G-protein involvement in muscarinic receptor-stimulation of inositol phosphates in longitudinal smooth muscle from the small intestine of the guinea-pig

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1 Aluminum fluoride (AlF), pertussis toxin (PTX) and cholera toxin (ChTX) have been used to examine the involvement of G-proteins during muscarinic acetylcholine receptor (ACHR) stimulation of inositol phospholipid hydrolysis in fragments of longitudinal smooth muscle from the small intestine of the guinea-pig.

2 Carbachol (CCh) induced time- and concentration-dependent increases in \(^{3}H\)-inositol monophosphates, \(^{3}H\)-inositol (1,4) biphosphates, \(^{3}H\)-inositol (1,3,4) trisphosphate, \(^{3}H\)-inositol (1,4,5) trisphosphate (\(^{3}H\)-Ins(1,4,5)P\(_3\)) and \(^{3}H\)-inositol tetrakisphosphates measured by h.p.l.c. These increases were inhibited >95% in the presence of the muscarinic ACHR antagonist atropine (0.5 \(\mu M\)).

3 AlF transiently increased the basal levels of \(^{3}H\)-Ins(1,4,5)P\(_3\) but increases in the levels of the other \(^{3}H\)-inositol phosphates occurred more slowly. CCh-induced increases in the levels of all the \(^{3}H\)-inositol phosphates were strongly inhibited in the presence of AlF.

4 PTX had no effect on basal levels of any of the \(^{3}H\)-inositol phosphates but reduced the effects of CCh on these; ChTX had no effects on either basal or CCh-stimulated levels.

5 It was concluded that muscarinic ACHR-stimulated increases in the levels of \(^{3}H\)-inositol phosphates occur via both a PTX-sensitive G-protein and a PTX-insensitive mechanism. The actions of AlF may suggest the involvement of an inhibitory G-protein in the regulation of muscarinic ACHR-stimulated inositol phospholipid turnover.

Keywords: Smooth muscle; G-proteins; aluminium fluoride; inositol phosphates; pertussis toxin; cholera toxin; muscarinic acetylcholine receptor

Introduction

In smooth muscle, muscarinic acetylcholine receptors (ACHRs) are linked via G-proteins to stimulation of inositol phospholipid hydrolysis and inhibition of adenylyl cyclase (Kamm & Stull, 1988; Noronha-Blob et al., 1989). These effects are thought to be mediated by different subtypes of receptor and in the longitudinal muscle of guinea-pig ileum, radioligand binding and contraction studies using selective antagonists have suggested that 70% of the muscarinic ACHRs are \(M_2\) and the other 30% are \(M_3\). The \(M_2\) subtype of the receptor mediates contraction (Giraldo et al., 1988; Michel & Whiting, 1990; Barocelli et al., 1993).

The G-protein \(\alpha\) subunits involved in phospholipase C (PLC) activation and adenylyl cyclase inhibition have been divided into two classes based on their sensitivity to PTX (Casey & Gilman, 1988). Studies using transfected marmalian cell lines have suggested that individual muscarinic ACHR subtypes evoke distinct cellular responses through differential interaction with these two classes of G-proteins (Peralta et al., 1988; Ashkenazi et al., 1989; Lechleiter et al., 1990). It has been suggested that the \(m_2\), \(m_3\) and \(m_5\) receptor subtypes primarily activate a PTX-insensitive G-protein pathway leading to a potent stimulation of PLC-mediated phosphoinositide hydrolysis (Peralta et al., 1988; Ashkenazi et al., 1989) whereas the \(m_2\) and \(m_3\) receptor subtypes primarily mediate both the activation of PLC and inhibition of adenylyl cyclase through PTX-sensitive pathways (Ashkenazi et al., 1989). Stimulation of phospholipase \(A_2\), phospholipase D, adenylyl cyclase, Ins(1,4,5)P\(_3\)-independent receptor-operated Ca\(^{2+}\) channels and Ca\(^{2+}\)-dependent tyrosine kinases by \(m_2\), \(m_3\) and \(m_5\) have also been reported (Nathanson, 1987; Baumgold & Fishman, 1988; Conklin et al., 1988; Stratton et al., 1989; Sandmann et al., 1991; Felder et al., 1993). Despite these results there is no conclusive evidence that an individual muscarinic ACHR subtype cannot link to several different G-protein or other signal transducing systems.

