British Journal of Pharmacology (1995) 115, 147-157

Effects of GTPγS on muscarinic receptor-stimulated inositol phospholipid hydrolysis in permeabilized smooth muscle from the small intestine

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1 Smooth muscle fragments from the longitudinal layer of the small intestine of the guinea-pig were permeabilized with *Staphylococcus aureus* alpha toxin (α-toxin) and used to investigate the role of G-protein activation in the regulation of muscarinic acetylcholine receptor (AChR)-stimulated inositol phospholipid hydrolysis.

2 The efficiency of α-toxin permeabilization was estimated by the release of [^{3}H]-2-deoxyglucose ([^{3}H]-2DG) after prior loading or lactate dehydrogenase (LDH) enzyme release from the smooth muscle fragments.

3 In α-toxin-permeabilized smooth muscle, but not in non-permeabilized muscle, GTPγS induced time- and concentration-dependent increases in labelled inositol phosphates. Carbachol (CCh) increased labelled inositol phosphates in both permeabilized and non-permeabilized muscle, although the increases were greater in non-permeabilized smooth muscle. The response to 100 μM CCh was severely reduced by 0.5 μM atropine.

4 In permeabilized muscle the effects of GTPγS or CCh on inositol phosphate levels were reduced by treatment with pertussis toxin (PTX) and completely inhibited by GDPβS.

5 GTPγS caused a concentration-dependent inhibition of the CCh-induced increases in the levels of labelled inositol phosphates. Dibutyryl cyclic AMP or Sp-cAMPs (adenosine-3',5'-cyclic phosphorothiolate-Sp) reduced the effects of CCh on inositol phosphate levels.

6 The results suggest that muscarinic AChR activation induces inositol phospholipid hydrolysis via more than one G-protein in this smooth muscle and that several mechanisms may contribute to the modulation of both stimulatory and inhibitory responses observed.

Keywords: Smooth muscle; permeabilization; inositol phosphates; G-proteins; GTPγS; GDPβS; pertussis toxin; cyclic AMP

Introduction

Stimulation of muscarinic acetylcholine receptors (AChRs) in longitudinal smooth muscle of small intestine results in an increase in inositol phospholipid hydrolysis by activation of a phospholipase C (PLC) producing inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (Jafferji & Michell, 1976; Best & Bolton, 1986). This effect is believed to be mediated by G-proteins. The Ins(1,4,5)P_3 causes Ca^{2+} release from the stores in the sarcoplasmic reticulum and this contributes to tension generations in this excitable smooth muscle (Pacaud & Bolton, 1992).

The structures of cloned muscarinic AChRs and the dependence of muscarinic AChR-induced responses on the presence of GTP in membranes and in permeabilized cells support the idea that agonist-induced increases in inositol phospholipid turnover are mediated by G-proteins (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985; Casey & Gilman, 1988). GTPγS is a slowly hydrolysable, non permeable analogue of GTP that causes stimulation of inositol phospholipid hydrolysis in many cell types including smooth muscle (Honkanen & Abdel-Latif, 1989). GTPγS also increases the Ca^{2+} sensitivity of myosin phosphorylation in both tonic and phasic smooth muscles but these effects may be separate from the effects of GTPγS on inositol phospholipid hydrolysis which have been investigated in this study (Kitazawa *et al.*, 1991; Ono *et al.*, 1992) although others believe they may be involved (Nishimura *et al.*, 1990).

Intact receptors and signal transduction systems are retained following permeabilization of smooth muscle with α-toxin and it has been used by various workers to investigate the mechanisms of excitation-contraction coupling (Nishimurar *et al.*, 1988; Kitazawa *et al.*, 1991). α-Toxin is a cytolytic exotoxin secreted by *Staphylococcus aureus*. Hexamerization of the α-toxin molecules results in the formation of pores of 2-3 nm in diameter in the plasma membrane (Füssle *et al.*, 1981; Hohman, 1988) but as the α-toxin monomer is 34 kDa it does not enter the cell and so its action is restricted to the plasma membrane (Bhakdi & Tranum-Jensen, 1987). The small pore size allows equilibration of cytoplasm, inorganic ions and small molecules such as GTP analogues, contained in experimental solutions, without losing larger molecules such as calmodulin, protein kinases and other enzymes.

We have shown previously that AlF interacts with G-proteins to inhibit muscarinic AChR-induced stimulation of inositol phospholipid hydrolysis (Prestwich & Bolton, 1995). The purpose of the present experiments was to examine further the role of G-proteins in the muscarinic AChR-induced increases in the levels of [^{3}H]-inositol phosphates observed in this tissue. The interaction between CCh and GTPγS was determined to see if GTPγS could potentiate the muscarinic AChR responses as described in other systems (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985; Smith *et al.*, 1985; Uhing *et al.*, 1986; Cockcroft, 1987; Cattaneo & Vicentini, 1989). For these experiments on smooth muscle fragments, attempts were made to permeabilize completely with α-toxin so that the increases in [^{3}H]-inositol phosphates

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observed occurred only in permeabilized fragments. These fragments were used to determine the effects of GTPγS and GDPβS (more stable analogues of GTP and GDP) and treatment with PTX, on the levels of [^{3}H]-inositol phosphates generated by muscarinic AChR activation with CCh. In addition to measurements of levels of [^{3}H]-inositol phosphates, levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) were determined to see if increases in cyclic AMP levels could account for some of the observed effects.

Methods

Guinea-pigs were stunned and exsanguinated and longitudinal muscle of the small intestine removed. Fragments were prepared by tissue chopping as previously described (Prestwich & Bolton, 1995).

Permeabilization of fragments

Preliminary experiments described in Results showed that incubation with 2500 u ml^{-1} α-toxin for 30 min in internal substitution solution (ISS, see below) resulted in satisfactory permeabilization of the smooth muscle fragments and this treatment was used in all experiments where permeabilization was required.

After washing the [^{3}H]-inositol-labelled fragments (fragments were incubated with 1.85 MBq ml^{-1} [^{3}H]-inositol for 20 h at 30°C) with Krebs ringer buffer (KRB) they were rinsed several times in ISS, suspended in 1 ml of this solution containing 2500 u ml^{-1} α-toxin and 925 kBq [^{3}H]-inositol and then incubated at 37°C for 30 min with occasional gentle mixing. At the end of 30 min incubation, the α-toxin was removed with several washes of 10 ml ISS.

Permeabilized fragments were aliquoted to give about 1 mg protein per pot into 'mesh pots' ('mesh pots' were 5 ml Gilson pipette tips which had been cut so that a small circle of nylon mesh could be placed over the end and secured with a ring and they would easily fit into a 5 ml beta vial); these were placed in a beta vial containing 3 ml ISS. The assay procedure was as follows: a mesh pot was removed, blotted onto blotting paper, and immersed in 3 ml ISS at 37°C for 30 s; this was repeated. The sample was then blotted and immersed in the test solution (500 μl). At the end of the incubation, 500 μl 20% trichloroacetic acid (TCA) and 50 μl 50 mM EDTA were added to stop the assay and after rinsing the fragments on the mesh with a small volume of buffer, the samples were vortexed, placed on ice for 20 min and then centrifuged at 4000 *g* for 10 min. The supernatant was removed and the TCA extracted by the addition of 4 × 5 ml water-saturated diethyl ether; the residual ether was removed with nitrogen and the pH of the samples adjusted to 7.0 with 1 M NaOH. The pellet was stored for protein determination by the method of Lowry *et al.* (1951) (there was no interference in the protein assay from anything leaching off the mesh). Samples were assayed for [^{3}H]-inositol phosphates by anion-exchange h.p.l.c. within 7 days.

In some experiments the tissue part was separated from the supernatant part at the end of the incubation period. For these experiments, at the end of the incubation period the inner pot was raised, 500 μl buffer pipetted into it to wash excess supernatant off the tissue and the tissue part was then immersed in a solution of 10% TCA. TCA was then added to the supernatant to give 10% final.

The composition of the high K^+ intracellular substitution solution (ISS) was as follows: (final, mM) K^+ aspartate 130, $MgCl_2$ 3.1, Na_2ATP 2, EGTA 2, creatine PO_4 20, HEPES 20, $CaCl_2$ 1 (free concentration = 0.137 μM) pH 7.2 with KOH. The pores in the plasma membrane made by α-toxin treatment did not allow molecules greater than 1000 Da to pass, therefore creatine kinase and calmodulin would be retained and so did not need to be added to the intracellular solution.

