Inhibition of muscarinic receptor-induced inositol phospholipid hydrolysis by caffeine, β -adrenoceptors and protein kinase C in intestinal smooth muscle

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1 The effects of caffeine, isoprenaline, dibutyryl cyclic AMP, isobutylmethylxanthine (IBMX), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or 1-oleoyl-2-acetylgllycerol (OAG), (protein kinase C (PKC) activators), 2-methoxy verapamil (D600), thapsigargin and ryanodine on muscarinic acetylcholine receptor (AChR)-stimulated inositol phospholipid hydrolysis were studied in smooth muscle fragments from the longitudinal layer of the small intestine of the guinea-pig.

2 Incubation of the fragments with the muscarinic agonist, carbachol (CCh) (100 μ M) resulted in rapid increases in the levels of all the inositol phosphate isomers with maximal increases in the [3 H]-inositol (1,4,5) trisphosphate ([3 H]-Ins(1,4,5)P₃) isomer occurring 10 s following incubation.

3 The β -adrenoceptor agonist, isoprenaline (10 μ M) and dibutyryl cyclic AMP (10 μ M), a membrane permeant analogue of cyclic AMP both reduced the CCh stimulation, but not the basal levels of [3 H]-inositol phosphates. This inhibition by dibutyryl cyclic AMP was enhanced in the presence of the phosphodiesterase inhibitor, IBMX. CCh inhibited the isoprenaline-induced increases in the levels of cyclic AMP and this was via a pertussis toxin (PTX)-sensitive G-protein mechanism.

4 TPA (1 μ M) and OAG (100 μ M) a 1,2-diacylglycerol (DAG) analogue both reduced the CCh-induced increases in [3 H]-inositol phosphates levels but neither affected basal values nor the basal levels of cyclic AMP.

5 D600 (10 μ M), which blocks voltage-dependent Ca²⁺ channels, also reduced the CCh-stimulated levels of [3 H]-inositol phosphates suggesting that some of the agonist-induced increases are due to a potentiating effect of Ca²⁺ entering the cell.

6 Caffeine (0.5–30 mM) significantly inhibited both the basal and CCh-induced increases in all the [3 H]-inositol phosphate isomers. Its inhibitory action was not due to increases in cyclic AMP since caffeine had no effect on the levels of cyclic AMP at concentrations up to 30 mM.

7 Incubation with thapsigargin (1 μ M) and ryanodine (10 μ M) had no effect on either basal or CCh-induced inositol phospholipid hydrolysis or cyclic AMP levels.

8 The results indicate a reciprocal inhibition by β -adrenoceptors and muscarinic AChRs of their effects on cyclic AMP and inositol phosphate levels respectively. Ca²⁺ entering the cell (but not the action of ryanodine or thapsigargin) potentiates while caffeine inhibits muscarinic AChR-induced rises in inositol phosphate levels. Diacylglycerols may exert a negative feedback inhibition on inositol phosphate production.

Keywords: Smooth muscle; inositol phosphates; caffeine; isoprenaline; protein kinase C; 2-methoxy verapamil (D600); cyclic AMP; pertussis toxin.

Introduction

Activation of muscarinic acetylcholine receptors (AChRs) on the longitudinal smooth muscle layer from the small intestine of the guinea-pig increases the activity of phospholipase C (PLC) via a G-protein causing inositol(1,4,5)trisphosphate (Ins(1,4,5)P₃) levels to increase (Best & Bolton, 1986; Prestwich & Bolton, 1994). The Ins(1,4,5)P₃ binds to its receptor on the sarcoplasmic reticulum (Ehrlich & Watras, 1988) which serves as a Ca²⁺ release channel and Ca²⁺ is released from stores down its concentration gradient into the cell; this contributes to the initial transient phase of contraction. Also, Ca²⁺-induced Ca²⁺ release may be activated via ryanodine receptors on the sarcoplasmic reticulum membrane. Muscarinic AChR activation also causes receptor operated channels in the plasma membrane to open, allowing the influx of cations (mainly Na⁺) (Bolton, 1979) and this activation is potentiated by Ca²⁺ released from the sarcoplasmic reticulum stores (Pacaud & Bolton, 1991). The resulting change in membrane potential triggers the opening of voltage-dependent Ca²⁺ channels resulting in Ca²⁺ influx and this contributes to the sustained phase of contraction.

