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Muscarinic cation current and suppression of Ca^{2+} current in guinea pig ileal smooth muscle cells

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Abstract

Cationic current (I_{cat}) and inhibition of the voltage-dependent Ca^{2+} current (I_{Ca}) evoked by muscarinic receptor activation with carbachol were studied using whole-cell patch clamp technique in smooth muscle cells isolated from longitudinal muscle of guinea pig small intestine. With low buffering of $[Ca^{2+}]_i$ (0.1 mM BAPTA [1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] in pipette solution) I_{cat} and I_{Ca} inhibitory responses had a rapid onset to an initial peak followed by a sustained phase. The sustained phase of I_{Ca} suppression was bigger than in the case when $[Ca^{2+}]_i$ was clamped to 100 nM, but decreased with repeated stimulation. Upon repeated stimulation with 50 μM carbachol in cells where $[Ca^{2+}]_i$ was clamped to 100 nM and when GTP was absent, I_{cat} amplitude decreased strongly and more substantially compared to I_{Ca} inhibition, but both responses declined only slightly when 1 mM GTP was present in the pipette solution. GDP-βS (1 or 5 mM) in pipette solution or pre-treatment of cells with pertussis toxin (6 μg/ml, for 4 h or longer) blocked I_{cat} more than I_{Ca} suppression by carbachol, whereas L-NAME (*N*-Ω-nitro-L-arginine methyl ester hydrochloride) (100 μM in pipette solution) affected neither of them significantly. We conclude that the cationic current and the suppression of the voltage-dependent Ca^{2+} current evoked by muscarinic receptor activation are mediated by pertussis toxin-sensitive G-protein(s) but the latter response was less sensitive to blockade by GDP-βS and to GTP deficiency in the cell. © 1998 Elsevier Science B.V.

Keywords: Smooth muscle, intestinal; Patch clamp; Ca^{2+} channel; Desensitization; G-protein; Pertussis toxin; Guanine nucleotide

1. Introduction

Activation of muscarinic receptors in intestinal smooth muscle causes intracellular Ca^{2+} release and membrane depolarization leading to smooth muscle contraction. The depolarization is caused by inward current of Na^+ through cationic channels (Benham et al., 1985; Inoue et al., 1987; Inoue and Isenberg, 1990a) and causes voltage-dependent Ca^{2+} channels to open. The signal from muscarinic receptors is conveyed in at least three directions: a) to cationic channel via pertussis toxin-sensitive G-protein (Inoue and Isenberg, 1990b; Komori et al., 1992), which also modulates the voltage dependence of the cationic channel (Zholos and Bolton, 1994); b) to phospholipase C via pertussis toxin-insensitive G-protein (Komori et al., 1992), which leads to production of $InsP_3$ and thus to the release of Ca^{2+} from intracellular stores (Komori and Bolton, 1991; Pacaud and Bolton, 1991) and c) to voltage-dependent Ca^{2+} channels (Mitsui and Karaki, 1990; Inoue and Isenberg, 1990c; Beech, 1993) via a G-protein, which was reported to be pertussis toxin-insensitive (Unno et al., 1995) and which suppresses I_{Ca}. It is likely that in intact cells this mechanism, together with hyperpolarizing current through Ca^{2+}-dependent K^+ channels, acts as a negative feedback to prevent Ca^{2+} overload of the cells.

I_{Ca} suppression, similar to I_{cat} generation, was shown to consist of a transient component which is due to intracellular Ca^{2+} release and a sustained component mediated by G-protein and requiring some level of $[Ca^{2+}]_i$ (Beech, 1993; Unno et al., 1995). This phenomenon is not restricted to intestinal smooth muscle or to muscarinic stimulation. Similar effects were observed upon administration of oestradiol in rabbit basilar artery cells (Ogata et al., 1996), with carbachol on pacemaker cells of rabbit sinoatrial node (Han et al., 1995), with acetylcholine on guinea pig gastric and tracheal myocytes (Wade et al., 1996) and on guinea pig ileal cells with histamine, bradykinin, substance P or acetylcholine (Beech, 1993).

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Prolonged or repeated exposure of isolated strips of guinea pig ileal smooth muscle to agonist leads to attenuation of the contractile response (Cantoni and Eastman, 1946; Paton and Rothschild, 1965; Joiner, 1973). In voltage-clamped intestinal smooth muscle cells it was demonstrated that cationic current (I_{cat}) also fades under these conditions and that this effect can be reduced if 1 mM GTP is introduced into the cell (Zholos and Bolton, 1996). Suppression of I_{Ca}, was suggested to participate in desensitization of force development to acetylcholine in guinea pig ileum (Himpens et al., 1991). Therefore, our aim was to investigate whether there are changes in the suppression of I_{Ca} as well, upon repeated muscarinic stimulation, and to elucidate the role of GTP and G-protein in this process when the effects due to increases in $[Ca^{2+}]_i$ were prevented.

2. Materials and methods

2.1. Cell preparation and pertussis toxin treatment

Male Dunkin–Hartley guinea pigs (350–500 g) were killed by cervical dislocation followed by exsanguination. Experiments were performed at room temperature (20–25°C) on single ileal smooth muscle cells obtained after collagenase treatment (1 mg/ml) at 36°C for 23–26 min and used within 10 h after isolation.

