G-protein control of voltage dependence as well as gating of muscarinic metabotropic channels in guinea-pig ileum

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1. Voltage-dependent properties of muscarinic receptor cationic current activated by carbachol in single smooth muscle cells have been studied using patch-clamp recording techniques. Cells were obtained by enzymic digestion from the longitudinal muscle layer of guinea-pig small intestine.

2. The inward cationic current showed a pronounced U-shaped current–voltage relationship (inward current negative). The relationship of cationic conductance to voltage could be described by a Boltzman distribution which was shifted 36 mV in the negative direction on the voltage axis by increasing fractional receptor occupancy (by increasing agonist concentration from 3 to 300 μM), and in the positive direction by desensitization during prolonged application of agonist. Cationic channels opened by low and high concentrations of carbachol at the same potential do not have identical properties.

3. Release of GTP within the cell, by flash photolysis of an inert caged precursor, had the same effect on the current–voltage relationship as increasing receptor occupancy by the agonist. Release of GDPβS by flash photolysis had the opposite effect.

4. These various results could be explained if cationic channel opening upon receptor activation required binding of at least one α-GTP subunit, but the position of the activation curve on the voltage axis depended critically on the concentration of activated G-protein α-subunits in the cell.

A wide variety of receptors activated by different ligands appear to mediate their effects via G-proteins. When activated these metabotropic receptors, as they are called, in many cases cause the opening of ion channels. Well-studied examples include the muscarinic receptors of the heart (Brown, 1993; Zang, Yu, Honjo, Kirby & Boyett, 1993). It is feasible that the system, receptor–G-protein–channel, is susceptible to various modulating influences in ways not available to ionotropic receptors, in which ligand binding site(s) and channel are part of the same complex molecule. We have studied the cationic current evoked by activation of muscarinic receptors with carbachol or acetylcholine in single smooth muscle cells from guinea-pig small intestine. These receptors appeared to be linked to the cationic channel by a pertussis toxin-sensitive G-protein (Inoue & Isenberg, 1990*a*; Komori, Kawai, Takewaki & Ohashi, 1992). The cationic current they evoke has an unusual voltage dependence, first increasing and then decreasing with increased negativity of the membrane potential, such that current is maximal around the resting membrane potential (approximately −55 mV; Benham, Bolton & Lang, 1985; Inoue & Isenberg, 1990*b*) and bears a U-shaped relationship to potential (inward current negative). In the course of investigating the origin of this peculiar voltage dependence, it became clear that not only is this channel gated by receptor activation via a G-protein, but also that the position and shape of the current–voltage relationship depends on the intensity of receptor activation. Further enquiry showed that this modulation could be explained if it depended critically on the concentration of activated G-protein α-subunits in the cell.

METHODS

Adult male guinea-pigs were killed by dislocation of the neck followed by exsanguination. Experiments were performed at room temperature on single ileal smooth muscle cells obtained after collagenase treatment (1 mg ml^{-1}, type IA from Sigma Chemical Co., Poole, Dorset, UK) at 36 °C for 25–30 min. Membrane current recordings were made from the whole cell using borosilicate patch pipettes (1–3 MΩ) and a List EPC-7 patch-clamp amplifier (List-elecronic, Darmstadt, Germany). Pipettes were filled with the following solution (mM): CsCl, 80; MgATP, 1; creatine, 5; glucose, 20; Hepes, 10; 1,2-bis-(*O*-aminophenoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA),

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10; $CaCl_2$, 4·6 (calculated $[Ca^{2+}]_i = 100$ nM); pH adjusted to 7·4 with CsOH. In experiments using caged compounds, caged GTP (guanosine-5′-triphosphate, P^3-1-(2-nitrophenyl)-ether ester; 2·5 mM) or GDPβS (guanosine-5′-*O*-(2-thiodiphosphate), 2-S-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl)thio ester; 2·5 mM) (obtained from Calbiochem or Molecular Probes) were added to the pipette solution. The external solution consisted of (mM): CsCl, 120; glucose, 12; Hepes, 10; pH adjusted to 7·4 with CsOH. Voltage-clamp pulses were generated and data captured using a Labmaster DMA TL-1-125 interfaced to a computer running the pCLAMP program (Axon Instruments Inc., Foster City, CA, USA). Data were analysed and plotted using MicroCal Origin software (MicroCal Software Inc., Northampton, MA, USA). Values are given as means ± S.E.M.

For photolysis of caged substances, light emitted from a xenon arc lamp as a flash of approximately 1 ms duration was filtered (300–380 nm) and focused on the cell. Relatively low light intensities were used which in control experiments (no caged compounds added to the pipette solution) had no effect on leak or voltage-dependent calcium currents in these cells.

