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INWARD CURRENT ACTIVATED BY CARBACHOL IN RAT INTESTINAL SMOOTH MUSCLE CELLS

BY SHIGEO ITO*, TOSHIO OHTA AND YOSHIKAZU NAKAZATO
From the Department of Pharmacology, Faculty of Veterinary Medicine, Hokkaido 060, Japan

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SUMMARY

1. Carbachol(0·1 mM or 10 μM)-evoked inward currents were studied with standard and perforated whole-cell patch clamp techniques in smooth muscle cells isolated from rat small intestine. The intracellular free Ca^{2+} concentration was monitored simultaneously with the fura-2 method.

2. With a K^+-containing pipette solution, carbachol produced an inward current at −60 mV and a large outward current at −20 mV.

3. When NaCl was substituted for KCl in the external and pipette solutions, carbachol elicited inward currents at holding potentials more inside-negative than 0 mV. The reversal potential of the carbachol-induced current altered when external chloride (−0·9 mV) was replaced by iodide (−21·2 mV), thiocyanate (−27·0 mV) and glutamate (18·2 mV). The carbachol-induced current at −60 mV was slightly decreased by the replacement of external NaCl with Tris-Cl.

4. The carbachol-induced inward current at −60 mV was accompanied by an increase in the intracellular concentration of free Ca^{2+}. Both responses to carbachol were observed 2 min after exposure of the cells to a Ca^{2+}-free solution containing 2 mM EGTA.

5. Intracellular application of heparin inhibited the inward current and Ca^{2+} transient responses to carbachol but not those to caffeine (10 mM). An inward current and Ca^{2+} transient were elicited after the patch membrane was ruptured at −60 mV, using a patch pipette containing inositol 1,4,5-trisphosphate ($InsP_3$).

6. It is concluded that the carbachol-induced inward current is due to increases in membrane Cl^- and Na^+ conductances. Ca^{2+} released from $InsP_3$-sensitive stores may play a role in increasing both conductances.

INTRODUCTION

In many types of smooth muscle acetylcholine acts on muscarinic receptors to cause depolarization and contraction. A number of conductance mechanisms are involved in the response to acetylcholine (Bolton, 1979). Depolarization induced by

* To whom correspondence should be addressed.

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muscarinic receptor stimulation has been shown to result from activation of non-selective cation channels in intestinal smooth muscle (Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987; Inoue & Isenberg, 1990*a*, *b*). On the other hand, it has been reported that α-adrenoceptor stimulation produced an increase in Ca^{2+}-dependent Cl^- conductance in the portal vein (Byrne & Large, 1988) and ear artery of the rabbit (Amédée, Benham, Bolton, Byrne & Large, 1990*a*) and the anococcygeus muscle (Byrne & Large, 1987) and portal vein of the rat (Pacaud, Loirand, Mironneau & Mironneau, 1989).

Recently, we demonstrated that caffeine increases the Cl^- conductance accompanied by the release of Ca^{2+} from intracellular stores in intestinal smooth muscle cells of the rat (Ohta, Ito & Nakazato, 1993). Muscarinic receptor activation increases the production of inositol 1,4,5-trisphosphate ($InsP_3$) (Gardner, Choo & Mitchelson, 1988; Secrest, Schoepp & Cohen, 1989; Parekh & Brading, 1991), which releases Ca^{2+} from intracellular stores in smooth muscle cells (Somlyo, Bond, Somlyo & Scarpa, 1985; Iino, 1990). It is therefore of interest to study the ionic mechanisms underlying the current response to muscarinic activation. In this study, we investigated the effect of carbachol on single cells enzymatically isolated from the rat intestine using standard and nystatin-perforated whole-cell patch clamp techniques. In some experiments, the intracellular concentration of free Ca^{2+} was simultaneously monitored with whole-cell membrane currents using cells loaded with fura-2.

METHODS

Male Wistar rats (200–250 g) were killed by stunning and bleeding, and the ileum removed. The longitudinal muscle layer was peeled from the underlying circular muscle layer and cut into small pieces (about 2 × 2 mm). Ileal smooth muscle cells were dispersed (Ohta, Ito, Noto, Tachibana, Nakazato & Ohga, 1992) using a combination of collagenase (0·5 mg ml^{-1}), papaine (0·5 mg ml^{-1}) and dithiothreitol (1 mM). The cells were suspended in normal physiological salt solution (PSS) containing 0·8 mM $CaCl_2$, placed on coverslips in a small aliquot of PSS and then kept in a moist chamber at 4 °C until use on the same day.

Whole-cell membrane currents were measured with the standard whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and the nystatin-perforated patch configuration of the whole-cell recording technique used to prevent the run-down of carbachol-induced responses (Horn & Marty, 1988) using a patch clamp amplifier (CEZ-2300, Nihon-Koden) at room temperature. Patch pipettes had a resistance of 2·5–4 MΩ. Data recording and illustration were carried out as described previously (Ohta *et al.* 1992). For the preparation of nystatin-perforated cells, nystatin (200 mg ml^{-1}) was dissolved in dimethylsulphoxide and then diluted with the pipette solution to final concentrations of 50–200 μg ml^{-1}. The patch pipette solution containing nystatin was sonicated. The experiments were carried out 5–10 min after formation of a gigaseal at which the access resistance decreased to 10–30 MΩ.