To investigate which type of G-protein mediates muscarinic suppression of I_{Ca}, the cells were incubated in physiological salt solution (PSS) containing 1.25 mM $CaCl_2$ and 0.6 mM $MgCl_2$ with 6 μg/ml of pertussis toxin or control added, at 36°C for 4–9 h. The incubation chamber was moist and saturated with oxygen. pertussis toxin was supplied a) in the form of lyophilized powder (Calbiochem-Novabiochem, Nottingham, UK), which was reconstituted in water to make stock solution, and b) in 50% glycerol solution (Speywood, Salisbury, Wiltshire, UK), which served as a stock solution. Stock solution of pertussis toxin was added to the cell suspension in PSS.

2.2. Membrane current recordings

Membrane current recordings were made from the whole cell using borosilicate patch pipettes (1–3 MΩ) and a Biologic RK 300 (Biologic Science Instruments, Claix, France) or Axopatch 200A (Axon Instruments, Foster City, CA, USA) voltage clamp amplifier. Voltage clamp pulses were generated and data captured on-line using a CED 1401 interface run under CED Patch and Voltage Clamp software (Cambridge Electronic Design, Cambridge, UK) or Labmaster DMA TL-1-125 interface run under pClamp program (Axon Instruments, Foster City, CA, USA). Series resistance was compensated by about 80%.

Carbachol was applied either focally, using pressure ejector and glass pipette roughly 10 μM in diameter placed in the vicinity of the cell and operated by pulses of N_2 of defined pressure and duration, or by fast exchange of external solution (Zholos and Bolton, 1995). The cells were exposed to a near maximally effective concentration of carbachol (50 μM) for 60–90 s, every 150–180 s. Voltage steps from −50 mV to 0 mV lasting 25 or 50 ms were applied every 5 or 10 s, respectively. Signals were sampled at 5 kHz and analyzed and plotted using MicroCal Origin software (MicroCal Software, Northampton, MA, USA). The experiments were begun only after the Ca^{2+} current stabilized, which was usually 5–6 min after establishing whole-cell configuration. Cationic current was measured by subtracting holding current in the absence of carbachol. Leakage compensation was not used.

2.3. Solutions and drugs

The physiological saline solution (PSS) contained (mM): NaCl 120, KCl 6, glucose 12, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1.2, pH adjusted to 7.4 with NaOH. In Ca^{2+}, Mg^{2+}-free PSS, $CaCl_2$ and $MgCl_2$ were omitted. In experiments where Na^+ was used as a charge carrier for Ca^{2+} channels, 1 mM EGTA was added to Ca^{2+}, Mg^{2+}-free PSS to bind the traces of divalent cations. Caesium-based saline solution (Cs^+-SS) contained (mM): CsCl 120, glucose 12, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1.2, pH adjusted to 7.4 with CsOH.

The cells were studied with Cs^+-based pipette solutions to eliminate K^+ outward currents. 'Low BAPTA' pipette solution contained (mM): CsCl 120, MgATP 1, creatine 5, glucose 20, HEPES 10, BAPTA 0.1, pH adjusted to 7.4 with CsOH. 'High BAPTA' pipette solution ($[Ca^{2+}]_i$ clamped at 100 nM) contained (mM): CsCl 80, MgATP 1, creatine 5, glucose 20, HEPES 10, BAPTA 10, $CaCl_2$ 4.6, pH adjusted to 7.4 with CsOH (total Cs^+ 124 mM). $[Cs^+]_o$ was made equal to $[Cs^+]_i$ to set I_{cat} reversal potential to 0 mV to avoid contamination of I_{Ca} by I_{cat} during voltage steps to 0 mV.

HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), $MgCl_2$, CsCl, EGTA (ethyleneglycol-bis-(β-aminoether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), creatine, BAPTA (1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), MgATP (magnesium salt of ATP), carbachol (carbamylcholine chloride), GTP (guanosine-5′-triphosphate, sodium salt), GDP-βS (guanosine-5′-*O*-(2-thiodiphosphate), trilithium salt), collagenase (type 1A), soybean trypsin inhibitor (type II-S), bovine serum albumin, L-NAME (*N*-Ω-nitro-L-arginine methyl ester hydrochloride) and atropine were all from Sigma (UK). NaCl, KCl, glucose and $CaCl_2$ were obtained from BDH (UK).

2.4. Statistics

Values are given as means ± S.E.M. I_{Ca} inhibition by carbachol was expressed as the reduction in I_{Ca} as a percentage of the control current before carbachol applica-

tion. Unless otherwise indicated, two-tailed Student's *t*-test for unpaired observations was used to compare the data and the differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Role of intra- and extracellular Ca^{2+} in I_{Ca} suppression

In single guinea-pig ileal cells held at −50 mV using 'Low BAPTA' pipette solution and depolarized to 0 mV every 5 s or 10 s to evoke voltage-dependent Ca^{2+} current, carbachol application caused two effects: (i) I_{Ca} inhibition (Fig. 1A) and (ii) cationic current generation (Fig. 1B, same cell) (note that −50 mV corresponds approximately