The concentration of free Ca^{2+}, Mg^{2+} and ATP were calculated with a computer programme called EQCAL (Biosoft, U.K.). This programme uses the stability constants for metal-chelate and metal-nucleotide complexes reported by Martell & Smith (1975).

Measurement of [^{3}H]-2DG release

Loss of ^{3}H from fragments which had been previously loaded with [^{3}H]-2-deoxyglucose ([^{3}H]-2DG) in the absence and presence of increasing concentrations of α-toxin was used to assess the degree of permeabilization by different concentrations of α-toxin. Permeabilization by this toxin was also compared with permeabilization by saponin, Triton-X-100 and by hypo-osmotic shock with pure water (distilled water passed through a Milli-Q deionization system). Release of the enzyme lactate dehydrogenase (LDH) was also determined to compare the effect of permeabilization by α-toxin with other permeabilizing agents.

Fragments from the longitudinal smooth muscle layer of the small intestine were incubated in KRB for 30 min at 37°C, followed by washing several times in KRB minus glucose and incubated with 185 kBq [^{3}H]-2DG in KRB minus glucose for 2 h at 37°C (based on the method of Olefsky, 1978). At the end of this period the fragments were washed several times in the high K^+ internal substitution solution (ISS). To estimate the extent of permeabilization of the fragments the escape of [^{3}H]-2DG was estimated as d.p.m. appearing in the supernatant or as d.p.m. remaining in the tissue fragments. Fragments were divided into mesh pots in a total volume of 1 ml containing either ISS alone or ISS in the presence of one of the following: α-toxin in concentrations from 250 u ml^{-1} to 8000 u ml^{-1}; saponin, 1 mg ml^{-1}; Triton-X-100, 1% or pure water to cause hypotonic lysis of cells. The temperature was 37°C (except that 22°C was used for saponin). At selected time intervals, 50 μl aliquots were removed to determine the amount of ^{3}H which had been released into the supernatant (Figure 1a). At the end of the incubation period residual d.p.m. in the supernatant were counted and 10% trichloroacetic acid added to the tissue portion to determine the number of d.p.m. remaining in the tissue. Hisafe 3 (LKB) (4 ml) was added to each pot and the d.p.m. determined in a Beckman scintillation counter. After correcting for the volume in the pot remaining at each time point, the amount of ^{3}H released (Figure 1a) or the ^{3}H remaining (Figure 1b) was calculated as a percentage of the total number of d.p.m. in the tissue at the beginning of the experiment.

Measurement of LDH release

The enzyme LDH was chosen for assay as an example of a molecule too large to escape through the membrane pores created by α-toxin. Smooth muscle fragments were rinsed several times after preparation in ISS and then incubated in either ISS alone, 5000 u ml^{-1} α-toxin, 1 mg ml^{-1} saponin or 1% Triton-X-100 for up to 60 min. At selected time intervals aliquots were removed, placed on ice and assayed immediately for LDH activity. At the end of the experiment the remaining LDH in the tissue was extracted with TCA.

The assay for LDH activity was based on the method of Bergmeyer & Bernt (1974). LDH activity was determined spectrophotometrically by observing the oxidation of NADH in the presence of pyruvate to produce lactate; 3 ml of 0.63 mM pyruvate and 50 mM phosphate buffer pH 7.5 was mixed with 50 μl of 11.3 mM NADH (buffered to pH 7.5 with $NaHCO_3$) and 100 μl of sample. The absorbance was read at 365 nm in a cuvette at 22°C, immediately after mixing and again exactly 5 min later in a Beckman spectrophotometer. The reduction in the optical extinction during this time was taken as a measure of LDH activity. The results are expressed as % of total LDH remaining at each time point.

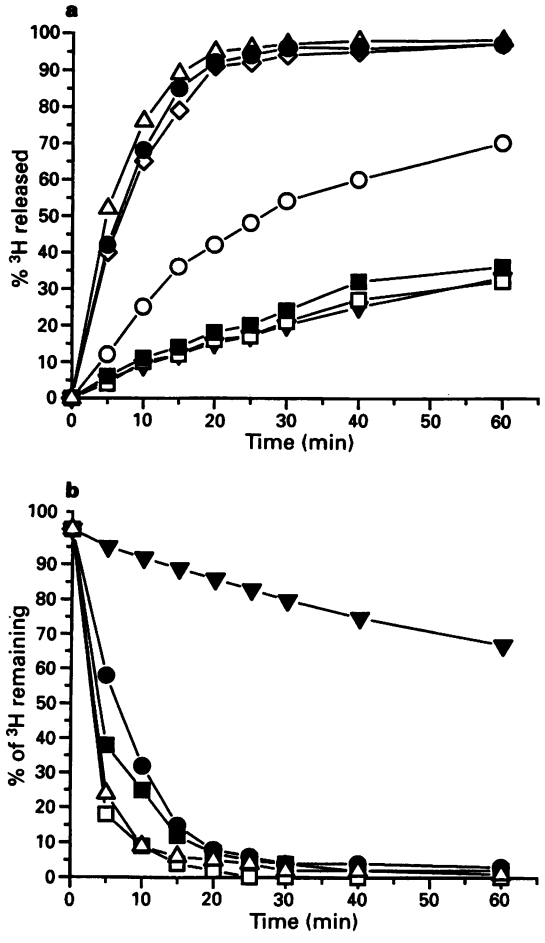

Figure 1 (a) Permeabilization of smooth muscle fragments with α-toxin. [^{3}H]-2DG-labelled smooth muscle fragments prepared as described, were incubated in the absence (ISS) (▼) or in the presence of ISS containing either 250 (□), 500 (■), 1000 (○), 2500 (◇), 5000 (●) or 8000 (△) u ml^{-1} α-toxin from zero time. The % of ^{3}H released with time during incubation with or without the α-toxin for up to 60 min at 37°C was determined. Results are expressed as the mean % release of ^{3}H into the supernatant compared to the total number of d.p.m. in each aliquot of tissue ($n = 2$). (b) Measurement of ^{3}H-efflux during permeabilization. [^{3}H]-2DG-labelled fragments were incubated in the absence (▼) ($n = 3$) of any permeabilizing agent (ISS alone) or in the presence of ISS containing either 2500 u ml^{-1} α-toxin (●) ($n = 3$), 1 mg ml^{-1} saponin (■) ($n = 2$), 1% Triton-X-100 (□) ($n = 2$) or pure H_2O (△) ($n = 2$) with no added solutes for up to 60 min at 22°C for the saponin and at 37°C for the rest. Results are expressed as the mean amount of ^{3}H remaining in the fragments at any time compared to the total d.p.m. in the aliquot of tissue. For abbreviatons in this and subsequent legends, see text.

Figure 2 shows the ability of the smooth muscle fragments to retain LDH under the different conditions.

Labelling and measurement of [^{3}H]-inositol phosphates by h.p.l.c.

The labelling (20 h incubation) and measurement of the [^{3}H]-inositol phosphate isomers was the same as in Prestwich & Bolton (1991; 1995). Briefly, chopped fragments (350 × 350 μm) were incubated in Krebs Ringer buffer of the following composition (final, mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, NaH_2PO_4 1.2, glucose 11.5, $MgCl_2$ 1.2, $CaCl_2$ 2.5 (pH was 7.25 when equilibrated) containing 1.85 MBq ml^{-1} [^{3}H]-inositol and kept in a humidified atmosphere containing 95% O_2/5% CO_2 at 30°C for 20 h. Contraction of the muscle to CCh could still be observed after this period of incubation.