Cells were superfused with the external solution at a flow rate of 2–3 ml min^{-1}. Carbachol (0·1 mM or 10 μM) or caffeine (10 mM) was applied to the cell through a pipette with a tip diameter of about 0·3 mm at a flow rate of 2–3 ml min^{-1}. The tip of the pipette was placed about 1 mm from the cell. To estimate the rate of change in the solution around a cell, the Ca^{2+} signal was measured when the solution containing fura-2 was applied to the cell through the pipette. The external solution around the cell completely changed within 0·5 s under these experimental conditions. The intracellular concentration of free Ca^{2+} was estimated from fura-2 fluorescence by the ratio method using dual-wavelength excitation and single emission (Grynkiewicz, Poenie & Tsien, 1985), as described previously (Ohta *et al.* 1993). Fura-2 was added to the pipette solution at a final concentration of 0·1 mM. In some experiments, fura-2AM (5 μM) was applied to the cells on coverslips for 30 min at room temperature and then the cells were stored in a moist chamber at 4 °C until used.

The ionic composition of the normal external solution was as follows (mM): NaCl, 126; KCl, 6; $MgCl_2$, 1·2; $CaCl_2$, 1·7; Hepes, 10; glucose, 14. The K^+-free external solution contained (mM): NaCl, 132; $MgCl_2$, 1·2; $CaCl_2$, 1·7; Hepes, 10; glucose, 14. In some experiments, the NaCl of the K^+-free external solution was replaced with Tris chloride (Tris-Cl), sodium glutamate, sodium iodide (NaI) or sodium thiocyanate (NaSCN). The pH was adjusted to 7·2 with NaOH, or when the external solution contained Tris-Cl, with Tris base. The K^+-containing pipette solution contained (mM): KCl, 132; $MgCl_2$, 1·2; $ATPNa_2$, 2; Hepes, 10; and the pH was adjusted to 7·2 with KOH. The K^+-free pipette solution was prepared by substitution of equimolar NaCl for KCl and the pH was adjusted to 7·2 with NaOH. All pipette solutions contained fura-2 or EGTA at a concentration of 0·1 mM.

Drugs

Chemicals used were $ATPNa_2$ (Boehringer, Germany), caffeine (Wako, Japan), dithiothreitol, carbamylcholine chloride (carbachol), collagenase, heparin, papaine (Sigma, USA), EGTA, fura-2, fura-2AM, Hepes and $InsP_3$ (Dojin, Japan).

Statistics

The results were expressed as mean values ± S.E.M. (n = number of observations), and statistical significance was assessed using Student's t test. P values of less than 0·05 were considered to indicate a significant difference between means.

RESULTS

Current responses to carbachol in the K^+-containing external and pipette solutions

Current responses to carbachol were observed using nystatin-perforated whole-cell recording which proved a useful technique to prevent the run-down of the responses on repeated drug challenge. Carbachol (0·1 mM) was applied for 10 s to a single smooth muscle cell maintained at various holding potentials in K^+-containing pipette and external solutions (Fig. 1). At a holding potential of −60 mV, carbachol evoked an inward current. In four out of eight cells, a small outward deflection occurred during the inward current. At a holding potential of −40 mV, the current response to carbachol became triphasic, consisting of a very transient inward current and then outward current followed by inward current in all cells examined (n = 8). At a holding potential of −20 mV, the outward current increased in amplitude and the initial but not the secondary inward current disappeared (n = 4). The outward currents elicited by carbachol were probably K^+ currents, as has been shown to be the case in intestinal smooth muscle cells of the rabbit (Komori & Bolton, 1990). In order to examine the ionic mechanisms underlying the inward current in response to carbachol, NaCl was substituted for KCl in the external and pipette solutions in the following experiments.

Current–voltage relationship of responses to carbachol

In the K^+-free external solution, a nystatin-perforated cell was maintained at various holding potentials by a patch pipette containing K^+-free and Na^+-containing solution. At holding potentials of −60 and −30 mV, application of carbachol (0·1 mM) for 5 s produced a transient inward current which subsequently exhibited fade despite the continued presence of the drug (Fig. 2). Membrane current noise slightly increased during and after the inward current. No outward current was seen during the inward current response because of the absence of K^+. The current response to carbachol was on the point of reversal at a holding potential of 0 mV.

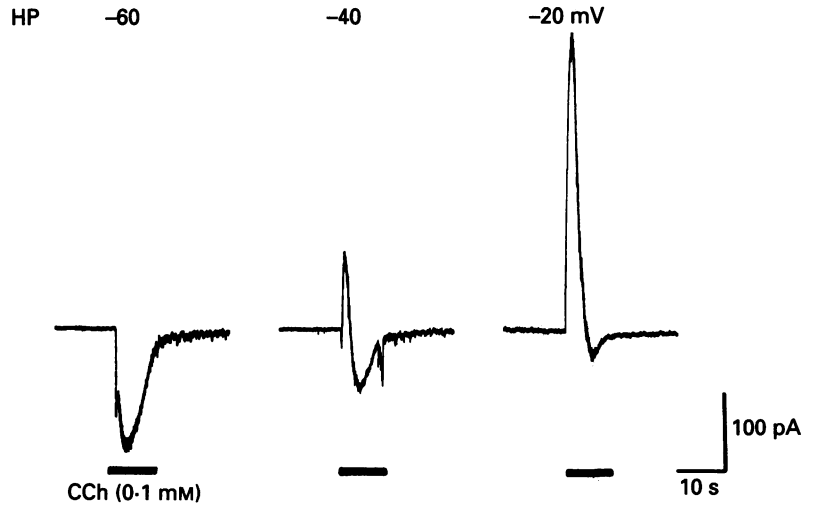

Fig. 1. Membrane current responses to carbachol at various holding potentials in K^+-containing pipette and external solutions. Membrane currents were recorded with nystatin-perforated whole-cell recording. Carbachol (CCh, 0·1 mM) was applied for 10 s at 4 min intervals (▬). Numbers shown above each current indicate holding potentials (HP).