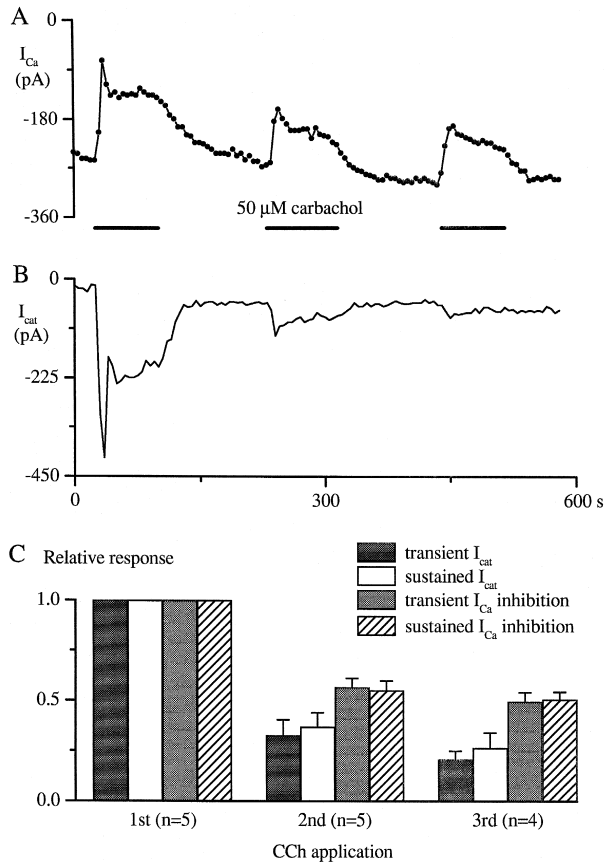

Fig. 1. Desensitization of the responses to carbachol in experiments with cells held with pipettes without added GTP and with low buffering of $[Ca^{2+}]_i$ (0.1 mM BAPTA in the pipette solution). Carbachol evoked biphasic I_{Ca} inhibition (A) and inward cationic current (B, same cell as in A) consisting of a transient and sustained phase. Both phases were attenuated upon repeated agonist administration. (C): summary data presented relative to the first response of the same type. Values normalized as 1.0 were: transient I_{cat} −433±119 pA, sustained I_{cat} −296±108 pA, transient I_{Ca} inhibition 68.6±4.1%, sustained I_{Ca} inhibition 49.6±9.8% with the number of observations indicated. All responses faded significantly ($P < 0.003$ at least). In this and all subsequent figures time zero on the abscissa corresponds to about 5–6 min after break-through.

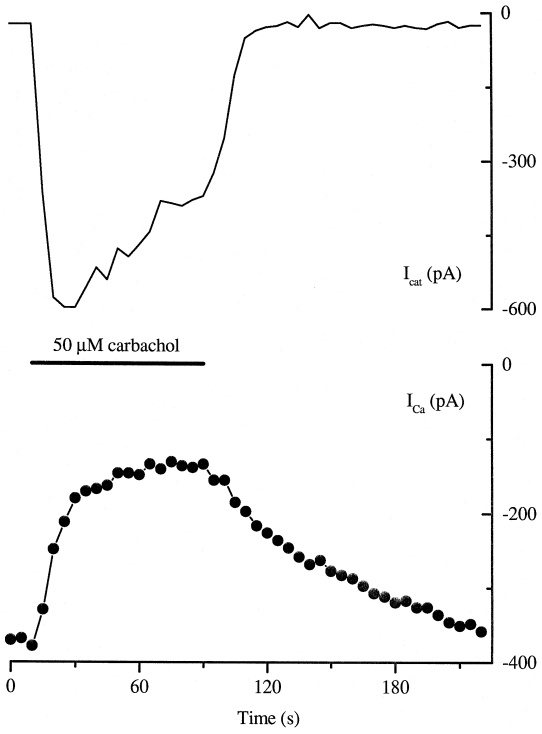

Fig. 2. Differences in the time course of the muscarinic receptor cationic current generation (top panel, measured at −50 mV) and voltage-dependent Ca^{2+} current inhibition (bottom panel, measured at 0 mV). Carbachol was applied to a single voltage-clamped guinea-pig ileal smooth muscle cell during the period indicated by the horizontal bar. Membrane potential was briefly stepped from −50 mV to 0 mV every 5 s to evoke I_{Ca}. I_{cat}, but not I_{Ca} inhibition, faded in the continuous presence of the agonist. Also note the significantly slower onset and offset rates of carbachol action on I_{Ca} in comparison to I_{cat} generation. GTP-free 'High-BAPTA' pipette solution was used with $[Ca^{2+}]_i$ clamped to 100 nM.

to the peak of I_{cat} bell-shaped *I–V* relationship at negative potentials (Zholos and Bolton, 1994)). The experimental protocol we employed allowed a reliable separation of these currents as at −50 mV most of the Ca^{2+} channels are deactivated whereas at 0 mV where I_{Ca} was measured possible contamination from I_{cat} was negligible because this is the I_{cat} reversal potential (e.g., Fig. 4A,B where similar reduction of I_{Ca} amplitude is seen irrespective of I_{cat} size during repeated carbachol applications). It should be noted also that cationic current when recorded in an analogue manner or when sampled digitally with a sufficiently high rate shows large noisy fluctuations, but in this and all other figures it was sampled every 5 or 10 s, hence its appearance is much smoother.