For the majority of the studies the [^{3}H]-inositol phosphate isomers were separated by h.p.l.c. with a method modified

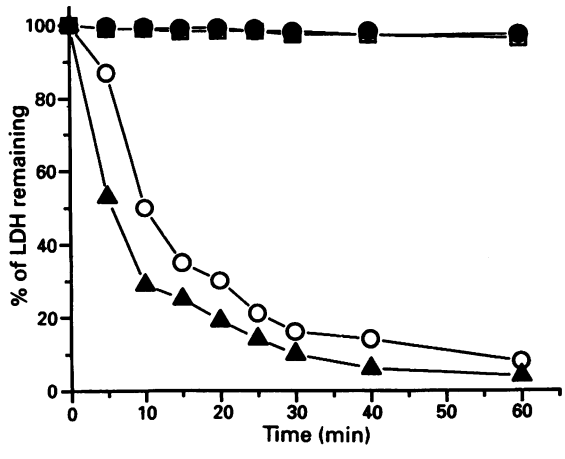

Figure 2 Measurement of loss of lactic dehydrogenase (LDH) during permeabilization. The smooth muscle fragments were incubated in ISS in the absence (●) ($n = 2$) or presence of 5000 u ml^{-1} α-toxin (□) ($n = 3$), 1 mg ml^{-1} saponin (○) ($n = 3$) or 1% Triton-X-100 (▲) ($n = 2$). Aliquots were removed at different times up to 60 min and the LDH activity determined. Results are expressed as the mean % of LDH remaining out of the total in each aliquot at each time point. The amount of LDH at time zero was calculated from the cumulative amount plus the total remaining at the end of the experiment.

from Irvine *et al.* (1985) and Batty *et al.* (1985). A Partisal SAX (strong anion exchange) column was used and the inositol phosphates eluted using an ammonium formate gradient from 0 to 2 M brought to pH 3.7 with H_3PO_4. The eluate was collected in a fraction collector. Elution profiles were determined by scintillation counting using a Beckman LS1701 scintillation counter. The identity of the various [^{3}H]-inositol phosphate isomers except for one of the [^{3}H]-inositol bisphosphates, was determined by comparing their elution profiles from the h.p.l.c. with those of labelled standards (see Prestwich & Bolton, 1991). From these standards the following inositol phosphate isomers were identified: [^{3}H]-inositol monophosphates ([^{3}H]-inositol (1) phosphate and [^{3}H]-inositol (4) phosphate) ($InsP_1$); [^{3}H]-inositol (1,4) bisphosphate ($Ins(1,4)P_2$); [^{3}H]-inositol (1,3,4) trisphosphate ($Ins(1,3,4)P_3$); [^{3}H]-inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$) and [^{3}H]-inositol tetrakisphosphates ($InsP_4$). From the elution profiles of other workers, the other [^{3}H]-inositol bisphosphate is likely to be [^{3}H]-inositol (3,4) bisphosphate (labelled $sInsP_2$ in the Figures). Agonist-stimulated responses were compared with control values and the results usually expressed as % change compared to control. For the studies using dibutyryl cyclic AMP and Sp-cAMPs the [^{3}H]-inositol phosphate isomers were separated on Agl-X-8 columns following the method of Downes & Michell (1981).

Measurement of cyclic AMP

Smooth muscle fragments were prepared and incubated as previously described (Prestwich & Bolton, 1991) when measuring [^{3}H]-inositol phosphates. Initial experiments to determine agonist responses (data not shown) were carried out in the absence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) but no changes in the basal cyclic AMP levels could be seen. Therefore the fragments were incubated in KRB containing 1 mM IBMX for the cyclic AMP experiments. After incubation with or without CCh for the desired time the reaction was terminated by addition of 10% (final) TCA. The TCA was removed by diethyl ether extraction, and the samples neutralized, lyophilized and reconstituted in water. The cyclic AMP formed was determined by competition binding assay with a radiolabelled kit from Amersham International Ltd.

Materials

GDPβS (trilithium salt), GTPγS (tetralithium salt), α-toxin and pertussis toxin (PTX) were obtained from Boehringer. Dibutyryl cyclic AMP and Sp-cAMPs were obtained from Calbiochem. [^{3}H]-2DG, [^{3}H]-inositol and the [^{3}H]-cyclic AMP assay kit were obtained from Amersham International Ltd. [^{3}H]-inositol contained a PT6 tablet which absorbed any radiolysis products, which meant no purification of the label was necessary before use. All other chemicals and drugs were obtained from Sigma Chemical Company Ltd or BDH.

Data analysis

The data are expressed as the mean ± s.e.mean of at least three paired experiments performed on different occasions. Intra-assay variation was very small and the main variability was seen between guinea-pigs. The statistical significance was assessed by use of Student's paired *t* test. *P* values that were less than 0.05 were considered to be significant. Calculations were performed using the computer programme INSTAT (GraphPAD software, U.S.A.).

Results

[^{3}H]-2DG experiments

The efflux of ^{3}H from smooth muscle fragments labelled with [^{3}H]-2DG increased with increasing concentrations of α-toxin reflecting the degree of permeabilization of the fragments (Figure 1a). In non-permeabilized fragments 33% of the total ^{3}H was released by the end of the 60 min incubation period. This may be because the ISS contains only 0.1 μM Ca^{2+} and this would make the fragments leaky. Incubation with up to 500 u ml^{-1} of α-toxin for up to 60 min at 37°C showed no increase in the rate of release of ^{3}H when compared with non-α-toxin treated fragments (Figure 1a). Increasing the concentration of α-toxin to 1000 u ml^{-1} resulted in an increased efflux of ^{3}H compared to control so that at 30 min, 50% of the ^{3}H had been released compared to only 20% in control. Incubation with concentrations of 2500 u ml^{-1} or greater resulted in more than 90% of the ^{3}H in the fragments being released by 20 min. Measurement of ^{3}H may include substances other than [^{3}H]-2DG: derived from the cell.

Measurement of the amount of ^{3}H remaining in the smooth muscle fragments showed that after 30 min incubation in ISS, the non-permeabilized fragments had lost only 20% of the ^{3}H taken up. However, following 30 min incubation with 2500 u ml^{-1} α-toxin, only 4% of the ^{3}H remained in the fragments (Figure 1b). After 30 min incubation with saponin (1 mg ml^{-1}, 20 min, 22°C), less than 10% of the ^{3}H remained and incubation with Triton-X-100 or distilled water resulted in almost complete loss of ^{3}H taken up by the end of the incubation period.

Since greater than 90% of the ^{3}H from [^{3}H]-2DG labelled fragments had been lost by 20 min when the fragments were permeabilized with 2500 u ml^{-1} or more α-toxin it was decided to use 2500 u ml^{-1} α-toxin and incubate for 30 min in order to permeabilize the fragments prior to the experiments.

LDH experiments

There was no detectable loss of LDH during incubation of smooth muscle fragments in ISS alone or during treatment with 5000 u ml^{-1} α-toxin (Figure 2) a concentration that has been shown to release more than 95% of the [^{3}H]-2DG taken up (Figure 1a) by the end of the 60 min incubation period. However, permeabilization of the fragments with Triton-X-

