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A role for inositol 1,4,5-trisphosphate in the initiation of agonist-induced contractions of dog tracheal smooth muscle

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1 To elucidate the role of inositol 1,4,5-trisphosphate (Ins-P_3) in the initiation of agonist-induced contraction of the smooth muscle cells of the dog trachea, we investigated the effects of acetylcholine (ACh) on the concentrations of Ins-P_3, phosphatidylinositol-4,5-bisphosphate (PI-P_2) or phosphatidic acid (PA). The effects of Ins-P_3 on the Ca^{2+} stored in the smooth muscle cells were also studied in saponin-permeabilized smooth muscle cells.

2 A half maximal or maximal Ca^{2+} accumulation into the cells was observed in the dispersed single, smooth muscle cells treated by saponin, in free Ca^{2+} concentrations of 4.6×10^{-7} or 5×10^{-5} M, respectively. The ATP-dependent Ca^{2+} accumulation was maximal at 0.63 nmol/10^5 cells.

3 Effects of Ins-P_3 on stored Ca^{2+} were observed at a free Ca^{2+} concentration of 3.7×10^{-7} M, which induces about half maximal ATP-dependent Ca^{2+}-accumulation. Ins-P_3 released the Ca^{2+} accumulated by ATP, in a dose-dependent manner. About 40% of the total Ca^{2+} was released following application of 3 μM Ins-P_3.

4 The release of stored Ca^{2+} induced by application of Ins-P_3 was followed by its re-uptake into the smooth muscle cells. Thus, the stored Ca^{2+} was repeatedly released with repetitive applications of Ins-P_3.

5 Application of ACh (10^{-5} M) to the dog trachea stimulated the production of Ins-P_3 in the soluble fraction and 10 s after this application, the relative amount of Ins-P_3 was 290% of the control value.

6 Concomitantly, ACh (10^{-5} M) either reduced or increased the contents of phosphatidyl inositol 4,5-biphosphate (PI-P_2) or phosphatidic acid (PA) in the lipid fraction of the smooth muscle cells to 60% or to 350% of the control value, respectively, thereby indicating that ACh stimulates the phosphodiesteric hydrolysis of PI-P_2.

7 5-Hydroxytryptamine (5-HT; 10^{-5} M) also reduced or increased the contents of PI-P_2 or PA to 80 or to 200% of the control values, respectively. However, neither histamine (10^{-5} M), in the presence or absence of cimetidine (10^{-5} M), nor prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 10^{-7} M) showed any effect on the contents of PI-P_2 or PA in the lipid fraction of the smooth muscle cells.

8 These results indicate that in muscle cells of the dog trachea, Ins-P_3 may play the role of intracellular second messenger in the initiation of ACh or 5-HT-induced contraction, but not in the case of histamine or $PGF_{2\alpha}$-induced contraction.

Introduction

Increases in cytoplasmic free Ca^{2+} (10^{-7} M) initiate contraction in smooth muscle cells, and the Ca^{2+} contributing to the activation of contractile proteins is of both extra and intracellular origins (see for example, Kuriyama *et al.*, 1982).

The airway smooth muscle cells have membrane properties distinct from those of other visceral smooth muscles, and it was reported that excitatory junction potentials (e.j.ps), caused by the release of acetylcholine (ACh) from the vagus nerve terminals, evoke twitch-like contractions when the amplitude exceeds 4 mV. The amplitude of contractions evoked by e.j.ps was much larger than that evoked by membrane depolarization elicited by outward-current pulses

made at the same level of membrane potential. Furthermore, exogenous ACh evoked phasic contractions in Ca^{2+}-free solution containing 2 mM EGTA, with only a slight reduction in the amplitude. These observations indicate that contractions evoked by activation of vagus nerve terminals, by transmural stimulation, or by application of exogenous ACh are mainly due to the release of Ca^{2+} from intracellular stores, and that the amount of Ca^{2+} stored in the smooth muscle cells of trachea exceeds that of other visceral smooth muscle cells (Ito & Itoh, 1984 a,b). The signalling system between the surface membrane (the location of muscarinic ACh receptors) and the intracellular Ca^{2+} stores is unknown. However, it was reported that histamine (acting through H_1-receptors) and adrenaline (acting through α-adrenoceptors) stimulate incorporation of ^{32}P into phosphatidylinositol in the smooth muscles of guinea-pig ileum (Jafferji & Michell, 1976) and rat vas deferens (Canessa de Scarnati & Lapetina, 1974), respectively. In these two smooth muscles, there was a significant decrease in the tissue phosphatidylinositol content on stimulation, thereby indicating that the increased phosphatidylinositol turnover may be related to the agonist-induced contraction of the smooth muscle cells (Jones *et al.*, 1979). Furthermore, in rabbit iris muscles, phosphodiesteric cleavage of phosphatidylinositol 4,5-bisphosphate (PI-P_2) to form inositol 1,4,5-trisphosphate (Ins-P_3) was documented, in response to muscarinic cholinoceptor or α_1-adrenoceptor stimulation (Abdel-Latif *et al.*, 1977; Akhtar & Abdel-Latif, 1980; Michell *et al.*, 1981).

