Early events in inositol phosphate metabolism in longitudinal smooth muscle from guinea-pig intestine stimulated with carbachol

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In longitudinal smooth muscle from guinea-pig intestine prelabelled with [^3H]inositol, carbachol produced a 3-fold increase in [^3H]inositol 1,4,5-trisphosphate within 2 s, and there was also a simultaneous increase in [^3H]inositol 1,4-bisphosphate. ^3H -labelling of inositol 1,3,4,5-tetrakisphosphate was not significantly increased until 60 s after carbachol stimulation, and the accumulation of [^3H]inositol 1,3,4-trisphosphate was relatively small.

INTRODUCTION

The early increase in intracellular calcium concentration that precedes the onset of neurotransmitter-evoked contraction of smooth muscle may be mediated in part by Ca^{2+} released from the sarcoplasmic reticulum by $\text{Ins}(1,4,5)\text{P}_3$ (for a review with special reference to smooth muscle see Abdel-Latif, 1986). This is supported by observations that $\text{Ins}(1,4,5)\text{P}_3$ can stimulate Ca^{2+} release and contraction in skinned vascular smooth muscle cells (Suematsu *et al.*, 1984; Yamamoto & van Breeman, 1985; Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986; Walker *et al.*, 1987). Occupation of muscarinic receptors by agonists is thought to result in $\text{Ins}(1,4,5)\text{P}_3$ generation through the modification of a GTP-binding protein which can in some way activate a $\text{PtdIns}(4,5)\text{P}_2$ -specific phospholipase C (reviewed by Gilman, 1987). A further inositol phosphate metabolite that has recently been proposed to modulate cellular calcium distribution is $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine & Moor, 1986; Morris *et al.*, 1987) formed by the Ca^{2+} -dependent phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$. Clearly, a requirement of this scheme is that the formation of these putative second messengers should be both of a sufficient magnitude and rapidity to account for the rise in intracellular Ca^{2+} after carbachol stimulation. Most earlier studies of smooth muscle have used long incubation times, included LiCl in incubations and have been unable to identify the inositol phosphate isomers formed. This is the first detailed report of h.p.l.c. analysis of smooth muscle extracts showing some of the initial events in inositol phosphate metabolism after carbachol stimulation.

EXPERIMENTAL

Materials

Myo-[2- ^3H]inositol (15–20 Ci/mmol), [^{32}P]ortho-phosphate and [^{14}C]inositol 1-phosphate were from Amersham International. AG1-X8 anion exchange resin (200–400 mesh) was from Bio-Rad. *myo*-inositol, ammonium formate and carbamoyl choline chloride

(carbachol) were purchased from Sigma. All other chemicals were 'AnalaR' grade from BDH. H.p.l.c. columns and Partisil SAX 10 anion exchange packing material were from Whatman, and h.p.l.c. equipment from Waters Division of Millipore.

Tissue preparation

Finely-chopped pieces of longitudinal smooth muscle were prepared from the small intestine of female guinea-pigs (250–350 g) and incubated in Krebs–Ringer bicarbonate-buffered saline as described by Best *et al.* (1985). Muscle fragments were incubated for 3 h at 37 °C with 50 μCi of *myo*-[^3H]inositol (previously purified on AG1-X8 resin, 200–400 mesh, in the formate form, 0.3 ml bed volume). Approximately equal weights of tissue (containing 2–7 mg of protein) were distributed in test tubes and stimulated for the times indicated at room temperature (22 ± 2 °C), without rinsing or addition of unlabelled inositol. This temperature was selected as suitable for carrying out the experiments not only because we wished to compare the time course of events with electrophysiological events previously published (Benham & Bolton, 1986) but also because attempts to perform experiments at 37 °C showed that only a 50 % increase in $\text{Ins}(1,4,5)\text{P}_3$ was found 2 s after carbachol stimulation, which declined thereafter. Incubations were ended by the addition of 50 % (w/v) trichloroacetic acid to a final concentration of 10 % (w/v) and placed on ice for 30 min. Approx. 1000 d.p.m. of ^{32}P -labelled $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ were added at this stage to determine recoveries of inositol phosphates. Samples were centrifuged, the supernatant was removed and washed five times with water-saturated diethyl ether to extract the acid. Extracts were finally adjusted to pH 7–8 with 1 M-NaOH before anion-exchange chromatography. Recovery of [4,5- ^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ through this extraction procedure and h.p.l.c. was 72.7 ± 4.7 % ($\pm \text{s.d.}$, $n = 24$). The residual precipitate was dissolved in 2 ml of 1 M-KOH at 60 °C and the protein content determined by the method of Lowry *et al.* (1951).