G-proteins have been shown to be directly activated by AlF. The AlF mixture gives AlF\(^{3-}\) (AlF) (Sternweis & Gilman, 1982) and Bigay et al. (1987) showed that the AlF complex, which has a chemical structure similar to that of PO\(_4^{3-}\), binds to the nucleotide binding site of the \(\alpha\)-subunit of the G-protein, close to the \(\beta\)-phosphate of GDP, and consequently activates the G-protein by dissociating the \(\alpha\) from the \(\beta\) subunits. Thus, when the nucleotide site contains GDP (as in the inactive state) AlF becomes associated with the GDP and mimics the \(\gamma\)-phosphate of GTP. In many cell types AlF has been shown to induce Ca\(^{2+}\) mobilization and phosphoinositide hydrolysis by direct activation of a G-protein coupled with PLC (Blackmore et al., 1985; Guillou et al., 1986; Paris & Pouyssegur, 1987; Toyjo et al., 1991) and to cause contraction in various smooth muscles (Marc et al., 1988; Zeng et al., 1989; Cushing et al., 1990; Hall et al., 1990; Ratz & Blackmore, 1990; Kawase & Van Bremen, 1992). AlF also affects cyclic AMP levels through G\(_i\) and G\(_s\), (Cook et al., 1985).

PTX and ChTX have both been used extensively in aiding the identification of the G-proteins linked to adenylyl cyclase (Stryer & Bourne, 1986) and have more recently been used to determine the involvement of G-proteins in PLC activity (Hepler & Gilman, 1992). In several cell types PTX blocks agonist-stimulated PLC activation (Brandt et al., 1985; Nakamura & Uj, 1985). In smooth muscle, variable effects of PTX have been found on agonist-stimulated phospholipase C activation e.g. responses have been shown to be markedly inhibited in the rabbit pulmonary artery (Hohlfeld et al., 1990) and in cultured cells from rat aorta (Bruns & Marmé, 1995).
1987) partially inhibited in rat and human myometrium (Anwer et al., 1989; Phaneuf et al., 1993) and not affected in pig coronary arteries (Sasaguri et al., 1986) a vascular smooth muscle cell line (Muldoon et al., 1989) or rat portal vein myocytes (Loirand et al., 1990). In guinea-pig myometrial smooth muscle, treatment with ChTX was shown to inhibit the muscarinic inositol phosphate response (Marc et al., 1988) and inhibit agonist-induced contractions in rat portal vein (Zhang et al., 1993). The fact that in some cells agonist-induced phosphoinositide breakdown is inhibited by ChTX whereas in others it is not led to the conclusion that more than one type of G-protein was associated with PLC (Simon et al., 1991).

In this study, both AIF, which by mimicking the γ phosphate of GTP activates all G-proteins in a non-selective way, and treatment with PTX and ChTX have been used to identify G-protein modulation of muscarinic AChR-induced increases in [3H]-inositol phosphate levels at early times.

**Methods**

**Preparation and assay of labelled fragments**

The preparation of the longitudinal layer from the small intestine of the guinea-pig is a modification of the method by Paterson & Zar (1968). Female guine-pigs (250-350 g) were killed by cervical dislocation and exsanguination. The small intestine was exposed and rinsed in pregressed Krebs Ringer buffer (KRB) and the longitudinal layer from the whole length of the small intestine except the terminal 5 cm, was carefully pulled away from the underlying tissue using fingers. Tissue which had been in contact with the fingers was discarded and the rest of the strips immersed in warm KRB solution. These strips were rinsed several times in fresh KRB. Each strip was examined under the microscope and any circular muscle pooled off. Chopped fragments of 350 μm x 350 μm were prepared with a McIlwain tissue chopper. Rinsed fragments were transferred to one or more wells of a 24-well multidish and incubated in 1 ml KRB containing 50 μM [3H]-inositol and kept in a humidified atmosphere containing 95% O₂/5% CO₂ at 30°C for 20 h. When required, smooth muscle fragments were incubated with 6 μg ml⁻¹ PTX or 5 μg ml⁻¹ ChTX for 20 h at 30°C (the activity of ChTX is impaired at temperatures above 30°C (Gill & Meren, 1983)).

