Journal of Physiology (1992), **450**, *pp.* 105–126
With 11 *figures*
Printed in Great Britain

GTP-BINDING PROTEIN INVOLVEMENT IN MEMBRANE CURRENTS EVOKED BY CARBACHOL AND HISTAMINE IN GUINEA-PIG ILEAL MUSCLE

BY S. KOMORI, M. KAWAI, T. TAKEWAKI AND H. OHASHI

From the Laboratory of Pharmacology, Department of Veterinary Science, Faculty of Agriculture, Gifu University, Gifu 501–11, Japan

(*Received 14 August 1991*)

SUMMARY

1. Single smooth muscle cells obtained by enzymic dispersion of the longitudinal muscle layer of guinea-pig ileum were used for recording membrane currents under whole-cell voltage clamp in response to carbachol (100 μM, unless otherwise stated) or histamine (100 μM) applied extracellularly.

2. At a holding potential of 0 mV, a transient outward current was evoked by carbachol and histamine. Responses to the two agonists were very similar in size and time course to the current response to caffeine (10 mM). The response to carbachol was virtually absent in the presence of histamine, and vice versa. Caffeine was without effect in the presence of either of these agonists. Inclusion of EGTA (10 or 20 mM) in the pipette abolished the responses to carbachol, histamine and caffeine. Thus, the outward current responses were considered to represent opening of Ca^{2+}-activated K^+ channels in response to a massive release of Ca^{2+} from the same stores by these three agents.

3. An inward current was evoked by carbachol and histamine, but not by caffeine at a holding potential of -40 mV, which was considered to represent opening of cationic channels. The carbachol-induced inward current was much longer in duration and larger in size than the histamine-induced inward current.

4. Inclusion of GDPβS (2 mM) in the pipette abolished the inward and outward current responses to histamine, but inhibited only part of those to carbachol.

5. When the holding potential was held at 0 mV with inclusion of GTPγS (0·1–1 mM) in the pipette, spontaneous transient outward currents appeared immediately after break-through but disappeared a few minutes later. Under these conditions, caffeine (10 mM) was almost without effect, suggesting that GTPγS had released Ca^{2+} stores. When the holding potential was held at -40 mV and GTPγS (0·1 or 0·2 mM) was present in the pipette, an inward current developed a few minutes after break-through. During the GTPγS-induced inward current, application of carbachol or histamine produced no further inward current. However, when 0·01 mM-GTPγS was included in the pipette solution, carbachol- and histamine-induced inward currents were potentiated.

6. Pretreated with 2–5 μg/ml pertussis toxin (PTX) did not change noticeably the

outward current responses to carbachol and histamine, but abolished or markedly reduced the inward current responses.

7. The results suggest that stimulation of muscarinic receptor or histamine receptor caused release of Ca^{2+} from storage sites and activation of cationic channels, and that regardless of the receptor type, calcium store release may be mediated via a PTX-insensitive G-protein, while the cation channels are activated via another G-protein which is sensitive to PTX.

INTRODUCTION

In many different cell types, a GTP-binding protein (G-protein) is suggested to be involved in the transduction of receptor stimulation into cellular responses (Stryer & Bourne, 1986; Gilman, 1987). In intestinal smooth muscles, stimulation of the muscarinic receptor produces membrane depolarization through activation of cationic channels leading to influx of Ca^{2+} from the extracellular fluid (Bolton, 1972; Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987) and release of Ca^{2+} out of the sarcoplasmic reticulum (Ohashi, Takewaki & Okada, 1974). The contractile response is mediated by an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting from these effects. However, it is still not certain whether the inward current elicited through activation of cationic channels in rabbit jejunal muscle cells by muscarinic agonist, carbachol (CCh) involves a G-protein system (Lim & Bolton, 1988; Komori & Bolton, 1990). In rabbit cells loaded with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), a GTP analogue resistant to hydrolysis, the inward current response to CCh is not sustained beyond the exposure period to the agonist and an inward current similar to CCh inward current is not evoked or only infrequently evoked (Komori & Bolton, 1990) by GTPγS itself. On the other hand, the Ca^{2+}-activated K^+ current elicited by CCh due to Ca^{2+} store release is reduced in a dose-dependent manner by guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS), a competitive inhibitor of GTP. Also, the release of Ca^{2+} stores are brought about by CCh and by GTPγS, and both effects are mediated by D-*myo*-inositol 1,4,5-trisphophate ($InsP_3$) formed by activation of phospholipase C (PLC). It is difficult to reconcile these observations with the idea that a single G-protein is involved in the transduction of the two muscarinic responses (Komori & Bolton, 1990). In guinea-pig ileal muscle cells, the inward current mediated via muscarinic receptors had been suggested to involve a G-protein system, since the effect was mimicked by GTPγS and blocked by GDPβS and pertussis toxin (PTX) which uncouples an agonist–G-protein link by ADP ribosylation of the α-subunit of G-protein (Inoue & Isenberg, 1990*b*). In this cell type, however, the involvement of a G-protein in muscarinic receptor-evoked Ca^{2+} store release has not been investigated. A species-related difference, or a difference in the experimental methods may be responsible for some of the discrepancies between previous works. Moreover, a question arises as to whether or not a single G-protein links the muscarinic receptor to the two cellular responses mentioned above.

The effects of histamine on intestinal smooth muscle are very similar to those of CCh (see review, Bolton, 1979). The contractile response is mediated by an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) which results from an influx of Ca^{2+} from the extracellular fluid and release of Ca^{2+} from storage sites in the cell. Membrane

depolarization related to an increase in membrane permeability to cationic ions is suggested to be brought about by activation of a cationic channel which is also activated by CCh (Bolton, Clark, Kitamura & Lang, 1981). Recently, P. Pacaud & T. B. Bolton (personal communication) provided evidence that histamine, like CCh, induces release of the Ca^{2+} store and an elevation of $[Ca^{2+}]_i$ in single smooth muscle cells. However, involvement of a G-protein in the transduction of stimulation of the histamine receptor into these cellular responses is not well understood yet.

The present study was made to investigate the effects of CCh and histamine on membrane currents in guinea-pig ileal muscle cells using standard patch-clamp techniques in an attempt to see whether or not G-proteins are involved in both activation of cationic channels and release of Ca^{2+} stores brought about by either muscarinic receptor or histamine receptor activation.

The present results indicate that both the inward current and the Ca^{2+} activated potassium current elicited by stimulation of histamine receptors are indistinguishable in pharmacological properties from those elicited by stimulation of muscarinic receptors, and suggest that a G-protein system appears to play a role in each of the two cellular responses of opening of cationic channels and release of Ca^{2+} stores, but a G-protein involved in the release of the Ca^{2+} store is characterized by insensitivity to PTX.

METHODS

Preparation of cells

Male guinea-pigs, weighing 350–450 g, were stunned and bled to death. A length of 10 cm of the ileum was removed and divided into two segments (each, about 5 cm long). The longitudinal muscle layer of the intestinal segments was peeled from the underlying circular muscle, washed in physiological salt solution (PSS; composition given below). The two muscle layers were cut into small pieces of about 1 cm square, placed in a test-tube containing 3 ml PSS to which calcium was not added (Ca-free PSS) and allowed to equilibrate for 10 min at 37 °C. The Ca-free PSS in the test-tube was replaced with 1 ml PSS to which 30 μM-calcium was added (low-Ca PSS). Two successive incubations of 15 min at 37 °C were carried out, each in a fresh low-Ca PSS containing collagenase (0·2–0·6 mg/ml), papain (0·3–0·6 mg/ml) and bovine serum albumin (5 mg/ml). After this enzyme digestion, the enzyme solution was removed. Tissue pieces were then placed in 2 ml PSS to which 120 μM-calcium was added, and agitated by drawing in and out of a blunt glass pipette 40–50 times. The solution in which isolated cells were suspended was removed and retained. The procedure was repeated several times at room temperature (20–25 °C). The cell-rich solutions obtained after the fifth to seventh steps were combined and centrifuged at about 700 r.p.m. for 2 min. The cells were resuspended in 3–5 ml PSS containing 0·5 mM added calcium, placed on ten to twenty cover-glasses as a small aliquot and kept in a moist atmosphere at 4 °C until use. The isolated cells were used for the experiments only on the same day as they were prepared.

Recording of membrane currents

A cover-glass with cells was placed in a small bath (0·8 ml) fixed on the stage of a Nomarski microscope (Nikon, TMD). The organ bath was filled with PSS, perfused with 5–10 ml of the same solution to wash away contaminants in the cell suspension, and equilibrated with the solution. Whole-cell membrane-current recordings were made at room temperature using standard patch-clamp techniques (Hamill, Marty, Neher, Sakman & Sigworth, 1981). Patch pipettes had a resistance of 4–7 MΩ when filled with a pipette solution. Membrane currents were amplified by a current amplifier (List, EPC-7 or Nihon Kohden, S-3666) and stored on FM tape using a recorder (Sony, FR-3215W) and replayed onto a thermal array recorder (Nihon Kohden, RTA-1100 M) for illustration and analysis.

Cells were dialysed with a potassium-rich solution (patch-pipette solution, composition given below) and membrane currents in response to drugs were recorded at a holding potential of 0 mV or −40 mV. At 0 mV, a potassium current can be recorded with almost no current through cationic

channels gated by stimulation of muscarinic receptors (Benham, Bolton & Lang, 1985), and at −40 mV, currents through potassium channels and the cationic channels can be recorded as an outward current and an inward current, respectively (Bolton & Lim, 1989; Komori & Bolton, 1990).