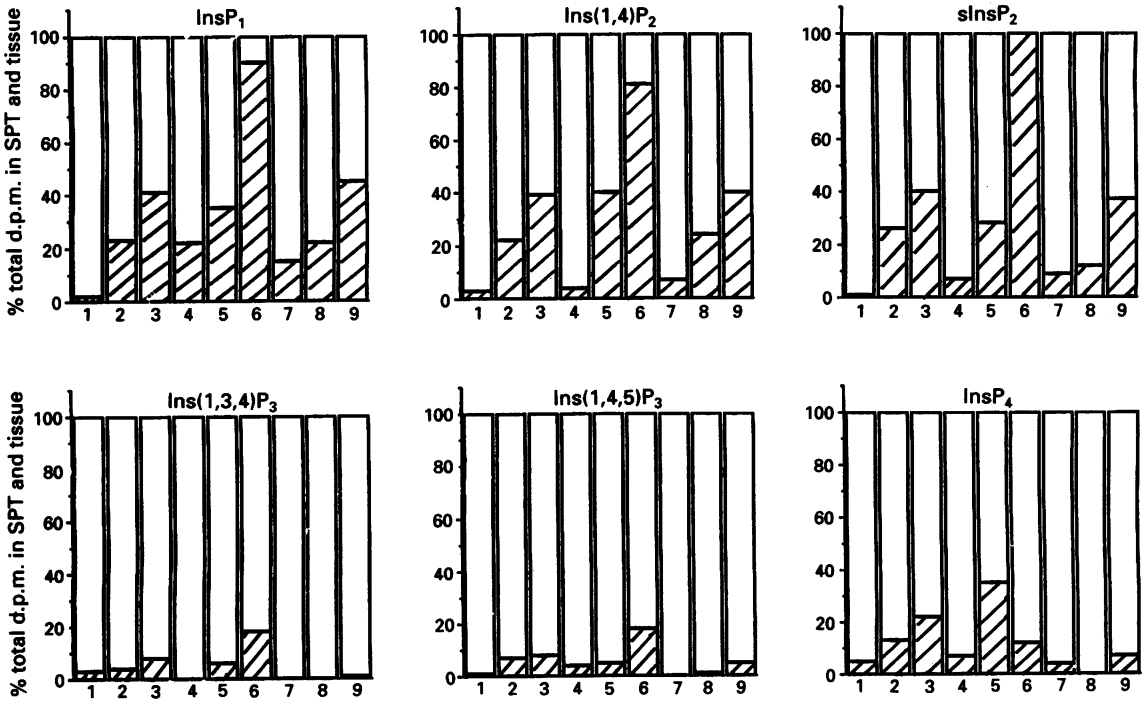

Figure 3 The distribution of d.p.m. between supernatant (SPT) and tissue in permeabilized fragments. Increases in the levels of [^{3}H]-inositol phosphates were determined in [^{3}H]-inositol-labelled fragments permeabilized with α-toxin in the presence of ISS alone, GTPγS or CCh for different times and the tissue part separated from the supernatant as described in the Methods. Results represent the % of the total d.p.m. for each isomer found in the supernatant (hatched) or tissue part (open). (1), (2), and (3) represent distribution of basal d.p.m. after incubation in ISS for 30 s, 5 min or 20 min respectively. (4), (5), and (6) represent distribution of d.p.m. after subtraction of basal levels following stimulation with 100 μM GTPγS for 30 s, 5 min or 20 min respectively and (7), (8), and (9) represent the distribution of d.p.m. after subtraction of basal levels following stimulation with 100 μM CCh for 30 s, 5 min or 20 min respectively. The results represent the values from one experiment. Separation of supernatant from the tissue and analysis of [^{3}H]-inositol phosphates was performed on many samples with similar results.

100 or saponin resulted in a rapid loss of the enzyme LDH, such that greater than 50% of the LDH was lost from the fragments in the first 5 min incubation with Triton-X-100 and 10 min incubation with saponin (Figure 2). Concentrations of α-toxin up to 8000 u ml^{-1} also failed to cause detectable release of LDH (data not shown).

Basal levels of [^{3}H]-inositol phosphates

In contrast to experiments on non-permeabilized fragments (Prestwich & Bolton, 1995) the basal levels of [^{3}H]-inositol phosphates increased with time after permeabilization. The increases were 83%, 59%, 36%, 59% and 75% for the [^{3}H]-$InsP_1$, [^{3}H]-Ins(1,4)P_2, [^{3}H]-Ins(1,3,4)P_3, [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-$InsP_4$ respectively at 5 min compared to the values at 10 s. The increase in inositol phosphates observed may be related to the contractile effects of mM ATP concentrations present in ISS on longitudinal muscle of guinea-pig ileum (Burnstock *et al.*, 1970). Also the loss of [^{3}H]-$InsP_1$ and [^{3}H]-Ins(1,4)P_2 through the 'pores' formed during permeabilization may cause a shift in the equilibrium resulting in increased intracellular turnover.

Distribution of d.p.m. between the supernatant and tissue

Measurement of the distribution of d.p.m. between the supernatant part and tissue part of a sample showed that in non-permeabilized fragments incubated in normal KRB for 10 min, no detectable amounts of radioactivity eluting on the h.p.l.c. corresponding to the [^{3}H]-inositol phosphates were observed. Only 3% of total radioactivity was found in the supernatant of non-permeabilized fragments after incubating in ISS for 10 min.

Following permeabilization, inositol phosphates escaped into the supernatant. Several experiments were performed to determine the distribution of inositol phosphates in the supernatant compared with the tissue portion. Saponin (1 mg ml^{-1}) also increased inositol phosphates in a similar way to α-toxin but the effects were much smaller. Measurement of individual [^{3}H]-inositol phosphates in the supernatant and tissue fractions from permeabilized fragments during different experimental conditions is shown in Figure 3. The first three columns are from control samples incubated for 30 s, 5 min and 20 min and it can be seen that there is a time-dependent increase in the percentage of d.p.m. found in the supernatant fraction for all the [^{3}H]-inositol phosphates. The other six columns have had the basal d.p.m. subtracted and represent the distribution during either GTPγS (100 μM) stimulation for 30 s (4), 5 min (5) or 20 min (6) or CCh (100 μM) stimulation for 30 s (7), 5 min (8) or 20 min (9). The general trend is that the longer the incubation the greater percentage of [^{3}H]-$InsP_1$, [^{3}H]-Ins(1,4)P_2 and [^{3}H]-sInsP_2 that appears in the supernatant but for the [^{3}H]-Ins(1,3,4)P_3, [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-$InsP_4$ the majority of the d.p.m. remained in the tissue part (Figure 3).

Effect of CCh and GTPγS on [^{3}H]-inositol phosphate levels

Incubation of α-toxin permeabilized, [^{3}H]-inositol-labelled, smooth muscle fragments with CCh (in the absence of added GTP) or GTPγS resulted in time- and concentration-dependent increases in the levels of all the [^{3}H]-inositol phosphates in combined tissue fragments and supernatant (Figures 4 and 5). The time courses and % increases produced by CCh and GTPγS in the levels of [^{3}H]-inositol phosphate isomers were very similar except for [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-$InsP_4$'s at the early times (Figure 4). Increases in the levels of [^{3}H]-Ins(1,4,5)P_3 were significantly less with GTPγS than with CCh at all time points ($P<0.05$). GTPγS (up to 1 mM) had no significant effect on [^{3}H]-inositol phosphate levels in non-permeabilized muscle fragments

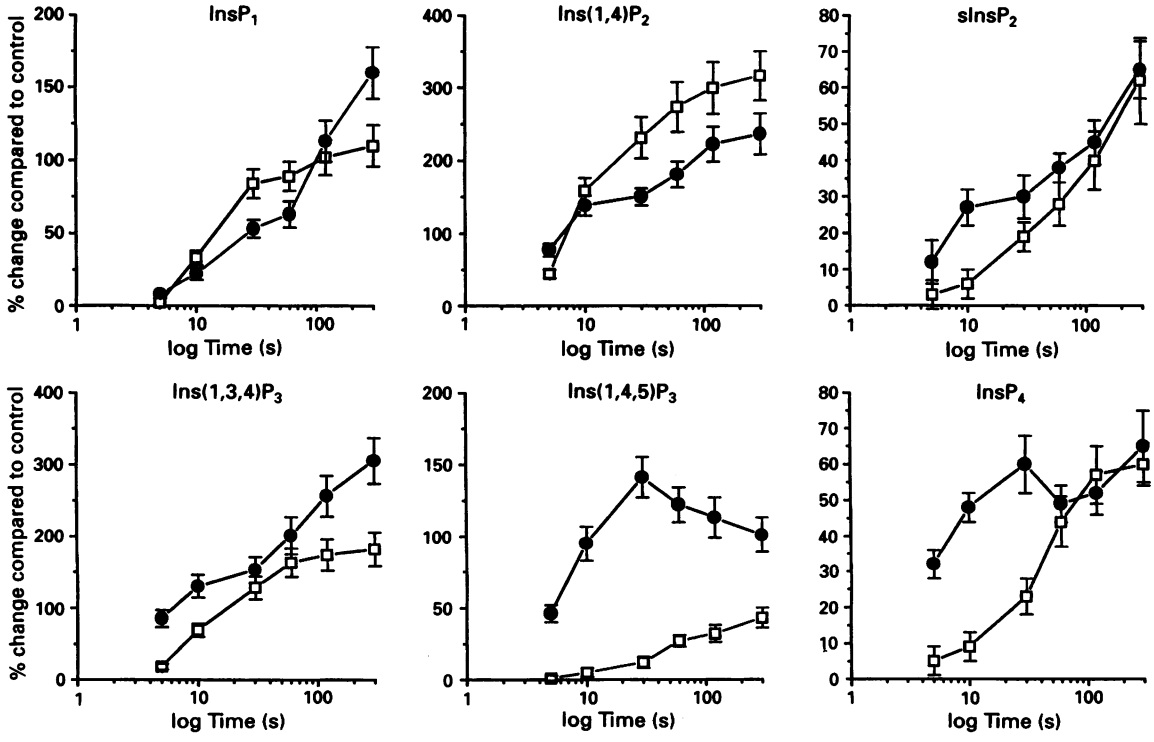

Figure 4 Effects of time of incubation with GTPγS or CCh on [^{3}H]-inositol phosphate levels in permeabilized smooth muscle fragments and their supernatant combined. [^{3}H]-inositol-labelled, α-toxin permeabilized fragments were incubated with 100 μM CCh (●) or 100 μM GTPγS (□) for 5 s, 10 s, 30 s, 60 s, 120 s or 5 min. Results represent the mean ± s.e.mean of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of added drug. Increases in the levels of [^{3}H]-$InsP_1$, [^{3}H]-Ins(1,4)P_2, [^{3}H]-sInsP_2, [^{3}H]-Ins(1,3,4)P_3, [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-$InsP_4$ are shown.

indicating that α-toxin treatment is essential to allow GTPγS access to the cell interior. Incubation of the permeabilized fragments with 0.5 μM atropine for 10 min prior to addition of 100 μM CCh reduced the CCh-induced increases in the levels of all the labelled inositol phosphates by an average of 79% (2 experiments). In non-permeabilized fragments the reduction of the response to 100 μM CCh was 95 ± 1.3% ($n = 6$). These effects are compatible with an equilibrium dissociation constant of atropine for the receptor of 3×10^{-9} M.

