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A novel GTP-dependent mechanism of ileal muscarinic metabotropic channel desensitization

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1 Cationic current (I_{cat}) was evoked in single isolated smooth muscle cells either by activating muscarinic receptors with the stable muscarinic agonist, carbachol (CCh), or by dialysing cells with GTP-γS. It was studied using patch-clamp recording techniques in cells obtained by enzymatic digestion from the longitudinal muscle layer of the guinea-pig small intestine.

2 I_{cat} appears only when muscarinic receptors or G-proteins are activated, but it is strongly voltage-dependent. Its activation could be described by the Boltzmann equation. During desensitization of I_{cat} evoked by 50 μM CCh, the slope factor, k, remained constant whereas the maximal conductance, G_{max}, slowly decreased and the potential of half-maximal activation, $V_{1/2}$, shifted positively by 32 mV during 4 min.

3 At peak response either to extracellular application of CCh (GTP-free, or 1 mM GTP-containing, pipette solution) or to intracellular application of GTP-γS (no CCh), the size and voltage-dependent properties of I_{cat} were similar. However, I_{cat} desensitization was slower in the presence of GTP (CCh applied) in the pipette solution and much slower with GTP-γS in the pipette (no CCh) compared to GTP-free pipette solution (CCh applied); the decrease in G_{max} with time was much delayed and the positive shift of the activation curve was inhibited. GDP-βS added to the pipette solution at 2 mM abolished I_{cat} in response to applied CCh; 50 μM did not prevent I_{cat} generation but significantly accelerated desensitization.

4 It was concluded that the rate of desensitization of the carbachol-evoked cationic current was due to a decline in the concentration of activated G-protein in the cell, which reduced the maximum number of channels which could be opened and shifted their activation range to less negative potentials.

Keywords: Smooth muscle; G-protein; GTP; GTP-γS; muscarinic cationic channels; desensitization

Introduction

The loss of cellular sensitivity and responsiveness following or during stimulation by an agonist is described generally as desensitization regardless of the quite diverse cellular processes involved (see Lohse, 1993, for a recent review).

Muscarinic cholinoceptors which are studied in the present experiments belong to the superfamily of GTP-binding, G-protein-coupled receptors. In intestinal smooth muscle cells they are linked via a Pertussis toxin-sensitive G-protein to the cationic channel which also has prominent intrinsic voltage-dependent gating (Benham *et al.*, 1985; Inoue & Isenberg, 1990a,b; Komori *et al.*, 1992; Zholos & Bolton, 1994). Single channel experiments showed that acetylcholine or carbachol activate only one single type of ion channel in guinea-pig ileal cells with 20-25 pS conductance and strong voltage-dependence of the probability of channel opening (Inoue *et al.*, 1987).

Activation of muscarinic receptors in visceral smooth muscles by the major excitatory neurotransmitters, acetylcholine, results in membrane depolarization due to cationic channels opening and muscle contraction. The contractile response of isolated strips of guinea-pig ileal smooth muscle declines during long-lasting or repeated exposures to the agonist (Cantoni & Eastman, 1946; Paton & Rothschild, 1965; Joiner, 1973). Desensitization does not alter either the apparent affinity, the total number of binding sites for CCh or the relative proportions of M_2 and M_3 muscarinic receptors in this tissue (Eglen *et al.*, 1992). This desensitization has been attributed to the electrogenic Na^+ pump extruding Na^+ entering during the action of ACh thus producing an outward current and membrane hyperpolarization (Paton & Rothschild, 1965; Bolton, 1973; Joiner, 1973). However, under voltage-clamp when hyperpolarization cannot be involved, well-developed desensitization of the cationic current evoked by muscarinic receptor activation was still observed (Benham *et al.*, 1985; Zholos & Bolton, 1994). This does not need Na^+ and K^+ ions and must involve some other mechanism which is investigated here. Desensitization to ACh in guinea-pig ileum was suggested to involve a G-protein mediated inhibition of voltage-gated Ca^{2+} channels (Himpens *et al.*, 1991).

The fading of the inward cationic current (I_{cat}) in the presence of ACh may be an important factor contributing towards the decline in muscle tension but its mechanisms are unclear. For G-protein-coupled receptors, several mechanisms of desensitization have been demonstrated (Lohse, 1993). It is perhaps surprising that G-protein inhibition due to GTP depletion was not found to play a role in desensitization of the muscarinic response in guinea-pig atrial myocytes: no difference in the rate of K-current desensitization was seen with no GTP, or up to 1 mM GTP, added to the pipette solution (Mubagawa *et al.*, 1994). The presence of GTP was important only for resensitization in these cells. However, both the affinity of G-proteins for GTP and the α-subunit GTPase activity, as well as the cell's ability to maintain GTP level may differ greatly in different cells. Involvement of G-proteins in I_{cat} desensitization seems likely as we have observed transient potentiation of I_{cat} in single ileal smooth muscle cells upon release of GTP from a caged precursor (Zholos & Bolton, 1994). We undertook this study in order to provide evidence whether G-protein, and specifically GTP depletion leading to a reduction in the availability of activated G-proteins, are involved in the desensitization of the muscarinic receptor cationic current in ileal smooth muscle cells.

Some of these results have been reported in abstract form (Zholos & Bolton, 1996).

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Methods

Experimental procedures were generally the same as already described (Zholos & Bolton, 1995). Male adult guinea-pigs (300–400 g) were killed by dislocation of the neck followed by immediate exsanguination. Experiments were performed at room temperature on single ileal smooth muscle cells. To bring about cell dispersal the longitudinal layer of the distal ileum was dissected and placed in physiological salt solution (PSS) of the following composition (in mM): NaCl 120, KCl 6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 12, HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) 10, pH adjusted to 7.4 with NaOH. The tissue was cut into small pieces which were transferred to Ca^{2+}- and Mg^{2+}-free PSS for about 5 min. The pieces were incubated in 2 ml of this solution containing collagenase, soybean trypsin inhibitor, and bovine serum albumin (all at 1 mg ml^{-1}) at 36°C for 20 min. The pieces were carefully washed in enzyme-free solution (Ca^{2+}- and Mg^{2+}-free PSS) and agitated in 1 ml of this solution until it became very cloudy. Cells were kept in low-Ca^{2+} PSS (0.8 mM) at 4°C until use.