Abbreviations used: $\text{Ins}(1)\text{P}$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$ are D-*myo*-inositol phosphates with the positional assignment of phosphoryl residues shown on the inositol ring; GroPIns , $\text{GroPIns}(4)\text{P}$ and $\text{GroPIns}(4,5)\text{P}_2$ are sn-glycerol-3-phosphoryl-1-D-*myo*-inositols with the indicated assignment of additional phosphate residues on the inositol nucleus.

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Inositol phosphate standards

[4- ^{32}P]Ins(1,4) P_2 and [4,5- ^{32}P]Ins(1,4,5) P_3 were prepared from ^{32}P -labelled human erythrocyte membranes, essentially as described by Downes *et al.* (1982). Glycerophosphoryl inositol phosphates were prepared from ^{32}P -labelled human erythrocyte membranes by deacylating the parent phospholipids with the methylamine procedure described by Berridge (1983).

[4,5- ^{32}P]Ins(1,3,4,5) P_4 was prepared by phosphorylation of [4,5- ^{32}P]Ins(1,4,5) P_3 using an homogenate prepared from guinea-pig cerebral cortex essentially as described by Irvine *et al.* (1986) except that 10 mM-2,3-bisphosphoglycerate was included in the phosphorylation incubation medium. [4- ^{32}P]Ins(1,3,4) P_3 was prepared by dephosphorylation of [^{32}P]Ins P_4 by an homogenate of intestinal smooth muscle in the absence of 2,3-bisphosphoglycerate and ATP.

Chromatography of inositol phosphates

H.p.l.c. was carried out using gradients developed from the method introduced by Irvine *et al.* (1985) and Batty *et al.* (1985) using a Partisil SAX 10 column and eluting with ammonium formate adjusted to pH 3.7 with orthophosphoric acid. Gradients were formed using a Waters 600 solvent delivery system and passed through a 25 cm $\times$ 0.4 cm column of SAX 10 equipped with a 5 cm $\times$ 0.4 cm guard column, packed with the same material. Samples were centrifuged at 13 000 *g* for 5 min before injection in volumes up to 2 ml whilst the column was eluted with water; 5 min later a linear gradient up to 0.2 M-ammonium formate over 10 min was initiated (the beginning of this gradient did not reach the fraction collector until 12–14 min after injection owing to a 7–9 min delay in the proportioning valves, pulse dampener, pump and columns). This was followed by a hyperbolic

Fig. 1. H.p.l.c. analysis of acid-soluble [^3H]inositol-labelled compounds from longitudinal smooth muscle

(a) Analysis of radioactively-labelled standards prepared from human erythrocyte membranes or obtained commercially. The gradient of ammonium formate/phosphoric acid is shown by the broken line. (b) Analysis of neutralized acid extracts from smooth muscle, prelabelled with [^3H]inositol and without stimulation with carbachol, and (c) the later part of the analysis shown in (b) but using an expanded vertical scale. 1, inositol; 2, Ins(1) P ; 3, GroPIns(4) P ; 4, Ins(1,4) P_2 ; 5, GroPIns(4,5) P_2 ; 6, Ins(1,3,4) P_3 ; 7, Ins(1,4,5) P_3 and 8, Ins(1,3,4,5) P_4 . The peaks denoted by letters are referred to in the text.

Fig. 2. Rapid changes in the profile of inositol polyphosphates found after carbachol stimulation of smooth muscle

[^3H]Inositol-labelled smooth muscle was stimulated with carbachol (0.1 mM) for 0 s (a), 2 s (b), 5 s (c) or 60 s (d). The Figure shows only the late part of the trace where inositol polyphosphates were eluted.

gradient (Waters curve 4) up to 0.4 M-ammonium formate over 10 min, followed by a gradient (Waters curve 4) over 10 min up to 1.0 M-ammonium formate and completed by a linear increase over 10 min to 2.0 M-ammonium formate (see Fig. 1a). The flow rate was $1.25 \text{ ml} \cdot \text{min}^{-1}$ and the eluate monitored for absorbance at 254 nm and ^{32}P β -emission with an on-line detector (Beckman model 170). Fractions were collected at 0.4 min intervals for scintillation counting or subsequent desalting of standards. Scintillation counting was carried

out, after the addition of 14 ml of Packard emulsifier/scintillant 299, and 2 ml of water to fractions containing in excess of 0.4 M-ammonium formate/phosphoric acid, using a program to calculate the d.p.m. of ^3H in the presence of ^{32}P .