RESULTS

When the steady-state inward current is plotted conventionally as a negative value, the inward cationic current evoked by muscarinic receptor activation shows a U-shaped dependence on membrane potential (Benham *et al.* 1985). Thus for moderate potential displacements from the reversal potential of the cationic current (in these experiments 0 mV) the current increases linearly with driving force, but with further hyperpolarization it failed to keep pace and the current actually decreased negative to −40 or −60 mV (Benham *et al.* 1985; Inoue & Isenberg, 1990*b*). At −100 mV, the muscarinic receptor-evoked current can be virtually lost. The U-shaped current–voltage (*I–V*) behaviour represents steady-state conditions, and the instantaneous *I–V* relationship is nearly linear. After a hyperpolarizing step, current relaxed to a new (quasi-) steady-state level, with a time constant in the range 30–430 ms at −120 mV (mean 171 ± 7 ms, $n = 83$). The U-shaped *I–V* curve could be quantitatively characterized by the decline in the conductance at negative potentials. This was measured in 106 cells when 50 μM carbachol (CCh) was applied and amounted to 34 ± 2% at −120 mV compared with its value at −40 mV. The relaxation did not depend on the permeant cation (Na^+, K^+ and Cs^+ were tested). However, extracellular Ca^{2+} and Mg^{2+}, as well as H^+, had some modulatory action on this process. Thus, to avoid possible complications from interactions of these cations with the channel, all experiments were performed in Ca^{2+}- and Mg^{2+}-free conditions in symmetrical 125 mM–125 mM Cs^+ solutions with an intracellular calcium concentration ($[Ca^{2+}]_i$) strongly buffered with BAPTA. Under these conditions carbachol evoked cationic current in a concentration-dependent manner and the conductance was half activated at −40 mV by 3·9 μM. With 50 μM carbachol, current amplitude at a holding potential of −40 mV was −698 ± 37 pA ($n = 140$) corresponding to 93% activation. The reversal potential of the cationic current did not vary with the concentration of agonist and was always at, or close to, 0 mV. In a series of twenty cells the concentration–effect relationship for carbachol was examined at −40 and −120 mV in the same cell; the EC_{50} was increased approximately 4-fold (to 16·4 μM) at the latter potential, which is equivalent to depolarization having a pronounced sensitizing action on the action of carbachol. This cannot be explained by voltage-dependent binding of carbachol to the muscarinic receptor (Marty & Tan, 1989), because both the decline and reactivation of the current are too rapid upon a step in potential with time constants in the order of several hundred milliseconds (see later). For comparison, activation and deactivation of the cationic current when carbachol was applied or removed from the bathing solution were 10–100 times slower. These metabotropic receptor responses also have a minimum latency of at least 100 ms and generally much more (Bolton, 1976) presumably due to the G-protein link between receptor and channel.

A lower concentration of carbachol (3 μM) increased cationic current within a few seconds when applied in the bathing solution. The *I–V* relationship was established by rectangular steps from −40 to +40, 0, −80 and −120 mV (Fig. 1*A* and *B*) or by slow ramps (3 s) from +80 to −120 mV (Fig. 2*A*). At this relatively low concentration the *I–V* relationship was almost linear over the range 0 to −40 mV but current did not increase further with greater hyperpolarization and actually declined somewhat (Figs 1*A a*, 1*B a*, 2*A a* and 2*B a*). If a much higher concentration of carbachol (300 μM) was applied to the same cell the *I–V* relationship was almost linear from 0 to −80 mV (some inward rectification compared with current at positive potentials was usual) but deviated from linear at more negative potentials (Figs 1*A b*, *B b*, 2*A b* and 2*B b*). Thus, the *I–V* relationship of the cationic current was affected by the concentration of agonist applied to the cell and, as the fractional occupancy of muscarinic receptors increased, the nearly linear portion of the *I–V* relationship was extended in the negative range (Figs 1*B* and 2*B*).

The relationship between the conductance opened by carbachol and the membrane potential was sigmoidal and could be fitted by a Boltzmann distribution (Fig. 2*C*). The half-activation point was shifted 36 mV (ranging from 17 to 89 mV) in the negative direction, from −70 ± 5 to −106 ± 5 mV, in sixteen experiments upon increasing the concentration of carbachol from 3 to 300 μM, whereas the slope remained unchanged (cf. Fig. 2*A–C*, responses *a* and *b*). The conductance changed e-fold for 19·3 ± 0·9 mV ($n = 14$) corresponding to about 1·25 charges exposed to the whole membrane potential.

If a higher concentration of carbachol was applied for several minutes, cationic current declined (desensitization; Figs 2*A* and 3*A*). The *I–V* relationship during this

desensitization process was studied. In the example shown in Fig. 2*A–C* the *I–V* relationship after 3 min in 300 μM carbachol approximated to that obtained about 20 s after applying 3 μM carbachol (Fig. 2; compare *Aa* and *Ba* with *Ac* and *Bc*). It was apparent that as the process of desensitization proceeded the activation range moved positively along the voltage axis (Fig. 2*C*). Application of a high concentration of agonist after desensitization seemed equivalent to a low concentration applied for a few seconds to the same cell.