Whole-cell membrane current was recorded with low-resistance borosilicate patch pipettes (1 to 3 MΩ) and Axopatch 200A (Axon Instruments Inc., Foster City, CA, U.S.A.) voltage-clamp amplifier. Voltage clamp pulses were generated and data were captured using a Labmaster DMA TL-1-125 interfaced to a computer running pClamp program (Axon Instruments Inc., Foster City, CA, U.S.A.). Recordings were also sampled at 48 kHz and stored on a digital tape recorder (DTR-1204, Biologic Science Instruments, Claix, France). Series resistance was compensated by about 80%. Data were analysed and plotted using MicroCal Origin software (MicroCal Software, Inc., Northampton, MA, U.S.A.) which uses the Levenberg-Marquardt nonlinear least square curve fitting algorithm. Values are given as the means ± s.e.mean. Student's *t* test was used for statistical comparison and differences were judged to be statistically significant for $P<0.05$.

Pipettes were filled with the following solution (in mM): CsCl 80, MgATP 1, creatine 5, glucose 20, HEPES 10, BAPTA 10, $CaCl_2$ 4.6 (calculated $[Ca^{2+}]_i = 100$ nM), pH adjusted to 7.4 with CsOH (total Cs^+ 124 mM). The basic external solution in which I_{cat} was recorded consisted of (in mM): CsCl 120, glucose 12, HEPES 10, pH adjusted to 7.4 with CsOH (total Cs^+ 124 mM). Cs^+ could conceivably give rise to small currents through ion channels other than muscarinic receptor cationic channels (e.g. K^+ channels). Such Cs^+ currents are likely to be neglibly small and, if present, would need to be significantly altered by CCh to affect the cationic current *I-V* relationships because current in the absence of CCh was subtracted. Background conductance was less than 4% of that activated by carbachol.

Chemicals used were: collagenase (type 1A), adenosine 5′ triphosphate (ATP, magnesium salt), guanosine 5′-triphosphate (GTP, sodium salt), guanosine 5′-O-(3-thiotriphosphate) (GTP-γS, tetralithium salt), guanosine-5′-O-(2-thiodiphosphate (GDP-βS, trilithium salt), creatine, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 1,2-*bis*(2-aminophenoxy) ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA), carbamylcholine chloride (carbachol), all from Sigma Chemical Co., Poole, Dorset.

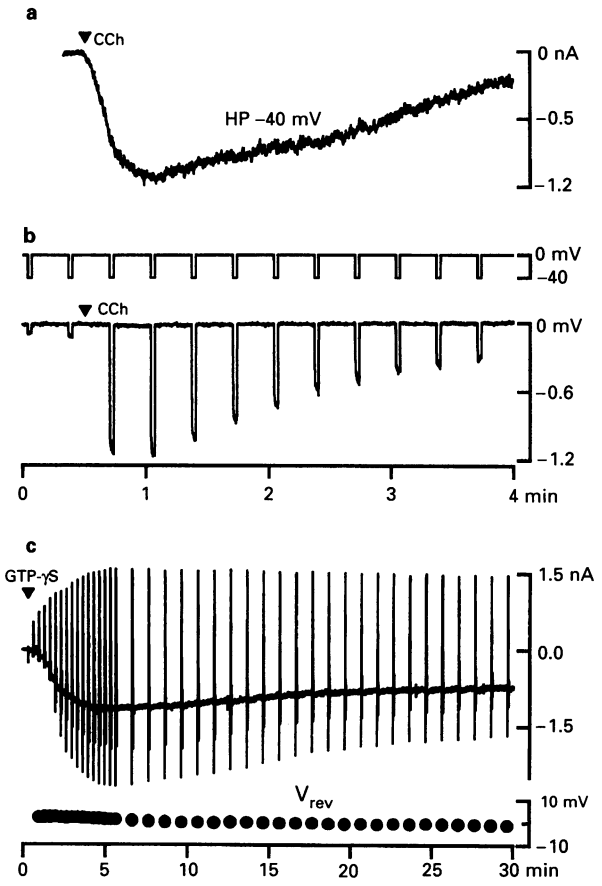

Figure 1 Inward current to carbachol showed desensitization whether (a) the cell was held continuously at −40 mV when inward I_{cat} was generated or (b), was held at the reversal potential for I_{cat} (0 mV). In this case briefly stepping to −40 mV indicated that desensitization was still occurring despite the absence of I_{cat} at 0 mV. (c) If I_{cat} was generated by adding GTPγS (200 μM) to the pipette solution only slight desensitization occurred over 30 min compared to substantial desensitization within 3 min when I_{cat} was evoked by applying carbachol to the cell; however, GTPγS-evoked I_{cat} was of comparable size. I_{cat} reversal potential plotted at the bottom was measured by applying voltage ramps at 20 s intervals during the rising phase and every 60 s thereafter. Note the difference in the time scale for CCh- and GTPγS-activated currents. In (a) and (b) the arrowhead indicates the point in time when the solution was changed to one which contained 50 μM carbachol (CCh) and this solution was applied for the remainder of the record. In (c) the arrowhead indicates the point at which whole-cell recording mode was achieved.

Results

General observations for I_{cat}

In the present experiments 50 μM carbachol (CCh), a close-to maximal concentration, was applied in the bathing solution at least 3 min after break-through to the whole-cell recording mode, a period which allowed equilibration of the cell interior with the pipette solution. If cells were held at −40 mV, which is close to the membrane potential existing in this tissue during muscarinic receptor activation (Bolton, 1972) then application of CCh to the cell evoked an inward current as the equilibrium potential (E_{Cs}) was 0 mV due to the use of 124 mM Cs^+ at both sides of the membrane and Cs^+ being the only permeant ion present. Inward current declined over a period of several minutes when the concentration of CCh was maintained constant (Figure 1a). This desensitization was also seen with acetylcholine but this was not used in these experiments because, unlike carbachol, it is not completely stable.