Recent studies have shown that Ins-P_3, a water soluble product of PI-P_2, may play a physiological role as a second messenger in the mobilization of intracellular Ca^{2+} in different cell types (see for example Berridge & Irvine, 1984). In the dog trachealis muscle, contraction induced by carbachol was associated with a decrease in the phosphatidylinositol (PI) pool, an increase in the phosphatidic acid and diacylglycerol pools, and an increase in the incorporation of $^{32}PO_4$ into PI (Baron *et al.*, 1984). These observations suggest that PI metabolism may play a physiological role in the signalling system between the drug-receptor reaction and intracellular Ca^{2+} stores. However, the changes in the content of polyphosphoinositides or inositol phosphates, including Ins-P_3, during the agonist-induced contraction, and the role of inositol phosphates in the initiation of contraction in the airway smooth muscles have not been clarified.

We have examined the effects of Ins-P_3 on the stored Ca^{2+} of tracheal smooth muscle cells made permeable with saponin to allow the metabolite access to the intracellular Ca^{2+} storage sites. The production of Ins-P_3 in the smooth muscle cells, as induced by the application of exogenous ACh, was also examined indirectly by measuring the hydrolysis of PI-P_2 or the production of PA in the lipid fraction, and directly by measuring the formation of [^{3}H]-Ins-P_3 in the water-soluble fraction. Finally, we compared the actions of histamine, 5-hydroxytryptamine (5-HT) or $PGF_{2\alpha}$ on the hydrolysis of PI-P_2 with that of ACh, in an attempt to obtain a clue as to whether Ins-P_3 is the universal second messenger for mobilization of intracellular calcium in the airway smooth muscle cells.

Methods

Preparation of muscle strips or single smooth muscle cells of the dog trachea

Adult mongrel dogs of either sex, weighing 10–15 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). Segments of cervical trachea were excised, and a dorsal strip of transversely running smooth muscle was separated from the cartilage. The mucosa and adventitial areolar tissues were carefully removed, under microscopic observation. Single smooth muscle cells from the trachea were prepared as described previously (Hirata *et al.*, 1981; Suematsu *et al.*, 1984).

Preparation of inositol 1,4,5-trisphosphate

Ins-P_3 was prepared from human erythrocyte ghosts according to the method described by Downes *et al.* (1982).

Assay of Ca^{2+} uptake and Ca^{2+}-release by inositol 1,4,5-trisphosphate in saponin-treated single smooth muscle cells

Ca^{2+} uptake and release were assayed by the filtration method described previously (Hirata *et al.*, 1984; Suematsu *et al.*, 1984). Briefly, Ca^{2+} was accumulated in the cells in a solution (10 ml) containing 100 mM KCl, 20 mM Tris-maleate (pH 6.8), 3 mM $MgCl_2$, 2 mM ATP, 10 mM NaN_3, 0.12 mM $CaCl_2$ (containing 1 μCi ml^{-1} ^{45}Ca), 0.44 mM EGTA (free Ca^{2+} concentration was calculated to be 3.7×10^{-7} M), and 1×10^5 ml^{-1} of saponin-treated muscle cells. Sodium azide was added to block the Ca^{2+} accumulation into mitochondria (Suematsu *et al.*, 1984; Hirata *et al.*, 1984). After 20 min incubation, 1 ml of the above mixture was passed through a glass fibre filter (Whatman GF/C; pore size; 1.2 μm). The filter was washed twice with 2 ml of the above solution without ^{45}Ca and cells, then dried and the radioactivity counted. The amount of $^{45}Ca^{2+}$ uptake could thus be determined. At 21 min, reagents in 1/100 volume of the reaction mixture were added, and the amount of ^{45}Ca in the cells was determined at various times, as described above.