Most smooth muscle relaxation is thought to occur by lowering the [Ca²⁺]_i and by dephosphorylation of myosin light chain (Gerthoffer & Murphy, 1983). Low levels of [Ca²⁺]_i are achieved following contraction by several mechanisms, reuptake of Ca²⁺ into the sarcoplasmic reticulum via Ca²⁺ ATPase, increased extrusion by the plasma membrane Ca²⁺ ATPase, Na⁺-Ca²⁺ exchange, and by the closure of voltage-operated calcium channels which occurs when the membrane potential is restored or when the membrane hyperpolarizes. An increase in [Ca]_i results in activation of Ca²⁺-dependent K⁺ channels which open causing the membrane to hyperpolarize and this helps to restore the membrane potential towards the resting level. Hyperpolarization caused by increases in K⁺ channel activity decreases the opening of the voltage-dependent Ca²⁺ channels that control Ca²⁺ influx and contributes to relaxation. Some of these mechanisms may also contribute to the switching off of inositol phospholipid hydrolysis and thus contribute to relaxation (Quast, 1993).

Hydrolysis of inositol phospholipids produces a bifurcating pathway and 1,2-diacylglycerol (DAG) is produced at the same time as the inositol phosphates (Rana & Hokin, 1990).

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The DAG (in conjunction with phosphatidylserine in the membrane) activates Ca^{2+} -dependent protein kinase Cs (PKCs) leading to phosphorylation of proteins (Nishizuka, 1992). PKC may be involved in the tonic component of the contractile response (Rasmussen *et al.* 1988). At the same time PKC may serve as a negative feedback regulator of inositol phospholipid hydrolysis thereby switching off not only the increases in inositol phosphates but also increases in DAG.

Application of caffeine to smooth muscle strips results in a transient contraction by releasing Ca^{2+} from intracellular stores (Matsumoto *et al.*, 1990). The mechanism is independent of $\text{Ins}(1,4,5)\text{P}_3$ release, since the effect of caffeine is not inhibited by heparin which prevents the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor (Komori & Bolton, 1991). In addition to these contractile effects, caffeine has potent relaxing ability on precontracted smooth muscles (Karaki *et al.*, 1987); it inhibits voltage-dependent Ca^{2+} channels (Hughes *et al.*, 1990); it causes hyperpolarization of the membrane (Van der Bent & Bény, 1991) and inhibits phosphodiesterase activity resulting in increases in the levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Butcher & Sutherland, 1962). Inhibition of $\text{Ins}(1,4,5)\text{P}_3$ levels have also been observed (Toescu *et al.*, 1992).

Ryanodine and thapsigargin also cause Ca^{2+} release from sarcoplasmic reticulum stores but by different mechanisms to caffeine. Ryanodine causes Ca^{2+} store depletion by maintaining the Ca^{2+} release channel in a partially open state (Fleischer *et al.*, 1985) and thapsigargin prevents the reuptake of Ca^{2+} into the sarcoplasmic reticulum by inhibiting the Ca^{2+} -ATPase pump of the sarcoplasmic reticulum (not the plasma membrane pump) in smooth muscle (Shima & Blaustein, 1992).

β -Adrenoceptors have been identified on longitudinal smooth muscle cells from the small intestine (Broadley & Grassby, 1985) and are of the β_3 -adrenoceptor type (Van der Vliet *et al.*, 1990). Isoprenaline, a β -adrenoceptor agonist has been shown to cause relaxation in smooth muscle as a result of increased protein kinase A activity through increases in cyclic AMP levels (Bülbring & Tomita, 1987) due to activation of adenylyl cyclase via Gs (Iyengar & Birnbaumer, 1981). Activation of β -adrenoceptors has also been shown to result in hyperpolarization of the membrane (Sadoshima *et al.*, 1988), inhibition of Ca^{2+} influx into cells (Abe & Karaki, 1988) and activation of specific kinases (β ARKs) (Richardson *et al.*, 1993).

The aim of this study was to determine the effects of drugs that might modify the muscarinic AChR-activated inositol phospholipid pathway. To do this [^3H]-inositol-labelled smooth muscle fragments were incubated in the presence of substances which can activate PKC, increase cyclic AMP levels, decrease $[\text{Ca}^{2+}]_i$ levels or alter the membrane potential, prior to muscarinic AChR activation. Since several of the substances used have not been shown to elevate cyclic AMP in smooth muscle from guinea-pig small intestine, changes in the levels of cyclic AMP were also determined to see if observed effects could be attributed to increases in adenylyl cyclase activity.