The decline in the current was not due in some way to a slow accumulation or depletion of ions on one or other side of the membrane because it occurred at all potentials including 0 mV where no current was generated (Figure 1b). At 0 mV where there is no net ion movement, so no change in ion concentration can occur, desensitization still occurred and could be demonstrated by stepping to −40 mV (Figure 1b). The decline in current under these latter circumstances amounted to 35 ± 9% ($n=5$) over 3 min compared to cells held at −40 mV where it was 45 ± 5% ($n=20$). These values are not

significantly different ($P>0.3$). Thus, despite the fact that in the case of cells held at 0 mV the period when ion redistribution could occur was only 10% of that during continuous holding at −40 mV, desensitization was the same.

Intracellular application of GTP-γS (without carbachol application to the cell) activates the same cationic current in these cells (Komori *et al.*, 1992) via direct G-protein activation, bypassing receptors. We found that I_{cat} desensitization was very slow in this case although a similar or even larger I_{cat} was flowing continuously over 30 min (Figure 1c). This experiment clearly shows that generation of large I_{cat} is not necessarily associated with its desensitization.

If further evidence against ion redistribution playing any part in the desensitization phenomena is needed, then measurements of reversal potential of I_{cat} should provide it. If ion redistribution were slow enough to explain a decline in I_{cat} over 4 min (Figure 1a), then it could hardly be expected that the change in ion gradient will be corrected within the time it takes to apply a voltage ramp (seconds as in Figure 1c) or a voltage step (microseconds as in Figure 2a) to another potential in order to estimate the reversal potential of I_{cat}. I_{cat} reversal potential, measured at regular intervals over 30 min by applying voltage ramps (which appear as deflections on the current trace in Figure 1c) showed no significant change

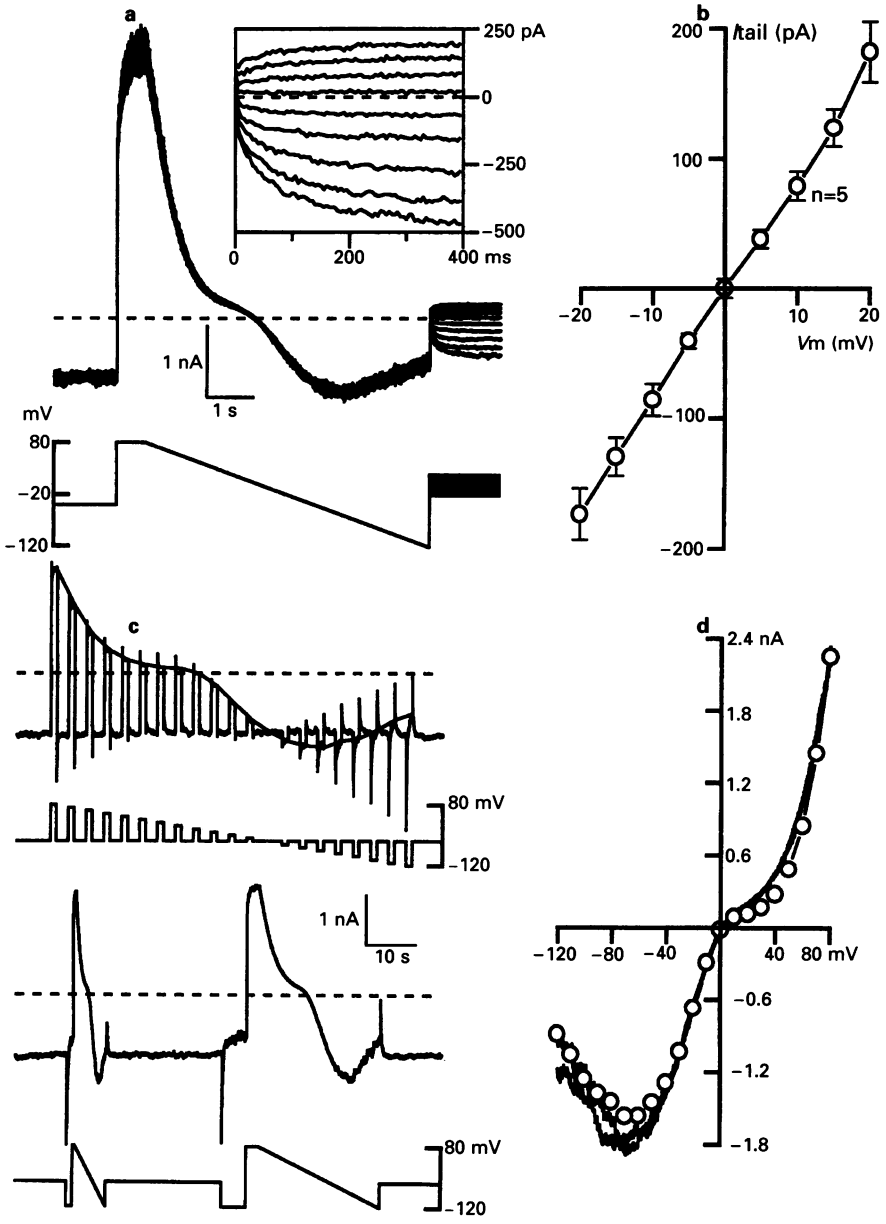

Figure 2 I_{cat} reversal potential is not affected by current flow during slow voltage ramp application in order to measure its steady-state *I-V* relationship. (a) An example of nine superimposed current traces obtained at 20 s intervals during GTP-γS-activated I_{cat} (above) and the voltage protocol used to measure E_{rev} (below). The inset shows the same traces on an extended scale following voltage step at the end of the ramp to various potentials (time zero); note that the current is flat at 0 mV. (b) I_{cat} tail current size (mean ± s.e.mean) measured in five cells at different test potentials immediately after the end of the slow voltage ramp, as shown in (a). (c,d) Slow voltage ramp produces I_{cat} *I-V* relationship similar to that measured by long voltage steps. (c) I_{cat} was activated by 200 μM GTP-γS applied intracellulary and its true steady-state *I-V* relationship was measured by stepping to various potentials (above, steady-state currents at the end of each pulse are connected as a visual aid) and compared to the (quasi)steady-state relation measured by slow ramps (below). Ramp duration was also increased from 6 s (left, our basic protocol in the present experiments) to 24 s (right) as an additional test (d) *I-V* relationships from panel (c) measured by voltage steps (○) or ramps (continuous lines).