The amount of transferred charge (size of current × time = coulombs) in response to drugs was estimated by measuring the area of the drug-induced current response. The values in the text are the means ± S.E. of the mean. Statistical significance was tested using a Student's unpaired t test and differences were considered significant when $P < 0.05$.

Application of drugs

Extracellular application of drugs such as carbachol, histamine and caffeine was made by introducing them into the recording chamber by replacing the bathing solution with the drug-containing solution several times. Intracellular application of GTPγS and GDPβS was performed as follows: a drug-filled pipette was used as a patch pipette, and by rupturing the patch membrane, the drug was allowed to diffuse into the cell under voltage clamp in the whole-cell mode.

Treatment with pertussis toxin (PTX)

Cells were suspended in PSS containing 0·5 mM added calcium and the cell suspension was divided into two parts. PTX was added to one part of the cell suspension to give a concentration of 2 or 5 μg/ml. Each cell suspension was placed on a cover-glass as a small aliquot and incubated at 37 °C for 5–8 h in a moisturized incubator. To evaluate the effects of PTX, cells pretreated with PTX, and cells which were incubated without PTX but actually handled in no different way from the PTX-treated cells, were used alternately for recording membrane currents.

Solutions and drugs

The PSS used in the experiments had the following composition (mM): NaCl, 126; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1·2; glucose, 14 and HEPES, 10·5 (titrated to pH 7·2 with NaOH). The patch-pipette solution had the following composition (mM): KCl, 134; $MgCl_2$, 1·2; MgATP, 1; EGTA, 0·05; glucose, 14; HEPES, 10·5 (titrated to pH 7·2). Pipette solutions containing GTPγS and GDPβS were prepared by adding the concentrated solution (dissolved in distilled water) in a small volume (less than 5% of its total volume), to give the desired concentration.

Drugs used were carbachol chloride (Tokyo Kasei), caffeine (Wako), histamine dihydrochloride (Wako), guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS; Sigma), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS; Sigma), glycol etherdiamine tetraacetic acid (EGTA; Wako), adenosine 5′-triphosphate magnesium (MgATP; Sigma) and pertussis toxin (PTX) (Kaken).

Concentrations in the text and figures are expressed as molar in the bathing solution or pipette solution.

RESULTS

Whole-cell currents at a holding potential of 0 mV in response to carbachol and histamine

In cells held under voltage clamp at a holding potential of 0 mV, spontaneous transient outward currents (STOCs; Benham & Bolton, 1986) appeared with varied durations (50–200 ms) and sizes (up to 0·6 nA). STOCs are considered to represent the opening of Ca^{2+}-activated K^+ channels brought about by spontaneous, sporadic release of Ca^{2+} from storage sites in the cell (Benham & Bolton, 1986). Thus, STOCs can be taken as an indicator of stored Ca^{2+} hence its presence in storage sites (Benham & Bolton, 1986; Bolton & Lim, 1989). Experiments were started 3–10 min after establishment of the whole-cell recording mode, during which STOCs usually reached a stable level in their mean size and discharge rate.

On application of carbachol (100 μM) or histamine (100 μM), a brief outward current was evoked, as shown in Fig. 1. The current size varied from 0·5 to 4·5 nA and the total duration varied from 3 to 15 s. In some cells, discharge of the brief outward

current was repeated several times but often decreased progressively in size, and this pattern of the current response was more frequently observed following carbachol application than following histamine application. STOCs usually disappeared following the brief outward current in response to carbachol or histamine, and, if the

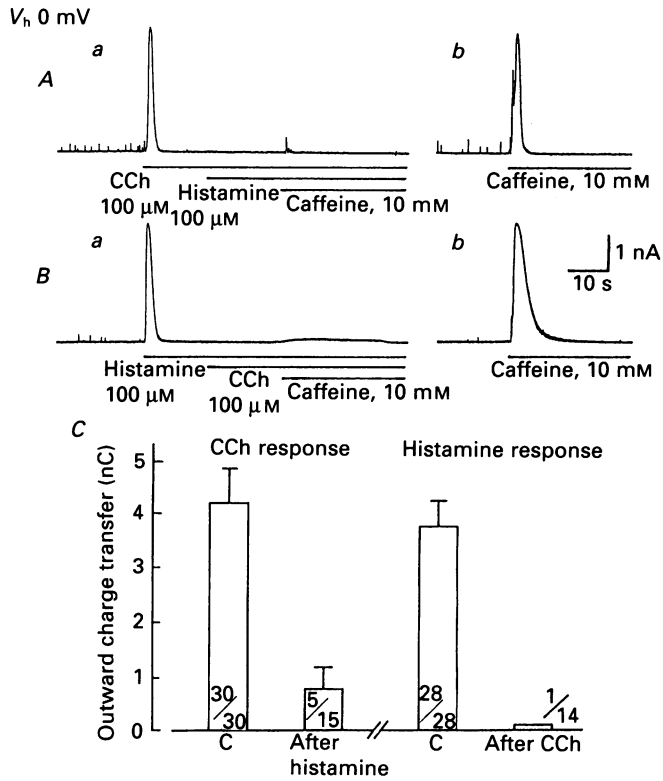

Fig. 1. Outward current responses to carbachol (CCh, 100 μM), histamine (100 μM) and caffeine (10 mM) of single ileal cells. Cells were held under voltage clamp at 0 mV (holding potential, V_h 0 mV). *A*: CCh evoked a brief outward current, but histamine and caffeine, applied in the presence of CCh, had virtually no effect (*a*). Caffeine elicited a brief outward current 7 min after replacing with drug-free solution (*b*). *B*: histamine evoked a brief outward current, but CCh and caffeine, applied in the presence of histamine were almost without effect (*a*). Caffeine elicited a brief outward current 9 min after replacing with drug-free solution (*b*). Current traces in *A* and *B* were obtained from two different cells. *C*, the mean outward charge transfer (in nanocoulombs, nC) upon CCh application and histamine application for cells which responded to these drugs: CCh alone (C), CCh after histamine (After histamine), histamine alone (C) and histamine after CCh (After CCh) in order of left to right. Each column represents the mean with one S.E. of the mean (vertical line). The fraction of responding cells is indicated in each column.

outward current response was small in size, small STOCs still occurred. The effects of these drugs were reversible and STOCs were restored a few minutes after removal of carbachol or histamine. Figure 1*C* shows the average outward charges transferred by the carbachol-induced current and histamine-induced current. The difference between the value for carbachol ($4{\cdot}18 \pm 0{\cdot}73$ nC, $n = 30$) and that for histamine

(3·70 ± 0·52 nC, $n = 28$) was not statistically significant ($P > 0·05$). From these results, together with the fact that with inclusion of EGTA (10 or 20 mM) in the pipette, the effects of carbachol and histamine were abolished, both the carbachol outward current and the histamine outward current were considered to represent the opening of Ca^{2+}-activated K^+ channels in response to a rise in cytosolic Ca^{2+} concentration due to a massive release of Ca^{2+} from its storage sites.

In order to determine whether carbachol and histamine act on a common store to release Ca^{2+}, carbachol (100 μM) was applied in the presence of, and 20–60 s after, histamine (100 μM), and vice versa. When a large outward current was elicited by histamine further application of carbachol was without effect on membrane current in ten out of fifteen cells (Fig. 1*B* and *C*) and evoked only a small outward current in the five remaining cells that was 0·86 ± 0·40 nC ($n = 5$) in terms of charge transfer (Fig. 1*C*). Application of histamine subsequent to carbachol evoked no outward current in thirteen out of fourteen cells (Fig. 1*C*) and only a small outward current in one cell. Caffeine (10 mM), when applied in the presence of histamine and carbachol, was ineffective, but caffeine-induced outward current reappeared several minutes after washing away histamine and carbachol (Fig. 1*A* and *B*).

The above results suggested that carbachol and histamine act on a common store to release Ca^{2+} which was also acted on by caffeine, and both drugs at a concentration of 100 μM are sufficient to produce a massive release of stored Ca^{2+}.

Whole-cell current at a holding potential of −40 mV in response to carbachol or histamine

At a holding potential of −40 mV, STOCs still appeared in half the cells, but they were smaller in size and in rate of discharge than those recorded at 0 mV holding potential. Carbachol or histamine was applied 3–10 min after establishment of the whole-cell recording mode.

Carbachol-induced inward current

High concentrations of carbachol (100 μM) elicited an inward current in all of thirty-six cells regardless of whether STOCs discharge occurred (Fig. 2*A*, *B* and *C*). The inward current response persisted until the drug was removed and it was preceded by a transient outward current in nineteen cells (Fig. 2*A* and *B*). In four cells current oscillations were observed (Fig. 1*B*). Inward charge transferred during the first 40 s of the current responses varied from 1·4 to 26·4 nC, giving an average of 7·70 ± 0·90 nC ($n = 36$).

