, 1988). In case of $< 1$ nM $[Ca^{2+}]_i$, ACh-induced inward current was very tiny but superimposed on a current noise. The ACh response was clearly facilitated when $[Ca^{2+}]_i$ was 158 nM. A still larger inward current was recorded with 1·4 $\mu$M $[Ca^{2+}]_i$ (Fig. 11*A*). When hyperpolarizing pulses were applied, $I_{ns,ACh}$ showed relaxation at all of these $[Ca^{2+}]_i$. The rate and the degree of relaxation was decreased by raising $[Ca^{2+}]_i$, but the shift of the steady-state activation curve was no more than 20 mV toward more negative potentials even at $[Ca^{2+}]_i$ of 1·4 $\mu$M. These results are consistent with those described in the case of facilitated tail current (see Fig. 5).

The experiments with 40 mM-Ca-EGTA were difficult to perform, especially those with $[Ca^{2+}]_i$ of $> 1$ $\mu$M. A $[Ca^{2+}]_i$ of 1 $\mu$M induced a contracture of the cells, often resulting in loss of the seal. After an exposure time longer than 5 min, the cell deteriorated, i.e. the cell membrane became leaky and it was impossible to reproduce the ACh response. These effects could result from the activation of $Ca^{2+}$-dependent protease (Belles, Heschler, Trautwein, Blomgren & Karlsson, 1988).

From a series of experiments using the ramp protocol (see inset), the *I–V* curves, the reversal potential and the maximal chord conductance (which appears close to the reversal potential, i.e. 0 mV) of ACh-sensitive currents were evaluated (Fig. 11*B*). In order to block $Ca^{2+}$ influx, 10 $\mu$M-nitrendipine was added to the PSS. With 40 mM-EGTA ($[Ca^{2+}]_i < 1$ nM), the conductance was $0{\cdot}23 \pm 0{\cdot}13$ nS per cell ($n = 6$), and the reversal potential was $1{\cdot}8 \pm 2{\cdot}2$ mV. As expected from the preceding results, the increase in $[Ca^{2+}]_i$ facilitated the conductance of $I_{ns,ACh}$ concentration dependently (○), while no significant shifts were observed in the reversal potentials. The maximal facilitation seemed to occur at $[Ca^{2+}]_i$ of $> 1$ $\mu$M. Assuming that the maximal facilitated conductance would be that obtained for tail $I_{ns,ACh}$ with 10 $\mu$M-EGTA (■ and the dashed line), half-maximal facilitation of $I_{ns,ACh}$ is observed at $[Ca^{2+}]_i$ between 100 and 300 nM. This value corresponds to the resting $[Ca^{2+}]_i$ of about 150 nM (Yamaguchi, 1986; Ito *et al.* 1988; Yagi *et al.* 1988), thus suggesting that nearly half of $I_{ns,ACh}$ would be ready to be open at this concentration. However, we