Methods

Preparation and assay of [^3H]-inositol-labelled fragments

Guinea-pigs were killed by dislocation of the neck and exsanguinated and longitudinal muscle of the small intestine removed. Contaminating circular muscle was removed and fragments ($350 \times 350 \mu\text{m}$) were prepared by tissue chopping as previously described (Prestwich & Bolton, 1994). The assay for the determination of the levels of [^3H]-inositol phosphates was the same as described by Prestwich & Bolton

(1991, 1994). Briefly, chopped fragments 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 [^3H]-inositol and kept in a humidified atmosphere containing 95% O_2 /5% CO_2 at 30°C for 20 h. When treated with toxin the fragments were incubated with pertussis toxin (PTX) $6 \mu\text{g ml}^{-1}$ for 20 h at 30°C . Functional responses of contraction of the fragments to CCh could still be observed after this long incubation period.

For the assay, the tissue (about 1 mg ml^{-1}) was incubated with or without the drug at 37°C . At the end of the incubation, $500 \mu\text{l}$ 20% trichloroacetic acid and $50 \mu\text{l}$ 50 mM EDTA were added to stop the assay. The trichloroacetic acid was extracted from the supernatant with water-saturated diethyl ether ($4 \times 5 \text{ ml}$); the residual ether was removed and the pH of the samples adjusted to 7.0 with 1 M NaOH ($5 \mu\text{l}$). Protein determination was done on the pellet using the method of Lowry *et al.* (1951). The elution profiles of the labelled inositol phosphate samples were assayed by anion-exchange high performance liquid chromatography (h.p.l.c.) within 7 days using a method modified from Irvine *et al.* (1985) and Batty *et al.* (1985) and described in Prestwich & Bolton (1991). The identity of the various [^3H]-inositol phosphate isomers 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: [^3H]-inositol monophosphates ([^3H]-inositol (1) phosphate and [^3H]-inositol (4) phosphate) (InsP_1); [^3H]-inositol (1,4) bisphosphate ($\text{Ins}(1,4)\text{P}_2$); [^3H]-inositol (1,3,4) trisphosphate ($\text{Ins}(1,3,4)\text{P}_3$); [^3H]-inositol (1,4,5) trisphosphate, ($\text{Ins}(1,4,5)\text{P}_3$) and [^3H]-inositol tetrakisphosphates (InsP_4). From the elution profiles obtained by other workers, the other [^3H]-inositol bisphosphate which is labelled s InsP_2 in the figures, is likely to be [^3H]-inositol (3,4) bisphosphate. After samples had been expressed as d.p.m. mg^{-1} protein, the agonist stimulated responses were compared with control values and the results expressed as % change compared to control. The effects the various agents had on basal and CCh-stimulated [^3H]-inositol phosphate formation were usually determined after a 10 min incubation at 37°C with the agent unless otherwise stated. Under these conditions CCh ($100 \mu\text{M}$) was added 10 s before the end of the 10 min incubation period.

Cyclic AMP determination

Smooth muscle fragments were prepared and incubated as previously described (Prestwich & Bolton, 1991) when measuring [^3H]-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 with isoprenaline. Therefore for the cyclic AMP measurements the fragments were incubated in KRB containing 1 mM IBMX. After incubation with or without drug (for 10 min) with and without CCh, ($100 \mu\text{M}$) 10 s before the end, the reaction was stopped with 10% (final) trichloroacetic acid. The trichloroacetic acid was removed by diethyl ether extraction, 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. The results are expressed as either pmol mg^{-1} protein or % change compared to control.

Materials

1-Oleoyl-2-acetyl glycerol (OAG), ryanodine, thapsigargin, 12-O-tetradecanoylphorbol-13-acetate (TPA) and pertussis toxin (PTX) were obtained from Calbiochem. [^3H]-inositol and the [^3H]-cyclic AMP assay kit were obtained from Amersham International Ltd. [^3H]-inositol contained a PT6 tablet which

absorbed any radiolysis products, 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

The average protein concentration in mg/tube was 1.1 ± 0.02 ($n = 20$). The data are expressed as the mean $\pm$ s.e. mean % change over control of at least three paired experiments performed on different occasions. Intra-assay variance was very small and highly significantly less ($P < 0.001$) than the variance between guinea-pigs. Tests of pair differences from zero were therefore applied. The statistical significance was assessed by use of a Student's t test. P values that were less than 0.05 were considered to be significant. Calculations were performed using the computer programme INSTAT (Graph-PAD software, U.S.A.).