Low concentrations of carbachol (2 μM), as shown in Fig. 2*D* and *E*, elicited oscillatory inward currents superimposed on a sustained inward current in eight of fifteen cells and in the remaining cells elicited sustained inward current with or without a preceding, transient inward current (Fig. 2*F*). The oscillations in current sometimes continued as long as carbachol was present in the bathing solution (Fig. 2*D*), but in other cells they decreased progressively in size and disappeared even in the presence of carbachol (Fig. 2*E*). Expressed as a charge transfer, the carbachol current responses varied between 0·3 and 12·1 nC, giving an average of 5·49 ± 0·90 nC ($n = 15$). This value was smaller than the corresponding value obtained with 100 μM-carbachol (7·70 ± 0·90 nC), but the difference between these two values was not

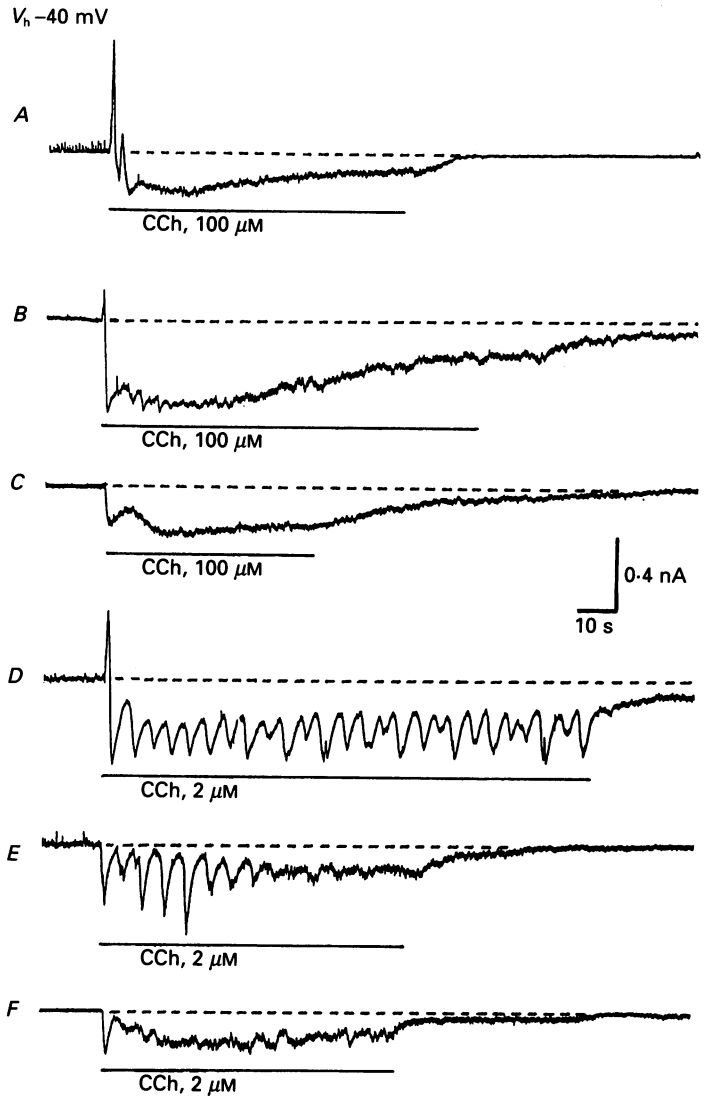

Fig. 2. Variations of current responses to carbachol (CCh) in cells voltage clamped at −40 mV (V_h −40 mV). *A–C*, CCh (100 μM) evoked a long-lasting inward current with (*A* and *B*) or without (*C*) a preceding outward current. *D–F*, CCh (2 μM) evoked oscillatory inward currents superimposed on a sustained inward current (*D* and *E*) or a transient inward current followed by a sustained inward current (*F*). Current traces in *A–F* were obtained from six different cells.

statistically significant ($P > 0{\cdot}05$). This may result from a preferential appearance of oscillatory inward currents elicited in response to carbachol at low concentrations (2 μM).

Histamine-induced inward current

Current responses to histamine (100 μM) were observed in fifty-six cells. Figure 3 shows recordings obtained in four different cells of this series. Inward current responses large enough to measure the charge transfer were obtained in only twenty-

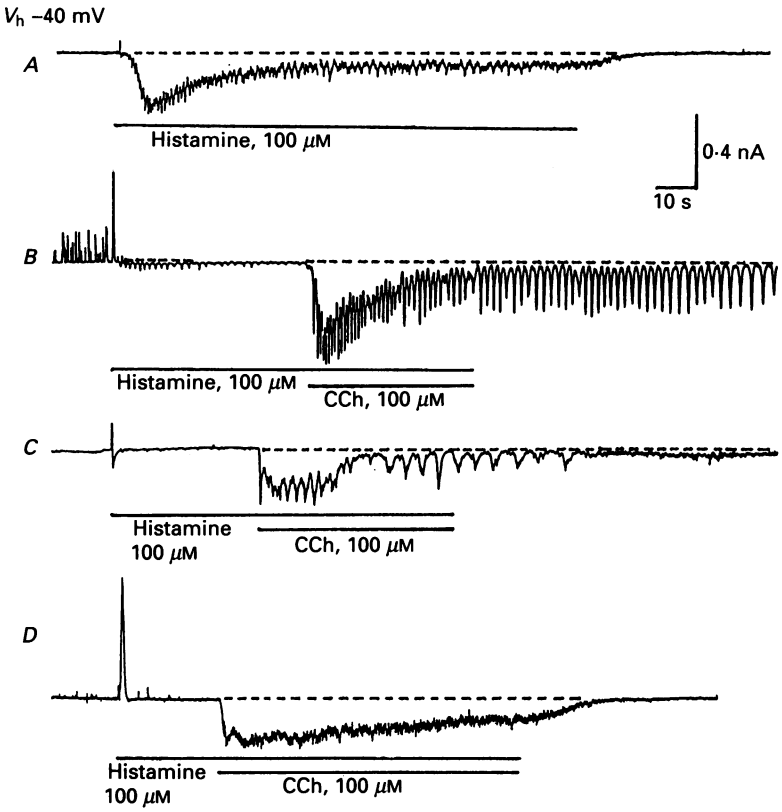

Fig. 3. Variations of current responses to histamine (100 μM) and to carbachol (100 μM) applied in the presence of histamine in cells voltage clamped at −40 mV (V_h −40 mV). Histamine evoked a long-lasting inward current similar to the inward current response to carbachol (*A*), a small but long-lasting inward current (*B*), a brief inward current (*C*), or no inward current (*D*). A brief outward current was elicited upon initial application of histamine (*A–D*). Further application of carbachol invariably evoked an inward current with (*B* and *C*) or without (*D*) oscillatory inward currents. See text for details. Current traces in *A–D* were obtained from four different cells.

four cells. The inward current responses in four cells were comparable in size with carbachol inward current responses (Fig. 3*A*). Eleven other cells responded by a small, sustained inward current (Fig. 3*B*, also see Fig. 5*D*) which was occasionally accompanied by a small oscillatory current. In the nine remaining cells, a brief inward current lasting for up to 5 s was elicited, as shown in Fig. 3*C*. The average charge movement measured from the current responses in the twenty-four cells was 1·15±0·33 nC (n = 24). Thirty-two cells out of fifty-six responded with a transient

outward current; rarely, an increase in current noise level was seen. In some cells, a spike-like inward current with a duration of about 1 s appeared immediately before or after a transient outward current.

Application of histamine in the presence of carbachol (100 μM) had no further effect on membrane current (7 cells), while carbachol (100 μM), applied in the