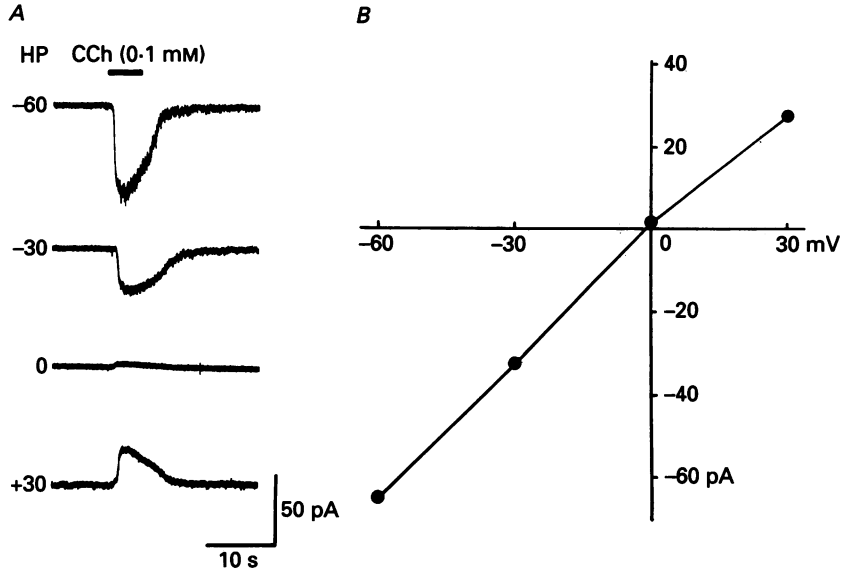

Fig. 2. Current responses to carbachol at various holding potentials in Na^+-containing, K^+-free pipette and external solutions. Whole-cell membrane currents were recorded with the nystatin-perforated whole-cell recording method. Na^+ in the patch pipette and external solutions was substituted for K^+ to inhibit K^+ currents in this and the following figures. *A*, carbachol (CCh, 0·1 mM) was applied for 5 s (▬). Holding potentials (HP) were −60, −30, 0 and +30 mV. *B*, the current–voltage plot was obtained from the cell illustrated in *A*.

Carbachol induced a significant outward current at a holding potential of +30 mV. The mean current response to carbachol at −60 mV was 105·8±39·2 pA and the mean reversal potential (E_r) was −0·3±0·4 mV ($n = 7$).

Ionic mechanisms underlying the inward current evoked by carbachol

Figure 3 shows current responses to carbachol recorded with the nystatin-perforated whole-cell recording method in the presence of various anions – iodide

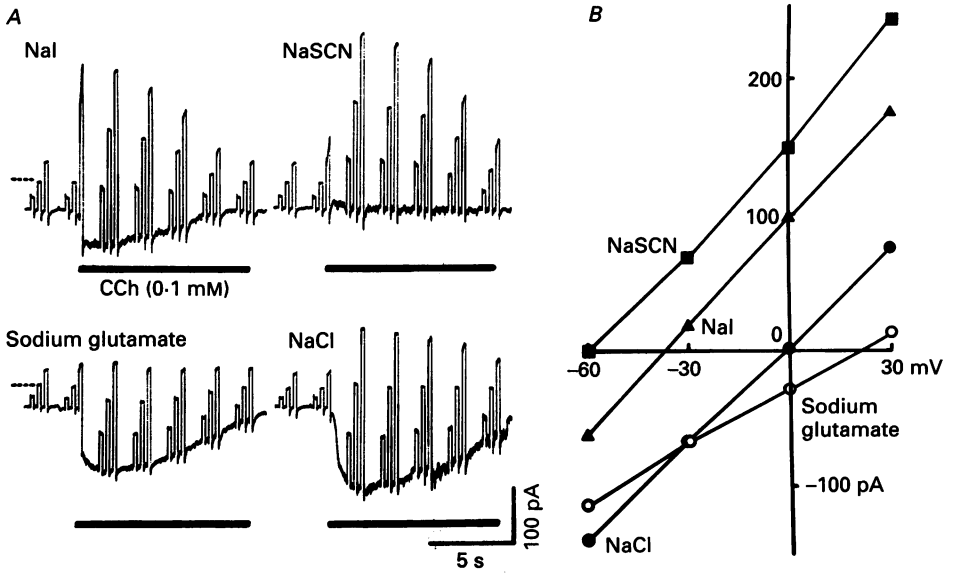

Fig. 3. Reversal potentials of the current response to carbachol in various external solutions. In *A*, the current responses were recorded with the nystatin-perforated whole-cell recording method in a single cell. The external solution was changed sequentially as follows: NaCl, sodium glutamate, NaI, NaSCN, sodium glutamate and NaCl. The membrane potential was held at −60 mV and the voltage was stepped to −30, 0 and +30 mV each for 200 ms at 2 s intervals. Carbachol (CCh, 0·1 mM) was applied for 10 s (▬). In the presence of NaCl, the amplitude of the first response to carbachol was 1·3 times larger than that of the last response. The second responses in the presence of NaCl and glutamate are shown. The dashed lines indicate the position of zero current. In *B*, the current–voltage plots in the presence of various anions were obtained from the cell illustrated in *A*. The measurements of current amplitude and direction were made when the carbachol-induced inward current was close to its peak amplitude. The amplitude of carbachol-induced current was calculated by subtracting the membrane current in the absence of carbachol from the values obtained in its presence.

(I^-), thiocyanate (SCN^-), glutamate$^-$ and chloride (Cl^-) – in the same cell. The membrane potential was maintained at −60 mV and the potential was stepped to −30, 0 and +30 mV each for 200 ms. When external NaCl was replaced by sodium glutamate, E_r had a positive value. Substitution of NaCl with NaI or NaSCN produced a shift of E_r to more inside-negative values. The mean E_r of the carbachol-induced current observed with the nystatin whole-cell method was −1·2±0·4 mV ($n = 12$) in external NaCl, −50·3±2·5 mV ($n = 7$) in NaSCN, −33·0±1·7 mV ($n = 5$) in NaI and 25·4±3·3 mV ($n = 7$) in sodium glutamate.