Both I_{Ca} suppression (Fig. 1A) and I_{cat} (Fig. 1B) had a transient and a sustained phase (Unno et al., 1995; also in case of histamine: Beech, 1993) the transient phase being due to intracellular Ca^{2+} release and a rise in $[Ca^{2+}]_i$ (Ganitkevich et al., 1987; Inoue and Isenberg, 1990c; Pacaud and Bolton, 1991; Komori et al., 1992). Upon repeated administration of carbachol with no GTP added to

the pipette solution the first sustained I_{Ca} suppression response was larger than in cells with $[Ca^{2+}]_i$ clamped to 100 nM and without GTP (see below) ('Low BAPTA': 49.6 ± 9.8%, $n = 5$ vs. 'High BAPTA': 27.1 ± 3.6%, $n = 14$; $P < 0.02$) but with repeated administrations it declined to levels of suppression comparable to those obtained when $[Ca^{2+}]_i$ was clamped to 100 nM ('Low BAPTA': 2nd administration: 28.6 ± 8.4%, $n = 5$, 3rd administration: 24.6 ± 3.7%, $n = 4$ vs. 'High BAPTA': 2nd administration: 23.1 ± 2.1%, $n = 14$, 3rd administration: 18.4 ± 2.5%, $n = 13$; $P > 0.3$ and $P > 0.2$, respectively). I_{Ca} suppression and I_{cat} responses expressed relative to the first responses of corresponding type, are shown in Fig. 1C.

Omitting $CaCl_2$ and $MgCl_2$ from the extracellular solution and adding 1 mM EGTA to bind residual Ca^{2+} and Mg^{2+} resulted in a several-fold larger current carried by Na^+ through modified Ca^{2+} channels (data not shown). In cells with $[Ca^{2+}]_i$ clamped to 100 nM and without GTP in the pipette solution, $I_{Na(Ca)}$ suppression was 8.7 ± 3.1% ($n = 5$, first application) and 8.4 ± 4.8% ($n = 5$, second application) which were not significantly different. In the presence of 1 mM GTP in the pipette solution, $I_{Na(Ca)}$ suppression was larger (34.4 ± 18.5%, $n = 5$) and also did not change significantly upon second administration of carbachol (29.6 ± 4.9%).

In cells held at −50 mV using 'High BAPTA' pipette solution to abolish all Ca^{2+}-release related effects, carbachol application produced only an initial and then declining I_{cat} and a developing and then sustained I_{Ca} inhibition (Fig. 2). Three major differences are notable in the kinetics of I_{cat} generation compared to I_{Ca} inhibition in the same cell. First, I_{cat} peaked much earlier, usually 5–10 s after agonist application, whereas I_{Ca} inhibition continued to increase up to 1–2 min. Second, in the continuous presence of carbachol I_{cat}, but not I_{Ca} inhibition, faded. Third, after carbachol was wash-out I_{cat} decayed within a few seconds but I_{Ca} recovery took at least 2–3 min. These findings may indicate that either different G-proteins mediate these two effects or, if the same muscarinic receptor and G-protein is involved, the requirement for the amount of activated G-proteins is different. To explore further these differences we compared these two effects of muscarinic receptor stimulation using repeated carbachol applications.

3.2. GTP-dependence of I_{cat} and I_{Ca} inhibition

I_{cat}, remained statistically unchanged even upon a third administration of carbachol to cells with 1 mM GTP added to the pipette solution (Fig. 3B,D) but declined in size upon repeated administration of carbachol to cells without added GTP (Fig. 4B,D). Suppression of I_{Ca} behaved similarly although the decline when GTP was not added to the pipette solution was less (Fig. 4A,D). These differences could be better illustrated by comparing the ratio of % I_{Ca} inhibition to I_{cat} size during repeated carbachol applica-