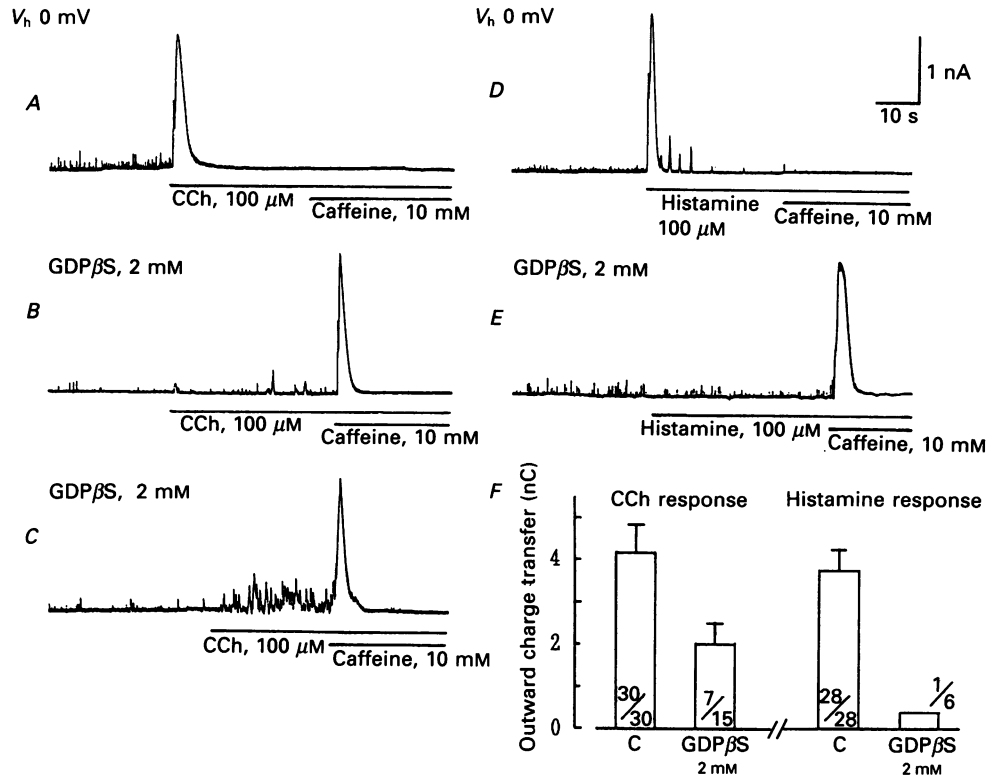

Fig. 4. Effects of GDPβS on outward current responses to carbachol (CCh, 100 μM) and to histamine (100 μM) in cells voltage clamped at 0 mV (V_h 0 mV). Cells were dialysed with normal or GDPβS (2 mM)-containing solution. *A*, a control experiment for the test of GDPβS effect on CCh responses. CCh elicited a brief outward current in a normal cell, and the response to caffeine (10 mM) in the presence of CCh was absent. *B* and *C*, current responses of two different GDPβS-filled cells to CCh and to caffeine (10 mM) applied in the presence of CCh. *D*, a control experiment for the test of GDPβS effect on histamine responses. *E*, current responses of GDPβS-filled cell to histamine and to caffeine (10 mM) in the presence of histamine. *F*, summary of the effects of GDPβS on the current responses to CCh (CCh response) and to histamine (Histamine response). Ordinate scale, the mean outward charge transfer (nC) during the current responses in normal (C) (data from Fig. 1*C* for the CCh and histamine responses) and GDPβS-filled cells which responded to the agonists. The fraction of responding cells is indicated in each column. Vertical lines indicate one S.E. of the mean. In GDPβS-filled cells, CCh- and histamine-induced outward currents were reduced (*F*) or virtually absent (*B*, *C* and *E*) and caffeine elicited an outward current.

presence of histamine, invariably elicited a sustained inward current (42 cells) (Fig. 3*B*, *C* and *D*). The inward current response to carbachol in the presence of histamine characteristically lacked a preceding outward current, and oscillations were prominent (observed in 40% of cells) which continued even after replacement of the bathing solution with drug-free solution in some cells. These carbachol current responses gave an average charge movement of $5{\cdot}88 \pm 0{\cdot}46$ nC ($n = 42$).

Effects of GDPβS on whole-cell currents in response to carbachol or histamine

Effects on the outward current response to carbachol or histamine

To allow sufficient GDPβS (2 mM) to enter the cell, the holding potential was set at −40 mV and the cell was placed in whole-cell recording mode for 7–10 min. After that, the holding potential was shifted gently to 0 mV by taking 20 s over this procedure and application of carbachol (100 μM) or histamine (100 μM) was made after STOCs reached a stable level in size and discharge rate.

When GDPβS was included in the pipette, there was no appreciable change in the size and discharge rate of STOCs. Carbachol-induced outward current was abolished in eight out of fifteen cells (Fig. 4*B*), but it was elicited in the other seven cells though its form varied from the almost normal to only an increase in size and discharge rate of STOCs (Fig. 4*C*). The average outward charge transferred during the carbachol responses was $2{\cdot}00 \pm 0{\cdot}46$ nC ($n = 7$), which was about 50% of the control (see Fig. 4*F*). The histamine-induced outward current was not obtained in five out of six cells (Fig. 4*E*), and one cell responded with a very small outward current (Fig. 4*F*). However, all of the cells in which carbachol and histamine were without effect responded with a brief outward current upon application of caffeine (10 mM) (Fig. 4*B* and *E*). The caffeine response was just like the current responses to carbachol and histamine in normal cells (Fig. 4*A* and *D*).

Effects on the inward current response to carbachol or histamine

With inclusion of GDPβS (2 mM) in the pipette, the holding potential was held at −40 mV and the cell was placed in the whole-cell current recording mode for 7–10 min before application of carbachol or histamine.

Carbachol (100 μM) did not elicit any inward current in three out of twelve cells. In five cells an oscillatory inward current was elicited and the effect was sustained until removal of the drug (Fig. 5*B*). Current responses of this type were often obtained in normal cells with carbachol at low concentrations (2 μM). Four cells responded by a small sustained inward current with or without a preceding transient inward current (Fig. 5*C*). The average charge transfer of $3{\cdot}31 \pm 0{\cdot}93$ nC ($n = 9$) was significantly smaller ($P < 0{\cdot}05$) than the corresponding value ($7{\cdot}70 \pm 0{\cdot}90$ nC, $n = 36$) in normal cells (Fig. 9).

Application of histamine (100 μM) had no effect on the whole-cell current in all of thirteen cells (Fig. 5*E* and *F*). Carbachol (100 μM) applied 30–60 s after histamine application elicited no inward current (6 cells; Fig. 5*E*) or a reduced inward current (7 cells; Fig. 5*F*) with or without an oscillatory component. The average charge transfer during the carbachol responses obtained in the presence of histamine in seven cells was $2{\cdot}95 \pm 0{\cdot}72$ nC ($n = 7$), which was significantly smaller ($P < 0{\cdot}05$) than the corresponding value in normal cells ($5{\cdot}88 \pm 0{\cdot}46$ nC, $n = 42$).

The inhibitory effects of GDPβS on the outward current and inward current in response to carbachol and histamine suggest a possible involvement of G-protein in Ca^{2+}-store release and activation of cationic channels mediated via muscarinic receptors or histamine receptors.

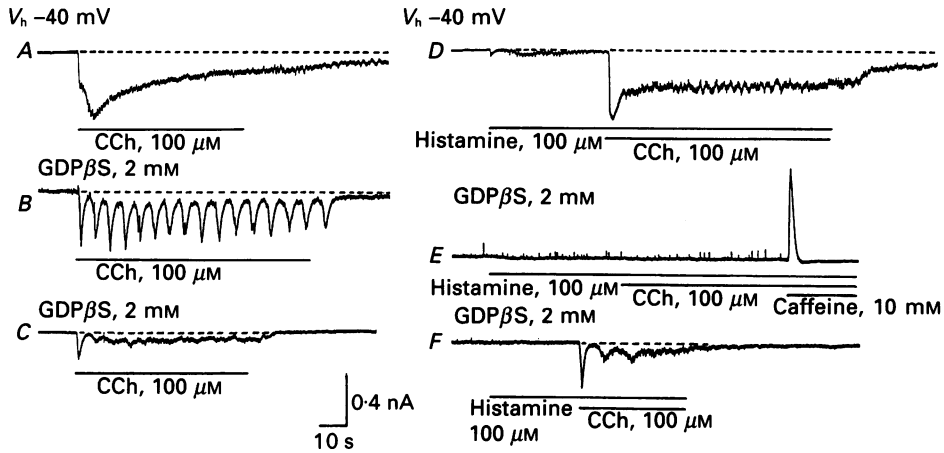

Fig. 5. Effects of GDPβS on inward current responses to carbachol (CCh, 100 μM) and histamine (100 μM) in cells voltage clamped at -40 mV (V_h -40 mV). Cells were dialysed with normal or GDPβS (2 mM)-containing solution. *A*, a control response of a normal cell to CCh. CCh elicited a large, long-lasting inward current. *B* and *C*, current responses of two different GDPβS-filled cells to CCh; oscillatory inward currents superimposed on a sustained inward current (*B*) and a transient inward current followed by a small, sustained inward current (*C*). *D*, control responses of another normal cell to histamine and to carbachol applied in the presence of histamine. *E* and *F*, responses of two different GDPβS-filled cells to histamine and to carbachol applied in the presence of histamine; no response to histamine and to CCh subsequent to histamine, but a brief outward current response to caffeine (*E*), and no response to histamine and a transient inward current followed by small oscillatory inward currents in response to CCh (*F*). In GDPβS-filled cells, CCh- and histamine-induced inward currents were relatively small in size or absent. See text for details.

Characteristics of the current response to GTPγS

The current response at a holding potential of 0 mV

With the inclusion of GTPγS (0·1–1 mM) in the pipette, STOCs appeared within several seconds after break-through and 2–7 min later they stopped discharging or decreased markedly in size and discharge rate (24 cells; Fig. 6*A*). A brief outward current with a 3–20 times longer duration than STOC duration appeared repeatedly in four cells, but it decreased in size with time and finally disappeared within 7 min. If caffeine (10 mM) was applied to the cells 3–10 min after break-through, no outward current (8 cells) or a small outward current (7 cells) was elicited. These small outward currents gave an average charge movement of $1{\cdot}26 \pm 0{\cdot}38$ nC ($n = 7$), which was significant smaller ($P < 0{\cdot}05$) than the value in normal cells ($4{\cdot}63 \pm 0{\cdot}75$ nC, $n = 8$).

The above results suggest that GTPγS causes release of Ca^{2+} from storage sites which are caffeine sensitive, as demonstrated in single smooth muscle cells from rabbit intestine and portal vein (Bolton & Lim, 1989; Komori & Bolton, 1989, 1990).