Analysis of inositol phospholipids and phosphatidic acid in the tracheal smooth muscle

The contents of inositol phospholipids and phosphatidic acid in the dog trachea were measured before and after treatment with various agonists including ACh, histamine, 5-HT or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). For this purpose, muscle strips 10 by 2–3 mm were labelled in a phosphate-free, HEPES-buffered Krebs solution (pH 7.4) containing 20 μCi ml^{-1} of ^{32}P-Pi (specific radioactivity, 30–40 Ci mmol^{-1}: Japan Atomic Energy Research Institute), at 37°C for 2 h, a time adequate for an isotopic equilibrium in terms of the inositol phospholipids. The strips were then washed three times with the above solution without ^{32}P-Pi, and were incubated with agonists for 10 to 300 s. The reaction was halted by adding a solvent containing chloroform, methanol and 12 M HCl (100:200:2, v/v), and the strips were homogenized in a glass-homogenizer. Crude lipid extracts in the solvent were chromatographed on Silica Gel 60 plates (Merck), according to the method of Billah & Lapetina (1982); the plates were then autoradiographed on Sakura X-ray film for 12–15 h. The fractions corresponding to phosphatidylinositol 4-phosphate (PI-P), PI-P_2 and PA on the plate were cut out, and counted for radioactivity in a liquid scintillation counter.

Assay of production of water-soluble inositol phosphates

For this purpose, the muscle strips prepared as described above, were labelled with 5 μCi ml^{-1} of myo-[2-^{3}H(N)]-inositol (specific radioactivity: 15.8 Ci/mmol; New England Nuclear) for 6 h at 37°C. The strips labelled with [^{3}H]-inositol were incubated with ACh (10^{-5} M) for 10 to 300 s, and the incubation then halted by immersing the strips in 2 ml of 5% (w/v) trichloroacetic acid (TCA). These tissues were then homogenized, the acid-soluble fraction was treated with diethylether to remove the TCA and then applied to a Dowex-formate column for analysis of inositol phosphates, according to the method of Berridge (1983).

Results

Effects of inositol 1,4,5-trisphosphate on Ca^{2+} stored in the saponin-treated smooth muscle cells of the trachea

To investigate whether or not Ins-P_3 mobilizes the Ca^{2+} stored in the smooth muscle cells of the dog trachea, we used saponin-treated single muscle cells to allow Ins-P_3 access to the intracellular Ca^{2+} store sites.

Figure 1 shows the effects of free Ca^{2+} concentrations on ATP-dependent Ca^{2+} accumulation into the store sites. The amount of Ca^{2+} accumulated by the ATP-dependent mechanism showed a dose-dependent increase at free Ca^{2+} concentrations ranging between 10^{-8}–5×10^{-5} M. The half maximal and maximal amounts of Ca^{2+} accumulation were 0.30 and 0.63 nmol per 10^5 cells, observed at a free Ca^{2+} concentration of 4.1×10^{-7} M and 5×10^{-5} M, respectively.

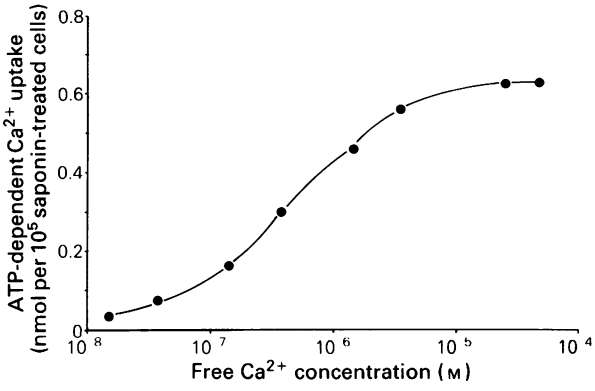

Figure 1 Effects of free Ca^{2+} concentrations ($10^{-8} - 5 \times 10^{-4}$ M) on the ATP-dependent Ca^{2+}-accumulation into saponin-treated single tracheal smooth muscle cells. The amount of Ca^{2+} stored in saponin-treated cells in the absence of ATP during Ca^{2+} accumulation was subtracted from that in the presence of ATP. Each point represents the mean of 5–8 experiments.

The effects of Ins-P_3 on the stored Ca^{2+} were observed at the free Ca^{2+} concentration of 3.7×10^{-7} M, a condition under which nearly half the maximal Ca^{2+} accumulation was obtained, since the Ca^{2+}-release from saponin-treated muscle cells by Ins-P_3 is inhibited when free Ca^{2+} is loaded in concentrations over 10^{-6} M (Suematsu *et al.*, 1984). Figure 2 shows the time course of Ca^{2+}-accumulation into or Ca^{2+}-release from the storage sites of saponin-treated single muscle cells by application of 3 μM Ins-P_3 in the presence of 3.7×10^{-7} M free Ca^{2+}. Ins-P_3 (3 μM) released Ca^{2+} within 1 min and the amount released was about 40% of the Ca^{2+} accumulated by ATP. The release of Ca^{2+} was transient, i.e. the initial release was followed by a re-uptake of Ca^{2+} into the cells. Thus, in response to the repetitive application of Ins-P_3 (3 μM), the Ca^{2+}-release occurred repeatedly.