Results

It was found previously, using the experimental protocol adopted here, that the maximum increase in the [3 H]-Ins(1,4,5) P_3 isomer during muscarinic AChR activation with a concentration of $100 \mu\text{M}$ CCh occurs at 10 s following application and thereafter the level returns towards the basal value (Prestwich & Bolton, 1994). Also, Konno & Takayanagi (1989) showed that $100 \mu\text{M}$ CCh was the concentration required to obtain a maximal release of Ca^{2+} from the sarcoplasmic reticulum for the guinea-pig ileum at 37°C . Therefore drugs were usually applied for the 10 min prior to application of $100 \mu\text{M}$ CCh for 10 s to determine the effects on CCh-induced increases in [3 H]-inositol phosphates.

Caffeine

Incubation of the smooth muscle fragments with 20 or 30 mM caffeine for 5 or 10 min caused a time-dependent decrease in the basal levels of all [3 H]-inositol phosphates (Figure 1). However, 1 min after application of caffeine a very small increase in the basal levels of all of the [3 H]-inositol phosphates except [3 H]-Ins P_1 and [3 H]-sIns P_2 occurred when compared to control. A 10 min incubation in either 20 or 30 mM caffeine significantly reduced basal levels of all the [3 H]-inositol phosphates (Figure 1). Concentrations of caffeine of 3 mM or more have been shown to release Ca^{2+} from intracellular stores; however, when smooth muscle fragments were incubated with different concentrations of caffeine from 0.5 mM to 20 mM for 10 min, although a small increase in levels of some [3 H]-inositol phosphates was seen at the lower concentrations of caffeine (0.5–1 mM), at higher concentrations (10 and 20 mM) there was a concentration-dependent decrease in basal levels of [3 H]-inositol phosphates (Figure 2). These effects of caffeine were not altered in the presence of $0.5 \mu\text{M}$ atropine, a concentration which inhibited more than 95% of the CCh-stimulated increases in the levels of the [3 H]-inositol phosphates in these fragments.

The presence of increasing concentrations of caffeine from 0.5 mM to 20 mM, prior to addition of the CCh, significantly inhibited in a concentration-dependent manner the CCh-induced increases (10 s, $100 \mu\text{M}$) in the levels of [3 H]-inositol phosphates (Figure 3). A 10 min incubation in the presence of 0.5 mM caffeine inhibited the CCh-induced production of [3 H]-Ins(1,4,5) P_3 by 34% and a 10 min incubation with 20 mM caffeine the CCh-induced production of all the [3 H]-inositol phosphates by $\geq 95\%$ (Figure 3).

Ryanodine ($10 \mu\text{M}$, 10 min) or thapsigargin ($1 \mu\text{M}$, 10 min) at concentrations shown to deplete Ca^{2+} stores in smooth muscle (Hisayama *et al.* 1990; Low *et al.* 1991) had no effect

Figure 1 The effect of incubation in caffeine for different times on basal levels of [3 H]-inositol phosphates. [3 H]-inositol-labelled smooth muscle fragments were incubated with 20 mM caffeine for 1 min (1), 5 min (2) or 10 min (3) or incubated with 30 mM caffeine for 1 min (4), 5 min (5) or 10 min (6). Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [3 H]-inositol phosphates compared to control where the control is in the absence of caffeine. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2 The effect of caffeine at various concentrations on basal levels of [³H]-inositol phosphates. [³H]-inositol-labelled smooth muscle fragments were incubated with 0.5 mM (1), 1 mM (2), 3 mM (3), 10 mM (4), and 20 mM (5) caffeine for 10 min. Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [³H]-inositol phosphates compared to control where the control is in the absence of caffeine.

Figure 3 The effect of caffeine on the carbachol (CCh)-induced increases in [³H]-inositol phosphates. [³H]-inositol-labelled smooth muscle fragments were incubated in the presence of different concentrations of caffeine for 10 min. CCh (100 μ M) was added for 10 s before the end and the reaction stopped. Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [³H]-inositol phosphate compared to control and except when caffeine was absent, the values were calculated from the formula $((Y - X)/X) \times 100\%$ where X is the d.p.m. in the presence of caffeine alone and Y is the d.p.m. in the presence of caffeine and CCh. When caffeine was absent Y and X are the d.p.m. in the presence and absence of CCh respectively.

on either the basal ($6.3 \pm 2.6\%$ change compared to control) or CCh-induced increase in the levels of [3 H]-inositol phosphates.