The current response at a holding potential of −40 mV

After break-through of the patch membrane with inclusion of GTPγS (0·1 or 0·2 mM) in the pipette, an inward current occurred in all of the cells, but it was variable in form, as shown in Figs 6*B* and *C*, 7*A* and *B* and 8*A*. In many cells, the

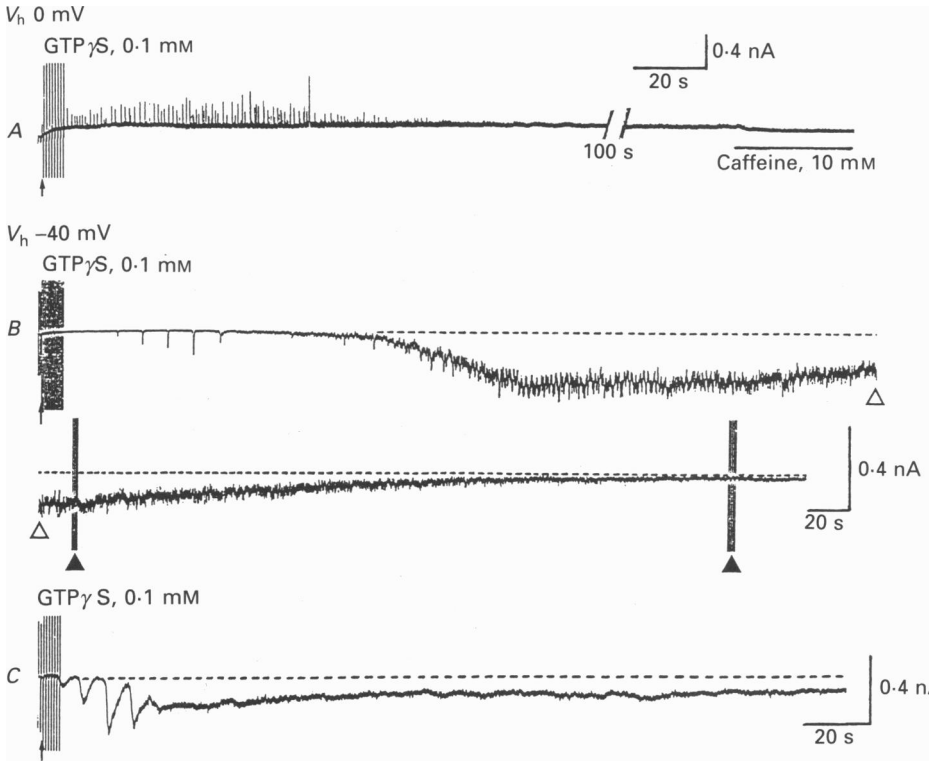

Fig. 6. Current responses to internal application of GTPγS in cells voltage clamped at 0 mV (V_h 0 mV) or −40 mV (V_h −40 mV). Cells were dialysed with GTPγS (0·1 mM-containing solution. Establishment of the whole-cell recording mode (↑) was confirmed by repetitive hyperpolarizing pulses (10 mV in intensity, 200 ms in duration) at an interval of 0·8 or 1 s. *A*, current record from a GTPγS-filled cell voltage clamped at 0 mV. Shortly after dialysis with GTPγS, STOCs were abolished and no response to caffeine (10 mM) was observed. The current trace was interrupted for 100 s as indicated. *B* and *C*, current records from two different GTPγS-filled cells voltage clamped at −40 mV. Dialysis with GTPγS resulted in the appearance of small, short-lasting inward currents (*B*) or oscillatory inward currents (*C*, also see Fig. 7*A* and *B*) followed by sustained inward current. In *B*, △ show continuity of the record, and access resistance was checked at ▲ using repetitive hyperpolarizing pulses with the same parameters as mentioned above. The GTPγS-induced inward current reached its peak, then decayed progressively and finally disappeared in 10 min or so (*B*). Dashed lines indicate a level of basal current immediately after establishment of the whole-cell recording mode.

current responses were oscillatory inward currents which appeared within 2·5 min after break-through, increased in size with time to reach a maximal level, then progressively decreased in size and finally disappeared to terminate in a sustained

inward current. The sustained inward currents varied in size from 0·05 to 0·8 nA in fifteen cells and decayed with a slow time course after reaching their peak and disappeared in 10–15 min (Fig. 6*B*).

When the GTPγS concentration in the pipette was reduced to 0·01 mM, an oscillatory inward current (2 cells), a sustained inward current (8 cells), or an

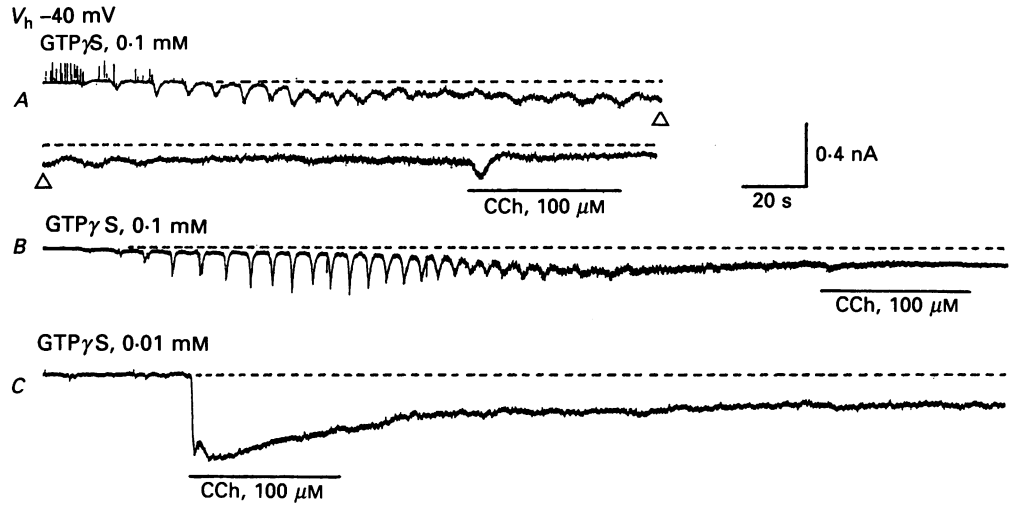

Fig. 7. Effects of GTPγS on current responses to carbachol (CCh, 100 μM) in cells voltage clamped at −40 mV (V_h −40 mV). Cells were dialysed with GTPγS-containing pipette solution. *A* and *B*, current records from two different cells dialysed with GTPγS (0·1 mM)-containing solution, CCh, applied at a peak of the GTPγS-induced inward current, evoked a small, brief inward current (*A*) or was almost without effect (*B*). In *A* △ show continuity of the record. *C*, current record from a cell dialysed with GTPγS (0·01 mM)-containing solution. CCh, applied about 4 min after dialysis with GTPγS evoked a large, long-lasting inward current and the effect was well preserved even after removal of CCh.

oscillatory inward current followed by a sustained inward current (3 cells) occurred within 3–8 min after break-through. The oscillations in current were usually lower in frequency, smaller in size and longer in discharge period, compared with those obtained with GTPγS at higher concentrations (0·1 and 0·2 mM). The peak current was no more than 0·3 nA.

These observations imply that GTPγS mimics both the release of Ca^{2+} from storage sites in the cell and activation of cationic channels mediated via muscarinic receptors or histamine receptors.

Effect of GTPγS on carbachol- and histamine-induced currents

Carbachol (100 μM) was applied to the cells at a time when the inward current elicited by GTPγS (0·1 or 0·2 mM) had reached a maximal level. No further inward current was elicited in six out of ten cells (Fig. 7*B*), but a small and brief increase in inward current was observed in the four remaining cells (Fig. 7*A*). Expressed as a charge transfer, the increased inward current gave 0·31 ± 0·12 nC ($n = 5$), which was

less than one-twentieth of the value obtained for the carbachol-induced inward currents in normal cells (Fig. 9). The histamine-induced inward current was also inhibited. Histamine (100 μM) was without effect in four out of six cells, but only a small and brief inward current was elicited in the two remaining cells (Figs 8*A* and 9).

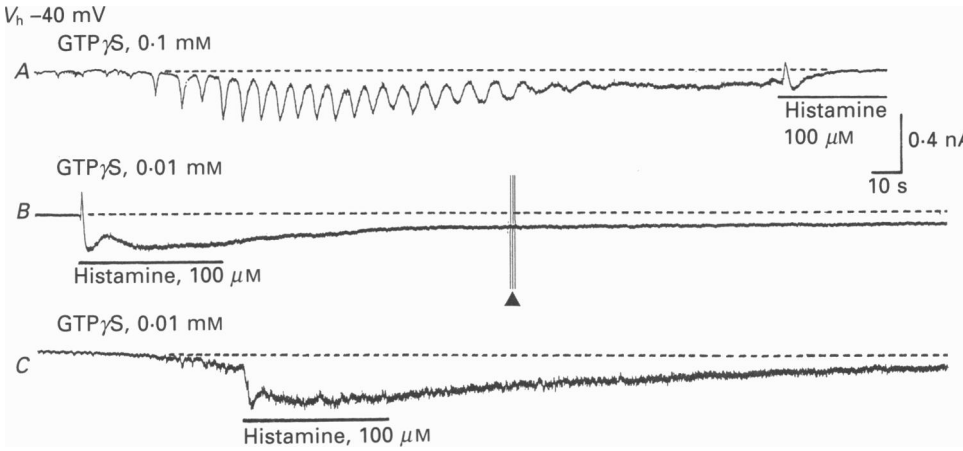

Fig. 8. Effects of GTPγS on current responses to histamine (100 μM) in cells voltage clamped at −40 mV (V_h −40 mV). *A*, current record from a cell dialysed with a GTPγS (0·1 mM)-containing solution. Histamine, applied at a peak of the GTPγS-induced inward current, evoked a brief outward current followed by a small inward current. *B* and *C*, current records from two different cells dialysed with a GTPγS (0·01 mM)-containing solution. Histamine, applied about 5 min (*B*) and 4 min (*C*) after dialysis with GTPγS, evoked a large, long-lasting inward current as did CCh (see Fig. 7*C*). In GTPγS (0·01 mM)-filled cells, histamine-induced inward currents were relatively large in size and long in duration.