Labelled and washed fragments were divided into aliquots to give about 1 μg of protein/tube. Experiments were performed at 37°C with or without the appropriate concentration of CCh or AIF (to give 10 μM aluminium chloride and 10 mM sodium fluoride final concentrations). When the effects of AIF were studied the concentration of CaCl₂ in the KRB solution was reduced to 0.8 mM; this was to prevent the formation of insoluble CaF₂ detected at 2.4 mM Ca²⁺ (Blackmore et al., 1985; Paris & Pouyssegur, 1987). This decrease in Ca²⁺ did not affect agonist-induced stimulation of [3H]-inositol phosphates. At the end of the test period the samples were stopped, processed, assayed by anion-exchange h.p.l.c. within 7 days and the elution profiles of the labelled inositol phosphate samples determined as described in Prestwich & Bolton (1991). The protein concentration of the samples was determined using the method of Lowry et al. (1951).

KRB solution was passed throughout with 95% O₂, 5% CO₂ and had the following composition (final, mM): NaCl 120, KCl 5.9, NaHCO₃ 15.4, Na₂HPO₄ 1.2, glucose 11.5, MgCl₂ 1.2, CaCl₂ 2.5 and the pH was 7.25 when equilibrated.

**Materials**

PTX and ChTX were obtained from Boehringer. [3H]-inositol was obtained from Amersham International Ltd. [3H]-inositol contained a PT6 tablet which absorbed any radioisotopes present, which meant no purification of the label was necessary before use. All the other chemicals and drugs were obtained from either Sigma Chemical Co. Ltd or BDH.

**Data analysis**

Results are expressed as d.p.m. mg⁻¹ protein. The average protein concentration was 1.10 ± 0.02 mg/tube (n = 20). AIF stimulated responses were compared with control values and the results expressed as % change compared to control (except Figure 2).

Data are expressed as the mean ± s.e.mean of at least three experiments performed on different occasions. The data presented are paired, i.e. each control was performed on the same day as the test, using tissue from the same animal unless otherwise stated. The statistical significance was assessed by use of a Student's t test of pair differences. 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.). Time in the figures is plotted on a logarithmic axis because of the wide range of incubation times employed in the experiments.

The identity of the various [3H]-inositol phosphate isomers except for one of the [3H]-inositol biphosphates, was determined by comparing their elution profiles from the h.p.l.c. with those of labelled standards (see Prestwich & Bolton, 1991). From the elution profiles of other workers, the other [3H]-inositol biphosphate is likely to be inositol (3,4) biphosphate.

**Results**

**Muscarinic AChR activation**

Addition of the stable muscarinic agonist carbachol (CCh) to fragments labelled with [3H]-inositol resulted in time- and concentration-dependent increases in the levels of all the [3H]-inositol phosphates measured (Figures 1 and 2) with an EC₅₀ of about 10 μM (Figure 3). In the presence of CCh (100 μM) a significant increase in the level of [3H]-Ins(1,4,5)P₃ was seen at the earliest time point measured, 2s (223 ± 27% above control, P<0.05) this peaked at 10 s (437 ± 54% above control, P<0.05) and was followed by a decline towards basal values (Figure 1). The [3H]-Ins(1,4,5)P₃ isomer rapidly increased upon stimulation with CCh and the increase was greatest at 30 s (651 ± 49% above control, P<0.01) and was less at later times. The levels of the other isomers measured rose more slowly than [3H]-Ins(1,4,5)P₃ and were still increasing at the end of the 5 min incubation period. The response of total [3H]-inositol phosphates to 100 μM CCh was reduced in the presence of 100 nM atripine by 45.8 ± 9.6% (n = 6) and by 95 ± 3.2% (n = 6) in the presence of 500 nM atropine.