Desensitization of the muscarinic receptor was most pronounced when the membrane potential was held at very negative potentials (e.g. −120 mV, Fig. 3*A*). If the cell was held at positive potentials (e.g. +80 mV, Fig. 3*A*) then current remained unchanged and no desensitization was apparent over the same period (2 min), during which current at −120 mV declined by 80%. At an intermediate potential (−40 mV) slight desensitization was seen. Desensitization was always less at positive potentials than at negative potentials and was often negligible over 2 min or more if cells were held at +30 to +80 mV (Fig. 4*B* shows an example of an unusual degree of desensitization at +80 mV). Desensitization may be explained by some internal process (e.g. a decline in the production of activated G-protein subunits) which reduces the effectiveness of activated muscarinic receptors to maintain channels in their open state at negative potentials; this same process might also explain the different *I–V* relationships obtained with low and high concentrations of carbachol, without invoking any voltage dependence of carbachol binding to the receptor.

The desensitization and appearance of a pronounced U-shaped *I–V* relationship with prolonged receptor activation (Fig. 2*B*) was associated with pronounced changes in the rate of relaxation of the current following a negative step (Fig. 3*B*). After 15 s in 50 μM carbachol, relaxation was small and slow upon stepping from −40 to −120 mV, in keeping with the nearly linear *I–V*

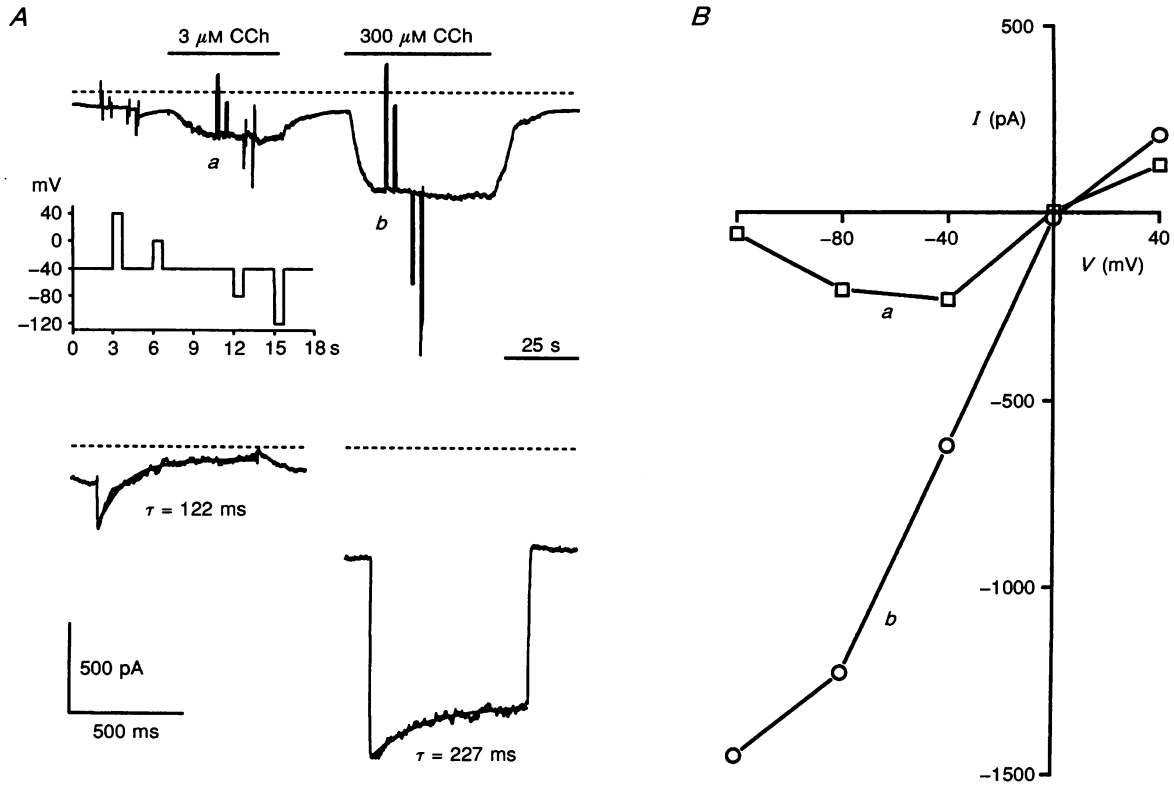

Figure 1. Cationic current evoked by muscarinic receptor activation in intestinal smooth muscle cells and the effect of agonist concentration and desensitization on its voltage dependence

A, membrane current–voltage relationships were measured three times, using the voltage protocol shown in the inset, before and during carbachol (CCh) application at low (3 μM; *a*) or high (300 μM; *b*) concentrations as indicated by the horizontal bars. Cationic current was obtained by subtracting current in the absence of carbachol. Relaxation of the cationic current in the presence of 3 μM (left) and 300 μM carbachol (right) during the 700 ms voltage steps from −40 to −120 mV is shown on an extended time scale below, fitted with a single exponential function using a least-squares method (superimposed continuous line). Dashed lines here and in all subsequent figures indicate zero current level. τ, time constant of relaxation. *B*, the last 50 ms of the steady-state cationic current at each pulse was averaged and plotted against membrane potential.