(Figure 1c, bottom). Its mean value was 0.3 ± 0.2 mV as averaged from 39 measurements in this experiment, very close to the calculated $E_{Cs} = 0$ mV. No change in I_{cat} reversal potential was also observed (although it was generally continuously monitored) throughout experiments with carbachol (e.g. Figure 3a). Current upon stepping to 0 mV from various holding potentials was flat and did not show any relaxation as would be expected if perturbation of ion gradient had occurred (e.g. Figure 1b).

It has been shown previously that I_{cat} in intestinal smooth muscle cells demonstrates a marked voltage-dependence; the conductance decreases with membrane hyperpolarization so that at very negative potentials I_{cat} can be virtually lost (Benham *et al.*, 1985; Inoue & Isenberg, 1990b; Zholos & Bolton, 1994). A further test was performed to see whether any of the non-linearity could be caused by an alteration in the ion gradient during the slow voltage ramp applied to measure the I_{cat} *I-V* relationship. Again, we used GTP-γS in these experiments because I_{cat} is stable and I_{cat} reversal potential was measured by applying steps to various potentials immediately after I_{cat} *I-V* relationship was established by slow voltage ramp; the protocol is illustrated at the bottom in Figure 2a. Current responses to slow ramps overlapped (nine superimposed traces in Figure 2a). Cationic current decreased in size with hyperpolarization negative to about -50 mV but measuring tail currents by stepping to various potentials (from -20 to $+20$ mV in 5 mV increments) at the end of the voltage ramp revealed no change in the reversal potential. Averaged tail current reversed at 0.1 mV (Figure 2b) and when E_{rev} was measured in individual cells it was found to be at 0.2 ± 1 mV ($n = 5$). Furthermore, during desensitization smaller currents deviated from linearity more at negative potentials (Figure 3). The above observations leave no doubt that neither I_{cat} desensitization nor its non-linear *I-V* arise from disturbances of the ion gradient.

Slow voltage ramps were used to measure steady-state *I-V* relationships. Figure 2(c-d) shows *I-V* relationships obtained in the same cell by slow voltage ramp (200 mV per 6 s as used throughout all experiments in this paper) or by voltage steps; steady-state current at the end of a long step showed only a slight difference attributable to small changes in I_{cat} with time. Increasing the ramp duration to 24 s did not produce any change in the *I-V* curve either (bottom trace in Figure 2c and two traces superimposed in Figure 2d) thus proving that 6 s ramp was sufficient to reach the steady-state conditions at each potential. Similar results were demonstrated in several cells.

I_{cat} *desensitization*

In the present experiments intracellular free Ca^{2+} concentration was strongly buffered at 100 nM but this did not affect the desensitization process. When $[Ca^{2+}]_i$ was weakly buffered by using a low EGTA concentration (50 μM) in the pipette solution and with 2.5 mM Ca^{2+} in the external solution, I_{cat} in response to 50 μM CCh declined to $53 \pm 4\%$ ($n = 5$) during 3 min, compared to $45 \pm 5\%$ ($n = 20$) with Ca-BAPTA buffer and Ca^{2+}-free external solution. The difference was not statistically significant. However, with weak $[Ca^{2+}]_i$ buffering I_{cat} oscillated (compare to Komori *et al.*, 1992; Zholos *et al.*, 1994) thus precluding measurement of its steady-state *I-V* relationship.

We have recently observed that desensitization of I_{cat} had equivalent effect on the *I-V* relationship to decreasing receptor occupancy by reducing the concentration of the agonist (Zholos & Bolton, 1994). Therefore in the present experiments we tested whether altering the level of G-protein activation would produce changes in the desensitization process. Cationic conductance activated in 20 different cells by 50 μM CCh ranged in size from about 10 to 50 nS but in different cells there was no obvious dependence of the rate of the cationic current decay over 100 s in the continuous presence of CCh on the maximal cationic conductance activated. This observation was also consistent with the previous conclusion that permeant ion redistribution did not occur during desensitization.

In order to see to what extent G-protein function affects the desensitization process, guanine nucleotides such as GDP-βS, GTP-γS or GTP, known to interact directly, were applied intracellularly. At the peak response CCh-activated current when 1 mM GTP was added to the pipette solution, or current activated by 200 μM GTP-γS added to the pipette (no CCh applied) showed no statistically significant differences compared to cells studied without guanine nucleotides. For example, I_{cat} amplitude at -40 mV was -732 ± 28 pA in control ($n = 226$), -832 ± 52 pA for GTPγS-activated current ($n = 79$) and -886 ± 185 pA with GTP in the pipette solution ($n = 8$). Similar shape of the *I-V* relationship was seen with current deviating from linearity at -120 mV on average by 65, 69 and 67%, respectively. Also, the rate of I_{cat} relaxation during voltage step to -120 mV (e.g. Figure 2c) due to voltage-dependent decrease in the channel open probability was similar with a mean time constant of 173 ± 6 ms (control, $n = 180$), 172 ± 11 ms (GTP-γS, $n = 78$) and 212 ± 42 ms (GTP, $n = 8$). No current could be activated by CCh under conditions when 2 mM GDP-βS was included in the pipette solution, an observation which supports the essential involvement of G-proteins. With a 10 times lower concentration of GDP-βS, an I_{cat} much smaller than in control was activated by CCh at the holding potential (-141 ± 45 pA, $n = 3$). In this case the current was also characterized by faster relaxation during a voltage step to -120 mV with a time constant of 44 ± 16 ms.

The major effects of guanine nucleotides were seen during the desensitization process. In three cells studied with a moderate concentration of GDP-βS (50 μM) added to the pipette solution desensitization kinetics were strongly accelerated (Figure 3b *vs* 3a): (i) current at -120 mV faded completely within 2 min and (ii) even current at $+80$ mV which was normally very stable decayed in the presence of GDP-βS.

The availability of activated G protein may decline during desensitization due to depletion of GTP. In support of this hypothesis we found that adding 1 mM GTP to the pipette solution reduced desensitization of the cationic current evoked by CCh, when compared with cells held with pipettes not containing GTP (Figure 3c *vs* 3a). Desensitization of the current activated by GTP-γS was even slower (Figure 3d). This could arise because GTP-γS forms a much more stable bond to the α-subunit so the concentration of activated G-protein declines much less with time.