We have already reported that caffeine causes an increase in Cl^- conductance in these cells (Ohta *et al.* 1993). Therefore, in various external solutions, the E_rs of membrane currents evoked by carbachol were compared with those evoked by caffeine in the same cells with a voltage-jump protocol using standard whole-cell recording (Table 1). As the first but not the second application of carbachol produced

TABLE 1. Reversal potentials (E_r) of the current responses to carbachol and caffeine in various external solutions obtained with the standard whole-cell recording method

		E_r (mV)	
External solution (132 mM)	Number of experiments	Carbachol (0·1 mM)	Caffeine (10 mM)
Sodium chloride	8	−0·9±0·9	−3·0±0·7
Sodium iodide	5	−21·2±2·2**	−37·7±0·7
Sodium thiocyanate	5	−27·0±4·8**	−60·6±3·2
Sodium glutamate	6	+18·2±3·0*	+30·0±3·2
Tris chloride	6	−0·2±1·5	+1·1±0·8

The reversal potentials were obtained with the voltage-jump protocol indicated in the legend to Fig. 3. Application of carbachol (0·1 mM) preceded that of caffeine (10 mM) in each cell in order to compare both responses. **, $P < 0·01$; *, $P < 0·05$ compared with the reversal potential of caffeine-induced currents.

an inward current under these conditions, carbachol was always administered before caffeine. When external Na^+ was replaced by $Tris^+$, there was no difference between the E_r values of inward currents induced by carbachol and caffeine. When external Cl^- was replaced by I^- or SCN^-, which are more permeable than Cl^- through Cl^- channels in vascular smooth muscle cells (Amédée, Large & Wang, 1990*b*), the E_r of the current response to carbachol shifted to more positive values than those to caffeine. On the other hand, when $glutamate^-$, which is considered to be a relatively impermeable anion, was substituted for Cl^-, the E_r of the current response to carbachol moved to a more negative value than that to caffeine. There was a significant difference between the E_r values of the current response to carbachol and caffeine in the presence of SCN^- ($P < 0·01$), I^- ($P < 0·01$) and $glutamate^-$ ($P < 0·05$) (Table 1). The results indicate that other conductance mechanisms in addition to an increase in Cl^- conductance are involved in the current response to carbachol.

It has been reported that muscarinic receptor activation increases non-selective cation conductance in intestinal smooth muscle cells of the rabbit and guinea-pig (Benham *et al.* 1985; Inoue *et al.* 1987; Inoue & Isenberg, 1990*a*, *b*). Therefore, we examined the effects of the replacement of external NaCl with Tris-Cl on the carbachol-induced inward current (Fig. 4). As already mentioned, carbachol (10 μM) produced an inward current accompanied by current noise in the presence of Na^+. Three minutes after the replacement of NaCl with Tris-Cl, the carbachol-induced inward current decreased in amplitude and the increase in current noise was no longer observed. The magnitude of the decrease in the carbachol-induced inward current varied from cell to cell (7–65%) and thus there was no significant difference in amplitude of the responses to carbachol in the presence and absence of Na^+. However, the smaller the initial current response to carbachol, the larger the

decrease in its amplitude caused by Na^+ replacement tended to be. The mean decrease in amplitude in the absence of Na^+ was estimated by subtracting the peak value of the current evoked by carbachol in the presence of Tris-Cl from the mean values of those evoked before and after the replacement of NaCl with Tris-Cl. The

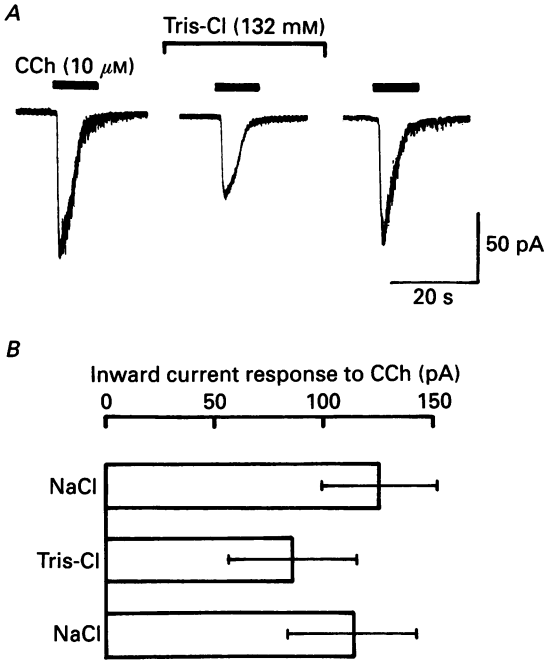

Fig. 4. The effects of removal of Na^+ on the carbachol-induced inward current. In *A*, inward current responses to carbachol (CCh, 10 μM) for 10 s (▬) were recorded with the nystatin-perforated whole-cell recording method at a holding potential of −60 mV. Three minutes after switching from external NaCl to Tris-Cl, carbachol (10 μM) was applied. The mean peak amplitudes (mean ± S.E.M., $n = 6$) of the inward current response to carbachol in the presence and absence of Na^+ are illustrated in *B*.

carbachol-induced inward current decreased by 34·2 ± 4·7 pA ($n = 6$) in the absence of Na^+.

Ca^{2+} transient and membrane current in response to carbachol

In our earlier paper, it was shown that caffeine-induced Cl^- current was accompanied by a simultaneous increase in intracellular concentration of free Ca^{2+} (Ohta *et al.* 1993). It was, therefore, determined with fura-2 microfluorometry whether or not carbachol also elicited an increase in intracellular free Ca^{2+} concentration in parallel with the inward current.