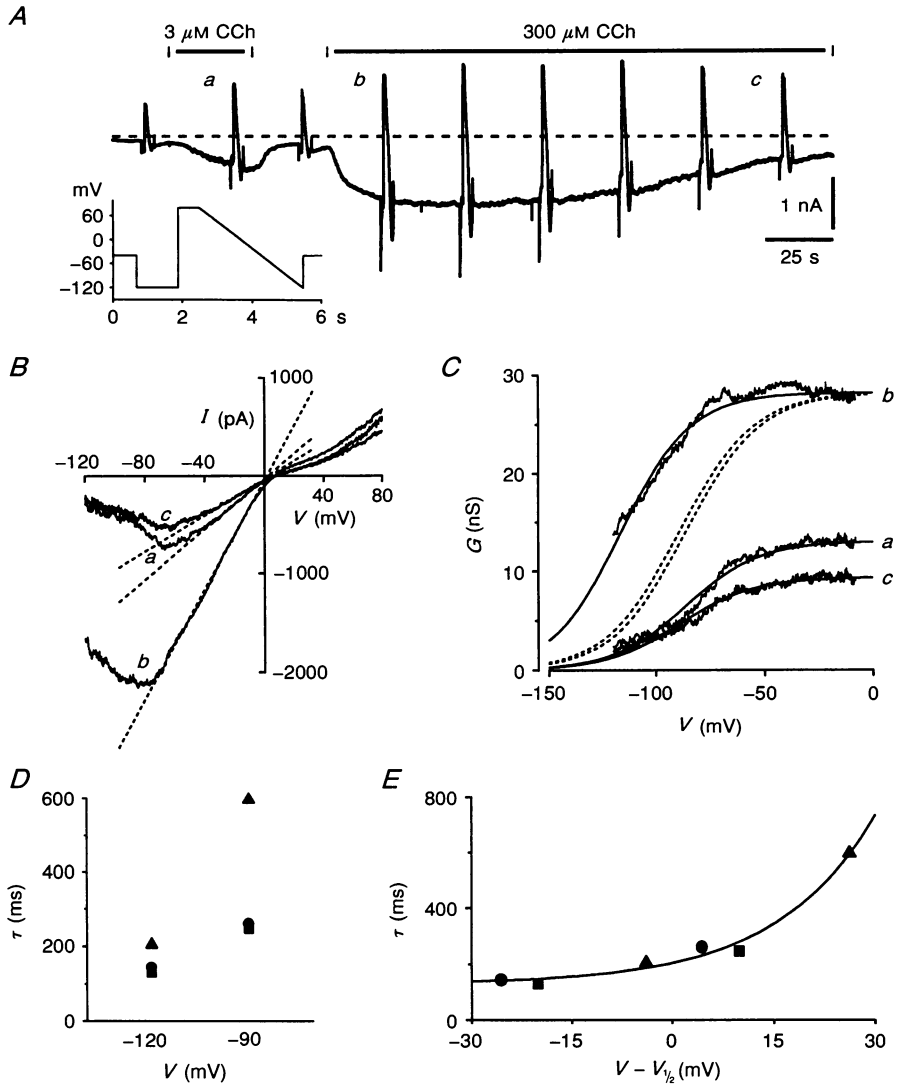

Figure 2. The effects of agonist concentration and desensitization on the cationic current voltage dependence and kinetics

A, carbachol (CCh) was applied at low concentration for 30 s and then at high concentration for 3·2 min to evoke muscarinic receptor desensitization; voltage-dependent properties of the cationic current were evaluated repeatedly at about 30 s intervals using the ramp voltage protocol shown in the inset. *B*, current–voltage relationships for the cationic current evaluated with the slow ramp pulses from +80 to −120 mV (3 s) at times indicated in *A* (*a*, 3 μM carbachol; *b* and *c*, 20 and 170 s after application of 300 μM carbachol, respectively). Dashed lines were fitted to linear portions of the *I–V* curves between −25 and −5 mV to obtain maximal slope conductance in each case. Note that deviation from linearity for trace *b* occurred at more negative potentials than in traces *a* and *c*. *C*, activation curves obtained by dividing the current amplitudes in *B* by the driving forces. Data were fitted by a Boltzmann distribution:

$$G = \frac{G_{max}}{1 + e^{(V - V_{½})/k}}$$

with the following best-fit parameters for conditions of *a*, *b* and *c*, respectively: the maximal chord conductance G_{max} was 13, 28 and 9 nS; the potential of half-maximal activation $V_{½}$ was −86, −116 and −89 mV; slope factor, *k*, was −17, −16 and −17 mV. The dashed lines show the activation curves *a* and *c* after scaling; note that they have the same slope but different $V_{½}$ than that in *b*. *D*, in another cell similar carbachol applications were made and the time constants of current relaxation during voltage steps from −40 to −90 and −120 mV were measured in 3 μM (●) and 300 μM carbachol at peak response (▲) and 4 min later (■). *E*, data from *D* when plotted *vs.* $V - V_{½}$ could be fitted by a single exponential function with an e-fold change per 14·3 mV (continuous line) which was comparable with the slope factor of −17·0 mV for the cationic current activation curve. $V_{½}$ values were −94·5 (3 μM), −116·2 (300 μM) and −100·0 mV (after 4 min desensitization).