Figure 4a further illustrates I_{cat} behaviour in time at three different potentials in the presence of these guanine nucleotides in the pipette solution. It can be seen that, firstly, desensitization is most pronounced at very negative potentials such as -120 mV (except for GTP-γS) and, secondly, the effects of the guanine nucleotides are also most pronounced at -120 mV. With depolarization they were attenuated and finally the difference became negligible at $+80$ mV except for GDP-βS for which strong desensitization developed even at $+80$ mV. These results can be explained by our previous observation (Zholos & Bolton, 1994) that a rather small concentration of activated G-protein is sufficient for channel opening at positive potentials, but progressively higher concentrations are necessary with hyperpolarization.

Cationic current activated either by external application of CCh with 1 mM GTP in the pipette solution or by GTP-γS without CCh application could be recorded for much longer time than shown in Figure 4a. The current measured at -120 mV under these conditions is also shown in Figure 4b on a longer time scale. After 30 min the current evoked by CCh when 1 mM GTP was present in the pipette decayed to the same level through the initial rate of decay was much slower for the GTP-γs-activated current. Note that maximum I_{cat} evoked by GTPγS in the pipette occurred later (3–5 min) than that evoked by CCh although relative currents were evoked at the same time with respect to the time of occurrence of the maximal current in each case.

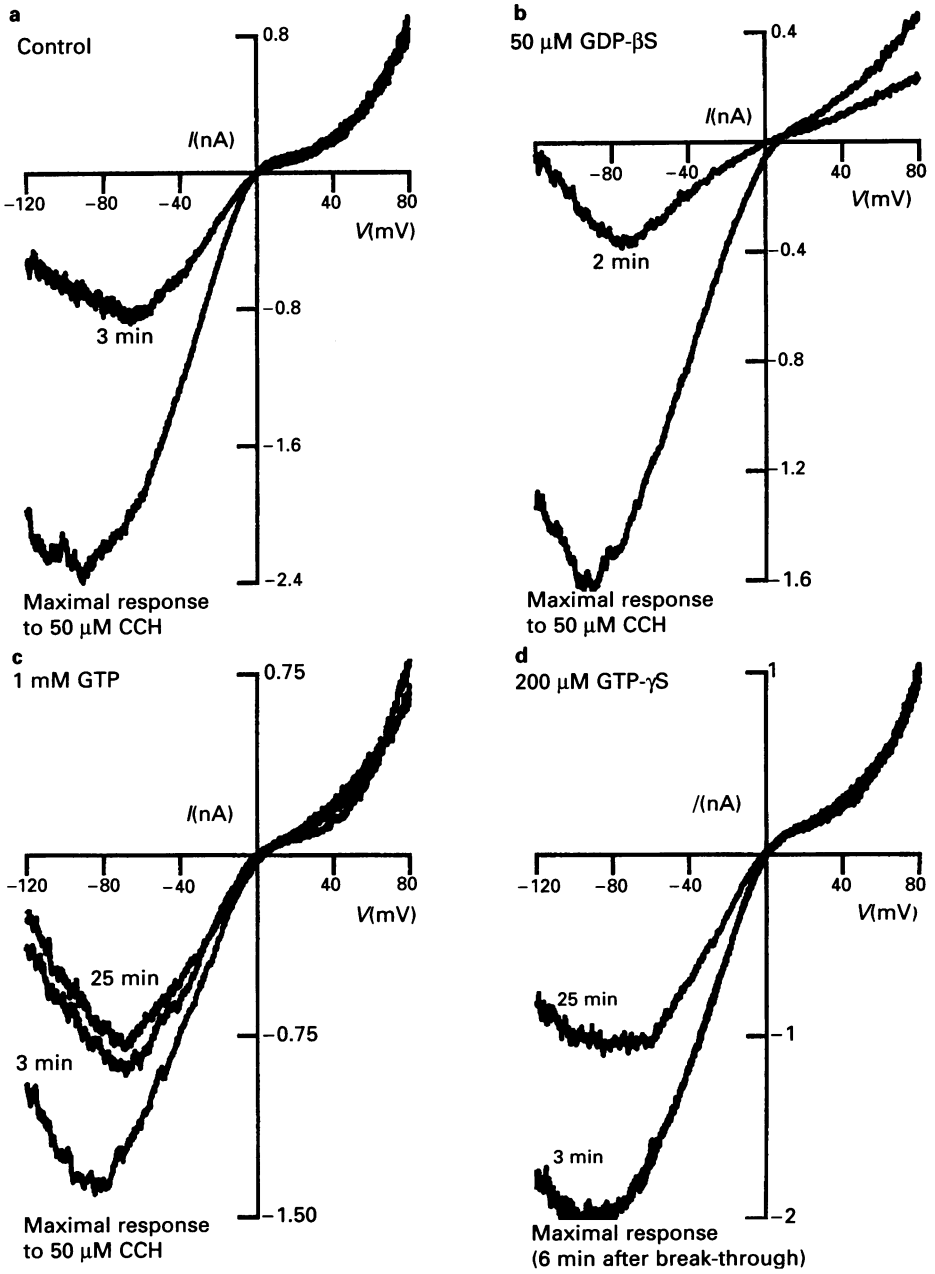

Figure 3 G-protein controls the desensitization properties of the muscarinic receptor cationic channels. (a-d) Typical examples of the *I-V* relations for I_{cat} activated either by 50 μM CCh application (a) with 50 μM GDP-βS (b) or with 1 mM GTP (c) added to the pipette solution or by 200 μM GTP-γS added to the pipette solution without CCh application (d). The *I-V* relations were obtained at times indicated.