Since PA was also produced in response to exogenously applied ACh in the dog trachea (see below) and it has been reported that PA has a Ca ionophore-like action (Salmon & Honeyman, 1980; Limas, 1980), we also examined the effect of PA on Ca^{2+} release from the saponin-treated single muscle cells. As shown in Figure 2, PA (Sigma) up to 50 μM in ethanol did not

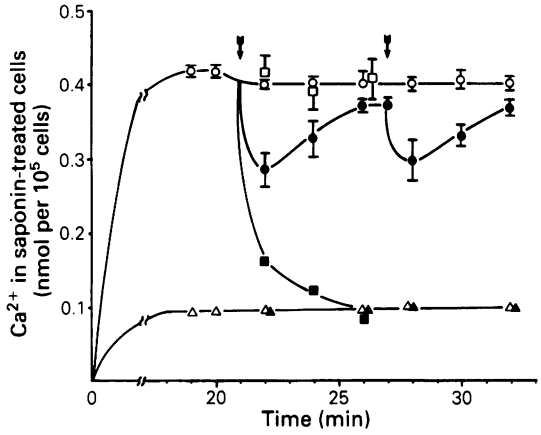

Figure 2 Effects of inositol 1,4,5-trisphosphate (Ins-P_3, 3 μM), phosphatidic acid (PA, 50 μM) or A23187 (1 μM) on the amount of Ca^{2+} accumulated in the saponin-treated single smooth muscle cells of the trachea. Saponin-treated cells (1×10^5 cells per ml) accumulated Ca^{2+} in a solution containing 0.1 M KCl, 20 mM Tris maleate (pH 6.8), 10 mM NaN_3, 3 mM $MgCl_2$, 2 mM ATP and 3.7×10^{-7} M free Ca^{2+} at 37°C for 20 min. At the time indicated by the arrows, Ins-P_3, PA or A23187 in 1/100 volume was added, and 1 ml of the above solution was passed through the glass fiber filter at the time indicated on the abscissa scale. (○) Control (the same volume of distilled water was added); (●) Ins-P_3 (3 μM); (■) A23187 (1 μM); (□) PA (50 μM). Triangles represent the Ca^{2+} uptake in the absence of ATP: (△) control; (▲) $InsP_3$ (3 μM). The vertical bars represent the s.e. for four experiments.

induce Ca^{2+} release. The Ca ionophore A23187 (1 μM) released all of the Ca^{2+} accumulated by ATP.

Figure 3 shows the dose-response relationship for Ins-P_3-induced Ca^{2+} release. In a concentration of

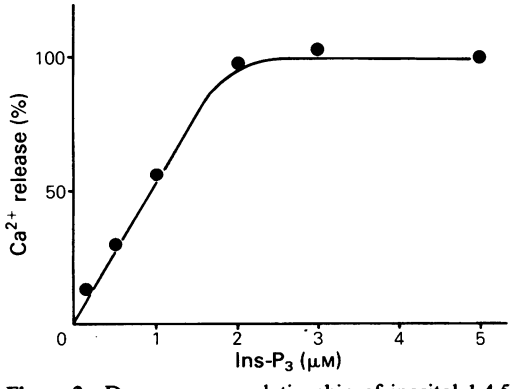

Figure 3 Dose-response relationship of inositol 1,4,5-trisphosphate(Ins-P_3)-induced Ca^{2+} release in saponin-treated single cells. The amount of Ca^{2+} released 1 min after the addition of Ins-P_3 was plotted against the concentration of Ins-P_3. Each point represents the mean of three experiments.

0.1 μM, Ins-P_3 evoked a significant Ca^{2+} release, and the maximal or half-maximal release of Ca^{2+} was observed at 2 and 0.8 μM Ins-P_3, respectively. This is comparable with findings in macrophages or in the porcine coronary artery (Hirata *et al.*, 1984, Suematsu *et al.*, 1984).

Effects of ACh on the amount of PI-P_2, PI-P or PA in lipid fraction, and of Ins-P_3 in soluble fraction of the smooth muscle of the trachea

On the assumption that endogenous Ins-P_3 in the smooth muscle cells plays a physiological role in the link between the surface membrane and intracellular Ca^{2+} storage sites, hydrolysis of PI-P_2 in the membrane would be the first and essential process in response to ACh (see Berridge & Irvine, 1984). Therefore, we observed the effects of ACh on the hydrolysis of PI-P_2. As shown in Figure 4, after application of 10^{-5} M ACh, the amount of PI-P_2 in the lipid fraction was gradually reduced in parallel with the time of incubation. The reduction in PI-P_2 content was apparent for the first 10 s, and 5 min after application of ACh, the relative amount of PI-P_2 was