RESULTS AND DISCUSSION

H.p.l.c. of [^3H]inositol-labelled compounds in acid extracts of smooth muscle

H.p.l.c. of trichloroacetic acid extracts of [^3H]inositol-labelled longitudinal smooth muscle resolved at least 12 ^3H -containing compounds. Six of these co-chromatographed with standards of known identity (Fig. 1a) and were tentatively identified as inositol, $\text{Ins}(1)\text{P}$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4)\text{P}_3$ (see Fig. 1), $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. Of the remaining six peaks, the one marked A eluted in a position expected of GroPIIns , but was not homogeneous since it could sometimes be resolved into two peaks. Preliminary experiments suggested that peak A comprised a compound containing a glycosidic linkage to $\text{Ins}(1)\text{P}$, since a major proportion was converted to $\text{Ins}(1)\text{P}$ by treatment with nitrous acid (250 mM- NaNO_2 in 50 mM-acetate, pH 4.0, at 50°C for 2.5 h). It could have a glucosaminyl- $\text{Ins}(1)\text{P}$ structure such as has been implicated in membrane protein anchors (reviewed by Low, 1987), but further studies will be required to confirm this idea. Peak B eluted in a position expected of $\text{Ins}(4)\text{P}$ [just after $\text{Ins}(1)\text{P}$]. Peaks C, D, E and F contained compounds of unknown identity, and were unaffected by nitrous acid treatment, but their retentions on SAX10 were similar to those of unidentified component(s) found in extracts of ^3H -labelled parotid glands (Hawkins *et al.*, 1986) and hepatocytes (Palmer *et al.*, 1987). Peak F eluted very close to $\text{Ins}(1,4)\text{P}_2$ and may have been another isomer of InsP_2 . In contrast with a report on guinea-pig longitudinal smooth muscle (Bielkiewicz-Vollrath *et al.*, 1987) in which peaks A, C, D and E were not resolved, we found them to be the major ^3H -labelled compounds in unstimulated tissue. In rabbit iris muscle, Akhtar *et al.* (1987) observed two peaks in this region which they identified as GroPIIns and inositol monophosphate(s) on the basis of their relationship to the retention of AMP. Both Bielkiewicz-Vollrath *et al.* (1987) and Akhtar *et al.* (1987) used very much more rapid, linear gradients and this probably accounts for their failure to achieve adequate resolution. Both we, and others (Palmer *et al.*, 1987; Hansen *et al.*, 1986; Dean & Moyer, 1987) have found it necessary to use complex gradients which provide a very slow increase in ammonium formate concentration over the region where isomers of InsP , InsP_2 and InsP_3 are eluted. However, carbachol stimulation did not change the ^3H content of peaks C, D, E, and F.

Effects of carbachol on inositol phosphates analysed by h.p.l.c.

After a delay of 5 s an increase in [^3H] $\text{Ins}(1,3,4)\text{P}_3$ was found and continued to increase up to 1 min (Figs. 2 and 3); it was also increased at 5 min after stimulation (results not shown). The early rise in $\text{Ins}(1,4,5)\text{P}_3$ accumulation between 2 and 5 s after exposure to carbachol (Figs. 2b, 2c and 3b) occurred at the time at which intracellular $[\text{Ca}^{2+}]$ has been reported to increase in ileal smooth muscle (Himpens & Casteels, 1987) and at the time when activation of Ca^{2+} -dependent K^+ currents

Fig. 3. Time-course of inositol phosphates formed in response to carbachol analysed by h.p.l.c.