When the GTPγS concentration in the pipette solution was reduced to 0·01 mM, a small oscillatory current or a small sustained current was elicited after breakthrough. In contrast to the above results, under these conditions the carbachol-induced inward current was increased in size and duration in all of five cells (Fig. 7*C*), and it persisted for a certain period of time even after the removal of carbachol. In two cells it took more than 30 min to decay completely. The average inward charge movement of 13·55 ± 1·53 nC ($n = 5$) was significantly ($P < 0·05$) greater than that in normal cells (7·70 ± 0·90 nC, $n = 36$; Fig. 9).

The inward current response to histamine was also potentiated, being a sustained one with or without an oscillatory component in all of twelve cells (Fig. 8*B* and *C*). The average charge movement of 3·76 ± 0·52 nC ($n = 12$) was significantly ($P < 0·01$) greater than the control (1·15 ± 0·33 nC, $n = 24$; Fig. 9).

The above results suggest that receptors for carbachol and histamine mediating the inward currents are coupled to a G-protein and both drugs do not produce further effect whenever the G-protein has been fully activated by GTPγS, but they may show a stronger effect under conditions in which the G-protein is more efficiently activated by these drugs in the presence of low concentrations of GTPγS.

Effect of PTX on carbachol- and histamine-induced currents

Pretreatment with 2–5 μg/ml PTX (see Methods) did not noticeably change the carbachol- and histamine-induced outward currents. The results obtained in PTX-treated cells were substantially similar to those in cells (PTX-untreated cells) which

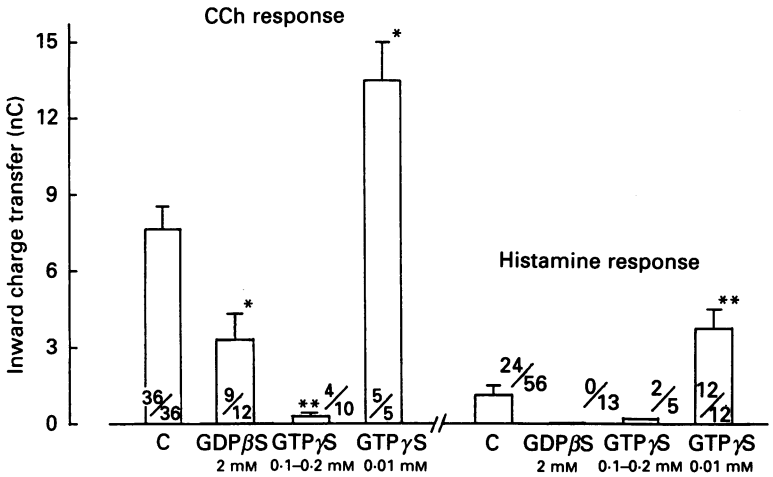

Fig. 9. Summary of the effects of GDPβS and GTPγS on the inward current responses to carbachol (CCh response) and to histamine (Histamine response) of cells voltage clamped at -40 mV (V_h -40 mV). Ordinate scale, the mean inward charge transfer (nC) during the first 40 s of the CCh- or histamine-induced inward currents in normal (C), GDPβS (2 mM)-filled, GTPγS (0·1–0·2 mM)-filled and GTPγS (0·01 mM)-filled cells which responded to CCh or histamine. The fraction of responding cells is indicated in each column. Vertical lines indicate one S.E. of the mean. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. In GDPβS-filled and GTPγS (0·1–0·2 mM)-filled cells, CCh- and histamine-induced inward currents were relatively small in size or absent. In contrast, in GTPγS (0·01 mM)-filled cells, these currents were relatively large in size.

were handled in the same way as PTX-treated cells except that PTX was not added to the cell suspension. The average outward charge movements during the carbachol current and histamine current were $1{\cdot}19 \pm 0{\cdot}40$ nC ($n = 10$) and $1{\cdot}10 \pm 0{\cdot}28$ nC ($n = 8$), respectively, in PTX-treated cells, which were not significantly different from $1{\cdot}31 \pm 0{\cdot}35$ nC ($n = 7$) and $1{\cdot}26 \pm 0{\cdot}32$ nC ($n = 9$) in cells not treated with PTX ($P > 0{\cdot}05$). Even in three cells treated with PTX at a higher concentration (20 μg/ml), carbachol was still effective in inducing an outward current followed by abolition of STOCs. In this series of experiments, the carbachol- and histamine-induced outward currents in either PTX-treated cells or PTX-untreated cells were smaller in size and shorter in duration than normal (cf. Figs 10 and 1). A similar change was also observed on the caffeine-induced outward current. These findings suggest that the Ca^{2+} store may be decreased in amount during the incubation at 37 °C.

In contrast, the inward current responses to carbachol and to histamine were almost completely blocked by PTX-treatment. Carbachol (100 μM) elicited no inward current in fourteen out of sixteen cells (Fig. 11*C*), but elicited a very small

inward current that amounted to less than 0·8 nC in terms of charge transfer in the two remaining cells (Fig. 11*B*). Histamine (100 μM) elicited no inward current in all of ten cells. However, carbachol and histamine elicited a brief outward current followed by an inhibition of STOCs or a short-lasting increase in size and/or

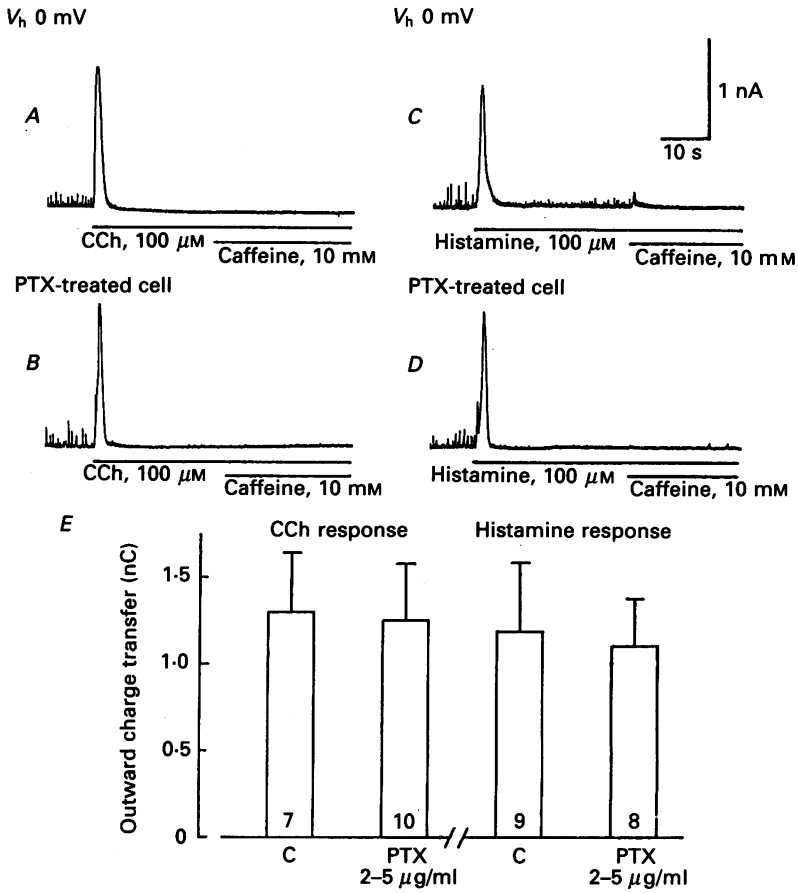

Fig. 10. Effects of pertussis toxin (PTX) on outward current responses to carbachol (CCh, 100 μM) and to histamine (100 μM) in cells voltage clamped at 0 mV (V_h 0 mV). Cells were incubated with or without PTX (2 or 5 g/ml) for 5–8 h (see Methods). Experiments were performed on cells treated with PTX and cells not treated with PTX. *A* and *C*, current records from two different cells not treated with PTX. *B* and *D*, current records from two different cells treated with PTX. In *A–D*, CCh and histamine evoked brief outward currents followed by inhibition of STOC discharges, and the responses to caffeine (10 mM) were virtually absent. *E*, the mean outward charge transfer (nC) during the CCh- or histamine-induced outward currents in cells not treated with PTX (C) and cells treated with PTX. The number of cells used is indicated in each column. Vertical lines indicate one S.E. of the mean. There was no difference in size of CCh- and histamine-induced outward currents between cells not treated with PTX and cells treated with PTX.

discharge rate of STOCs, in about half of the PTX-treated cells held at a holding potential of −40 mV (Fig. 11*C* and *F*). Further application of carbachol in the presence of histamine was without effect (6 cells; Fig. 11*F*).