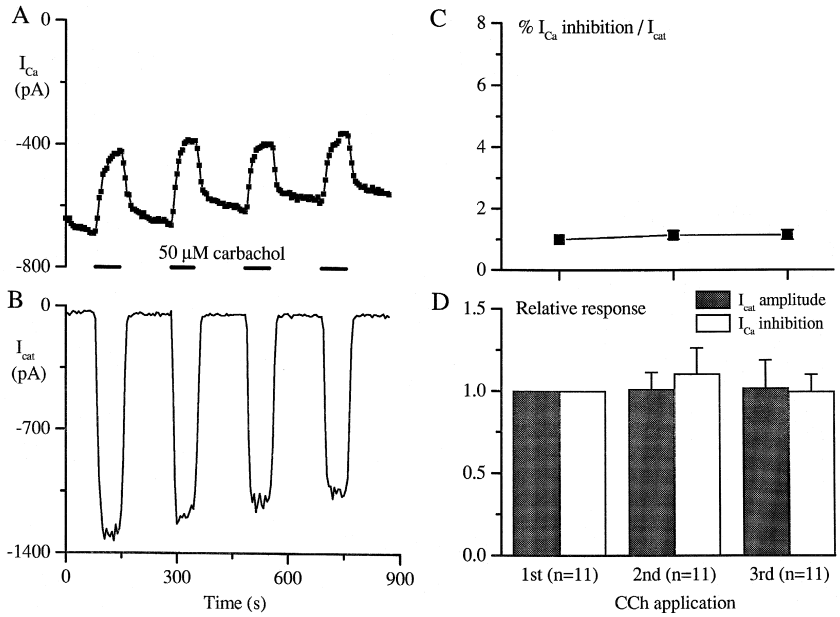

Fig. 3. Relation between I_{Ca} inhibition and I_{cat} generation during repeated carbachol applications in cells held with pipettes containing 1 mM GTP and with $[Ca^{2+}]_i$ clamped at 100 nM. (A), (B): peak I_{Ca} and I_{cat}, respectively, measured in the same cell during 50 μM carbachol applications shown by the horizontal bars. (C): in each individual cell % I_{Ca} inhibition was related to I_{cat} amplitude during the same carbachol application. I_{cat} amplitude during the first response to carbachol was normalized as 1.0. The number of observations is the same as shown on the abscissa in panel D. Under these conditions no change in the ratio was seen. Note that the same scale applies as in Fig. 4C. (D): averaged I_{Ca} inhibition and I_{cat} size with repeated carbachol applications. Values normalized as 1.0 during the first response to carbachol were −633 ± 90 pA ($n = 11$) for I_{cat} amplitude and 35.5 ± 3.4% ($n = 11$) for I_{Ca} inhibition. No statistically significant changes in these two responses were observed.

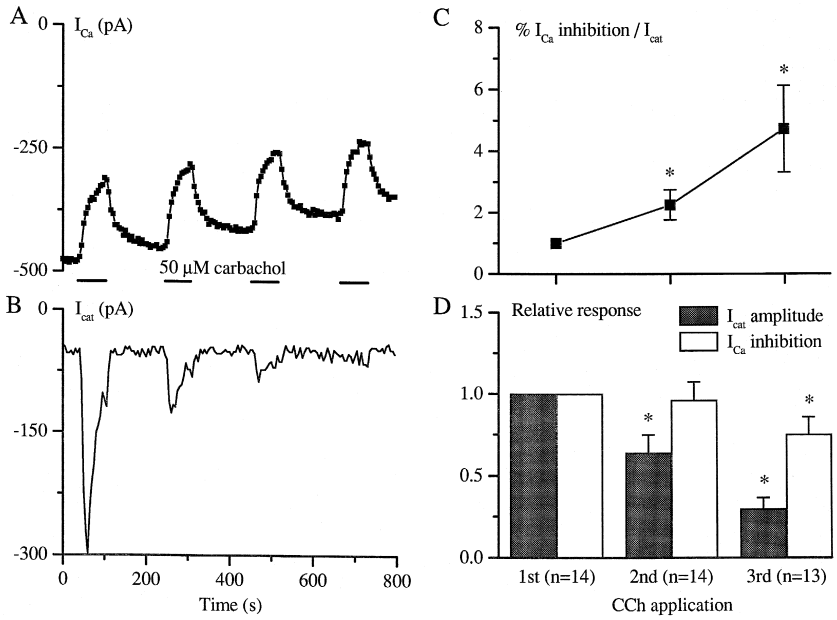

Fig. 4. Relation between I_{Ca} inhibition and I_{cat} generation during repeated carbachol applications in an experiment similar to that shown in Fig. 3 but with GTP-free pipette solution. (A), (B): peak I_{Ca} and I_{cat}, respectively, measured in the same cell during 50 μM carbachol applications shown by the horizontal bars. (C): the ratio of % I_{Ca} inhibition to I_{cat} size was calculated as described for Fig. 3C and plotted on the same scale. $*$ denotes $P < 0.05$ compared to the first response. (D): averaged changes in I_{Ca} inhibition and I_{cat} size with repeated carbachol applications. Values measured during the second and third responses were normalized to the corresponding values during the first response in the same cell which were -341 ± 61 pA ($n = 14$) for I_{cat} amplitude and $27.1 \pm 3.6\%$ ($n = 14$) for I_{Ca} inhibition. $*$ denotes $P < 0.05$.

tions to the same cell. The ratio remained unchanged in cells with 1 mM GTP in the pipette solution (Fig. 3C) but increased significantly in cells without GTP added to the pipette solution (Fig. 4C). I_{cat} size but not I_{Ca} suppression was significantly greater in cells with 1 mM GTP added than in cells perfused with pipette solution without GTP (I_{cat}: +GTP: -633 ± 90 pA, $n = 11$; no GTP: -341 ± 61 pA, $n = 14$; $P < 0.02$. I_{Ca} suppression: +GTP: $35.5 \pm 3.4\%$, $n = 11$; no GTP: $27.1 \pm 3.6\%$, $n = 14$; $P > 0.1$). Control I_{Ca} amplitude (immediately before application of