**Effect of aluminium fluoride**

Incubation of smooth muscle fragments with AIF (10 mM sodium fluoride plus 10 μM aluminium chloride) from 20 s up to 30 min resulted in increases in the levels of the [3H]-inositol phosphates (Figure 1) but the increases in the isomers were less than seen with 100 μM carbachol except in the case of [3H]-Ins(1,4,5)P₃ and [3H]-Ins(1,4)P₂. AIF caused a significant increase in [3H]-Ins(1,4,5)P₃ levels within 20 s (44 ± 7%, P<0.05) and also at 60 s (69 ± 9% above control, P<0.01). By 5 min the levels were returning towards basal (25 ± 7% above control) but still significantly increased (P<0.05); by 10 min the levels of [3H]-Ins(1,4,5)P₃ had almost returned to basal values (10 ± 3% above control) and by 30 min incubation with AIF, the levels were less than basal values (Figure 1). A similar profile was seen for the [3H]-inositol tetrakisphosphate isomers (55 ± 6% above control at 60 s, P<0.01) and by 5 min the levels were returning towards basal.
Figure 1 Comparison of [3H]-inositol phosphate levels in the presence of AlF or carbachol (CCh). [3H]-inositol-labelled smooth muscle fragments were incubated for 20 s, 1 min, 5 min, 10 min or 30 min in the presence of AlF (10 mM NaF plus 10 μM AICAR) (■) or 2 s, 5 s, 10 s, 30 s, 1 min, 2 min or 5 min in the presence of 100 μM CCh (●). Results represent the mean ± S.E.M (where it exceeds symbol width) of three experiments performed on separate occasions and are expressed as the % change in [3H]-inositol phosphates compared to control where the control was in the absence of AlF or CCh. These experiments were not paired. Increases in the levels of [3H]-inositol monophosphates, (InsP1); [3H]-inositol (1,4)bisphosphate (Ins(1,4)P2); [3H]-inositol (3,4) bisphosphate (sInsP2); [3H]inositol (1,3,4) trisphosphate, (Ins(1,3,4)P3); [3H]-inositol (1,4,5) trisphosphate, (Ins(1,4,5)P3) and [3H]-inositol tetrakis-phosphates (InsP4) are shown.

Figure 2 The effect of AlF (10 mM sodium fluoride and 10 μM aluminium chloride) on carbachol (CCh)-induced increases in [3H]-inositol phosphate levels. [3H]-inositol-labelled smooth muscle fragments were stimulated with 100 μM CCh for 10 s, 60 s or 5 min in the absence (●) or in the presence of AlF added 5 min (■) or 30 min. (△) before CCh. Results represent the mean ± S.E.M (where it exceeds symbol width) of three experiments performed on separate occasions and are expressed as absolute d.p.m. mg⁻¹ protein. See also legend to Figure 1.
(34 ± 3% above control, *P* < 0.05) and by 30 min the levels had returned to basal. However, AIF caused a gradual increase in the other [3H]-inositol phosphates. These increased very slowly over the first 5 min incubation, then increased more rapidly and were still increasing at the end of the 30 min incubated period. [3H]-inositol monophosphates increased 43 ± 4% above control by 60 s (*P* < 0.01) and 953 ± 111% above control by 30 min (*P* < 0.01). The [3H]-inositol (1,4) bisphosphate increased 45 ± 7% above control at 60 s (*P* < 0.01) and 323 ± 38% above control by 30 min (*P* < 0.01). The increase seen in the [3H]-inositol (1,3,4) trisphosphate isomer was 84 ± 7% above control by 60 s (*P* < 0.01) and 403 ± 58% above control by 30 min, (*P* < 0.01). A comparison between the time courses of increases of [3H]-inositol phosphates in the presence of CCh (100 μM) and AIF (Figure 1) shows, however, that the increases in [3H]-inositol phosphates in response to AIF were much slower than for CCh.