β -Adrenoceptor activation

Incubation with the β -adrenoceptor agonist, isoprenaline ($10 \mu\text{M}$, 10 min) had no significant effect on the basal levels of [3 H]-inositol phosphates ($5.3 \pm 3.4\%$ decrease compared to control). However, incubation with isoprenaline prior to application of CCh ($100 \mu\text{M}$) for 10 s resulted in a significant decrease in the CCh-induced increases in the levels of all the [3 H]-inositol phosphates compared to CCh alone (Figure 4). Since isoprenaline increases the levels of cyclic AMP in smooth muscle and other tissues (Iyengar & Birnbaumer, 1981) fragments were incubated with a cyclic AMP analogue, dibutyryl cyclic AMP ($10 \mu\text{M}$, 10 min) under the same conditions as for isoprenaline and this also reduced the CCh-stimulation of [3 H]-inositol phosphates by almost the same amount (Figure 4). IBMX (1 mM) alone caused a small inhibition of the CCh effects on labelled inositol phosphates (Figure 4). When smooth muscle fragments were incubated in the presence of dibutyryl cyclic AMP ($10 \mu\text{M}$) plus IBMX (1 mM) the response of most isomers to CCh was almost completely inhibited (Figure 4).

Protein kinase C

To determine whether increased PKC activity, which may occur following muscarinic AChR activation and inositol phospholipid hydrolysis, could affect the CCh-induced increases in the levels of the [3 H]-inositol phosphates, fragments were incubated with the phorbol ester, TPA. Incubation of

smooth muscle fragments for 10 min with TPA ($1 \mu\text{M}$) had no effect on basal release of [3 H]-inositol phosphates (data not shown) but significantly inhibited the time-dependent increases in the levels of [3 H]-inositol phosphates seen in the presence of CCh ($100 \mu\text{M}$) (Figure 5) (at least $P < 0.05$ at all time points). A 10 min incubation with TPA prior to application of CCh for 10 s, also significantly inhibited increases in the levels of all the [3 H]-inositol phosphates produced by 0.1 – $100 \mu\text{M}$ CCh (Figure 6). When smooth muscle fragments were incubated for 2 h with TPA ($1 \mu\text{M}$) prior to addition of CCh, the inhibition of CCh-induced increases in levels of [3 H]-inositol phosphates was greater (Table 1). Incubation with the DAG analogue, OAG ($100 \mu\text{M}$), for either 10 min or 2 h resulted in similar inhibitions of CCh-induced increases in the levels of [3 H]-inositol phosphates as seen with TPA (Table 1).

2-Methoxyverapamil (D600)

Incubation of the smooth muscle fragments with the voltage-dependent Ca^{2+} channel antagonist D600 ($10 \mu\text{M}$) for 10 min prior to addition of CCh ($100 \mu\text{M}$) significantly reduced the early (2 s to 120 s) CCh-stimulated increases in the levels of all the [3 H]-inositol phosphates (Figure 7). The peak CCh-induced response of [3 H]-Ins(1,4,5) P_3 was significantly inhibited by 41% at 10 s ($P < 0.05$) and by 62% at 2 min ($P < 0.05$).

Cyclic AMP measurements

IBMX (1 mM , 10 min) increased the basal levels of cyclic AMP from $2.01 \pm 0.26 \text{ pmol mg}^{-1}$ protein to $36 \pm 2.7 \text{ pmol mg}^{-1}$ protein ($n = 5$). Changes in basal cyclic AMP levels

Figure 4 The effect of isobutylmethylxanthine (IBMX), isoprenaline, dibutyryl cyclic AMP and dibutyryl cyclic AMP with IBMX on levels of [3 H]-inositol phosphates in carbachol (CCh). [3 H]-inositol-labelled smooth muscle fragments were incubated for 10 min in the absence or presence of either isoprenaline, dibutyryl cyclic AMP or dibutyryl cyclic AMP with IBMX; 10 s before the end of the incubation period $100 \mu\text{M}$ CCh was added for 10 s and the reaction stopped. The histograms represent (1) CCh alone, (2) 1 mM IBMX plus CCh, (3) $1 \mu\text{M}$ isoprenaline plus CCh, (4) $10 \mu\text{M}$ dibutyryl cyclic AMP plus CCh, (5) $10 \mu\text{M}$ dibutyryl cyclic AMP with 1 mM IBMX plus CCh. Results represent the mean $\pm$ 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 and in the presence of drug. Isoprenaline, IBMX alone, dibutyryl-cyclic AMP and dibutyryl-cyclic AMP plus IBMX had no effect on the basal production of [3 H]-inositol phosphates (data not shown).