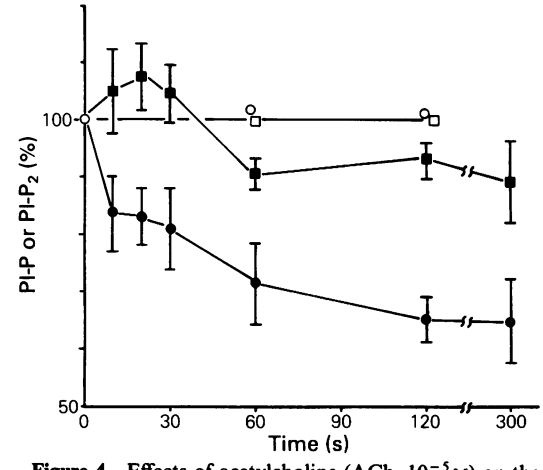

Figure 4 Effects of acetylcholine (ACh, 10^{-5} M) on the contents of phosphatidylinositol 4-phosphate (PI-P) or phosphatidylinositol 4,5-bisphosphate (PI-P_2) in the membrane fraction of the dog trachea. The ^{32}P-labelled dog trachea was treated with ACh 10^{-5} M and the tissue was homogenized in an organic solvent containing chloroform, methanol and HCl (100:200:2, v/v), at the time indicated on the abscissa scale. Following thin layer chromatography and autoradiography of the lipid extracts, the contents of PI-P and PI-P_2 were measured. Circles indicate the relative contents of PI-P_2 in the presence (●) or absence (○) of 10^{-5} M ACh, and squares the contents of PI-P in the presence (■) or absence (□) of 10^{-5} M ACh, respectively. A hundred percent of PI-P and PI-P_2 was 3019 ± 177 and 12662 ± 396 c.p.m. mg^{-1} of protein (± s.e.; $n = 5$), respectively. The vertical bars represent the s.e.mean for five experiments.