Mechanisms of desensitization

Although muscarinic receptor activation (or G-protein activation) is obligatory for I_{cat} to appear, I_{cat} is, nevertheless, strongly voltage-dependent. To analyse mechanisms responsible for I_{cat} desensitization, *I-V* relationships like those illustrated in Figure 3 were converted into cationic conductance *vs.* membrane potential relations (activation curves) by dividing current amplitude at each potential by the voltage driving force. Figure 5a (top panel) shows two activation curves obtained at the peak response to agonist and 4.3 min later; changes in the *I-V* relationship and in the reversal potential were continuously monitored in all experiments ($n=20$) by applying ramp voltage commands every 20 s. It was previously demonstrated (Benham *et al.*, 1985; Inoue & Isenberg, 1990b; Zholos & Bolton, 1994) that the relationship between the muscarinic cationic conductance, G, and membrane potential, V, could be described by a Boltzmann distribution in the following form:

$$G = \frac{G_{max}}{1 + \exp((V - V_{1/2})/k)} \quad (1)$$

where $V_{1/2}$ is the potential of half-maximal activation ($G=0.5\ G_{max}$) and k is the slope factor. Thus, the activation curves were fitted to this equation (continuous smooth lines in Figure 5a, upper panel). Their analysis (lower panel) shows that two processes developed during desensitization: (i) a decrease in the maximal conductance and (ii) a parallel shift of the activation curve towards less negative membrane potentials. The latter phenomenon is more evident after an appropriate scaling of the activation curve obtained at 4.3 min

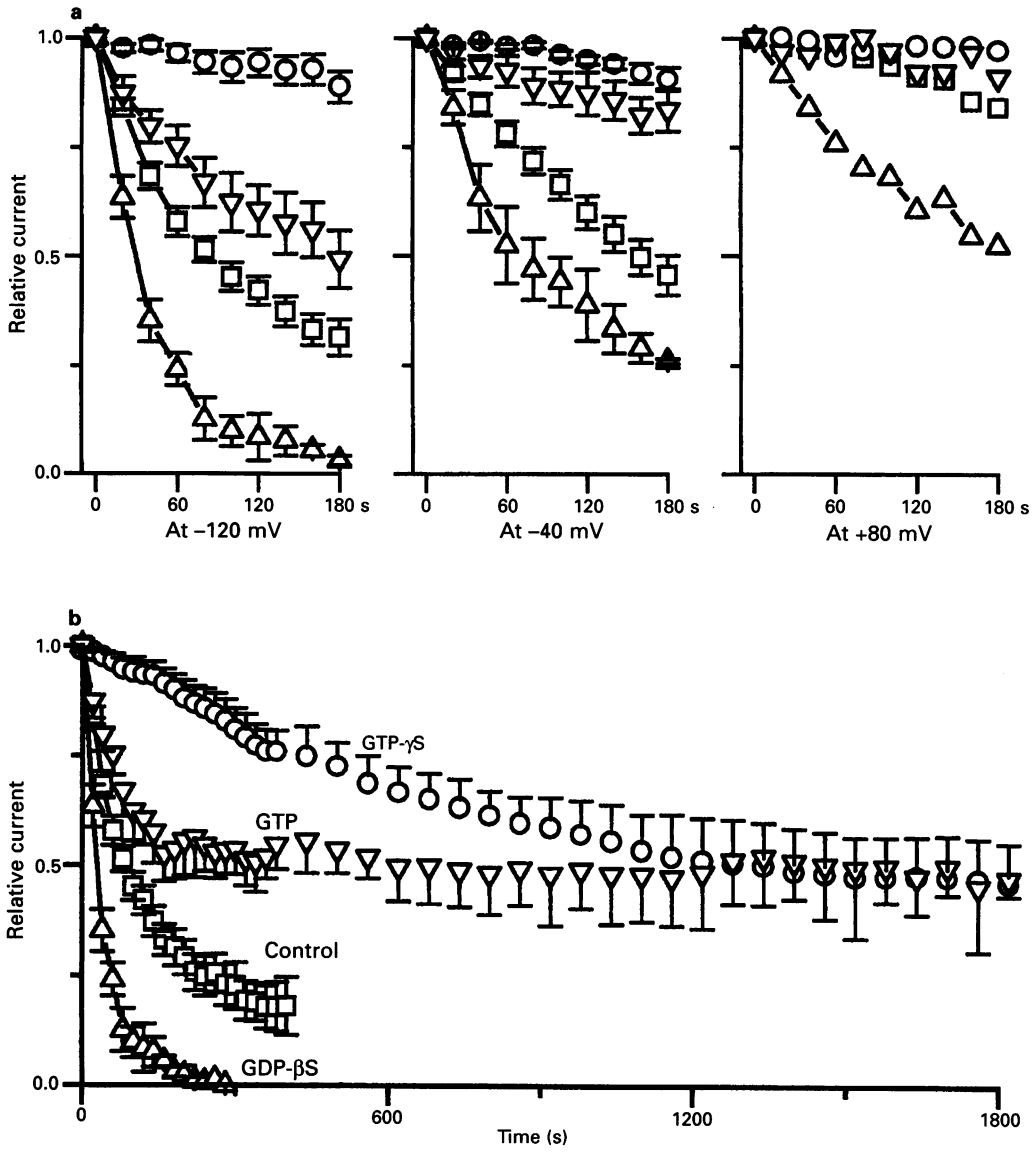

Figure 4 I_{cat} desensitization is affected by the presence of GTP, GDP-βS or GTP-γS in the pipette solution. (a) Mean relative current amplitude at three different potentials is plotted during 3 min period after maximal response to 50 μM CCh (□, $n=20$); 1 mM GTP (▽, $n=5$); 50 μM GDP-βS (△, $n=3$) or 200 μM GTPγS (○, $n=10$); s.e.mean values for +80 mV are omitted as they mostly intersect. (b) Measurements at −120 mV are plotted on a longer time scale to illustrate the time course of I_{cat} over 30 min with GTP (carbachol 50 μM applied) and GTP-γS (no carbachol applied). In both (a) and (b) maximum I_{cat} was expressed as a relative value of 1.0 but it occurred within a few seconds when CCh was applied but after several minutes when GTP-γS was present in the pipette.

(lower panel). The $V_{1/2}$ values are also shown by the vertical dotted lines to illustrate that a parallel positive shift by 32 mV had developed during this time.

In similar experiments but with 1 mM GTP added to the pipette solution ($n=6$), desensitization was considerably slowed down as already described. Activation curves plotted in Figure 5b (upper panel) show that the decrease in the maximal cationic conductance in the presence of GTP still developed though it was considerably slower (10 min of desensitization in b compared to 4.3 min in a). It was remarkable, that the positive shift of the activation curve on the voltage axis was completely ($n=2$) or very strongly ($n=4$) suppressed when GTP was added to the pipette so that after scaling the activation curves overlapped (lower panel in b). Similar observations could be made for GTP-γS-activated cationic current (Figure 5c). When the $V_{1/2}$ and G_{max} values at peak response in each cell were normalized as 100% it could be seen that without GTP in the pipette the change in the $V_{1/2}$ and G_{max} values was up to 25 and 75% ($n=8$), respectively, during 3–4 min of desensitization in the presence of 50 μM CCh. With GTP added to the pipette solution (CCh-activated current, $n=6$), or with GTP-γS-activated current ($n=5$), the changes in the $V_{1/2}$ and G_{max} values remained less than 7 and 30%, respectively, during 10 min of desensitization.