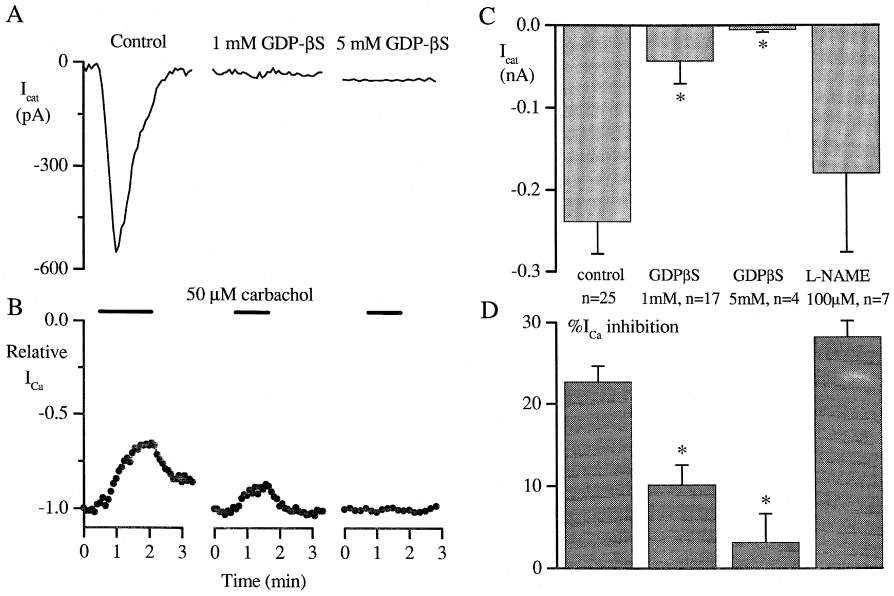

Fig. 5. Original records and summary data from the experiments with GDP-βS and L-NAME. Each substance was added to Cs^+-based pipette solution without GTP and with $[Ca^{2+}]_i$ clamped to 100 nM. 50 μM carbachol was administered focally for 60–90 s, 7–8 min after establishing the whole-cell configuration. $*$: statistically significant, $P < 0.05$. (A): cationic current and (B): Ca^{2+} current measured in three different cells in control and with 1 or 5 mM GDP-βS added to the pipette solution as indicated. In B Ca^{2+} current amplitude before carbachol application was normalized as 1.0 to facilitate comparison (from left to right it was -359, -481 and -229 pA). (C): effects on carbachol-evoked I_{cat}. (D): effects on carbachol-evoked I_{Ca} inhibition.

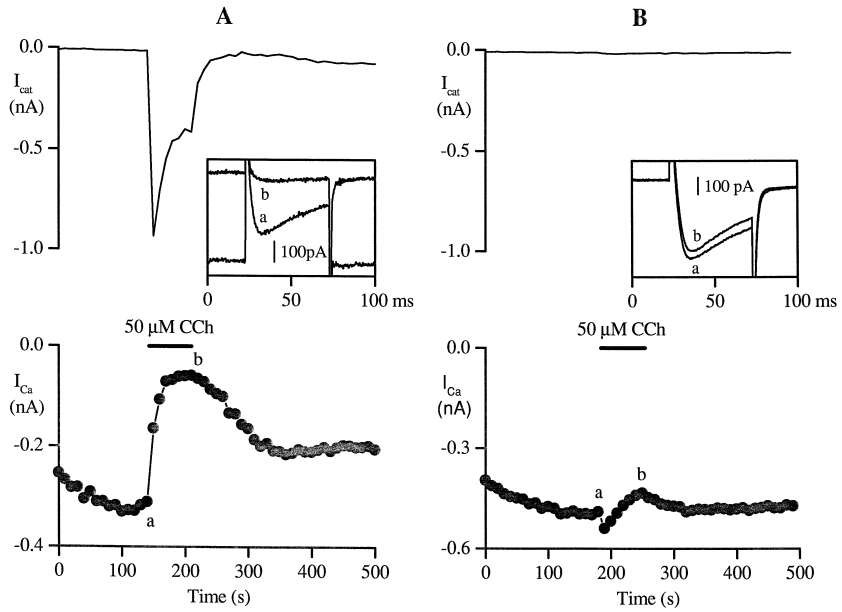

Fig. 6. Original traces of I_{cat} (upper row) and time courses of peak I_{Ca} (lower row) in A, control and B, cells treated by pertussis toxin supplied in 50% glycerol. The cells were bathed in Cs^+-SS and perfused with Cs^+-based pipette solution without GTP and with $[Ca^{2+}]_i$ clamped to 100 nM. Insets: original traces of I_{Ca} obtained at times indicated in the bottom panels.

carbachol) was not affected by GTP (+GTP: -553 ± 47 pA, $n = 11$; no GTP: -493 ± 46 pA, $n = 14$; $P > 0.3$).

GDP-βS is a non-hydrolyzable analogue of GDP known to block the function of G-proteins and 1 mM GDP-βS in the pipette solution abolished or significantly suppressed I_{cat} (Fig. 5A,C). Of 17 cells tested, I_{Ca} suppression was completely prevented in 7 cells, whereas in 10 cells I_{Ca} suppression was partially blocked. 5 mM GDP-βS blocked both I_{cat} and I_{Ca} inhibition by carbachol.

The inhibitor of nitric oxide synthase, L-NAME (100 μM in pipette solution) did not affect either I_{cat}, or I_{Ca} suppression significantly (Fig. 5C,D).