The current responses to carbachol and histamine in cells not treated with PTX and held at −40 mV were very similar in form but somewhat smaller in size than in normal cells (Fig. 11*A*, *D* and *E*). The average charge movement during the carbachol-induced inward current responses (5·44 ± 0·80 nC, $n = 14$) was about 70 %

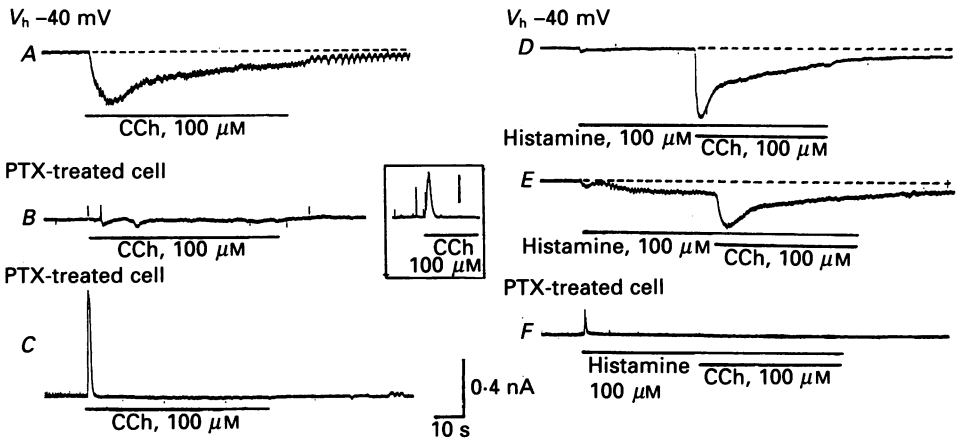

Fig. 11. Effects of pertussis toxin (PTX) on inward current responses to carbachol (CCh, 100 μM) and to histamine (100 μM) in cells voltage clamped at −40 mV (V_h −40 mV). Experiments were performed on cells not treated with PTX and cells treated with PTX, as in Fig. 10. *A*, *D* and *E*, current records from three different cells not treated with PTX. CCh evoked an inward current in the absence (*A*) and presence of histamine (*D* and *E*), and histamine evoked a small inward current (*D* and *E*). *B*, *C* and *F*, current records from three different cells treated with PTX. CCh and histamine did not evoke any appreciable inward current, but they did evoke a brief outward current (*C* and *F*). Inset in *B* shows a brief outward current in response to a second application of CCh 8 min after the first CCh; calibration indicates 0·5 nA. In cells treated with PTX, CCh- and histamine-induced inward currents were absent.

of the normal (7·70 ± 0·90 nC, $n = 36$). Similar results were obtained with histamine. Application of carbachol subsequent to histamine invariably elicited an inward current. The average charge movement (4·48 ± 1·15 nC, $n = 5$) was about 76 % of the normal (5·88 ± 0·46, $n = 42$).

These results suggest that the inward current responses, but not the outward current responses, to carbachol and histamine are mediated via their G-protein-coupled receptors which are inhibited by PTX treatment.

DISCUSSION

In this paper, we provide evidence that carbachol and histamine produce release of Ca^{2+} stores (which in turn leads to activation of Ca^{2+}-activated K^+ channels) and activation of cationic channels in smooth muscle cells of guinea-pig ileum. These effects are mediated via GTP-binding protein (G-protein)-coupled receptors. In the cases of both muscarinic receptors and histamine receptors, the G-protein involved in release of Ca^{2+} stores is insensitive to PTX, while the G-protein involved in activation of cationic channels is sensitive to this toxin.

Release of Ca^{2+} stores by carbachol and histamine

The concentrations (100 μM) of carbachol and histamine employed were supramaximal and expected to cause a rapid and massive release of Ca^{2+} stores. This is supported by the finding that the outward currents evoked by these drugs were usually followed by cessation of STOC discharge which is related to the loss of available Ca^{2+} from the stores (Benham & Bolton, 1986). Carbachol, histamine and caffeine appear to act on common Ca^{2+} stores; application of carbachol subsequent to histamine elicited virtually no outward current, and vice versa. In rabbit jejunal muscle cells, the Ca^{2+}-activated K^+ current mediated via muscarinic receptors is blocked by heparin (Komori & Bolton, 1990), and involvement of D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) in the muscarinic effects is extremely likely (Komori & Bolton, 1991). Histamine as well as carbachol has been reported to accelerate inositol phospholipid hydrolysis in guinea-pig ileum to produce various inositol phosphates including $InsP_3$ (Donaldson & Hill, 1985; Mallows & Bolton, 1987; Bielkiewicz-Vollrath, Carpenter, Schulz & Cook, 1987; Prestwich & Bolton, 1991). Therefore, it is very probably that stimulation of histamine receptors produces an increase in $InsP_3$, $InsP_3$ releases Ca^{2+} from intracellular stores, and this in turn activates Ca^{2+}-activated K^+ current. The contractile effect of histamine on a chemically-skinned muscle from guinea-pig ileum, which is mediated via its action to release Ca^{2+} from intracellular storage sites, was inhibited by heparin (K. Fukami, S. Komori, T. Takewaki & H. Ohashi, unpublished data).

Histamine (1 mM) was reported to increase the total amount of inositol phosphates to 2-hold and carbachol (0·1 mM) to 4-hold (Mallows & Bolton, 1987). Recently, measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using a fluorescent dye, Indo-1, demonstrated that carbachol and histamine cause an increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from storage sites in single smooth muscle cell of guinea-pig jejunum and the peak magnitude of the intracellular Ca^{2+} transient in response to histamine at 0·3 mM was about 35% of that in response to carbachol at 0·1 mM (personal communication, P. Pacaud & T. B. Bolton). The smaller effects of histamine are consistent with the presence of the smaller number of binding sites for histamine in this tissue (Hill, Young & Marrian, 1977). In the present experiments, however, histamine-induced outward current was not significantly different in size from the carbachol-induced outward current. There may not be a linear relationship between $[Ca^{2+}]_i$ and the Ca^{2+}-activated K^+ current we measured and the concentration of histamine used (0·1 mM) may increase intracellular Ca^{2+} concentration beyond the level required for full activation of the Ca^{2+}-activated K^+ channels. New techniques which allow Ca^{2+} signals and membrane currents to be simultaneously measured may help provide an answer to this problem.

Activation of inward cation current by carbachol and histamine

The histamine-induced inward current did not flow at 0 mV holding potential which is close to the equilibrium potential for the cationic channels activated by muscarinic agonists (Benham *et al.* 1985). The histamine inward currents were much smaller than the carbachol currents. The marked difference in size of the inward current responses to histamine and carbachol is consistent with the difference in

number between histamine and muscarinic receptors (Hill *et al.* 1977). The histamine effect on inward current was observed less consistently than that on outward current. The most likely explanation for this is a weak linkage between the histamine receptor and the G-protein which is involved in activation of cationic channels. When histamine was applied at a peak of the inward current for carbachol, it caused no further development in current. Thus, the effect of histamine was not additive to that of carbachol. It seems, therefore, likely that histamine activates only a small portion of cationic channels which can be fully activated by carbachol. This view is supported by the findings that the muscarinic receptor and histamine receptor in guinea-pig ileal cells are each coupled to a G-protein (see below) and these two G-proteins are at present indistinguishable. Carbachol and histamine produce membrane depolarization in intestinal smooth muscle and the effects have been suggested to be brought about by the opening of ion channels with the same ionic selectivity (Bolton *et al.* 1981). Recently, activation of cationic channels by acetylcholine in guinea-pig ileal cells was found to be dependent on $[Ca^{2+}]_i$ (Inoue & Isenberg, 1990*a*) and on membrane potential (Inoue & Isenberg, 1990*c*), and cationic channels gated by histamine should be also.

In the present experiments, the number of cells which responded to histamine with a large inward current comparable to the carbachol inward current was about 8% of the total number of cells. If such cells are distributed in a tessellated manner in the longitudinal muscle layer of guinea-pig ileum, the maximal increase of membrane conductance produced by histamine would be much smaller than that produced by carbachol, as previously reported (Bolton *et al.* 1981).

Involvement of G-protein in the receptor-mediated responses

If receptors for carbachol and histamine are linked to G-proteins, the effects of activation of the receptors should be mimicked by GTPγS and the effects of either of the agonists and GTPγS would be saturative, not additive. This was the case for the high concentrations (0·1 or 0·2 mM) of GTPγS: full activation of the cationic channels via G-proteins by the GTP analogue blocked further effect of stimulation of the receptors. The inward current responses were potentiated and prolonged over the period of exposure to the agonists (and thereby rendered very long lasting) by lower concentrations of GTPγS, and they were inhibited by GDPβS, a competitive inhibitor of GTP for binding on G-proteins, suggesting involvement of G-proteins in the receptor-mediated responses. Thus, such behaviour of the carbachol- and histamine-induced inward currents in guinea-pig ileal cells is typical of a G-protein-mediated response, as in other cells in which a G-protein linkage is believed to be involved in the receptor-mediated currents (atrial myocyte: Kurachi, Nakajima & Sugimoto, 1987; submucous plexus neurone: Mihara, North & Surprenant, 1987). Inoue & Isenberg (1990*b*) also concluded that a G-protein was involved in the muscarinic inward current in the cells, based on the results obtained under different ionic environments from those in our experiments. However, in rabbit jejunal cells which are held under substantially similar conditions to the present experimental conditions, the muscarinic inward current was found to behave anomalously for a G-protein-mediated response (Lim & Bolton, 1988; Komori & Bolton, 1990). Even at high concentrations GTPγS did not regularly evoke inward current and only

partially blocked inward current to carbachol on most occasions; also carbachol in the presence of GTPγS produced a less sustained, rather than a more sustained response (Komori & Bolton, 1990). A role of G-protein in activating the muscarinic inward current in rabbit jejunal cells may be not as simple as in other cells, as previously discussed (Komori & Bolton, 1990).