For standard whole-cell recording, fura-2 (0·1 mM) was loaded into the cells through patch pipettes and for nystatin-perforated whole-cell recording, cells were pretreated with the membrane-permeable fura-2 analogue, fura-2AM (5 μM) (Fig. 5). With standard whole-cell recording, carbachol (0·1 mM) evoked an inward current

(135·6 ± 30·2 pA, $n = 13$) and an increase in intracellular free Ca^{2+} concentration (0·76 ± 0·11 μM, $n = 13$). Caffeine (10 mM), which was applied to the cells for 10 s about 4 min after carbachol, also elicited both types of response (107·8 ± 35·2 pA, 0·6 ± 0·05 μM, $n = 13$) and these were of similar magnitude to those evoked by

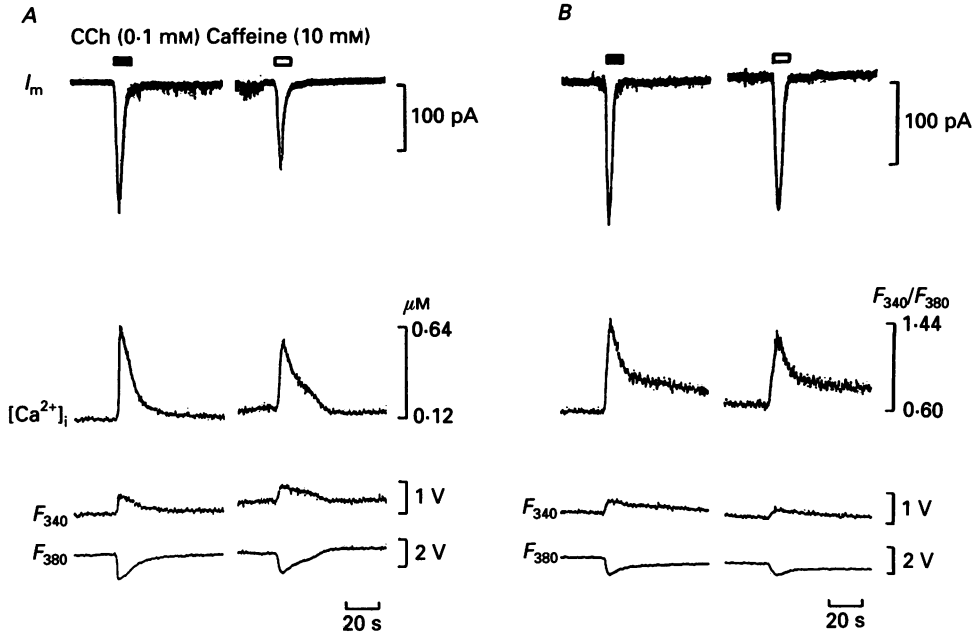

Fig. 5. Simultaneous measurements of intracellular free Ca^{2+} concentration and membrane current in response to carbachol and caffeine combining the fura-2 method with the standard and nystatin-perforated whole-cell recording methods. For whole-cell patch recording (*A*), fura-2 (0·1 mM) was introduced into the cell through a patch pipette. For nystatin-perforated whole-cell recording (*B*), the cells were pretreated with fura-2AM (5 μM) for 30 min at room temperature. At a holding potential of −60 mV, cells were stimulated by carbachol (CCh, 0·1 mM, ▬) and then by caffeine (10 mM, ▭) for 10 s about 4 min after the wash-out of carbachol. Traces are, from top to bottom: membrane currents (I_m), intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$: *A*, μM; *B*, F_{340}/F_{380}) and fluorescent signals excited by 340 (F_{340}) and 380 (F_{380}) nm.

carbachol. With nystatin-perforated whole-cell patch recording, inward currents were elicited by carbachol (104·6 ± 18·0 pA, $n = 12$) and caffeine (90·3 ± 12·4 pA, $n = 12$), the amplitudes of which were comparable to those obtained with standard whole-cell recording. There was no significant difference in the peak currents obtained from the two different methods of current recording. Carbachol and caffeine also increased the intracellular free Ca^{2+} concentration by 1·04 ± 0·15 and 0·70 ± 0·1 (F_{340}/F_{480}, $n = 12$), respectively.

Ca^{2+} dependence of the responses to carbachol

Experiments were carried out to examine whether carbachol-induced inward currents were associated with the increase in intracellular free Ca^{2+} concentration. In order to decrease the intracellular Ca^{2+} concentration, the cells were dialysed with

the pipette solution containing fura-2 and 10 mM EGTA. Under these conditions, carbachol failed to elicit any inward current or Ca^{2+} transient in the cells at a holding potential of −60 mV ($n = 8$, data not shown).

In the cells pretreated with fura-2AM, the current and Ca^{2+} transient in response to carbachol were monitored simultaneously with nystatin-perforated whole-cell