3.3. Type of G-protein involved in muscarinic I_{Ca} suppression

To establish which type of G-protein mediates muscarinic suppression of I_{Ca} we used pertussis toxin, which is known to ADP-ribosylate the α-subunit of G-proteins belonging to G_i/G_o families (Katada and Ui, 1982), thus uncoupling them from the receptors (Gilman, 1987). The cells had $[Ca^{2+}]_i$ strongly buffered to 100 nM and no GTP was added. Pertussis toxin from both sources blocked I_{cat} and did not affect significantly the control amplitude of I_{Ca} in the absence of carbachol (Fig. 6). In 23 cells studied I_{Ca} in control group was 474 ± 48 pA ($n = 11$) and after the treatment with pertussis toxin supplied in 50% glycerol I_{Ca} amplitude was 384 ± 55 pA ($P > 0.2$). I_{cat} in these cells was 776 ± 154 pA ($n = 11$) and 29 ± 12 pA, respectively ($P < 0.0001$). Before and after the treatment with pertussis toxin supplied as lyophilized powder I_{Ca} amplitude was 397 ± 103 pA ($n = 4$) and 402 ± 74 pA ($n = 5$) ($P > 0.9$) whereas I_{cat} amplitude decreased from 910 ± 316 pA ($n = 4$) to 30 ± 14 pA ($n = 5$) ($P < 0.02$).

Upon administration of carbachol, I_{Ca} suppression in pertussis toxin-incubated cells was significantly reduced in comparison to control cells (Fig. 6). The level of attenuation of I_{Ca} suppression was, however, different. pertussis toxin supplied in 50% glycerol solution decreased I_{Ca} suppression from $61.9 \pm 10.2\%$ ($n = 11$) to $8.9 \pm 2.2\%$ ($n = 12$), while pertussis toxin supplied as lyophilized powder decreased I_{Ca} suppression from $73.0 \pm 5.4\%$ ($n = 4$) to $42.9 \pm 10.6\%$ ($n = 5$). While control I_{Ca} suppressions were not statistically different between these two groups of cells the effects of pertussis toxin from the two different commercial sources were significantly different one from another ($P < 0.0003$).

Generally, the cells incubated with pertussis toxin for less than 3–4 h still showed some I_{Ca} suppression by carbachol. It was interesting that I_{Ca} inhibition in these cells produced by carbachol declined in contrast to control cells where no desensitization was observed. With longer times of incubation muscarinic stimulation had little or no effect on I_{Ca} which suggests that a long time of incubation with a relatively high concentration is needed for pertussis toxin to exert its effect on G-proteins regulating Ca^{2+} channels.

4. Discussion

A number of differences were detected between the behaviour of the cationic current and the inhibition of the

voltage-dependent Ca^{2+} current, both evoked by activation of the muscarinic receptor; these differences imply that the signal transduction mechanisms, probably G-proteins, involved differ in some way.

The inhibition of I_{Ca} produced by muscarinic receptor activation developed more slowly than the cationic current and declined more slowly on washout; it desensitised significantly less on repetition and was more resistant to inhibition by GDP-βS added to the pipette solution and to pertussis toxin treatment of the cells. In these experiments we did not find as others have found (Unno et al., 1995) that the inhibition of I_{Ca} was resistant to pertussis toxin, so it seems likely that both I_{cat} and the inhibition of I_{Ca} by carbachol via muscarinic receptors are mediated by G-proteins G_i and/or G_o. Nevertheless, it seems possible that there are different G-protein types mediating these two responses; since a number of G-proteins are pertussis toxin-sensitive, but presumably not equally so, different pertussis toxin-sensitive G-proteins might mediate these two muscarinic effects.

In most of these experiments $[Ca^{2+}]_i$ was 'clamped' using a $CaCl_2$/BAPTA combination at 10^{-7} M free Ca^{2+} concentration. This manoeuvre effectively removed a major effect of muscarinic receptor activation, release of Ca^{2+} from stores in the cell; this as shown in Fig. 1 strongly potentiates both I_{cat} and inhibition of I_{Ca} (see also Beech, 1993; Unno et al., 1995). We did not, however, choose to buffer $[Ca^{2+}]_i$ to very low levels because this interferes in some way with G-protein-mediated effects in these cells (Beech, 1993). In the presence of GTP added to the pipette solution the modulation of I_{Ca} by carbachol was about the same whether Ca^{2+} or Na^+ was the charge carrier ($35.5 \pm 3.4\%$, $n = 11$ vs. $34.4 \pm 18.5\%$, $n = 5$).

Han et al. (1995) reported that the incubation of isolated pacemaker cells of rabbit sinoatrial node with L-NAME (0.2–1 mM) abolished carbachol-induced I_{Ca} suppression. In guinea pig gastric myocytes, acetylcholine decreased Ca^{2+} channel open probability in the cell-attached patch mode, when Ba^{2+} was used as a charge carrier. The involvement of a diffusible cytosolic messenger was postulated (Wade et al., 1996, also Unno et al., 1995). To avoid relatively long incubation with L-NAME, which has to enter the cell and become deesterified, and to test the possible involvement of NO as a cytosolic messenger mediating I_{Ca} suppression we used 100 μM L-NAME added to the pipette solution. The fact that it did not affect I_{Ca} suppression suggests that a) NO is not a diffusible messenger mediating I_{Ca} suppression, or b) 7–8 min (this was the time after establishment of the whole-cell configuration after which carbachol was applied) is not sufficient for L-NAME to act on NO-synthase. In case of GTP or GDP-βS, the effects were obvious 5–6 min after breakthrough, showing that this time is sufficient for molecules of similar size to diffuse into the cell. Also, GTPγS was found to fully activate I_{cat} within 3–5 min after breakthrough (Zholos and Bolton, 1996).