Figure 5 The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the time course of carbachol (CCh)-induced increases in the levels of [3 H]-inositol phosphates. [3 H]-inositol-labelled smooth muscle fragments were incubated for 10 min in the absence or presence of 1 μ M TPA. CCh (100 μ M) was added for either 5 s, 10 s, 30 s, 60 s or 120 s before the end of the incubation. Results are shown as the time-dependent increases in [3 H]-inositol phosphates in the presence of CCh alone (●) or in the presence of CCh plus TPA (□). TPA had no effect on the basal levels of [3 H]-inositol phosphates. Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [3 H]-inositol phosphates compared to control where the control is in the absence of CCh.

could not be detected in the presence of several different substances including isoprenaline, in the absence of IBMX. Therefore all experiments were performed with IBMX in the incubation buffer. Under these conditions isoprenaline (10 μ M) increased the levels of cyclic AMP by $335 \pm 43\%$ ($n = 5$). CCh (100 μ M, 10 min) had no significant effect on the basal levels of cyclic AMP (28.9 ± 6.7 pmol mg^{-1} protein) but significantly reduced the isoprenaline-induced increases by $44.8 \pm 5.3\%$ ($P < 0.05$, $n = 5$). Treatment with PTX (6 μ g ml^{-1} 20 h at 30°C) did not affect the basal levels of cyclic AMP but prevented the CCh-induced inhibition of isoprenaline-induced increases in cyclic AMP levels.

The effects of several other substances (under conditions where inhibition of CCh-induced increases in the levels of [3 H]-inositol phosphates had been observed) on the basal levels of cyclic AMP and in the presence of CCh (100 μ M) were measured. However, caffeine (up to 30 mM), TPA (1 μ M), D600 (10 μ M) and aluminium fluoride (10 μ M aluminium chloride and 10 mM sodium fluoride) had no effect on the basal levels of cyclic AMP in the presence or absence of IBMX and had no effect on the levels of cyclic AMP in the presence of CCh (100 μ M).

Discussion

Except for thapsigargin and ryanodine, the substances used in this study inhibited the CCh-induced increases in the levels of all the [3 H]-inositol phosphates. However, only caffeine, or dibutyryl cyclic AMP in the presence of IBMX, could completely inhibit the effects of CCh and only caffeine affected the basal levels of the [3 H]-inositol phosphates.

Depletion of Ca^{2+} from the stores is unlikely to account

for inhibition by caffeine of basal or agonist-induced changes in levels of [3 H]-inositol phosphates since neither thapsigargin nor ryanodine had any effects on [3 H]-inositol phosphate levels. The decrease in the basal levels of [3 H]-inositol phosphates by caffeine (Figures 1 and 2) may be due to its ability to inhibit PLC directly. However, the small increase in basal levels after 1 min incubation with caffeine may be due to the transient increase in $[\text{Ca}^{2+}]_i$ caused by the release of Ca^{2+} from sarcoplasmic reticulum stores. This small increase in $[\text{Ca}^{2+}]_i$ may be sufficient to increase the basal activity of PLC and may also be sufficient to activate Ca^{2+} -dependent K^+ channels in the plasma membrane which would result in hyperpolarization. This has recently been shown to inhibit agonist-induced inositol phospholipid hydrolysis (Yamagishi *et al.*, 1992; Yanagisawa *et al.*, 1993). Caffeine has been shown to induce a transient contraction in coronary artery smooth muscle followed by a sustained relaxation which occurs simultaneously with the relaxation caused by a hyperpolarization of the membrane (Van der Bent & Bény, 1991).