The effect of several concentrations of NaF on this muscle have been studied (Watson *et al.*, 1988) so we chose to study the effects of a maximally effective concentration at numerous time points to follow progressive activation of G-protein effects on inositol phosphates levels in these experiments. Incubation with AIF for 1 min prior to addition of 10 or 100 μM CCh for 10 s reduced the CCh-induced increases in the levels of [3H]-InsP(1,4,5)P3 by 8.4 ± 2.3% (not significant) but significantly reduced all the other [3H]-inositol phosphates by an average of 56 ± 4.4 (*P* < 0.05). However, when fragments were incubated for 5 min or 30 min in the presence of AIF and 100 μM CCh added either for 10 s, 60 s or 5 min, the CCh-induced increases in the levels of all the [3H]-inositol phosphates including [3H]-Ins(1,4,5)P3 were almost completely inhibited at each time point by an average of 86 ± 2.5% for a 5 min incubation with AIF and 97 ± 1.2% for a 30 min incubation with AIF (*P* < 0.01) (Figure 2). It will be seen that the effects of AIF on all inositol phosphate isomers levels were less when 5 min incubation was used than the effects of CCh (100 μM) after 5 min incubation (Figure 1).

Application of AIF for 5 min increased the measured levels of labelled inositol phosphates and 30 min incubation produced further increases in all inositol phosphates measured except [3H]-Ins(1,4,5)P3 and [3H]-InsP2 (Figure 1). Although there was no increase in basal levels of these two isomers upon 30 min incubation with AIF, the effects of CCh on these were completely inhibited; 5 min incubation with AIF produced substantial reduction in the effect of CCh on [3H]-Ins(1,4,5)P3 (Figure 2). In the case of the levels of [3H]-Ins(1,4)P2 and [3H]-Ins(1,3,4)P2, modest rises were produced by AIF, less than the effects of CCh alone, but nevertheless the action of CCh was still abolished. In the case of [3H]-InsP3 the effect of incubation with AIF for 5 min was comparable to, and the effect of 30 min much greater than, the effect of CCh alone and incubation with AIF completely prevented (30 min AIF) or substantially reduced (5 min incubation) the effects of CCh.

**Pertussis and cholera toxin treatment**

The increases in the levels of all the [3H]-inositol phosphates observed following a 10 s stimulation with increasing concentrations of CCh were significantly reduced after PTX treat-

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**Figure 3** The effects of pertussis toxin (PTX) and cholera toxin (ChTX) treatments on carbachol (CCh)-induced increases in [3H]-inositol phosphate levels. [3H]-inositol-labelled smooth muscle fragments were incubated in either normal KRB or KRB with 6 μg ml⁻¹ PTX or 5 μg ml⁻¹ ChTX for 20 h at 30°C. At the end of the incubation period each set of fragments was washed and divided and the increases in the levels of [3H]-inositol phosphates in the presence of increasing concentrations of CCh for 10 s was determined. The symbols represent the effect of CCh with no treatment (Δ) after treatment with PTX (■) and after treatment with ChTX (▲). Results represent the mean ± s.e.mean (where it exceeds symbol width) 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. PTX and ChTX had no significant effect on basal [3H]-inositol phosphate production. Statistical analysis comparing the increases in labelled inositol phosphates in the presence of CCh alone and after treatment with PTX are shown in the figure and represent the following: *P* < 0.05; **P* < 0.01.
Application of muscle. The actual concentration of phosphates (basal d.p.m. mg\(^{-1}\) protein for \(^{3}H\)-Ins(1,4,5)\(P_3\), in control fragments was 976 ± 8 and in PTX treatment fragments was 884 ± 140, and for total \(^{3}H\)-inositol phosphates this was 26592 ± 3758 and 290004 ± 4202 without and with PTX treatment respectively). The peak response to \(^{3}H\)-Ins(1,4,5)\(P_3\) still occurred at 10 s but the % stimulation after treatment with PTX was significantly less (258 ± 82%) compared to the response in fragments which had not been treated (516 ± 49%, \(P<0.01\)) (Figure 4). Despite the fact that cells were incubated with a high concentration of PTX (6 \(\mu\)g ml\(^{-1}\)) and for a long period (20 h) part of the CCh-induced increases in \(^{3}H\)-inositol phosphate levels was PTX-insensitive. This indicates that a PTX-insensitive G-protein or a G-protein-independent mechanism is involved in mediating part of the CCh-induced increases in the levels of \(^{3}H\)-inositol phosphates. In contrast to PTX, treatment with ChTX had no effect on either the basal levels of \(^{3}H\)-inositol phosphates or on CCh-induced increases in \(^{3}H\) inositol phosphates levels (Figure 3).