Effect of simultaneous addition of GTPγS and CCh on the levels of [^{3}H]-inositol phosphates

Although CCh-induced increases in the levels of [^{3}H]-inositol phosphates could be observed in permeabilized fragments and their supernatants in the absence of any added GTP, the absolute increase in d.p.m. was less than with non-permeabilized fragments for some of the isomers (Figure 6) and the maximum increase in [^{3}H]-Ins(1,4,5)P_3 was later in permeabilized fragments (30 s in permeabilized fragments, 10 s in non-permeabilized) and did not decline. The [^{3}H]-Ins(1,4)P_2 isomer increased slowly up to 2 min incubation, compared with the non-permeabilized fragments where the response peaked at 30 s (Figure 6).

Addition of 20 μM GTPγS at the same time as different concentrations of CCh, followed by incubation for 2 min did not further increase the levels of any of the [^{3}H]-inositol phosphates over the effect of CCh alone (data not shown). Incubation of permeabilized fragments with 100 μM GTPγS and various concentrations of CCh for 5 min however, significantly inhibited the CCh-induced increases in the levels of all the [^{3}H]-inositol phosphates ($P < 0.05$) (Figure 7). Incubation with 3 μM CCh for 5 min increased the levels of all the [^{3}H]-inositol phosphates and these increases were inhibited in the presence of 1–100 μM GTPγS when added at the same time as the CCh. The IC_{50} for GTPγS inhibition was between 5 μM and 8 μM for all the [^{3}H]-inositol phosphate isomers and at 100 μM GTPγS, almost all (95 ± 4.2%) the CCh response was inhibited.

In fragments permeabilized with saponin (1 mg ml^{-1} for 20 min at 22°C) CCh alone (without GTP) increased inositol phosphate levels as did GTPγS. The effects of CCh or GTPγS were generally smaller on the inositol phosphate isomers measured than when α-toxin was used.

The effect of incubation with GTPγS prior to CCh addition

When permeabilized smooth muscle fragments were incubated in the presence of increasing concentrations of GTPγS for 5 min before the addition of 100 μM CCh for a further 30 s, GTPγS above 1 μM caused a significant inhibition of the CCh-induced increases in the levels of all the [^{3}H]-inositol phosphates (Figure 8).

Effect of PTX treatment on CCh and GTPγS effects in permeabilized fragments

The CCh-induced increases and the GTPγS-induced increases in the levels of [^{3}H]-inositol phosphates were greatly reduced following treatment with PTX (6 μg ml^{-1} for 20 h at 30°C) (Figures 8 and 9). GTPγS inhibited the effect of CCh on [^{3}H]-inositol phosphate levels and this was, in general, also true when the effect of CCh was substantially reduced by PTX pretreatment. However, in different experiments there was some variation in the effects of GTPγS on the PTX-insensitive CCh response (compare Figures 8 and 9).

Effect of GDPβS

GDPβS (5 mM) had no effect on the basal levels of the [^{3}H]-inositol phosphates. However it almost completely inhibited (by 92% and 96% respectively) the CCh-(100 μM)

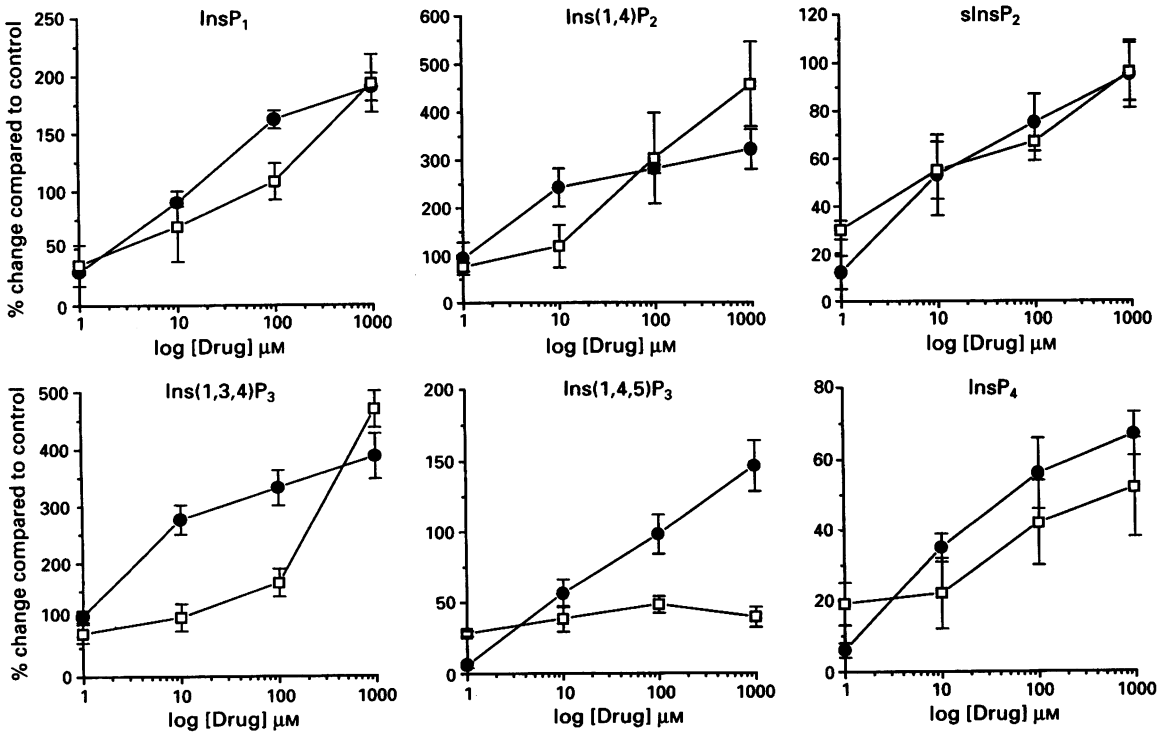

Figure 5 Concentration-response curves for the effects of CCh and GTPγS on [^{3}H]-inositol phosphates in permeabilized smooth muscle fragments and their supernatant combined. Permeabilized fragments were incubated in the presence of different concentrations of CCh (●) or GTPγS (□) for 5 min. Results represent the mean ± s.e.mean of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of added drug.