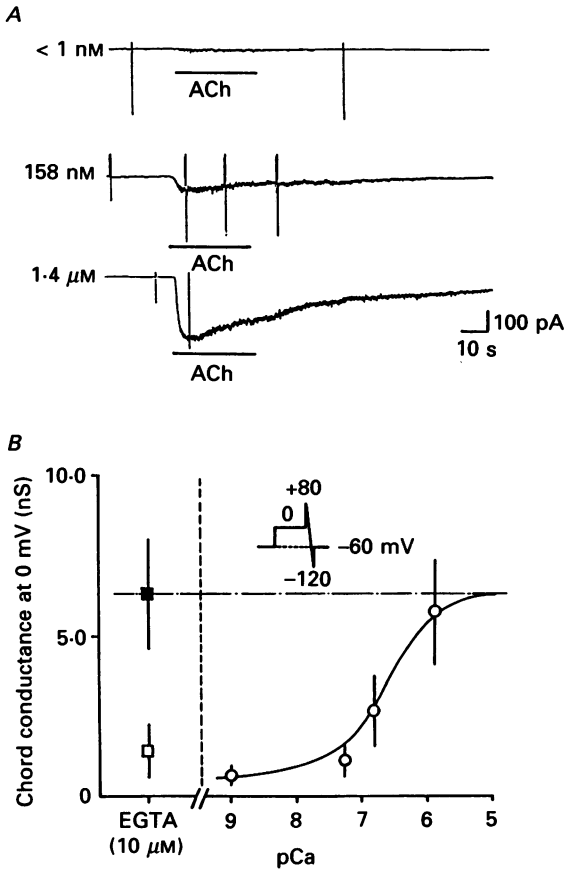


Fig. 11. Amplitude of $I_{ns,ACh}$ depends on $[Ca^{2+}]_i$ of the pipette solution was adjusted with 40 mM-Ca-EGTA to the indicated concentration. *A*, pen recording demonstrates the effect of $[Ca^{2+}]_i$ on ACh response. Three representative traces with different $[Ca^{2+}]_i$ ( < 1 nM, 158 nM and 1·4 μM). *B*, influence of $[Ca^{2+}]_i$ of chord conductance at 0 mV of $I_{ns,ACh}$ (○, evaluated close to the reversal potential with ramp signals; see inset). $Ca^{2+}$ influx via $I_{Ca}$ was blocked by 10 μM-nitrendipine. Mean ± S.E.M. from six to ten cells. The line is drawn by eye. The squares on the left side of the plot show the maximally facilitated chord conductances at 0 mV obtained from the experiments with 10 μM-EGTA, in the absence (■) or in the presence (□) of 10 μM-nitrendipine or D600.

did not interpret the data further because the variation in this experiment was rather large, presumably due to the cell deterioration described above.

### *$Ca^{2+}$ release from SR may contribute to facilitation of $I_{ns,ACh}$*

ACh is known to induce a break-down of phosphatidylinositides that increases $[Ca^{2+}]_i$ by mobilizing $Ca^{2+}$ from the SR (Hashimoto, Hirata & Ito, 1985; Gillo, Lass, Nadlen & Oron, 1987). Therefore, facilitation of $I_{ns,ACh}$ may play a role even at the resting membrane potential (−50 to −60 mV) where $Ca^{2+}$ influx via voltage-operated channels is unlikely. Since caffeine and ryanodine are thought to impair the

SR function (Endo, 1977; Fleischer, Ogunbunmi, Dixon & Fleer, 1985; Iino, Kobayashi & Endo, 1988), we investigated the interaction of these drugs with $I_{ns,ACh}$.

When 300 $\mu$M-ACh was added to the bath in the continuous presence of 20 mM-caffeine, $I_{ns,ACh}$ current was reduced by 50–70% (holding potential of −60 mV, $n$ = 4). Pre-treatment of the cells with 10 $\mu$M-ryanodine did not change the amplitude of $I_{ns,ACh}$ over 30 min ($n$ = 3). However, the presence of ryanodine prevented the recovery of $I_{ns,ACh}$ from caffeine treatment. It might be possible that the ryanodine effect needs the releasable channels to be opened by caffeine ('use-dependent' effect, see Fleischer *et al.* 1985). These results support the view that $Ca^{2+}$ released from the SR by ACh facilitates the effect of ACh near the internal side of the channel protein.

Caffeine also altered the facilitation of tail $I_{ns,ACh}$ due to depolarization and $I_{Ca}$. Tail $I_{ns,ACh}$ was not only reduced in amplitude but also accelerated in time course of decay. The extent of this inhibition was rather variable from cell to cell; in the most extreme case the amplitude of tail $I_{ns,ACh}$ was reduced by 50%. Provided that caffeine does not severely reduce $I_{Ca}$, reduction of tail $I_{ns,ACh}$ by caffeine suggests a component of $Ca^{2+}$ release from SR that could be triggered by $I_{Ca}$ ($Ca^{2+}$-induced $Ca^{2+}$ release; Endo, 1977; Saida, 1982) which might be depressed after caffeine depletion of the $Ca^{2+}$ stores.

## DISCUSSION

The results of this paper support the hypothesis that the ACh-activated non-selective cation channel of the ileal smooth muscle cells, once activated by an agonist, is modulated by changes in the intracellular calcium concentration $[Ca^{2+}]_i$. We studied the effect of $[Ca^{2+}]_i$ either by cell dialysis with Ca-EGTA mixtures of known pCa or by grading the amount of $Ca^{2+}$ influx through voltage-operated $Ca^{2+}$ channels.