The Ca^{2+}-activated outward K^+ currents due to release of Ca^{2+} stores, mediated via muscarinic receptors and histamine receptors, were inhibited by GDPβS, though the histamine receptor-mediated response was more susceptible to the GDP analogue. The residual part of the current responses is not necessarily considered to be mediated by a signal transducer other than G-protein, since GDPβS inhibited only part of the carbachol-induced inward current which proved to be a G-protein-mediated response in the present study and in the previous study (Inoue & Isenberg, 1990*b*). The lesser susceptibility to GDPβS of the two muscarinic receptor-mediated responses may be related to a high density of muscarinic receptors in the smooth muscle (Hill *et al.* 1977). GDPβS in the intracellular fluid may not reach a level sufficient to completely prevent the α-subunit of the G-protein from being activated by carbachol. GTPγS inhibited STOCs and virtually abolished caffeine-induced outward current, suggesting an action to deplete Ca^{2+} stores. It has been shown in rabbit jejunal cells that GTPγS activates a G-protein associated with PLC and the resulting increase in $InsP_3$ causes Ca^{2+} store release (Komori & Bolton, 1990). In this respect, GTPγS mimics carbachol and histamine. These properties of the outward currents suggest involvement of a G-protein in a transduction of these responses. The failure of GTPγS to evoke an outward current is probably due to a slow release of Ca^{2+} from storage sites by GTPγS. As Ca^{2+} is buffered in the cytosol, a rise of cytoplasmic Ca^{2+} concentration, especially in the vicinity of Ca^{2+}-activated K^+ channels may not exceed the threshold level for activation of the K^+ channels (Bolton & Lim, 1989; Komori & Bolton, 1990).

PTX-sensitive and -insensitive G-proteins

Pretreatment with PTX blocked the inward cationic current but not the outward current in response to carbachol and histamine. The selective blockade by PTX of the inward current, taken together with the results with GTPγS and GDPβS, indicates that both receptors are of the G-protein-coupled receptor family. In contrast, the resistance to PTX of the outward currents suggests that a PTX-insensitive G-protein may couple muscarinic and histamine receptors to PLC activation which leads to $InsP_3$ production and Ca^{2+}-store release. It has been suggested in other smooth muscles that the G-proteins associated with receptor-regulated PLC do not serve as substrates for PTX (Sasaguri, Hirata, Itoh, Koga & Kuriyama, 1986; Loirand, Pacaud, Mironneau & Mironneau, 1990).

Possible interactions of G-proteins with receptors of a signalling system have been described by Neer & Clapham (1988): one G-protein can be activated by more than one type of receptor and a single receptor can activate one or more G-proteins. Muscarinic receptors in guinea-pig ileal smooth muscle have been characterized as an M2-subtype (Hammer & Giachett, 1984) and histamine receptors as an H1-subtype (Hille *et al.* 1977; Hill & Young, 1981; Leurs, Brozius, Smit, Bast & Timmerman, 1991). Thus, it is possible that a single type of receptor of the M2-type or H1-type links to two different G-proteins, a PTX-sensitive G-protein and a PTX-insensitive

G-protein. However, the existance of an M3-subtype of muscarinic receptor (Michel & Whiting, 1990) and of a non-H1-subtype of histamine receptor (Donaldson & Hill, 1985) has been suggested in guinea-pig ileal muscle. Therefore, it is also possible that each of two subtypes of the muscarinic receptor (e.g. M2 and M3) or of the histamine receptor (e.g. H1 and non-H1) links to one specific G-protein (a PTX-sensitive or -insensitive G-protein).

We would like to thank Professor T. B. Bolton (London) for his critical reading of the manuscript.

REFERENCES

BENHAM, C. D. & BOLTON, T. B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *Journal of Physiology* **381**, 385–406.

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.

BIELKIEWICZ-VOLLRATH, B., CARPENTER, J. R., SCHULZ, R. & COOK, D. E. (1987). Early production of 1,4,5-inositol trisphosphate and 1,3,4,5-inositol tetrakisphosphate by histamine and carbachol in ileal smooth muscle. *Molecular Pharmacology* **31**, 513–522.

BOLTON, T. B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *Journal of Physiology* **220**, 647–671.

BOLTON, T. B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiological Reviews* **59**, 606–718.

BOLTON, T. B., CLARK, J. P., KITAMURA, K. & LANG, R. J. (1981). Evidence that histamine and carbachol may open the same ion channels in longitudinal smooth muscle of guinea-pig ileum. *Journal of Physiology* **320**, 363–379.

BOLTON, T. B. & LIM, S. P. (1989). Properties of calcium stores and transient outward currents in single smooth muscle cells of rabbit intestine. *Journal of Physiology* **409**, 385–401.

DONALDSON, J. & HILL, S. J. (1985). Histamine-induced inositol phospholipid breakdown in the longitudinal smooth muscle of guinea-pig ileum. *British Journal of Pharmacology* **85**, 499–512.

GILMAN, A. G. (1987). G proteins: transducers of receptor-generated signals. *Annual Reviews in Biochemistry* **56**, 615–649.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HAMMER, R. & GIACHETTI, A. (1984). Selective muscarinic receptor antagonists. *Trends in Pharmacological Sciences* **5**, 18–20.

HILL, S. J. & YOUNG, J. M. (1981). Characterization of [^{3}H]-mepyramine binding to the longitudinal muscle of guinea-pig small intestine. *Molecular Pharmacology* **19**, 379–387.

HILL, S. J., YOUNG, J. M. & MARRIAN, D. H. (1977). Specific binding of ^{3}H-mepyramine to histamine H1 receptors in intestinal smooth muscle. *Nature* **270**, 361–363.

INOUE, R. & ISENBERG, G. (1990*a*). Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. *Journal of Physiology* **424**, 73–92.

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

INOUE, R., KITAMURA, K. & KURIYAMA, H. (1987). Acetylcholine activates single sodium channels in smooth muscle cells. *Pflügers Archiv* **410**, 69–74.

KOMORI, S. & BOLTON, T. B. (1989). Actions of guanine nucleotides and cyclic nucleotides on calcium stores in single patch-clamped smooth muscle cells from rabbit portal vein. *British Journal of Pharmacology* **97**, 973–982.

KOMORI, S. & BOLTON, T. B. (1990). Role of G-proteins in muscarinic receptor inward and outward currents in rabbit jejunal smooth muscle. *Journal of Physiology* **427**, 395–419.

KOMORI, S. & BOLTON, T. B. (1991). Calcium release induced by inositol 1,4,5-trisphophate in single rabbit intestinal smooth muscle cells. *Journal of Physiology* **433**, 495–517.

KURACHI, Y., NAKAJIMA, T. & SUGIMOTO, T. (1987). Short-term desensitization of muscarinic K^+ channel current in isolated atrial myocytes and possible role of GTP-binding proteins. *Pflügers Archiv* **410**, 227–233.

LEURS R., BROZIUS, M. M., SMIT, M. J., BAST, A. & TIMMERMAN, H. (1991). Effects of histamine H1-, H2- and H3-receptor selective drugs on the mechanical activity of guinea-pig small and large intestine. *British Journal of Pharmacology* **102**, 179–185.

LIM, S. P. & BOLTON, T. B. (1988). A calcium-dependent rather than a G-protein mechanism is involved in the inward current evoked by muscarinic receptor stimulation in dialysed single smooth muscle cells of small intestine. *British Journal of Pharmacology* **95**, 325–327.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1990). GTP-binding proteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein myocytes. *Journal of Physiology* **428**, 517–529.

MALLOWS, R. S. E. & Bolton, T. B. (1987). Relationship between stimulated phosphatidic acid production and inositol lipid hydrolysis in intestinal longitudinal smooth muscle from guinea pig. *Biochemical Journal* **244**, 763–768.

MICHEL, A. D. & WHITING, R. L. (1990). The binding of [^{3}H]4-diphenylacetoxy-*N*-methylpiperidine methiodide to longitudinal ileal smooth muscle muscarinic receptors. *European Journal of Pharmacology* **176**, 197–206.

MIHARA, S., NORTH, R. A. & SURPRENANT, A. (1987). Somatostatin increases an inwardly rectifying potassium conductance in guinea-pig submucous plexus neurones. *Journal of Physiology* **390**, 335–355.

NEER, E. J. & CLAPHAM, D. E. (1988). Role of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

OHASHI, H., TAKEWAKI, T. & OKADA, T. (1974). Calcium and the contractile effect of carbachol in the depolarized guinea-pig taenia caecum. *Japanese Journal of Pharmacology* **24**, 601–611.

PRESTWICH, S. A. & BOLTON, T. B. (1991). Measurement of picomole amounts of any inositol phosphate isomer separable by h.p.l.c. by means of a bioluminescence assay. *Biochemical Journal* **274**, 663–672.

SASAGURI, T., HIRATA, M., ITOH, H., KOGA, T. & KURIYAMA, H. (1986). Guanine nucleotide binding protein involved in muscarinic responses in the pig coronary artery is insensitive to islet-activating protein. *Biochemical Journal* **239**, 567–574.

STRYER, L. & BOURNE, H. R. (1986). G proteins: a family of signal transducers. *Annual Reviews of Cell Biology* **2**, 391–419.