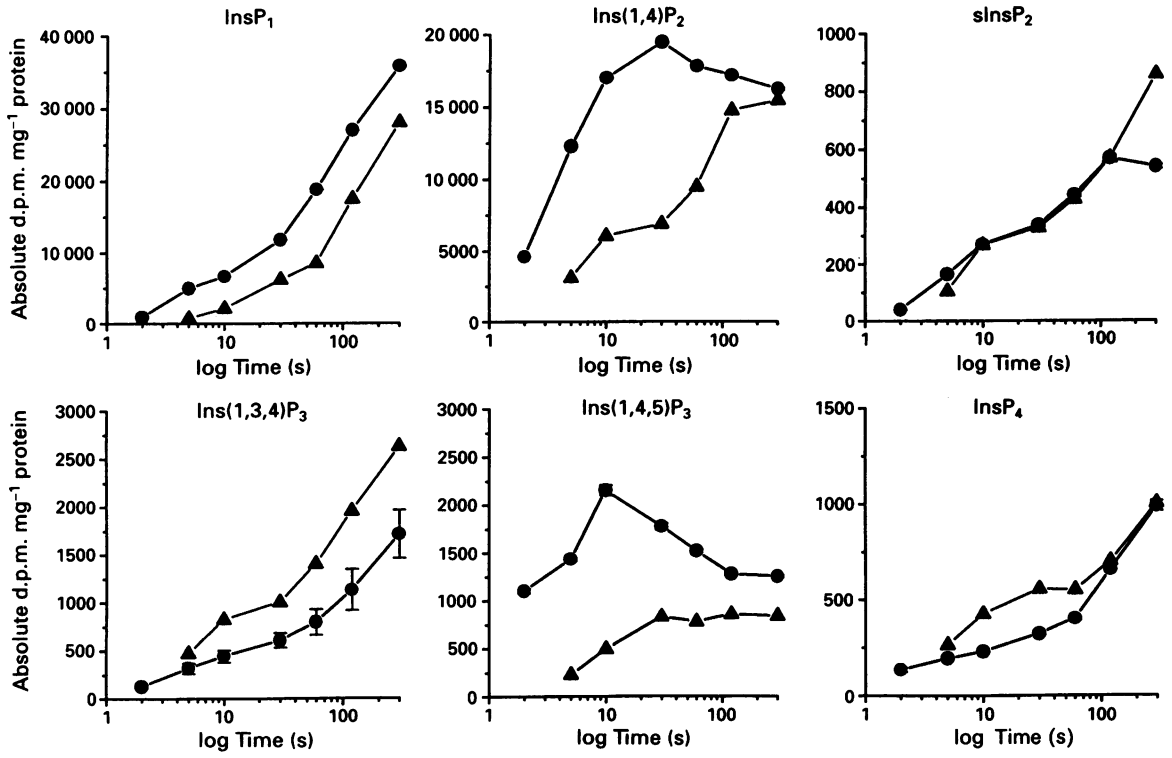

Figure 6 Comparison of CCh-induced increases in [^{3}H]-inositol phosphates in non-permeabilized and permeabilized fragments with their supernatant. CCh-induced increases in the levels of [^{3}H]-inositol phosphates were determined in non-permeabilized fragments and fragments permeabilized with 2500 u ml^{-1} α-toxin for 30 min. Results show the time-dependent increases in the levels of [^{3}H]-inositol phosphates in the presence of 100 μM CCh in non-permeabilized fragments (●) or permeabilized (▲) fragments and are the mean ± s.e.mean (where it exceeds symbol width) of three unpaired experiments performed on separate occasions, on separate guinea-pigs and are expressed as absolute d.p.m. minus basal values.

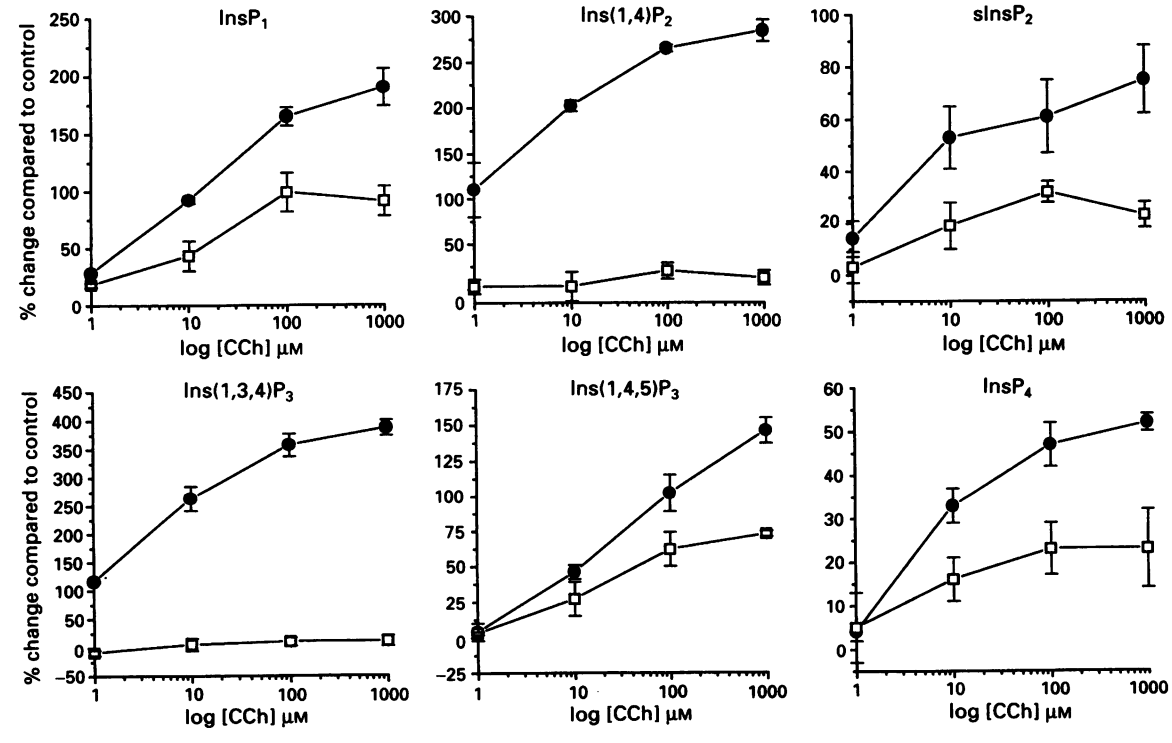

Figure 7 Effect of GTPγS on the response to CCh in permeabilized fragments. Permeabilized fragments were incubated with either 1 μM, 10 μM, 100 μM or 1 mM CCh for 5 min in the absence (●) or presence (□) of 100 μM GTPγS for 5 min added with the CCh. Results represent the mean ± s.e.mean of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of CCh.

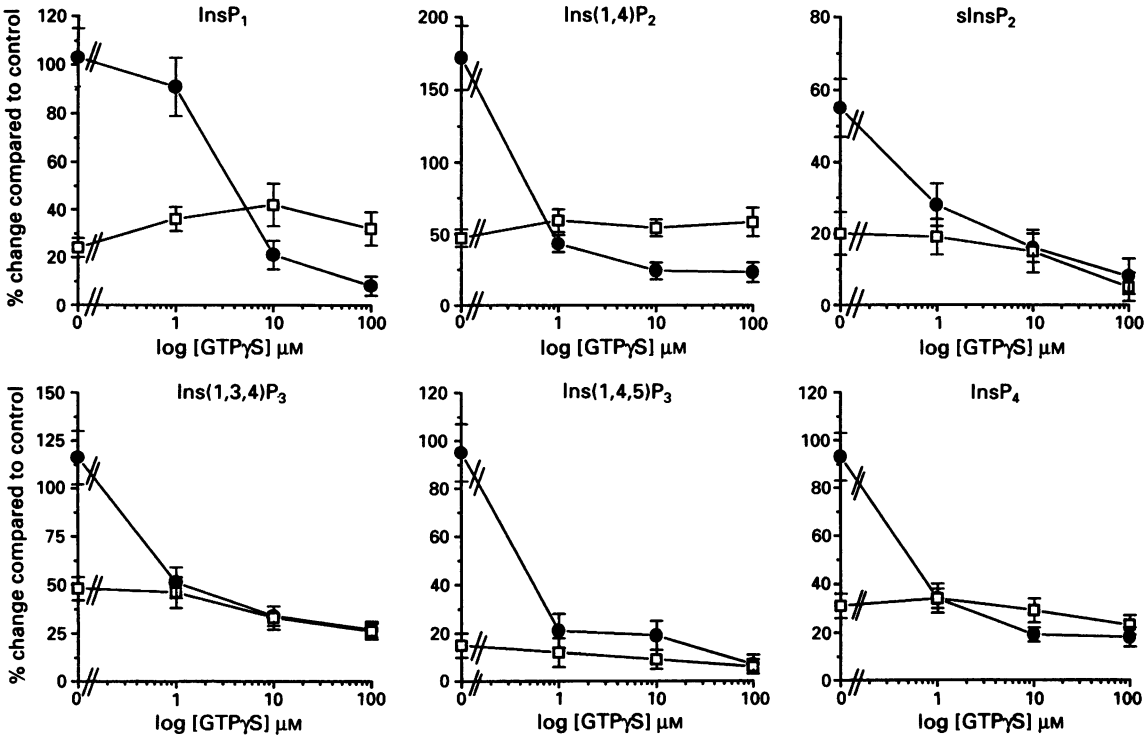

Figure 8 GTPγS inhibition of the CCh response and the effect of PTX on this. Smooth muscle fragments were incubated for 20 h at 30°C in either normal KRB or KRB containing 6 μg ml^{-1} PTX; [^{3}H]-inositol was present in the incubation solutions. The fragments were then permeabilized and incubated for 5 min in the absence or in the presence of 1 μM, 10 μM or 100 μM GTPγS. At the end of the 5 min incubation period, 100 μM CCh was added for a further 30 s and the reaction stopped. Results are shown as the % increase over the level of [^{3}H]-inositol phosphate without CCh. The inhibitory effect of GTPγS on the % increase of the response to 100 μM CCh in fragments with no PTX (●) and with PTX treatment is shown (□). Results represent the mean ± s.e.mean of three experiments performed on separate occasions.