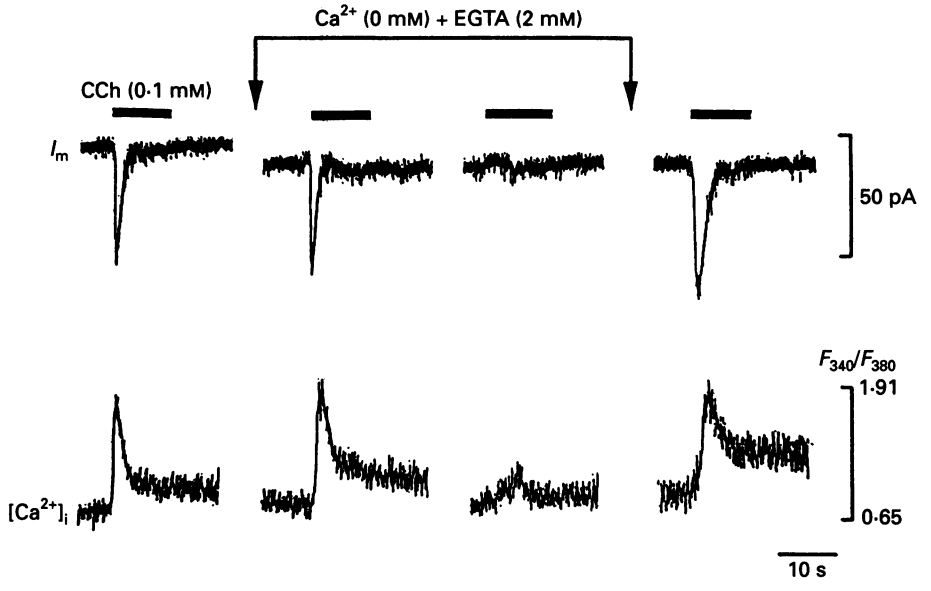

Fig. 6. Effect of removal of external Ca^{2+} on the inward current and Ca^{2+} transient in response to carbachol. With the nystatin-perforated whole-cell recording method at a holding potential of −60 mV, carbachol (CCh, 0·1 mM) was applied for 10 s (▬) to the cells pretreated with fura-2AM (5 μM). Carbachol was applied before, during (at 2 and 6 min) and after exposure to the Ca^{2+}-free external solution containing 2 mM EGTA. Traces are: membrane currents (I_m) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$, F_{340}/F_{380}).

recording at a holding potential of −60 mV in the presence and absence of external Ca^{2+} (Fig. 6). Two minutes after switching from the normal external solution to the Ca^{2+}-free solution containing 2 mM EGTA, carbachol evoked an inward current (93·9 ± 7·4 %) and Ca^{2+} transient (87·4 ± 8·9 %, $n = 7$) with amplitudes comparable to those obtained in the presence of external Ca^{2+}. In four out of seven cells, both responses to carbachol disappeared 6 min after exposure to the Ca^{2+}-free solution. In the other three cells, carbachol was still effective in producing both responses, but their amplitudes were greatly attenuated to varying degrees (current, 32·9 ± 20·8 %; Ca^{2+} transient, 18·3 ± 8·9 % $n = 3$). When Ca^{2+} was reintroduced into the external solution, current and Ca^{2+} transient responses partly recovered by 82·2 ± 12·5 and 42·0 ± 9·9 % ($n = 7$), respectively.

Effects of heparin on the responses to carbachol

Muscarinic activation has been shown to elicit the production of InsP_3 (Gardner *et al.* 1988; Secrest *et al.* 1989; Parekh & Brading, 1991) resulting in Ca^{2+} release from

the intracellular stores. Heparin has been proposed to act as an inhibitor of Ca^{2+} release induced by $InsP_3$ in smooth muscle cells (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989; Komori & Bolton, 1991; Ohta *et al.* 1992).

To examine the effect of heparin on the membrane current and Ca^{2+} transient in response to carbachol, carbachol (0·1 mM) was continuously applied for 1 min and

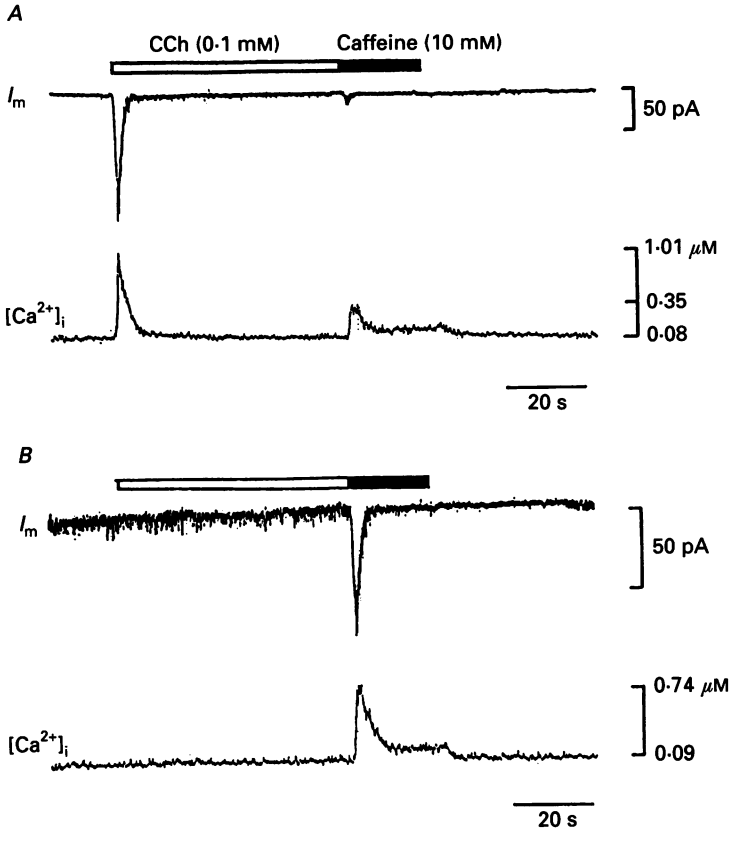

Fig. 7. Effects of heparin on the inward current and Ca^{2+} transient in response to carbachol. The current responses were observed with the standard whole-cell recording method using a patch pipette containing fura-2 (0·1 mM) without (*A*) and with (*B*) heparin (5 mg ml^{-1}) at a holding potential of −60 mV. Carbachol (0·1 mM) was applied for 1 min, and subsequently caffeine (10 mM) for 20 s. Traces are membrane currents (I_m) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$, μM).