Discussion

Application of \(\mu\)M concentrations of CCh results in a rapid and transient increase in Ins(1,4,5)\(P_3\) levels in this smooth muscle. The Ins(1,4,5)\(P_3\) causes Ca\(^{2+}\) release from the stores (Krogsgaard-Larsen et al., 1991) in the sarcoplasmic reticulum and this contributes to the initial transient phase of contraction. The actual concentration in the muscle of Ins(1,4,5)\(P_3\) is determined by the balance between synthesis and degradation. Thus the rapid decay of the Ins(1,4,5)\(P_3\) signal could be due either to a slowing of the initially elevated rate of synthesis of Ins(1,4,5)\(P_3\) or to an accelerated removal by phosphorylation and dephosphorylation. One feature of the muscarinic AChR-induced Ins(1,4,5)\(P_3\) response is that, even when the agonist is continuously present, the intracellular Ins(1,4,5)\(P_3\) levels peak and then start to return towards basal levels within a short period of time. Although phosphorylation by one or more specific kinases may play a major role in this desensitization, feedback mechanisms involving G-protein subunits may also contribute to the termination or slowing down of the signalling response.

Aluminium fluoride

AIF has been shown to increase [Ca\(^{2+}\)]\(_{i}\), increase Ca\(^{2+}\) influx, deplete phosphatidylinositol (4,5) bisphosphate and increase Ins(1,4,5)\(P_3\) levels (Blackmore et al., 1985). This suggests that AIF can mimic the effects of Ca\(^{2+}\)-mobilizing agents by activating a G-protein involved in the coupling of receptors to PLC-mediated hydrolysis of phosphatidylinositol (4,5) bisphosphate.

The concentration of AIF used in this study (10 mM sodium fluoride plus 10 \(\mu\)M aluminium chloride) has previously been shown on single cells from longitudinal smooth muscle of the small intestine to abolish the transient outward currents probably caused by spontaneous release of calcium from stores within 2 min of application in a similar way to that of GTP\(\gamma\)S and CCh (Bolton & Lim, 1989). This suggests it causes a depletion of calcium from the internal stores via Ins(1,4,5)\(P_3\) release in these cells. Using longitudinal smooth muscle from the small intestine of the guinea-pig, NaF was found to cause a concentration-depen-

![Figure 4](image-url)
dent increase in the levels of total inositol phosphates (although lithium was present and only total inositol phosphates were measured at one time point of 20 min) and this was maximal at 10 mM NaF. No further increase in PLC was produced by the addition of AICl₃, suggesting that contamination with Al³⁺ ions was sufficient to activate inositol phosphate formation maximally when NaF was added alone (Watson et al., 1988). AICl₃ with 10 mM NaF has also been shown to result in maximal increases in inositol phosphates in other smooth muscle cells (Marc et al., 1988).