The results from cell dialysis suggest that, in a range between 10 nM and 1 $\mu$M, elevated $[Ca^{2+}]_i$ enhances the maximal chord conductance along a sigmoid curve. Half-maximal facilitation appeared with 200 nM $[Ca^{2+}]_i$, close to the $[Ca^{2+}]_i$ of the resting cells of 150 nM ($Ca^{2+}$-selective electrode: Yamaguchi, 1986; fluorescent dyes: Ito *et al.* 1988; Yagi *et al.* 1988). Since the narrow space between the SR and the inner surface of the sarcolemmal membrane would give only limited diffusional access to Ca-EGTA, the $[Ca^{2+}]_i$ close to the ACh-activated channel protein may differ from $[Ca^{2+}]_i$ in the patch pipette, thus, the pCa labelled in Fig. 11 is only a rough estimate.

In the present context, we called the effect of the elevated $[Ca^{2+}]_i$ on $I_{ns,ACh}$ 'facilitation'. This was used in order to make clear that $[Ca^{2+}]_i$ did not activate the channel (or the current through it) by itself. Figure 11*A* clearly shows that the net current is almost zero in the absence of ACh, and that the increase in inward current requires activation of the channel via ACh as a pre-requisite. This necessity of activation by the agonist demonstrates that the ACh-activated non-selective cation channels are not the $Ca^{2+}$-activated non-selective cation channels that have been described for a variety of cells (Colquhoun *et al.* 1981; Yellen, 1982; Ehara, Noma & Ono, 1988).

In physiological situations, the facilitation of the $I_{ns,ACh}$ by $[Ca^{2+}]_i$ can be expected when $[Ca^{2+}]_i$ is increased either by $Ca^{2+}$ release from the SR or by $Ca^{2+}$ influx through the sarcolemma. Here, we studied in detail the facilitation of $I_{ns,ACh}$ as it results from

$Ca^{2+}$ influx via $I_{Ca}$. In most experiments, the depolarizing pulses were applied during the sustained ACh-response, then $I_{Ca}$ was followed by a large 'tail $I_{ns,ACh}$'. Tail $I_{ns,ACh}$ was apparently split into two parts. A minor part (about 20% on average) persisted in the presence of $Ca^{2+}$ antagonists and could be attributed to the effects of voltage-dependent gating of $I_{ns,ACh}$ (Inoue & Isenberg, 1990). The larger part of tail $I_{ns,ACh}$ was blocked by $Ca^{2+}$ antagonists, and this part relates to $Ca^{2+}$ influx via $I_{Ca}$. When $I_{Ca}$ was large, it facilitated $I_{ns,ACh}$ to an extent which was nearly maximal, i.e. close to that recorded on cell perfusion with 1 $\mu$M-free $Ca^{2+}$. Here, a rough estimate may demonstrate that $Ca^{2+}$ influx via $I_{Ca}$ is large enough to increase $[Ca^{2+}]_i$ sufficiently. Integration of $I_{Ca}$ over the time from the beginning ($t_0$) until the end ($t_1$) of depolarization allows estimation of the transported charge, which is about 10 pC per cell during 200 ms. This number is divided by the Faraday constant $F = 96500$ C mol$^{-1}$, the equivalence charge $z = 2$ and the cell volume ($V = 4{\cdot}1 \pm 1{\cdot}3$ pl, $n = 20$), and we obtain:

$$\Delta[Ca^{2+}]_{i,tot} = \frac{1}{zFV}\left(\int_{t_0}^{t_1} I_{Ca}\,dt\right) \doteqdot 12\ \mu\text{M}.$$

$\Delta[Ca^{2+}]_{i,tot}$ estimates an apparent increment in the total cellular calcium concentration. Nearly 95% of this calcium would be bound to the $Ca^{2+}$-binding proteins or sequestrated into compartments and about 5% would be ionized (compare Isenberg & Wendt-Gallitelli, 1989). Thus, during the 200 ms long depolarization, free $[Ca^{2+}]_i$ may increase by 600 nM or from 150 to 750 nM. This value is close to the 600 nM that was recorded during electrical stimulation from Fura-2-loaded smooth muscle cells (Yagi *et al.* 1988).