then caffeine (10 mM) was applied for 20 s to cells dialysed with fura-2 in the presence and absence of heparin (5 mg ml^{-1}). In spite of the continuous application of carbachol, carbachol produced a transient increase in the current (102·2 ± 15·1 pA) and intracellular free Ca^{2+} concentration (0·95 ± 0·1 μM, $n = 6$), which returned to basal levels within 10 s (Fig. 7*A*). These values were not significantly different from those obtained with application of carbachol for 10 s. However, subsequent application of caffeine caused smaller increases in the inward current (13·7 ± 5·8 pA) and Ca^{2+} transient (0·36 ± 0·04 μM) than those evoked by caffeine 4 min after wash-

out of carbachol (Fig. 5*A*). On the other hand, in the cells dialysed with heparin and fura-2, carbachol failed to produce an inward current and Ca^{2+} transient, but subsequent application of caffeine increased both types of response as much as $159{\cdot}7 \pm 16$ pA and $0{\cdot}69 \pm 0{\cdot}12$ μM ($n = 6$) (Fig. 7*B*). These values were comparable to those obtained with caffeine alone (Fig. 5). In half of the cells, carbachol increased current noise in the presence of heparin.

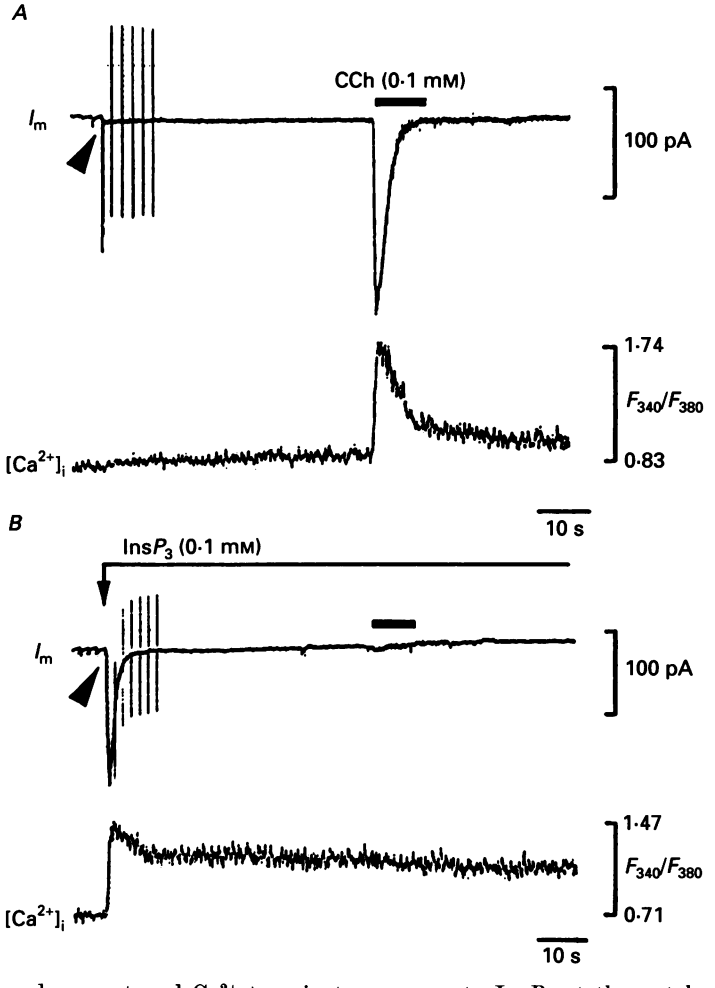

Fig. 8. Inward current and Ca^{2+} transient responses to InsP_3 at the patch membrane break-through at a holding potential of -60 mV. The pipette contained InsP_3 (0·1 mM) in *B* but not in *A*. The cells were pretreated with fura-2AM (5 μM) and the patch membrane potential was held at -60 mV. The transition to the whole-cell mode (arrowhead) resulted in increases in the capacitative currents. One minute after the break-through of the patch membrane, carbachol (CCh, 0·1 mM) was applied to the cell for 10 s (▬). Traces are: membrane currents (I_m) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$, F'_{340}/F_{380}).

Effects of inositol 1,4,5-trisphosphate included in the pipette

It has been shown that in the pipette solution containing InsP_3, a marked outward current was evoked just following break-through of the patch membrane at a holding potential of 0 mV in gastrointestinal smooth muscle of the rabbit (Komori & Bolton, 1991) and rat (Ohta *et al.* 1992). In the cells pretreated with fura-2AM, no response was observed with InsP_3-free pipette solution after break-through from the cell-attached patch to the whole-cell recording mode at a holding potential of −60 mV (Fig. 8*A*). Carbachol (0·1 mM) evoked an inward current (139·3 ± 32·8 pA) and Ca^{2+} transient (1·17 ± 0·13, F_{340}/F_{380}, $n = 7$) 1 min after break-through. On the other hand, a prominent inward current (84·5 ± 16·5 pA) and increase in intracellular free Ca^{2+} concentration (0·80 ± 0·12, F_{340}/F_{380}, $n = 7$) were seen using the pipette solution containing InsP_3 (0·1 mM) just after the break-through of the patch membrane (Fig. 8*B*). The subsequent responses to carbachol no longer occurred in two out of seven cells dialysed with InsP_3. Carbachol caused increases in current noise in two cells, and a small inward current (5·4 ± 3·8 pA) and Ca^{2+} transient (0·1 ± 0·1, F_{340}/F_{380}) in the other three cells.