Although the muscarinic AChR-stimulation of [H]-inositol phosphate levels occurs within seconds, the AIF response is much slower, taking several minutes before increases could be seen in most of the isomers (Figure 1). Such a slow or gradual development of the response has been observed by others in several cell types (Matzoki et al., 1988; Baukal et al., 1988) and in smooth muscle cells (Marc et al., 1988). The increases in the levels of the [H]-inositol phosphates were generally smaller with AIF than with CCh and after 10 min incubation only the [H]-inositol monophosphate isomer levels and [H]-Ins1P₃ reached a value close to that of CCh at 5 min. The peak increase in the levels of [H]-Ins(1,4,5)P₃ and [H]-Ins(1,4,5)P₅ at 60 s in the presence of AIF are only 69 ± 9% above control compared to 437 ± 54% at 10 s and 308 ± 19% at 60 s in the presence of CCh (Figure 1). However, although AIF may act by stimulating a G-protein linked to [H]-phosphatidylinositol(4,5)bisphosphate hydrolysis, it also may have other effects which inhibit phosphatidylinositol(4,5)bisphosphate hydrolysis with time such as inhibition of the kinases that convert phosphatidylinositol(4)phosphate to phosphatidylinositol(4,5) bisphosphate (Chang et al., 1990) resulting in a depletion of the (labelled) phosphatidylinositol(4,5) bisphosphate pool or inhibition of ATP hydrolysis (Biffen & Martin, 1989). The response of Ins(1,4,5)P₃ to AIF may then decrease whereas increases in lower inositol phosphates may still occur.

AIF inhibited the CCh-induced increase in [H]-inositol phosphate levels (Figure 2). This inhibition was evident when the absolute increases in d.p.m. mg⁻¹ protein in the presence of CCh alone were compared with the absolute increases with CCh in the presence of AIF. AIF alone had modest and slowly developing effects on [H]-Ins(1,4,5)P₃ and [H]-Ins(1,4,5)P₅ (Figure 1) but severely inhibited the effects of CCh (100 µM) on these; it also inhibited the effects of CCh on [H]-Ins(1,4,5)P₃ and [H]-Ins(1,4,5)P₅ after 5 min incubation (Figure 2) despite having a much smaller effect on the Ins(1,4,5)P₃ response than CCh (Figure 1). Only in the case of [H]-Ins(1,4,5)P₅ did AIF alone have a comparable effect to CCh alone; again AIF blocked the effect of CCh. It may be that depletion of phosphatidylinositol(4,5) bisphosphate (Biffen & Martin, 1989; Claro et al., 1990) in AIF prevents much action of PLC to form higher inositol phosphate isomers either when AIF is present alone or when CCh is added in addition. However, persistent action of PLC on phosphatidylinositol may allow the formation of [H]-Ins(1,4,5)P₅ in increasing amount throughout 30 min incubation with AIF although CCh may under these conditions be unable to cause further increases in PLC activity. However, if AIF stimulates PLC maximally in this way, it does not explain why it does not increase [H]-Ins(1,4,5)P₅ at early times although CCh does so.

Inhibition is perhaps better explained if AIF stimulated an inhibitory G-protein linking muscarinic AChRs to PLC as suggested by other workers (Godfrey & Watson, 1988; Geet et al., 1990). A 1 min incubation with AIF (which causes a small, but its maximal, stimulation of [H]-Ins(1,4,5)P₅) had no significant effect on the CCh-induced increases in the levels of [H]-Ins(1,4,5)P₅ but inhibited the increased incorporation of all other [H]-inositol phosphates. This may indicate the existence of both stimulatory and inhibitory G-proteins, such that, with longer incubation in AIF, inhibitory G-protein effects become apparent which block the action of CCh. Released βγ subunits combining with α subunits due to the non-selectivity of AIF or a direct effect of the βγ subunit on the PLC enzyme itself may also occur and both inhibition of agonist-stimulated inositol phosphate production (Moriarty et al., 1989; Boyer et al., 1989) and stimulation of the PLC by AIF have recently been identified (Clapham & Neer, 1993). Other mechanisms can be envisaged. With time the levels of the [H]-inositol phosphates other than [H]-Ins(1,4,5)P₅ continue to increase and the levels of 1,2-diacylglycerol will continue to rise resulting in the activation of protein kinase Cs, which may act as negative feedback regulators (Katada et al., 1985; Olianas & Onali, 1993).