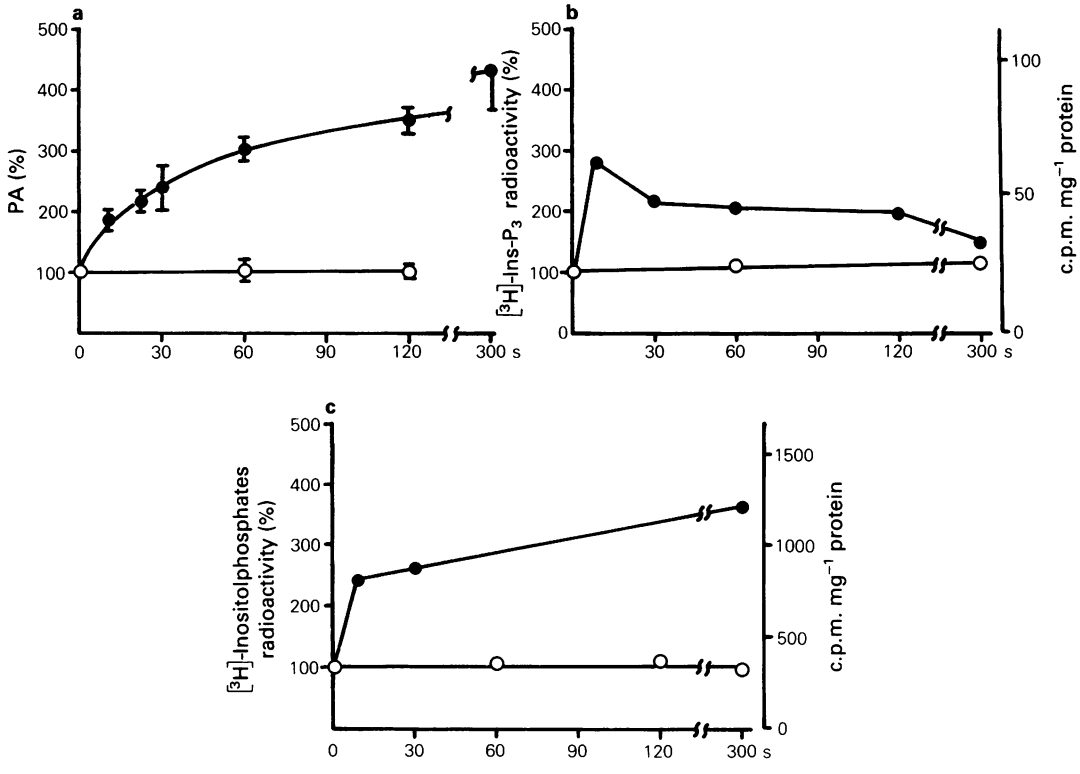

Figure 5 Production of phosphatidic acid (PA) (a) in lipid fraction or [^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-Ins-P_3) (b) and [^{3}H]-inositolphosphates (c) in the water-soluble fraction of smooth muscle cells of the dog trachea, induced by acetylcholine (ACh) (10^{-5} M). (a) The ^{32}P-labelled dog trachea was used for the measurements of PA: (○) control; (●) ACh (10^{-5} M). A hundred percent of PA was 2313 ± 345 c.p.m. mg^{-1} of protein (± s.e., $n = 5$). The vertical bars represent the s.e.mean for five experiments. (b) Production of [^{3}H]-Ins-P_3: the dog trachea labelled with [^{3}H]-inositol was used for the measurements of inositol phosphates. Inositol phosphates in water-soluble fraction were applied on a Dowex formate column. Circles indicate relative contents of Ins-P_3 in the presence (●) or absence (○) of 10^{-5} M ACh. A hundred percent of Ins-P_3 was 21.8 c.p.m. mg^{-1} of protein. Each point represents the mean for three experiments. (c) Production of [^{3}H]-inositolphosphates including [^{3}H]-Ins-P_3, [^{3}H]-inositol biphosphate and [^{3}H]-inositolmonophosphate in the presence (●) or absence (○) of 10^{-5} M ACh, respectively. A hundred percent of [^{3}H]-inositolphosphates was 333 c.p.m. mg^{-1} of protein. Each point represents the mean for three experiments.

64 ± 6% (s.e., $n = 5$) of the initial value. The amount of PI-P was little affected by treatment with ACh, although a slight reduction was observed after treatment with 60 s.

To determine whether or not the hydrolysis of PI-P_2 is due to a phosphodiesteric cleavage induced by ACh, we measured the amount of PA in the smooth muscle cells after application of ACh. ACh stimulated the synthesis of PA (Figure 5a), and 2 min after application, the amount of PA was 350 ± 22% ($n = 5$) of the control value. This indicates that PA is formed through diacylglycerol, one of the phosphodiesteric products of phospholipid. Thus, reduction in the content of PI-P_2 in the smooth muscle cells is due to its hydrolysis by the phosphodiesterase, as activated by exogenously applied ACh.

To confirm the above observations that exogenous ACh stimulates the phosphodiesteric hydrolysis of PI-P_2 (Figures 4 and 5a), we also measured the production of inositol phosphates in the water soluble fraction using [^{3}H]-inositol-labelled trachea. As shown in Figure 5b, application of ACh (10^{-5} M) stimulated the synthesis of Ins-P_3 and the relative content was increased to 290% of the control value, within 10 s. The content of Ins-P_3 gradually reverted to the original level with prolonged incubation (300 s). On the other hand, total inositol phosphates (inositol-monophosphate, inositol-bisphosphate and Ins-P_3) increased up to 370% of the initial value at 300 s after application of ACh (Figure 5c), indicating that Ins-P_3 phosphatase may contribute to the reduction of Ins-P_3 during the prolonged incubation with ACh.

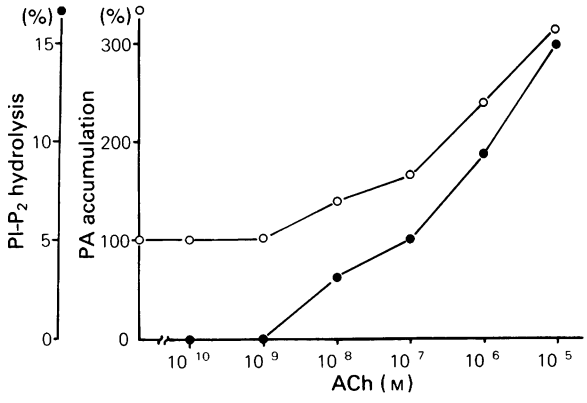

Figure 6 Dose-response relationship of acetylcholine (ACh)-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI-P_2) or production of phosphatidic acid (PA). The ^{32}P-labelled dog trachea was stimulated by various concentrations of ACh for 30 s, and quantities of PI-P_2 and PA were determined as described in Figure 4.

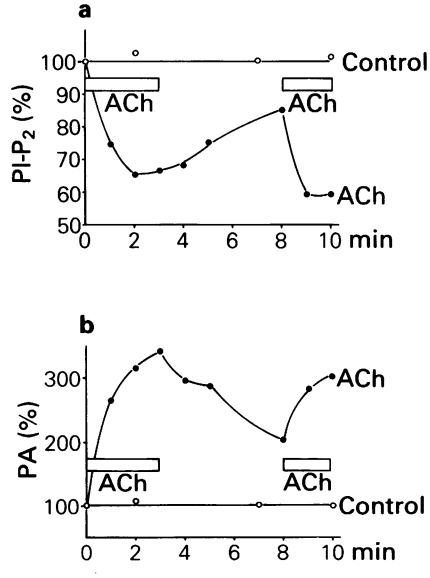

Figure 7 Effects of acetylcholine (ACh)-withdrawal on the contents of phosphatidylinositol 4,5-bisphosphate (PI-P_2) or phosphatidic acid (PA). The ^{32}P-labelled dog trachea was treated with 10^{-5} M ACh for 3 min, and then the tissue was rinsed by Krebs solution without ACh for 5 min and again treated with ACh-containing solution for 2 min. The contents of PI-P_2 or PA were determined as described in Figure 4. A hundred percent of PI-P_2 or PA was 12346 and 2350 c.p.m. mg^{-1} of protein, respectively: (○) control; (●) ACh. Each point represents the mean for two experiments.