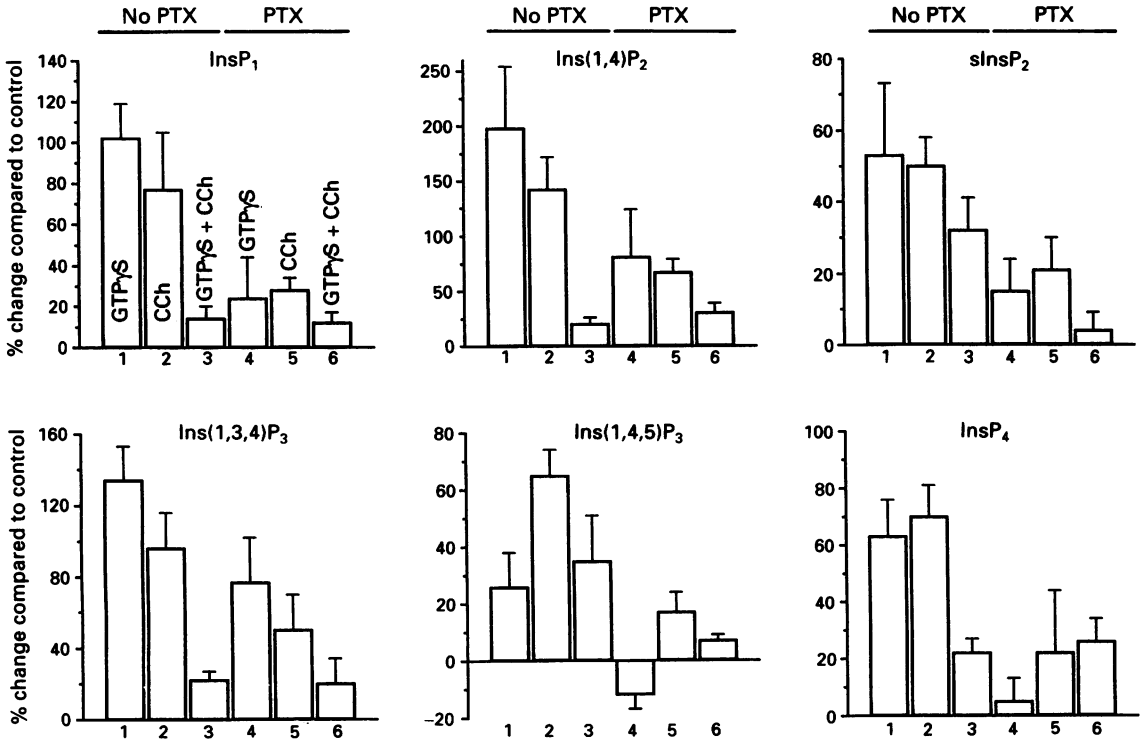

Figure 9 Effect of PTX on the interaction between CCh and GTPγS. [^{3}H]-inositol-labelled smooth muscle fragments were incubated for 20 h at 30°C in either normal KRB or KRB containing 6 μg ml^{-1} PTX. The fragments were then permeabilized and incubated in 100 μM GTPγS for 5.5 min, incubated for 5 min in ISS alone followed by 100 μM CCh for 30 s or 100 μM GTPγS for 5 min followed by 30 s application of 100 μM CCh. Columns (1), (2) and (3) are without PTX and (4), (5), and (6) are with PTX treatment. (1) and (4) represent 100 μM GTPγS for 5 min, (2) and (5) represent 100 μM CCh for 30 s and (3) and (6) represent 30 s application of 100 μM CCh in the presence of 100 μM GTPγS previously present for 5 min. Results represent the mean ± s.e.mean of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of CCh for (2), (3), (5) and (6) and the absence of GTPγS for (1) and (4).

and GTPγS-(100 μM) induced increases in the levels of [^{3}H]-$InsP_1$ and [^{3}H]-Ins(1,4,5)P_3. The increases in [^{3}H]-Ins(1,4)P_2, [^{3}H]-Ins(1,3,4)P_3 and [^{3}H]-$InsP_4$ levels due to GTPγS stimulation were inhibited by 82%, 81% and 82% respectively and increases due to CCh stimulation were inhibited by 81%, 80% and 96% respectively.

Effect of dibutyryl cyclic AMP and Sp-cAMPs on CCh-induced increases in [^{3}H]-inositol phosphate levels

Incubation of permeabilized fragments in the presence of either 10 μM dibutyryl cyclic AMP or 10 μM Sp-cAMPs for 10 min prior to application of CCh (100 μM) for a further 10 s resulted in a substantial reduction in the response to CCh. The average % reduction ($n = 3$) in the CCh response for [^{3}H]-$InsP_1$, [^{3}H]-$InsP_2$, [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$ isomers in the presence of dibutyryl cyclic AMP was 93 ± 11, 89 ± 13, 86 ± 11 and 69 ± 11 and in the presence of Sp-cAMPs was 56 ± 6, 78 ± 6, 71 ± 7 and 79 ± 7 respectively.

Cyclic AMP measurements

Basal cyclic AMP values for non-permeabilized smooth muscle fragments incubated in the absence of added IBMX rose from 0.98 to 2.12 pmol mg^{-1} protein during a 10 min incubation period. Changes in these levels in the absence of IBMX upon β-adrenoceptor activation or following treatment of fragments with ChTX could not be observed (data not shown). Basal cyclic AMP levels incubated in the presence of 1 mM IBMX increased from 10 to 36 pmol mg^{-1} protein over a 10 min incubation period. This indicates that there is very efficient metabolism of cyclic AMP by phosphodiesterases present in these fragments. Increases in the levels of cyclic AMP were observed when a β-adrenoceptor agonist was added if 1 mM IBMX was present. However, up to 100 μM CCh (for 10 min) had no significant effect on the levels of cyclic AMP (20 ± 6 pmol mg^{-1} protein, $n = 6$) whereas incubation with 100 μM GTPγS (for 10 min) significantly increased cyclic AMP levels to 174 ± 18 pmol mg^{-1} protein ($P < 0.01$, $n = 3$). However, 10 mM NaF/10 μM $AlCl_3$ had no significant effect on cyclic AMP levels (28 ± 8 pmol mg^{-1} protein, $n = 3$). GDPβS (5 mM, for 10 min) had no effect on the basal levels of cyclic AMP but significantly inhibited the increases produced by 100 μM GTPγS by 55 ± 9.2% ($P < 0.05$, $n = 3$) (incubated together for 10 min).

Discussion

Permeabilization

The studies on the loss of [^{3}H]-2DG and LDH indicate that a sufficient concentration of α-toxin will permeabilize the cell membranes allowing small, but not large, molecules to escape. Further evidence for this came from the loss of [^{3}H]-inositol phosphates which also escaped from the tissue after α-toxin treatment; in fragments not so treated, escape of [^{3}H]-inositol phosphates was negligible. Also, GTPγS normally without effect on [^{3}H]-inositol phosphate levels, increased these substantially after α-toxin treatment indicating that adequate access to the interior of cells was achieved. The question of whether some cells in the tissue fragments remained unpermeabilized is an important one, because if permeabilized and non-permeabilized cells are both present in significant numbers, the total tissue response will represent events taking place in two separate populations and not, as is required for the interpretation of these experiments, in the same cells. Increasing α-toxin concentration from 2500 to 8000 u ml^{-1} only marginally increased the rate of loss of [^{3}H]-2DG over the first 5 min. Thus increasing the rate of formation of membrane pores more than 3 fold by increasing the α-toxin concentration had only a marginal effect on exchange. It is possible that treatment with 2500 u ml^{-1} or more of α-toxin for only 5 min is sufficient to produce exchange of small molecules at a near maximum rate although this possibility was not tested and α-toxin was routinely applied for 30 min. Presumably, at concentrations above 2500 u ml^{-1} other factors limit the escape of small molecules from the tissue fragments. Triton-X-100 (1%) and saponin (1 mg ml^{-1}) caused a greater loss of [^{3}H]-2DG but these agents also caused loss of large molecules such as LDH. The loss of these larger molecules and the resultant alterations in the cell molecular architecture may explain the faster loss of [^{3}H]-2DG seen with these detergents. Saponin and Triton-X-100 have been used to permeabilize smooth muscle (Endo *et al.*, 1977; Obara & Yamada, 1984) but loss of receptor function (Itoh *et al.*, 1983; Somlyo *et al.*, 1985) and destruction of intracellular membranes have been observed (Knight & Scrutton, 1986). However, agonist-induced contraction has been shown to occur in smooth muscle permeabilized with saponin in the absence of GTP (Fermum *et al.*, 1991). In the present study, both GTPγS and CCh increased inositol phosphate levels in saponin-treated fragments although the effects were less than in α-toxin treated tissue.