Toxin treatment

The results using ChTX (Figure 3) suggest that G₄α, the βγ subunits produced from activation of Gs, or the increases in cyclic AMP that would be expected to occur following ChTX treatment are not involved in muscarinic AChR activated inositol phospholipid hydrolysis. However, PTX did partially inhibit the CCh-induced [H]-inositol phosphate production by 53% at 2 s and by 50% at 10 s for [H]-Ins(1,4,5)P₅. Komori et al. (1992) showed that although incubation with PTX (6 µg ml⁻¹ for 6 h) inhibited the agonist-induced cationic current, CCh-induced release of Ca²⁺ from stores was not affected (which has been shown in these cells to be due to an increase in Ins(1,4,5)P₃). This suggests that sufficient [H]-Ins(1,4,5)P₅ is still produced following PTX treatment to cause Ca²⁺ store release with high concentrations of CCh. Similar results have been found in myometrial cells (Phaneuf et al., 1993). Long incubations with PTX (20 h) may result in the ADP-ribosylation of a slowly riibosylated PTX-sensitive G-protein (Neylon et al., 1994) and down regulation of the β subunit of G-proteins, resulting in a non-specific inhibition of all G-proteins present (Watkins et al., 1989). Hence, although the PTX effects in the present study may be due to the presence of a G-protein, the altered component of the G-protein is not necessarily an identified PTX-sensitive α subunit.

In many of the studies where a PTX-sensitive G-protein has been shown to be involved in agonist-induced inositol phospholipid hydrolysis, the presence of only one G-protein has been assumed to link the receptor with the PLC. Partial inhibition of muscarinic AChR agonist-induced production of inositol phosphates has been observed in reconstitution studies (Ashkenazi et al., 1989; Lazareno et al., 1993). A PTX-sensitive muscarinic agonist-induced production of [H]-inositol phosphates has been observed in identifiably PLC-negative cells (Marc et al., 1990). This may suggest that certain PLC-negative cells are also PLC-negative cells which are insensitive to agonist stimulation of PLC.

In the present study both the PTX-sensitive and PTX-insensitive G-proteins may be linked to the M₄ receptors which are thought to be the predominant receptors involved with contraction in this smooth muscle. However M₂ receptors associated with PTX-sensitive G-proteins may also have a role in the agonist increased in the levels of inositol phosphates (Thomas et al., 1993). The identification of the muscarinic receptor subtype linked to the inositol phosphates response is not a trivial problem and although both M₂ and M₄ subtypes have been suggested to exist in this tissue, their individual roles in contraction via increases in inositol phosphates have been determined mainly by the use of antagonists which show only modest selectivity for any one subtype (Caulfield, 1993; Doods et al., 1994; Egger et al., 1994).

The identification of the G-protein α subunit linking the PTX-sensitive activation of PLC mediating muscarinic AChRs, and several other types of G-protein-coupled receptor has proved elusive although recently Ga₂ and Ga₃ have been shown to be coupled to transfected muscarinic AChR m₂ and the stimulation of PLC (Dell'Acqua et al., 1993).
Recent reports describing βy subunit activation of PLCβ2 raises the possibility that the PTX sensitive PLC activation in these experiments may be mediated by βy subunits (Clapham & Neer, 1993). Muscarinic AChR-expressing COS cells activate PLCβ2 only in the presence of Gβy, indicating that mAChR can initiate signalling cascades working through Gβy (Katz et al., 1992). However, different regions of PLCβ2 are activated by α and βy subunits and so it is possible that α and βy subunits may independently modulate a single PLCβ2 molecule at the same time. (Bong Lee et al., 1993). If some PLCβ2 subunits have abilities to stimulate or inhibit a particular effector, then the forms of βy associated with different G-proteins that interact with individual receptors will be an important determinant of agonist regulation.

**References**


**Conclusion**

The inhibitory effect of AIF on CCh-induced increases in labelled inositol phosphates may indicate the activation by AIF of an inhibitory G-protein although other possibilities cannot as yet be excluded. Muscarinic AChR responses are coupled to PLC via PTX-sensitive G-protein(s) and by a PTX-insensitive mechanism.

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