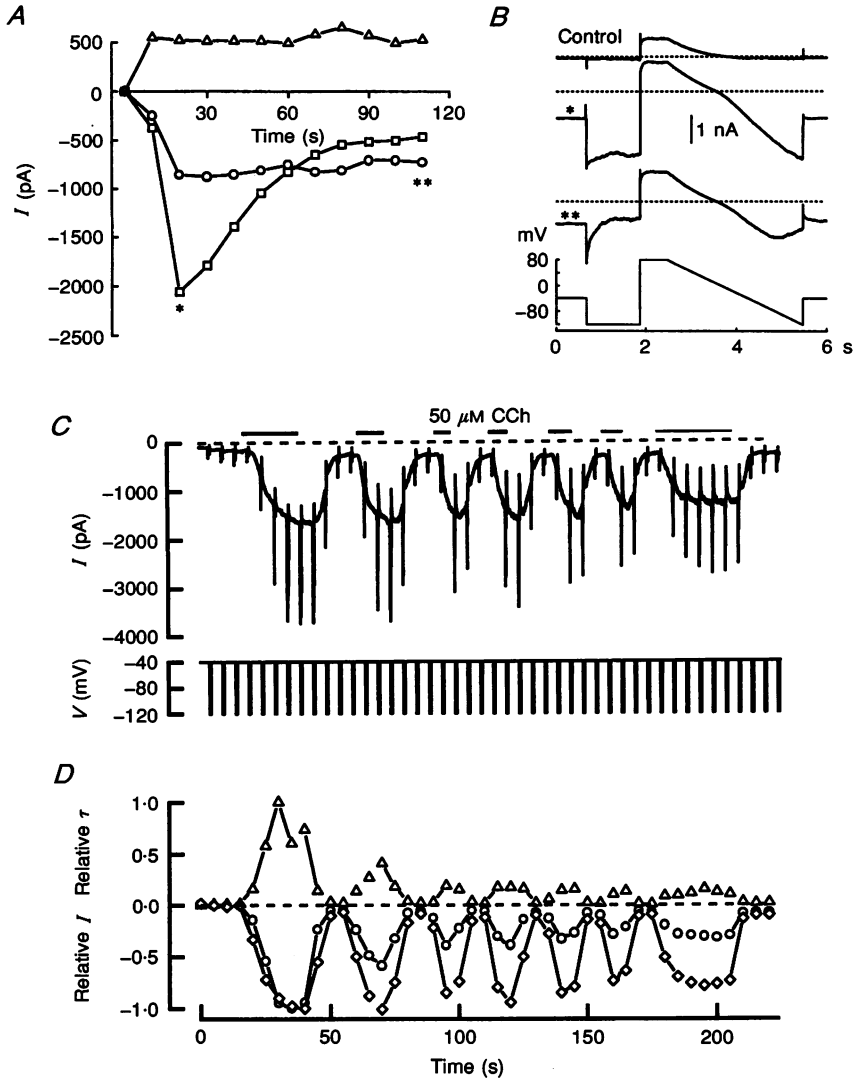

Figure 3. Cationic current voltage dependence and relaxation kinetics during muscarinic receptor desensitization evoked by prolonged or repeated short exposures to carbachol
A, carbachol (CCh; 50 μM) added to the bath at time zero resulted in cationic current activation which was monitored at 80 (△), −40 (○) and −120 mV (□). Notice the 'crossover' of the current sizes at −40 and −120 mV which occurred typically after 40–200 s (129 ± 21 s mean, $n = 9$) which may be explained by the positive shift of the activation curve (compare Fig. 2*C*). *B*, current traces obtained before and in the presence of carbachol at the times indicated by asterisks in *A* illustrate that considerable acceleration of current relaxation upon stepping to −120 mV developed with time and desensitization. *C*, in another cell, 50 μM carbachol was applied repeatedly during periods indicated by horizontal bars while membrane potential was stepped from −40 to −120 mV at 0·2 Hz as the lower trace shows. *D*, from the experiment illustrated in *C*, steady-state current amplitudes at −40 (◇) and −120 mV (○) as well as the time constants (△) of the current relaxation at −120 mV were measured and plotted as relative values. Note that desensitization affects the kinetics of relaxation and cationic current amplitude at −120 mV more than at −40 mV. Current at −120 mV and τ showed parallel changes, with a correlation coefficient in the range 0·87–0·94. Note also that current is activated more rapidly at −40 mV when compared with −120 mV (first application of carbachol in *D*) and even more so at +80 mV (Figs 3*A* and 4*B*, for example).

relationship. After 100 s, relaxation was more rapid and much greater; at this time steady-state current at −120 mV was less than that at −40 mV (Fig. 3*A* and *B*), whereas at 15 s it was about 2·5 times greater. The 'crossover' of the current sizes, after about 60 s at −40 and −120 mV, seen in Fig. 3*A*, is particularly striking. The rapid relaxation of current, following a negative step in potential after desensitization to a high concentration of carbachol, was comparable with the rapid relaxation of current seen upon such a step in the presence of a low