DISCUSSION

The present experiments indicate that carbachol elicits an inward current and Ca^{2+} transient at a holding potential of −60 mV in intestinal smooth muscle cells of the rat. Under the same experimental conditions, we have already shown that caffeine evokes an inward current resulting from an increase in Cl^- conductances, which is activated by the intracellular Ca^{2+} released from the intracellular stores (Ohta *et al.* 1993). When external Cl^- was replaced with SCN^-, I^- or glutamate$^-$, the E_r of carbachol-induced currents obtained with the voltage-jump protocol shifted in the same sequence as those of caffeine-induced currents. These results suggest that carbachol increases Cl^- conductance in smooth muscle cells of the rat intestine.

With standard whole-cell patch recording, however, each E_r value of the current response to carbachol obtained with the voltage-jump protocol in the presence of foreign anions was shifted towards 0 mV as compared with that to caffeine. It appears that there is involvement of other conductance mechanisms in the current response to carbachol. Carbachol has been shown to increase the non-selective cation conductance accompanied by an increase in membrane current noise in gastrointestinal smooth muscle cells of the rabbit (Benham *et al.* 1985), guinea-pig (Inoue & Isenberg, 1990*a*, *b*) and dog (Vogalis & Sanders, 1990; Sims, 1992). In rat intestinal smooth muscle cells, the inward current response to carbachol tended to be attenuated by the replacement of Na^+ with $Tris^+$ at a holding potential of −60 mV. In addition, current noise was superimposed on the inward current response to carbachol in the presence of Na^+ but not in its absence. Therefore, it is suggested that an increase in Na^+ conductance is also partly involved in the inward current response to carbachol in intestinal smooth muscle cells of the rat. The difference between E_rs of the current response to carbachol and caffeine may be explained by the increased Na^+ conductance in response to carbachol but not to caffeine. It seems likely that

non-selective cation channels in addition to Cl^- channels were activated by carbachol in the smooth muscle cells of the rat intestine. Both conductance mechanisms have been shown to be involved in the current response to noradrenaline in the portal vein (Byrne & Large, 1988; Wang & Large, 1991) and ear artery of the rabbit (Amédée *et al.* 1990*a*) and the current response to acetylcholine in the trachea of the dog and guinea-pig (Janssen & Sims, 1992).

With the voltage-jump protocol of nystatin-perforated whole-cell recording, anion substitution of the external solution produced marked changes in E_r of the carbachol-induced current compared to those obtained with the standard whole-cell recording method. In smooth muscle cells of the rabbit ear artery, it has been shown that the membrane current activated by noradrenaline appears to be solely due to an increase in Cl^- conductance with no perceptible contribution from an increase in cation conductance when the nystatin technique is used (Amédée *et al.* 1990*b*). Although the reason for this remains unclear, these results appear to be similar to those in the present experiments.

The inward current response to carbachol seems to depend on the increase in intracellular free Ca^{2+} concentration, because it is inhibited by a high concentration of EGTA in the pipette solution. Furthermore, even when cells were exposed to Ca^{2+}-free solution containing EGTA for 2 min, carbachol, like caffeine (Ohta *et al.* 1993), elicited an inward current and Ca^{2+} transient comparable to those observed in the presence of Ca^{2+}, and both responses were simultaneously attenuated by further exposure of cells to the Ca^{2+}-free solution. It therefore seems likely that the inward current response to carbachol occurs as a consequence of a rise in intracellular free Ca^{2+} which was released from intracellular stores in smooth muscle cells of the rat intestine. The increase in intracellular free Ca^{2+} has been shown to play a role in activation of non-selective cation conductances in gastrointestinal smooth muscle cells (Inoue & Isenberg, 1990*b*; Pacaud & Bolton, 1991; Sims, 1992) and chloride conductances in various smooth muscle cells (Byrne & Large, 1987; Pacaud *et al.* 1989; Amédée *et al.* 1990*a*, *b*; Wang & Large, 1991; Janssen & Sims, 1992; Wang, Hogg & Large, 1992). The inward current responses to carbachol involve activation of not only Cl^- but also Na^+ conductances in rat intestinal smooth muscle cells. Carbachol, like caffeine, seems to activate the Cl^- conductance by increasing intracellular free Ca^{2+} concentration. However, caffeine, which released Ca^{2+} from the intracellular stores, opened the Ca^{2+}-activated Cl^- channels but not cation channels (Ohta *et al.* 1993). This indicates that the cation channels are not stimulated directly by Ca^{2+}. It seems likely that the cation channels, once opened by carbachol, are modulated by intracellular free Ca^{2+}.

InsP_3 is considered to be a second messenger substance producing the release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). The release of Ca^{2+} from the stores by activation of muscarinic receptors has been proposed to be due to the production of InsP_3 in rabbit jejunal smooth muscle cells (Komori & Bolton, 1990, 1991). This seems to be the case in rat intestinal smooth muscle cells, because inward current and Ca^{2+} transient responses to carbachol, but not those to caffeine, were inhibited by internal application of heparin which competes with InsP_3 for receptor sites in skinned smooth muscles (Kobayashi *et al.* 1989; Ohta *et al.* 1992). It has been

shown that InsP_3 introduced into a cell through the patch pipette produces outward K^+ currents resulting from Ca^{2+} released from the stores at a holding potential of 0 mV (Komori & Bolton, 1991; Ohta *et al.* 1992). Similarly, InsP_3 elicited an inward current and Ca^{2+} transient in rat small intestinal smooth muscle cells at a holding potential of −60 mV when introduced into the cell through the patch pipette. In addition, carbachol failed to cause either response in cells dialysed with InsP_3. It therefore appears that the Ca^{2+} transient in response to carbachol is attributed to InsP_3-induced Ca^{2+} release from intracellular stores. There seems to be an overlap in carbachol- and caffeine-sensitive Ca^{2+} stores since caffeine followed by carbachol application produces only a small Ca^{2+} transient in smooth muscle cells of the rat intestine.

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