Discussion

Muscarinic receptor cationic current in smooth muscle cells is quite different from other metabotropic receptor currents because it has marked voltage-dependence being deactivated by membrane hyperpolarization (hence I_{cat} decay during negative voltage steps); this intrinsic voltage-dependence is further modulated by G-protein so altering the voltage range for channel gating (Zholos & Bolton, 1994).

When I_{cat} was activated either by a submaximal CCh concentration or by 200 μM GTP-γS, amplitude and voltage-dependent properties of I_{cat} at peak response were similar. GTP added to the pipette solution at 1 mM also did not have any significant effect on I_{cat} properties at the maximal response

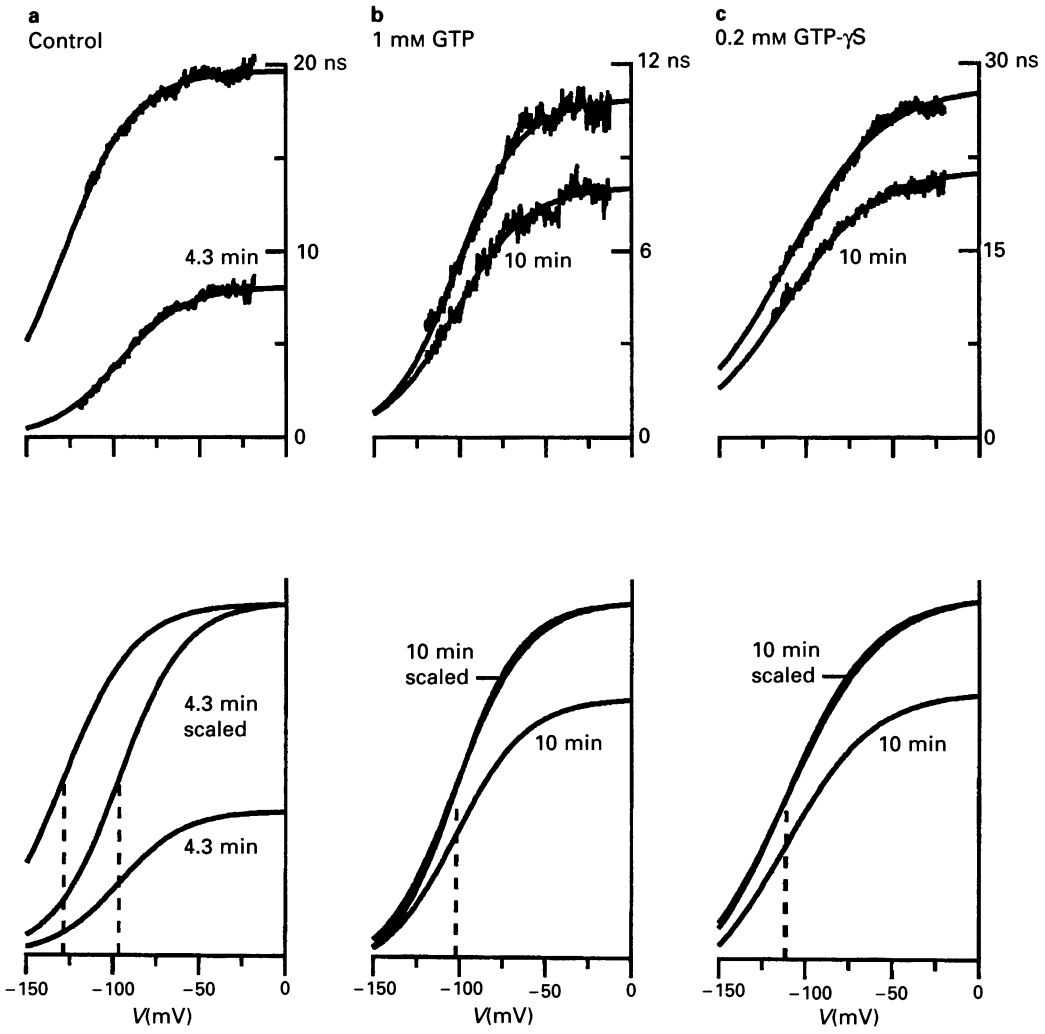

Figure 5 Typical examples of the cationic conductance activation curves in control (50 μM CCh) (a) and in the presence of 1 mM GTP (b) or 200 μM GTP-γS (no CCh) (c) in the pipette solution. Activation curves in the upper panels were fitted by the eq. 1 with the following parameters:

	G_{max} (nS)	$V_{1/2}$ (mV)	k (mV)
Control: maximal response	19.7	-128.8	-20.6
4.3 min	8.1	-96.5	-19.1
GTP present: maximal response	10.9	-102.0	-19.0
10 min	8.0	-102.3	-20.8
GTP-γS: maximal response	28.1	-111.2	-27.4
10 min	21.4	-111.7	-25.6

Lower panels show the same activation curves also after scaling of the curve obtained during desensitization. Dashed lines indicated the position of the $V_{1/2}$ value for each curve.

to 50 μM CCh. However, later on during desensitization, the effects of guanine nucleotides known to affect G-protein function became very pronounced. GDP-βS at 50 μM considerably accelerated I_{cat} desensitization (Figure 3b). Conversely, desensitization was slowed down when GTP was added to the pipette solution or if I_{cat} was activated by GTP-γS (Figure 3c,d). The effect of GTP, the natural guanine nucleotide, is obviously physiologically relevant and was studied in detail.