Isoprenaline causes hyperpolarization of smooth muscle through cyclic AMP-dependent protein kinase (Kume *et al.*, 1989) or directly via the α subunit of Gs (Scornik *et al.*, 1993). K^+ -channel opening drugs which have been shown to inhibit agonist-induced inositol phosphate increases (Yamagishi *et al.* 1992), also cause hyperpolarization of the membrane and relaxation which is independent of changes in cellular cyclic nucleotide concentrations (Berry *et al.* 1991). So, hyperpolarization may be a major mechanism by which various substances may inhibit CCh-induced inositol phospholipid hydrolysis.

Although caffeine has been shown to inhibit phospho-

Figure 6 The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on concentration-response curve to carchol (CCh). [^3H]-inositol-labelled smooth muscle fragments were incubated in the absence or presence of $1\text{ }\mu\text{M}$ TPA for 10 min; 10 s before the end of the incubation period either $0.1\text{ }\mu\text{M}$, $1\text{ }\mu\text{M}$, $10\text{ }\mu\text{M}$, or $100\text{ }\mu\text{M}$ CCh was added for 10 s and the incubation stopped. Results are shown in the presence of CCh alone (●), or CCh plus TPA (□). Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [^3H]-inositol phosphates compared to control where the control is in the absence of CCh.

diesterase activity (Butcher & Sutherland, 1962) thereby increasing the levels of cyclic AMP, this was not how caffeine inhibited the levels of [^3H]-inositol phosphates in this muscle. Under conditions where caffeine completely inhibited the CCh-induced increases in [^3H]-inositol phosphates it had no effect on the levels of cyclic AMP, when assayed either in the presence or absence of the phosphodiesterase inhibitor, IBMX. Thus, it may also be that caffeine can inhibit PLC activity directly.

However, when increases in the levels of cyclic AMP do occur, these may contribute to the observed inhibition of CCh-induced increases in the levels of [^3H]-inositol phosphates. Isoprenaline increased the levels of cyclic AMP in this study. Cyclic AMP has been shown to decrease $[\text{Ca}^{2+}]_i$ as a result of hyperpolarization due to stimulation of Ca^{2+} -activated K^+ channel opening (Sadoshima *et al.*, 1988), inhibition of Ca^{2+} influx into cells (Abe & Karaki, 1988), increased Ca^{2+} extrusion from cells through stimulation of the membrane Ca^{2+} -pump (Bülbring & den Hertog, 1980) and increased Ca^{2+} uptake into intracellular stores (Saida & Van Breemen, 1984). However, functional antagonism and crosstalk have been shown to occur following muscarinic AChR and β -adrenoceptor activation (Roffel *et al.*, 1993). The ability of isoprenaline to relax tone due to muscarinic AChR activation has been shown to be increased following treatment with PTX (Mitchell *et al.*, 1993), possibly due to loss of CCh inhibition of isoprenaline-induced increases in cyclic AMP following treatment with PTX. It is possible that the isoprenaline inhibition of CCh-induced inositol phospholipid hydrolysis may have been greater following PTX treatment, but this was not tested.

The membrane permeable analogue of cyclic AMP, dibutyryl cyclic AMP, also reduced CCh-stimulated [^3H]-inositol

phosphate levels. The addition of IBMX (1 mM) which is a phosphodiesterase inhibitor (Smellie *et al.* 1979) with dibutyryl cyclic AMP resulted in a further inhibition. This suggests that when the hydrolysis of endogenous cyclic AMP is inhibited, a further increase of cyclic AMP inside the cell (from dibutyryl cyclic AMP) is enough to completely inhibit increases in [^3H]-inositol phospholipid levels following CCh-induced stimulation. However, direct inhibition of PLC activity by phosphodiesterase inhibitors has also been shown (Hall *et al.*, 1990) and non specific effects of the dibutyryl moiety have been suggested to occur in smooth muscle (Bülbring & Hardman, 1975). IBMX is a non selective phosphodiesterase inhibitor and inhibits cyclic GMP-dependent phosphodiesterase as well. However, the levels of cyclic GMP were not measured in this study.

β -Adrenoceptor activation, however, also increases the activity of β -adrenoceptor kinases (βARKs) and in the presence of $\beta\gamma$ subunits they may phosphorylate the muscarinic AChR or the G-proteins involved in inositol phospholipid hydrolysis serving to uncouple the receptor from the G-protein (Richardson *et al.*, 1993). Other protein kinases exist distinct from $\beta\text{-ARK}$, PKC or other putative second messenger regulated kinases, which can phosphorylate the m3 AChR in CHO transfected cells (Tobin & Nahorski, 1993).