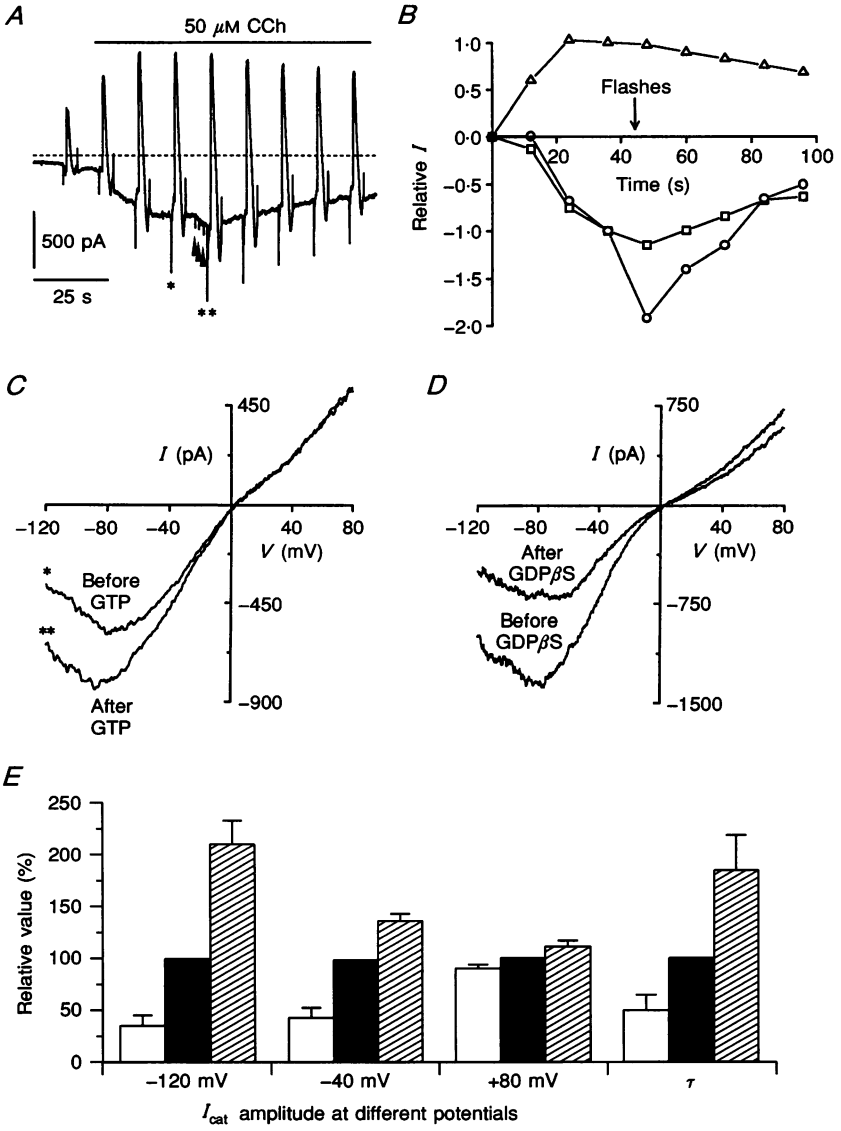

Figure 4. Effects of flash-released GTP and GDPβS

A, a representative example from nine experiments in which a pipette solution containing 2·5 mM caged GTP was used and three light flashes were applied at the peak response to 50 μM carbachol (CCh; arrowheads, also seen as short artifacts on the current trace). Voltage pulses identical to those shown in Fig. 2*A* were applied at 12 s intervals to monitor both the kinetics of current relaxation at −120 mV and *I–V* relationships during slow ramps. *B*, steady-state current amplitudes of the cationic current evoked by carbachol at 80 (△), −40 (□) and −120 mV (○) from the trace in *A* are shown relative to maximal values at the same potentials. Current amplitudes were measured by averaging the last 50 ms segment of the steady-state cationic current during step pulses (same voltage protocol as in Figs 2*A* and 3*B*). *C*, *I–V* curves for cationic current obtained with the slow ramps at times indicated by asterisks in *A*. *D*, *I–V* curves obtained in a similar experiment but with 2·5 mM caged GDPβS added to the pipette solution. The *I–V* relationship was evaluated first at the peak response to 50 μM carbachol followed by single light flash to release about 180 μM GDPβS and the *I–V* relationship measured 10 s later. The time constant of the current relaxation at −120 mV was reduced by flash from 224 to 178 ms. *E*, mean data for the effects of flash-released GTP and GDPβS on the cationic current amplitude (I_{cat}) at three different potentials and time constant of its relaxation during a voltage step to −120 mV. Each column and bar represents mean and S.E.M., respectively, from 9 cells with GTP (▨) and 5 cells with GDPβS (□) in relationship to control (■; before flash) normalized as 100% for each cell. All mean values are significantly different from control (Student's *t* test, $P < 0{\cdot}05$) except for current amplitude at +80 mV which was hardly affected by GTP ($P = 0{\cdot}08$). In control experiments, cells were loaded with 2·5 mM caged ATP to test possible effects of the flash and release of the caged group (1-(2-nitrophenyl)-ethyl) itself. Similar flashes resulted in small and inconsistent changes, for example 7 ± 8% increase of the current at −120 mV and 8 ± 2% decrease at −40 mV ($n = 4$). No significant effects of flash-released GTP or GDPβS were seen in the absence of agonist.