During I_{cat} desensitization two processes were identified: a reduction in limiting conductance G_{max} and a positive shift of the $V_{1/2}$ value (e.g. Figure 5a). It is notable that the latter can quantitatively account for the much greater desensitization at very negative voltages (Figure 4a): when the activation curve moves positively this has an effect on I_{cat} steady-state amplitude first at very negative potentials such as -120 mV and only later and to a lesser extent at less negative potentials as shown in Figure 4a. Reduction in G_{max} and shift in $V_{1/2}$ develop gradually, so it seemed that they might have a common origin. However, one of the most important observations in the present experiments is that these two processes can be separated by adding a saturating concentration of GTP (1 mM) to the pipette solution. Under these conditions a positive shift of $V_{1/2}$ was suppressed more strongly than the decrease in the maximal conductance which occured more slowly than in the absence of GTP added to the pipette but still developed over 10 min (Figure 5).

Several components, perhaps three, of the desensitization process could be identified. Only one of these seems to be G-protein dependent. As I_{cat} in response to GTP-γS declines steadily over 30 min to about 50% of its maximum size (Figure 4b) there is some desensitization which may reflect a decline in cell function or loss of essential components (e.g.

G-protein or channels) in dialysed cells. CCh application with GTP in the pipette resulted in a rapid 40% decline in I_{cat} within 3 min or so; as this occurred while GTPγS-evoked I_{cat} was increasing (to peak at 3–5 min) this component may be obscured in the case of GTP-γS. Afterwards, however, CCh-evoked I_{cat} with saturating GTP was almost constant and achieved the same relative mean value as GTPγS-evoked I_{cat} after 30 min. Without GTP in the pipette the initial decline in CCh-evoked I_{cat} was greater suggesting that the cell has a component of desensitization due to its inability to produce GTP at a sufficiently high rate; this was borne out by the effect of flash-released GTP which increased I_{cat} at this time (Zholos & Bolton, 1994). Addition of GDP-βS to the pipette accelerated desensitization presumably by competing with endogenous GTP. Desensitization was also accelerated by increasing the CCh concentration presumably by placing increasing discrepancy between GTP synthesis and GTPase activity. Thus, there is at least a component of desensitization due to a limited supply of GTP by the cell, and also another component which occurs initially and is not due to this lack but to some other cause.

Though desensitization has been observed for nearly all receptor-mediated responses with only a few exceptions, such as the inositol trisphosphate receptor (Oldershaw *et al.*, 1992) its mechanisms in different systems are understood to very different extents. The best studied examples include the β_2-adrenoceptor of the superfamily of G-protein-coupled receptors, and the muscle type nicotinic acetylcholine receptor representing ionotropic (ion-channel) receptors. In the present experiments we have studied the process of desensitization in the system muscarinic receptor /G-protein/ cationic channel in guinea-pig ileal smooth muscle cells, for which very little is known so far. The closest counterpart, from the sub-group of G-protein-coupled receptors that open an ion channel where desensitization was studied in much more detail, is the muscarinic receptor G_K inwardly rectifying K^+ channel system ($I_{K,ACh}$) in atrial cells. In this system desensitization was shown as $I_{K,ACh}$ fade during GTP-γS-induced activation of the current without agonist binding to the receptor (Kurachi *et al.*, 1987). $I_{K,ACh}$ showed biphasic decay and more recently it was established that the initial fast phase involves phosphorylation/dephosphorylation of G_K or the K^+ channel, whereas the slow phase involves the muscarinic receptor (Zang *et al.*, 1993). Resensitization in this system is very sensitive to the GTP level in the cell and no recovery of the response was seen with ATP- and GTP-free pipette solution whereas less than 10% recovery was seen with GTP-free, ATP-containing solution presumably due to GTP synthesized from ATP (Mubagawa *et al.*, 1994). However, the desensitization process itself was not affected by GTP (0–1 mM).

In atrial myocytes the lack of effects of GTP were explained by the cell's ability to maintain a GTP level sufficient for G-protein function which was estimated at 25–50 μM. By analogy, we can suggest that it is not the case in smooth muscle cells due to differences either in the rate of GTP synthesis or the rate of GTP hydrolysis, or both. The α-subunit has an intrinsic GTPase activity which determines the lifetime of the activated α-subunit in the active GTP-bound form. It was shown that as the GTPase activity decreases, the steady-state level of α-GTP dramatically increases (Mahama & Linderman, 1994). It is also known that the GTPase activity varies greatly in different cell systems ranging from about 2 to 200 min^{-1} (1.7 min^{-1} for purified β-adrenoceptor - G_S, Brandt & Ross, 1986; 2 min^{-1} in isolated membranes or solubilized preparations of G-proteins, Higashijima *et al.*, 1987; 2.1 min^{-1} for muscarinic receptor - G_i, Tota *et al.*, 1987; 3–5 min^{-1} for α_1-adrenoceptor - G-protein from rat liver membranes, Im & Riek, 1990; 120 min^{-1} in intact platelet membranes, Thomsen & Neubig, 1989; 135–200 min^{-1} in cardiac myocytes, Breitwieser & Szabo, 1988). In our system the rate of GTPase activity associated with the cationic current generation could be estimated using the approach suggested by Breitwieser & Szabo (1988) for $I_{K,ACh}$ in atrial myocytes. Washout of CCh results in I_{cat} deactivation with the mean time constant of 8.3 s (our unpublished observations), thus the rate of GTPase activity can be calculated to be about 7 min^{-1}. This activity is much lower than that for G_K in cardiac cells where, nevertheless, no effects of GTP depletion on $I_{K,ACh}$ was seen. However, in ileal smooth muscle cells there are at least two other G proteins linking the muscarinic receptor to phospholipase C activation and voltage-dependent Ca^{2+} channel inhibition which may also contribute to GTP depletion. Another possible explanation could be lower affinity of G-protein for GTP in smooth muscle cells compared to atrial myocytes. In this case similar reduction in GTP level would have larger effect on G-protein activation.

In conclusion, two major mechanisms are involved in the G-protein dependent I_{cat} desensitization in guinea-pig ileal smooth muscle cells; one is presumably due to reduction in the maximal number of functional channels which can be opened (i.e. decline in G_{max}) and another one is due to positive shift of the cationic conductance activation curve so that progressively stronger depolarization is required to open the same proportion of the channels. The former process is slowed and the latter is strongly inhibited by an excess of GTP in the pipette solution. The results demonstrate that G-proteins play a role not only in enabling but also in modulating the muscarinic response.

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