Facilitation of $I_{ns,ACh}$ was specific for $Ca^{2+}$, i.e. it could not be mimicked by $Ba^{2+}$ or $Sr^{2+}$. Our data cannot distinguish whether $Ca^{2+}$ binds directly to the channel protein or exerts its effect more indirectly. The Michaelis constant for $[Ca^{2+}]_i$-mediated facilitation is of the same order as that of $Ca^{2+}$ binding to calmodulin (Carafoli, 1987). Thus, the $[Ca^{2+}]_i$-mediated facilitation could result from stimulation of an intracellular $Ca^{2+}$-regulated enzyme, e.g. $Ca^{2+}$–calmodulin-stimulated phosphodiesterase (Green & Gillette, 1988) or protein kinase C (regulation of $K^+$ currents; Tohse, Kameyama & Irisawa, 1987). However, in those experiments the effect of elevated $[Ca^{2+}]_i$ only appeared with a delay of several minutes which does not compare to the present situation. Thus, we favour a direct interaction of $Ca^{2+}$ with the channel protein.

Intracellular $Ca^{2+}$ is known to modulate ionic channels by a variety of mechanisms. For example, in case of $Ca^{2+}$-activated 'maxi'-$K^+$ channels (Singer & Walsh, 1987), an elevated $[Ca^{2+}]_i$ shifts the voltage–open probability relationship to more negative potentials, although the maximal open probability is almost independent of $[Ca^{2+}]_i$. This scheme of the $Ca^{2+}$-activated $K^+$ channel does not apply to our results. Channel activation requires ACh. If ACh is present the channel is gated by voltage and modulated by $[Ca^{2+}]_i$. We found only modest shifts in the gating parameters (steady-state activation curve, time constants curve for relaxation). Even a large increment in $[Ca^{2+}]_i$ seemed to shift these parameters by no more than 15 mV, which is much less than in the case of the maxi-$K^+$ channel. Also, $[Ca^{2+}]_i$ increased the maximal chord conductance of $I_{ns,ACh}$ severalfold, whereas it has less effect on the $Ca^{2+}$-activated maxi-$K^+$ channel.

In the case of the GABA-activated $Cl^-$ current, an increase in $[Ca^{2+}]_i$ decreased the amplitude of the current by reduction in the open time and prolongation in the closed time intervals or by modulation of the receptor affinity for agonist (Inoue *et al.* 1986; Maruyama, Oomura, Sadoshima, Tokutomi, Behrends & Akaike, 1988). In our case such modulation of channel kinetics and receptor affinity by $[Ca^{2+}]_i$ seem to make only a minor contribution since the changes in the voltage-dependent parameters or those in concentration–response curve for ACh are small. Therefore, the above-mentioned mechanisms can explain only a minor part of our results, at best. We suggest that facilitation of $I_{ns,ACh}$ by $[Ca^{2+}]_i$ may result from an increased number of the functional channels, analogous to the model that was suggested for cyclic AMP-dependent regulation of voltage-gated $Ca^{2+}$ channels in cardiac cells (Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Heschler & Trautwein, 1989). Single-channel recordings are needed to prove such a suggestion.

The ACh-induced depolarization seems to be very sensitive to spontaneous action potentials (Fig. 1), i.e. not only is the depolarization attenuated by the absence of action potentials (either abolished by $Ca^{2+}$ removal or blocked by $Ca^{2+}$ antagonists), but also action potentials appear to induce the enhancement of ACh-induced depolarization (see for example Fig. 7 in Inoue & Isenberg, 1990). According to a study using Fura-2 in the same preparation (Himpens & Somlyo, 1988), spontaneous contractions are well synchronized to the rhythmic increase in $[Ca^{2+}]_i$ which presumably results from the voltage-dependent $Ca^{2+}$ influx during the spike. Furthermore, the omission of the extracellular $Ca^{2+}$ or the addition of the $Ca^{2+}$ antagonists not only abolishes such rhythmic increases but also decreases the resting level in $[Ca^{2+}]_i$ in tens of seconds. Therefore, if $Ca^{2+}$ influx via voltage-operated $Ca^{2+}$ channels is a main contributor for the contraction during the application of ACh (Grider & Makhlouf, 1988), the $[Ca^{2+}]_i$ sensitivity of $I_{ns,ACh}$ channels might work as a very effective positive feedback mechanism to maintain the $Ca^{2+}$ influx by sustaining depolarization. Further analysis should be performed to examine any role of sarcoplasmic reticulum.

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