concentration of carbachol (Fig. 1*A*). For example, the time constants upon stepping from −40 to −120 mV in the same cell were 94 ± 12 ms (3 μM carbachol), 178 ± 14 ms before and 112 ± 13 ms after desensitization (300 μM carbachol; $n = 20$) when current had declined to a size equal to that evoked by 3 μM carbachol. Again, low concentrations without desensitization, and high concentrations when desensitization had developed, seemed equivalent. There was also a striking parallelism in the behaviour of the time constant of relaxation (τ) following a negative step and the steady-state current size at −120 mV, as if a single process determined both τ and the *I–V* relationship (Fig. 3*C* and *D*).

The relationship between conductance and potential, which can be described by a Boltzmann equation, is explained in the same way as for other voltage-dependent channels by the existence of charges on the channel protein which are subject to part or all of the membrane field. We postulated that the positive shift of the activation curve along the voltage axis that occurs during desensitization or with a reduction in the concentration of agonist, could be due to a lower concentration of activated G-protein subunits in the cell, i.e. it could be due respectively to a decline in the cell's ability to produce G-protein subunits with time if agonist application is prolonged or to a lower fractional receptor occupancy. The slope of the Boltzmann distribution was unchanged when this was shifted in a negative direction by increasing the concentration of agonist. Because of the negative shift of the Boltzmann distribution with increasing fractional occupancy of the receptors, steps from −40 to −120 mV would not be expected to produce equivalent effects at different agonist concentrations (Fig. 2*C*). Thus, we supposed that with low concentrations of agonist, τ was small and *I–V* relationships were U-shaped (Fig. 1*A* and *Ba*) due to the low level of production of the activated G-protein, whereas with high concentrations of agonist, when the rate of production of G-proteins and their concentration in the cell were higher, τ was greater and the linear portion of the *I–V* relationship was extended in the negative direction (Fig. 1*A* and *Bb*).

We examined equivalent changes in activation at different concentrations of agonist, by choosing potentials at equivalent positions on the Boltzmann curve; similar changes in τ were observed, as expected, and τ changed exponentially along the Boltzmann curve with an e-fold change per 14–16 mV ($n = 2$; Fig. 2*D* and *E*). Thus, both the voltage-dependent behaviour of the cationic channel and its kinetics upon a step change in potential, seem to be determined by its charge. However, the range of potential over which equivalent changes are observed is apparently a function of the fraction of receptors occupied by agonist or, better, the concentration of activated G-protein to which the cationic channels are exposed internally at any instant. Thus, cationic channels opened by a low concentration of carbachol are different from those which open at the same potential in the presence of a high concentration because the latter close more slowly than the former upon the same negative step; thus channel properties are a function of fractional receptor occupancy.

We speculated that the activating portion of the G-protein may be an α-subunit in the GTP-bound form, and that the availability of these α-GTP units declines during desensitization due to both α-subunit and GTP depletion. In support of this, when 1 mM GTP was added to the pipette solution, desensitization and all related phenomena, such as a positive shift of the activation curve, were strongly retarded ($n = 5$, data not illustrated). To investigate the mechanism of desensitization further we filled pipettes with GTP-free solution which contained the inert 'caged' GTP precursor. Photolysis of the precursor to release around 0·4 mM GTP inside the cell had the expected effect of extending the linear part of the *I–V* relationship (Fig. 4*A–C*). The effect was, as predicted, especially pronounced at −120 mV (111 ± 23% increase, $n = 9$), less so at −40 mV (37 ± 7%) , and absent in the example shown at +80 mV (a mean increase of only 11 ± 5% on average). Release of GTP in the cell by flash photolysis also increased current during muscarinic receptor activation in cells held for longer periods at −120 mV (it had no effect in the absence of receptor activation) and slowed the rate of decay of the cationic current upon a hyperpolarizing step: the time constant of a fitted exponential increased from 112 to 154 ms (Fig. 4*A* and *E*). It is remarkable, however, that the *I–V* relationship between +80 and −20 mV was almost unaltered (Fig. 4*A* and *C*). Flash release of GDPβS from a 'caged' precursor had the opposite effect to release of GTP: cationic current was reduced especially at negative potentials and the decay of current upon a negative step accelerated (Fig. 4*D* and *E*).