These observations indicate that treatment of the tissue with ACh enhances the phosphodiesteric hydrolysis of PI-P_2, and results in an increase in Ins-P_3 and PA, in smooth muscle cells.

Figure 6 shows the relationships between ACh-doses and relative amounts of PI-P_2 as well as the PA accumulation in lipid fraction of the dog trachea. ACh, in concentrations of over 10^{-8} M apparently stimulated the breakdown of PI-P_2 and the production of PA.

Effects of ACh withdrawal on the contents of PI-P_2 or PA

In the dog trachea, Ins-P_3 evoked repeated release of Ca^{2+} from saponin-treated single smooth muscle cells in the presence of 3.7×10^{-7} M Ca^{2+} and 2 mM ATP (Figure 2). However, in the presence of ACh, there was no recovery in the amounts of PI-P_2 in the lipid fraction (Figure 4), and this recovery would relate to the contraction-relaxation cycle in the muscle cells, if Ins-P_3 actually does play a key role in the initiation of contraction.

Therefore, we observed the effects of ACh-withdrawal on the contents of PI-P_2 or PA in the muscle cells. As shown in Figure 7, application of ACh reduced or increased the content of PI-P_2 or PA to 65% or to 340% of the initial value in 3 min, respectively. After removal of ACh, the content of PI-P_2 gradually increased to 85% of the control value 5 min after the withdrawal of ACh, although complete recovery did not occur. Similarly, the contents of PA decreased from 340 to 200% of the initial value 5 min after the removal of ACh. While, the complete recovery in the contents of PI-P_2 or PA was not observed after withdrawal of ACh, repeated applications of ACh did evoke a reduction in the contents of PI-P_2 or increase in PA, thereby indicating that PI-P_2/Ins-P_3 cycles continue in the presence or absence of ACh.

Effects of histamine, 5-hydroxytryptamine or $PGF_{2\alpha}$ on PI-P_2 and PA contents in the muscle cells

In the airway smooth muscle cell, chemical mediators including ACh, histamine, 5-hydroxytryptamine (5-HT), PGF series or leukotrienes play a major role in the initiation of contractions. Therefore, it was of interest to observe the effects of such chemical mediators on the PI-P_2 content in the smooth muscle cells.

Figure 8 shows the effects of histamine (10^{-5} M), 5-HT (10^{-5} M) and $PGF_{2\alpha}$ (10^{-7} M) on the relative amount of PI-P_2 (a) or PA (b). Histamine (10^{-5} M) had no effects on the content of PI-P_2 or PA, while 5-HT (10^{-5} M) decreased the content of PI-P_2 to 80% of the initial value and increased the PA content to 200% of the control. H_1- and H_2-histamine-receptors (H_1 or H_2-

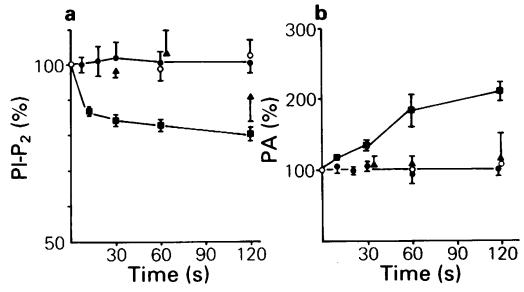

Figure 8 Effects of histamine (● 10^{-5} M), 5-hydroxytryptamine (5-HT, ■ 10^{-5} M) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, ▲ 10^{-7} M) on the content of phosphatidylinositol 4,5-bisphosphate (PI-P_2) (a) or phosphatidic acid (PA) (b). Open circles show controls. The ^{32}P-labelled dog trachea was treated with histamine, 5-HT or $PGF_{2\alpha}$, then the quantity of PI-P_2 or PA was determined as described in Figure 4. A hundred percent of PI-P_2 or PA was 10978 or 1906 c.p.m. mg^{-1} of protein, respectively. Vertical bars represent the s.e.mean for four experiments.

receptors) distributed in the airway smooth muscle cells mediate muscle contraction and relaxation, respectively (see for example, Okpaka *et al.*, 1978). Therefore, experiments were carried out in the presence of cimetidine, an H_2-antagonist. However, histamine had no effect on the content of PI-P_2 or PA in the muscle cells. $PGF_{2\alpha}$ (10^{-7} M) was also without effect on the content of PI-P_2 or PA.