TPA can substitute for DAG in the activation of PKC even in the absence of increases in $[\text{Ca}^{2+}]_i$ (Castagna *et al.*, 1982) and inhibition of agonist-induced inositol phosphate formation in smooth muscle by phorbol esters has been shown (Olianas & Onali, 1993). Although in some studies activation of PKC has been shown to increase the levels of cyclic AMP (Katada *et al.*, 1985; Yoshimasa *et al.*, 1987) TPA had no effect on cyclic AMP levels in this study. Several

Table 1 Average percentage inhibition of the effects of a 10 s stimulation with 100 μ M carbachol (CCh) on [3 H]-inositol phosphate formation following (a) a 10 min incubation with 1 μ M TPA (b) a 2 h incubation with 1 μ M TPA (c) a 10 min incubation with 100 μ M OAG and (d) a 2 h incubation with 100 μ M OAG

	(a) 10 min TPA + CCh (n = 3)	(b) 2 h TPA + CCh (n = 2)	(c) 10 min OAG + CCh (n = 3)	(d) 2 h OAG + CCh (n = 2)
[3 H]-InsP ₁	55**	91	54*	84
[3 H]-Ins(1,4)P ₂	42**	72	23*	62
[3 H]-sInsP ₂	33*	52	25*	65
[3 H]-Ins(1,3,4)P ₃	53*	79	42*	74
[3 H]-Ins(1,4,5)P ₃	77**	92	55*	89
[3 H]-InsP ₄	76**	88	52*	86

* $P < 0.05$ and ** $P < 0.01$. For abbreviations, see text.

Figure 7 The effect of 2-methoxyverapamil (D600) on the carbachol (CCh)-induced increases in levels of [3 H]-inositol phosphates. [3 H]-inositol-labelled smooth muscle fragments were incubated in the absence or presence of 10 μ M D600 for 10 min; 100 μ M CCh was added at 2 s, 5 s, 10 s, 30 s, 60 s or 120 s before the end of incubation. Results are shown in the presence of CCh alone (●) or CCh plus D600 (□). Results represent the mean $\pm$ s.e. mean of three experiments performed on separate occasions and are expressed as the % change in [3 H]-inositol phosphates compared to control where the control is in the absence of CCh.

other effects of PKC activation could have contributed to the inhibition of the CCh effects: for example, phosphorylation of muscarinic AChRs resulting in desensitization of muscarinic AChR-mediated stimulation of inositol phospholipid hydrolysis and internalization of receptors (Orellana *et al.*, 1985; Creazzo & Wreen, 1988) or phosphorylation of PLC β (Ryu *et al.*, 1990). Phorbol esters have also been shown to inhibit agonist-induced contraction (Holzer & Lippe, 1989) and decrease CCh-induced increases in [Ca^{2+}]_i in several smooth muscles (Sasaguri & Watson, 1990; Mitsui & Karkai, 1993). It was suggested that this may occur via a direct inhibition of inositol phosphates or via activation of Na⁺-K⁺-ATPase resulting in membrane hyperpolarization and inhibition of voltage-dependent Ca²⁺ channels. However, the direct inhibition of Ca²⁺ channels by phorbol esters has also been reported (Sherman *et al.*, 1989).

Potential of the inositol phosphate responses due to Ca²⁺ influx (Sladeczek *et al.*, 1988) suggests that the effect of D600 on the levels of [3 H]-inositol phosphates measured

during CCh-stimulation could be via its block of voltage-dependent Ca²⁺ channels and thereby inhibition of Ca²⁺ influx. Therefore some of the inhibition of CCh-induced increases in the levels of [3 H]-inositol phosphates by caffeine, PKC, D600 and isoprenaline, may also result from the inhibition of voltage-dependent Ca²⁺ channels and of Ca²⁺ influx either directly or as a result of hyperpolarization. However in permeabilized portal vein, D600 has also been suggested to uncouple the G-protein/PLC interaction (Kobayashi *et al.*, 1991).

Previous studies suggested that a major mechanism regulating muscarinic AChR-induced increases in inositol phospholipid turnover in this smooth muscle was via G-protein α and $\beta\gamma$ subunits (Prestwich & Bolton, 1994). However changes in the levels of [Ca^{2+}]_i, membrane potential changes and alterations due to activation of protein kinases may also have important regulatory functions.

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