It is apparent that muscarinic receptor activation evokes at least three identified G-protein mediated pathways in guinea-pig longitudinal intestinal smooth muscle: pertussis toxin-sensitive G-proteins mediate cationic channel opening (and possibly modulate the voltage-dependent properties of this current, Zholos and Bolton, 1994) and inhibition of voltage-dependent I_{Ca} while pertussis toxin-insensitive G-protein mediates PLC activation and diacylglycerol formation; inositol trisphosphate simultaneously formed releases stored Ca^{2+} which normally elevates $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ has several effects including potentiation of I_{cat} and suppression of I_{Ca}; it also may facilitate opening of large-conductance Ca^{2+}-activated K^+-channels (BK channels) and contribute to contraction.

In conclusion, Ca^{2+} channels seem to be more sensitive to G-protein or its subunits than cationic channels so that the sustained phase of I_{Ca} suppression remains functional even at low levels of GTP in the cell. This phenomenon is mediated by a pertussis toxin-sensitive G-protein and is modulated by GTP and by $[Ca^{2+}]_i$, which facilitates it.

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References

Beech, D.J., 1993. Inhibitory effects of histamine and bradykinin on Ca^{2+} current in smooth muscle cells isolated from guinea-pig ileum. J. Physiol. (London) 463, 565–583.

Benham, C.D., Bolton, T.B., Lang, R.J., 1985. Acetylcholine activates an inward current in single mammalian smooth muscle cells. Nature 316, 345–347.

Cantoni, G.L., Eastman, G., 1946. On the response of the intestine to smooth muscle stimulants. J. Pharmacol. Exp. Ther. 87, 392–399.

Ganitkevich, V.Ya., Shuba, M.F., Smirnov, S.V., 1987. Calcium-dependent inactivation of potential-dependent calcium inward current in an isolated guinea-pig smooth muscle cell. J. Physiol. (London) 392, 431–449.

Gilman, A.G., 1987. G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56, 615–649.

Han, X., Shimoni, Y., Giles, W.R., 1995. A cellular mechanism for nitric oxide-mediated cholinergic control of mammalian heart rate. J. Gen. Physiol. 106, 45–65.

Himpens, B., Droogmans, G., Casteels, R., 1991. Carbachol-induced non-specific desensitization in guinea-pig ileum. Naunyn-Schmiedeberg's Arch. Pharmacol. 343, 580–587.

Inoue, R., Isenberg, G., 1990a. Effect of membrane potential on acetylcholine-induced inward current in guinea-pig ileum. J. Physiol. (London) 424, 57–71.

Inoue, R., Isenberg, G., 1990b. Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. Am. J. Physiol. 258, C1173–C1178.

Inoue, R., Isenberg, G., 1990c. Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. J. Physiol. (London) 424, 73–92.

Inoue, R., Kitamura, K., Kuriyama, H., 1987. Acetylcholine activates single sodium channels in smooth muscle cells. Pflügers Arch. 410, 69–74.

Joiner, P.D., 1973. Studies on the loss of acetylcholine sensitivity in ileal muscle. J. Pharmacol. Exp. Ther. 186, 552–561.

Katada, T., Ui, M., 1982. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. J. Biol. Chem. 257, 7210–7216.

Komori, S., Bolton, T.B., 1991. Calcium release induced by inositol 1,4,5-trisphosphate in single rabbit intestinal smooth muscle cells. J. Physiol. (London) 433, 495–517.

Komori, S., Kawai, M., Takewaki, T., Ohashi, H., 1992. GTP-binding protein involvement in membrane currents evoked by carbachol and histamine in guinea-pig ileal muscle. J. Physiol. (London) 450, 105–126.

Mitsui, M., Karaki, H., 1990. Dual effects of carbachol on cytosolic Ca^{2+} and contraction in intestinal smooth muscle. Am. J. Physiol. 258, C787–C793.

Ogata, R., Inoue, Y., Nakano, H., Ito, Y., Kitamura, K., 1996. Oestradiol-induced relaxation of rabbit basilar artery by inhibition of voltage-dependent Ca channels through GTP-binding protein. Br. J. Pharmacol. 117, 351–359.

Pacaud, P., Bolton, T.B., 1991. Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. J. Physiol. (London) 441, 477–499.

Paton, W.D.M., Rothschild, A.M., 1965. The changes in response and in ionic content of smooth muscle produced by acetylcholine action and by calcium deficiency. Br. J. Pharmacol. 24, 437–448.

Unno, T., Komori, S., Ohashi, H., 1995. Inhibitory effect of muscarinic receptor activation on Ca^{2+} channel current in smooth muscle cells of guinea-pig ileum. J. Physiol. (London) 484, 567–581.

Wade, G.R., Barbera, J., Sims, S.M., 1996. Cholinergic inhibition of Ca^{2+} current in guinea-pig gastric and tracheal smooth muscle cells. J. Physiol. (London) 491, 307–319.

Zholos, A.V., Bolton, T.B., 1994. G-protein control of voltage dependence as well as gating of muscarinic metabotropic channels in guinea-pig ileum. J. Physiol. (London) 478, 195–202.

Zholos, A.V., Bolton, T.B., 1995. Effects of divalent cations on muscarinic receptor cationic current in smooth muscle from guinea-pig small intestine. J. Physiol. (London) 486, 67–82.

Zholos, A.V., Bolton, T.B., 1996. A novel GTP-dependent mechanism of ileal muscarinic metabotropic channel desensitization. Br. J. Pharmacol. 119, 997–1012.