Samples were prepared as described in Fig. 1 and analysed by h.p.l.c. (a) $\text{Ins}(1,4)\text{P}_2$, (b) $\text{Ins}(1,4,5)\text{P}_3$ (■) and $\text{Ins}(1,3,4)\text{P}_3$ (□), and (c) $\text{Ins}(1,3,4,5)\text{P}_4$. ^3H found in each peak which co-eluted with the appropriate standard was summed and corrected for recovery and protein content, before expression as a percentage of control values. Control (zero time) values were $(13.04 \pm 0.17) \times 10^3$, $(2.70 \pm 0.25) \times 10^3$, $(3.31 \pm 0.12) \times 10^3$ and $(1.98 \pm 0.36) \times 10^3$ d.p.m./10 mg of protein ($\pm$ S.E.M., $n = 3$) for $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ respectively. Each point is the mean derived from three separate incubations of muscle from different animals, $\pm$ S.E.M. Differences found in the presence of carbachol were compared with the zero-time point for statistical significance using Student's t test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

apparent delay before the increase in $\text{Ins}(1,4)\text{P}_2$ after carbachol stimulation (Figs. 2 and 3), suggesting that either appreciable dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ occurs at times earlier than 2 s or that both $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}(4)\text{P}$ may be substrates for a receptor-activated phospholipase C. Since Berridge (1983) first noted the rapid appearance of both InsP_2 and InsP_3 in blowfly salivary glands stimulated with 5-hydroxytryptamine, similar observations have been made in other cell systems. The idea that the early appearance of $\text{Ins}(1,4)\text{P}_2$ is due to rapid hydrolysis of $\text{PtdIns}(4)\text{P}$ rather than a rapid dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ is suggested by the observation that at times as short as 0.5 s the rise in $\text{Ins}(1,4,5)\text{P}_3$ did not precede that of $\text{Ins}(1,4)\text{P}_2$, when GH₃ cells were stimulated with thyrotropin-releasing hormone (Tashjian *et al.*, 1987). Although no role as a second messenger has been proposed for $\text{Ins}(1,4)\text{P}_2$, if it accumulated to a sufficiently high concentration it might inhibit the dephosphorylation of other inositol phosphates [e.g. inhibition by $\text{Ins}(1,4)\text{P}_2$ of $\text{Ins}(1,3,4)\text{P}_3$ dephosphorylation in LiCl-treated hepatocytes, as shown by Shears *et al.* (1987a)].

$\text{Ins}(1,3,4,5)\text{P}_4$ metabolism in longitudinal smooth muscle

In view of the proposed actions of $\text{Ins}(1,3,4,5)\text{P}_4$, in combination with $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(2,4,5)\text{P}_3$, to alter intracellular calcium distribution (Irvine & Moor, 1986; Morris *et al.*, 1987) it was of interest to assess the extent of $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ accumulation in response to calcium-mobilizing stimuli in smooth muscle. After 2 s stimulation with carbachol the increase in $\text{Ins}(1,3,4,5)\text{P}_4$ was not statistically significant (Fig. 3c), and even the largest increase observed (172 %, shown in Fig. 2d) was much less than observed at early times after stimulation of other tissues with carbachol [e.g. 3.5-fold in rat cerebral cortex (Batty *et al.*, 1985); > 10-fold in parotid slices (Hawkins *et al.*, 1986)]. A significant increase in $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was not found until 60 s after stimulation of smooth muscle with carbachol (Fig. 3c), at which time another small peak closely following $\text{Ins}(1,3,4,5)\text{P}_4$ was sometimes found (Fig. 2d), and may correspond with the compound of similar retention on SAX10 recently observed in extracts of hepatocytes (Shears *et al.*, 1987b) and adrenocortical cells (Balla *et al.*, 1987) and suggested to be $\text{Ins}(1,3,4,6)\text{P}_4$.

The slower and less extensive accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ in smooth muscle and some other cell types could be accounted for by an accelerated rate of degradation of $\text{Ins}(1,3,4,5)\text{P}_4$ or a decreased rate of InsP_3 3-kinase activity. The former alternative seemed less likely, since the accumulation of $\text{Ins}(1,3,4)\text{P}_3$ was also less in smooth muscle (Figs. 2 and 3b) and in unpublished experiments it was found that formation of $\text{Ins}(1,3,4,5)\text{P}_4$ from $\text{Ins}(1,4,5)\text{P}_3$ by homogenates was some eight times slower in guinea pig longitudinal smooth muscle than in guinea-pig cerebral cortex.

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has been observed in single jejunal smooth muscle cells under voltage-clamp (Benham & Bolton, 1986).

In contrast to analysis using 1-X8 resins (see Bielkiewicz-Vollrath *et al.*, 1987; Hashimoto *et al.*, 1986; our unpublished work), h.p.l.c. showed that there was no

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