The increase in most [^{3}H]-inositol phosphate isomers in α-toxin permeabilized fragments was less than in non-permeabilized fragments. After permeabilization, $[Ca^{2+}]_i$ was held at 0.13 μM by the use of EGTA and there is presumably no membrane potential change. Phospholipase C (PLC) is an enzyme stimulated by Ca^{2+} (Low *et al.*, 1986; Ryu *et al.*, 1987) and depolarization of the membrane may increase inositol phosphate formation (Jafferji & Michell, 1976; Best & Bolton, 1986); both these effects may contribute in the non-permeabilized fragments to the increase in [^{3}H]-inositol phosphate levels produced by CCh. Also, increases in the levels of all the [^{3}H]-inositol phosphates following incubation of permeabilized fragments with CCh occurred in the absence of any added GTP. However, commercial ATP may be contaminated with GTP (Ross & Gilman, 1980) and ATP may also be transphosphorylated to GTP (Sternweis & Gilman, 1982; Otero, 1990).

From the distribution of [^{3}H]-inositol phosphates between the supernatant and tissue of the samples it was found that as the basal levels increase with time so does the percentage of d.p.m. found in the supernatant (Figure 3). As the levels of [^{3}H]-$InsP_1$, [^{3}H]-Ins(1,4)P_2 and [^{3}H]-s$InsP_2$ increase with time when GTPγS is present a substantial proportion of these isomers move into the supernatant part suggesting the levels rose in the cytosol of the cells. Although the time-dependent increases in these [^{3}H]-inositol phosphates are similar in the presence of CCh or GTPγS, the movement of isomers into the supernatant is not as great in the presence of CCh (Figure 3). This may reflect a faster turnover of the [^{3}H]-inositol phosphates in the presence of CCh so that they are not so available for diffusion out of the cell. Also the d.p.m. for the higher [^{3}H]-inositol phosphates, [^{3}H]-Ins(1,3,4)P_3, and [^{3}H]-Ins(1,4,5)P_3 stay mainly in the tissue fragments in the presence of both GTPγS or CCh. This may also reflect a higher turnover of these isomers and indicates that these isomers are readily metabolized and therefore do not have a chance to pass out of the cell and/or there is some compartmentalization within the cell. The changeable results in the distribution of the [^{3}H]-$InsP_4$ isomer may reflect a possible mixture of isomers which change with time.

GTPγS and CCh

GTPγS inhibited the action of CCh when added at the same time and was an even more effective inhibitor when added prior to CCh application. Previously we have shown that although AlF increased basal levels of [^{3}H]-inositol phosphates, it completely inhibits the CCh-induced increases in [^{3}H]-inositol phosphates in non-permeabilized fragments (Prestwich & Bolton, 1995). In this study, GTPγS also both increased the levels of [^{3}H]-inositol phosphates in perme-

abilized fragments and inhibited the CCh response suggesting that both AlF and GTPγS may act through G-proteins both to stimulate basal levels of [^{3}H]-inositol phosphates and to inhibit CCh-induced increases in [^{3}H]-inositol phosphate levels.

However, incubation with GTPγS also increased basal levels of cyclic AMP. Increases in cyclic AMP result in increased activity of protein kinase A and phosphorylation of proteins so that part of the inhibition of CCh-stimulated increases in the levels of [^{3}H]-inositol phosphates by GTPγS could be due to activation of protein kinase A. Increases in protein kinase A activity via activation of Gsα could result in phosphorylation of the muscarinic AChR, G-protein or PLC mimicking a negative feedback mechanism. This mechanism for GTPγS action seems likely since incubation of the smooth muscle fragments in the presence of either of the cyclic AMP analogues, dibutyryl cyclic AMP or Sp-cAMPs, also substantially reduced the CCh-induced stimulation of labelled inositol phosphates. However, incubation with AlF under conditions which completely inhibited CCh-induced increases in labelled inositol phosphates (Prestwich & Bolton, 1995) had no effect on the levels of cyclic AMP. Therefore other mechanisms for this inhibition by both G-protein activators must also be considered.

Activation of PLC by GTPγS or AlF would also increase 1,2-diacylglycerol levels and activate protein kinase C. Protein kinase C activation could result in the phosphorylation of the G-protein (Katada *et al.*, 1985) or the muscarinic AChR itself (Woods *et al.*, 1987) resulting in a reduced ability of the system to increase [^{3}H]-inositol phosphates in response to further agonist being present. However G-protein-mediated inhibition of PLC activity itself has also been observed (Bizzarri *et al.*, 1990; Geet *et al.*, 1990; Gutowski *et al.*, 1991).

Recent studies have suggested that both βγ subunits and α subunits can both stimulate and inhibit the activity of PLC (Sternweis & Smrcka, 1992). PLCβ2 can be modulated independently by α and βγ subunits acting at different sites (Bong Lee *et al.*, 1993). Both α and βγ subunits can regulate cardiac K^+ channel opening (Logothetis *et al.*, 1988). Thus there are several ways α and βγ can modulate PLC: α alone, βγ alone, or both acting independently; both acting synergistically or both acting antagonistically.

PLC exists in several isoforms some of which e.g. PLCβ1-4 are regulated by G-proteins whereas others are not e.g. PLCγ. PLCβ forms 1-3 are regulated both by Gα subunits of the Gq class, and by Gβγ subunits (Katz *et al.*, 1992; Park *et al.*, 1993). PLCβ4 is not regulated by Gβγ. Activation of PLCβ isoforms by Gαq/11 and Gβγ is independent and not conditional on priming by either subunit. However, the concentration of βγ subunits required to activate PLCβ3 was shown to be much higher (40 fold) than that of α subunits required to activate PLCβ1 (Park *et al.*, 1993). In longitudinal muscle of guinea-pig small intestine it is conceivable that the PTX-insensitive component of muscarinic AChR activation of inositol phospholipid hydrolysis may occur via α subunits from a G-protein of the Gq family interacting with a PLCβ1 isozyme, and PTX-sensitive component arise from the interaction of the muscarinic AChR with Gi (associated with adenylyl cyclase inhibition) freeing up βγ subunits to interact with the PLCβ2 isozyme.

As well as reducing the CCh-induced increases in [^{3}H]-inositol phosphate levels, PTX treatment also reduced the increases produced by GTPγS, which is a receptor-independent process. This may be explained by failure of αβγ subunits to dissociate into α and free βγ following α subunit ribosylation. The carboxyl terminal (where ADP-ribosylation by PTX of a cysteine residue occurs) is thought to be involved in binding to the receptor and the major effect of ADP-ribosylation by PTX is an uncoupling of the receptor from the G-protein. This sensitivity of both CCh and GTPγS to PTX suggests that PTX not only uncoupled the muscarinic AChR from PLC but also uncoupled the G-protein from PLC activity.

Muscarinic AChR types

The effects observed due to muscarinic AChR activation may be mediated by only one type or several types of muscarinic AChRs. It has been suggested that M_2 and M_3 receptors are present in this tissue (Michel & Whiting, 1990) and contraction via stimulation of mainly the M_3 type has been suggested to occur (Barocelli *et al.*, 1993). However transfection studies have shown that stimulation of the m3 AChR can result in inositol phospholipid hydrolysis and activation of the m2 AChR can result in both adenylyl cyclase inhibition and inositol phospholipid hydrolysis (Ashkenazi *et al.*, 1989). The response to activation of both transfected m2 and m3 receptors in oocytes was inhibited by heparin suggesting that both receptors acted through an Ins(1,4,5)P_3-dependent pathway. The precise type of muscarinic AChR involved in this study awaits either highly selective antagonists or transfection and analysis of the specific subtypes present in this tissue.

The authors thank the MRC for financial support.

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(Received October 20, 1994
Revised January 12, 1995
Accepted January 17, 1995)