DISCUSSION

The major novel conclusion from the present experiments is that the position of the activation curve on the voltage axis depends critically upon the concentration of activated α-subunits generated at any time in the cell. The relationship between the conductance evoked by muscarinic receptor activation and potential could be well fitted by a Boltzmann-type equation consistent with the idea that charged groups on the molecule determine the probability of channel open state. However, no channel opening seems possible without initial binding of an activated α-subunit and a negative shift in the Boltzmann distribution occurred, without change in slope, as fractional occupancy of the muscarinic receptor, and hence presumably concentration of α-subunits, increased. In such a system, the binding of at least one α-GTP subunit would be needed before channel opening is possible; binding of two (or more) subunits may be also possible and may have the effect of increasing channel lifetime at any potential. At short times and when fractional receptor occupancy by

agonist is high, two or more α-GTP subunits may bind to a single channel. Under such conditions, relaxation of current upon a negative step may reflect channel lifetimes with one, two, or more α-GTP subunits bound. Even at a concentration of 3 μM carbachol, there may be a significant proportion of channels with two or more α-GTP subunits bound as it is close to the EC_{50}. Alternative models to the one we have suggested are possible; for example, a second α-GTP-dependent channel modulatory process.

The shifting of the voltage dependence of a current upon ligand binding seems to be a widespread and fundamental mechanism by which ion channels are controlled. Indeed, this has been established for such diverse channel types as the large-conductance Ca^{2+}-activated K^+ channel (maxi-K^+ channel) where Ca^{2+} acts as a ligand (e.g. McManus & Magleby, 1991), the cationic channel (i_f) activated by hyperpolarization where voltage dependence is affected by cyclic AMP (DiFrancesco & Tortora, 1991; DiFrancesco & Mangoni, 1994) and the potential-dependent Ca^{2+} channel which is controlled by neurotransmitters (more specifically by G-proteins) (Bean, 1989; Grassi & Lux, 1989; Kasai & Aosaki, 1989; Elmslie, Zhou & Jones, 1990; Lopez & Brown, 1991; Pollo, Taglialatela & Carbone, 1991; Brown, 1993). Our present findings show that metabotropic receptor channels can also be controlled in a similar or analogous manner. The physiological significance of this control is obvious: depolarization evoked by acetylcholine through muscarinic receptor cationic channel openings would lead to a more tight 'coupling' between channels and G-protein α-subunits resulting in the amplification of the effect of receptor activation, increased probability of channel opening and further depolarization. The molecular basis of this control, however, remains unclear and further work is needed to elucidate details of the mechanisms involved.

REFERENCES

Bean, B. P. (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**, 153–156.

Benham, C. D., Bolton, T. B. & Lang R. J. (1985). Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature* **316**, 345–346.

Bolton, T. B. (1976). On the latency and form of the membrane responses of smooth muscle to the iontophoretic application of acetylcholine or carbachol. *Proceedings of the Royal Society* B **194**, 99–119.

Brown, A. M. (1993). Membrane-delimited cell signalling complexes: direct ion channel regulation by G proteins. *Journal of Membrane Biology* **131**, 93–104.

DiFrancesco, D. & Mangoni, M. (1994). Modulation of single hyperpolarization-activated channels (i_f) by cAMP in the rabbit sino-atrial node. *Journal of Physiology* **474**, 473–482.

DiFrancesco, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145–147.

Elmslie, K. S., Zhou, W. & Jones, S. W. (1990). LHRH and GTP-γ-S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* **5**, 75–80.

Grassi, F. & Lux, H. D. (1989). Voltage-dependent GABA-induced modulation of calcium currents in chick sensory neurons. *Neuroscience Letters* **105**, 113–119.

Inoue, R. & Isenberg, G. (1990*a*). Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. *American Journal of Physiology* **258**, C1173–1178.

Inoue, R. & Isenberg, G. (1990*b*). Effect of membrane potential on acetylcholine-induced inward current in guinea-pig ileum. *Journal of Physiology* **424**, 57–71.

Kasai, H. & Aosaki, T. (1989). Modulation of Ca-channel current by an adenosine analog mediated by a GTP-binding protein in chick sensory neurons. *Pflügers Archiv* **414**, 145–149.

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. *Journal of Physiology* **450**, 105–126.

Lopez, H. S. & Brown, A. M. (1991). Correlation between G protein activation and reblocking kinetics of Ca^{2+} channel currents in rat sensory neurons. *Neuron* **7**, 1061–1068.

McManus, O. B. & Magleby, K. L. (1991). Accounting for the Ca^{2+}-dependent kinetics of single large-conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. *Journal of Physiology* **443**, 739–777.

Marty, A. & Tan, Y. P. (1989). The initiation of calcium release following muscarinic stimulation in rat lacrimal glands. *Journal of Physiology* **419**, 665–687.

Pollo, A., Taglialatela, M. & Carbone, E. (1991). Voltage-dependent inhibition and facilitation of Ca channel activation by GTP-γ-S and Ca-agonists in adult rat sensory neurons. *Neuroscience Letters* **123**, 203–207.

Zang, W.-J., Yu, X.-J., Honjo, M. S., Kirby, M. S. & Boyett, M. R. (1993). On the role of G-protein activation and phosphorylation in desensitization to acetylcholine in guinea-pig atrial cells. *Journal of Physiology* **464**, 649–679.

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