Discussion

The present results can be summarized as follows; (i) micromolar concentrations of Ins-P_3 released Ca^{2+} from intracellular store sites in saponin-treated smooth muscle cells, followed by re-accumulation of Ca^{2+} into the cells; (ii) application of ACh to muscle strips of the dog trachea enhanced the phosphodiesteric hydrolysis of PI-P_2 resulting in the production of PA and water-soluble Ins-P_3; (iii) the content of PI-P_2 and PA recovered after withdrawal of ACh from the incubation medium, and repetitive application of ACh enhanced the phosphodiesteric hydrolysis of PI-P_2 repeatedly; (iv) 5-HT stimulated the hydrolysis of PI-P_2, but histamine and $PGF_{2\alpha}$ did not.

In guinea-pig macrophages (Hirata *et al.*, 1984) or smooth muscle cells of porcine coronary artery (Suematsu *et al.*, 1984), the Ca^{2+} released by Ins-P_3 was not re-accumulated into the intracellular storage sites. The lack of re-uptake of the released Ca^{2+} in the macrophages was attributed to the remaining Ins-P_3 in the incubating medium (un-hydrolysed Ins-P_3 after short application of 5-μM Ins-P_3) (Hirata *et al.*, 1984). On the other hand, the re-accumulation of Ca^{2+} released by Ins-P_3 was observed in rat hepatocytes (Joseph *et al.*, 1984) or insulinoma (Biden *et al.*, 1984), and these authors concluded that the re-accumulation of Ca^{2+} is due to the degradation of Ins-P_3 by Ins-P_3-phosphomonoesterase. The present observations indicate that in contrast to porcine coronary artery, the re-accumulation of Ca^{2+} into intracellular storage sites after application of Ins-P_3 in micromolar concentrations occurs preferentially in the smooth muscle cells of dog trachea. The discrepancy observed in the dog trachea and porcine coronary artery in the re-accumulation process of Ca^{2+} may be due to the difference in the activity of Ins-P_3-phosphomonoesterase in the two muscle types.

The amount of ATP-dependent Ca^{2+} accumulation into the single smooth muscle cells of the dog trachea was 2.0–2.5 times larger than that observed in the porcine coronary artery in free Ca^{2+} concentrations between 10^{-8} to 5×10^{-5} M (Suematsu, Hirata, Sasaguri, Hashimoto & Kuriyama, unpublished observations indicate that the capacity of Ca^{2+} storage sites in the smooth muscle cells of the dog trachea is by ATP did not differ in the trachea (4.1×10^{-7} M) or porcine coronary artery (4.5×10^{-7} M). These observations indicate that the capacity of Ca^{2+} store sites in the smooth muscle cells of the dog trachea is 2.0–2.5 times larger than in porcine coronary artery.

A variety of agonists, including ACh and 5-HT, induce the hydrolysis of inositol phospholipids in various tissues (see for example Berridge & Irvine, 1984). On the other hand, micromolar concentrations of Ins-P_3 release Ca^{2+} from the intracellular non-mitochondrial storage sites in various cell types, including rat pancreatic acinar cells (Streb *et al.*, 1983), hepatocytes (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Dawson & Irvine, 1984), macrophages (Hirata *et al.*, 1984), cardiac microsomes (Hirata *et al.*, 1984) and smooth muscle cells of the porcine coronary artery (Suematsu *et al.*, 1984).

In the present experiments, ACh or 5-HT induced hydrolysis of PI-P_2 resulting in the synthesis of Ins-P_3, and exogenously applied Ins-P_3 released stored Ca^{2+} from the saponin-treated smooth muscle cells. Thus, Ins-P_3 may play a role in the initiation of ACh or 5-HT-induced contraction, as an intracellular second messenger in airway smooth muscle cells.

The minimum concentrations of ACh required to evoke the synthesis of Ins-P_3 or to induce contraction were in the same range, between 10^{-9}–10^{-8} M (Ito & Itoh, 1984a), and the amount of Ins-P_3 or the amplitude of ACh-induced contractions showed a dose-dependent increase. Furthermore, hydrolysis of PI-P_2 occurred within 10 s after the application of ACh. These observations also support the above view that Ins-P_3 may be the intracellular second messenger inducing contraction in the airway smooth muscle.

It was reported that 5-HT and histamine, which provoke contraction, also elicit an increase in phos-

phatidyl inositol turnover in the longitudinal smooth muscle of guinea-pig ileum through the same receptor (Jafferji & Michell, 1976). However, in the present experiments, the former but not the latter induced the hydrolysis of PI-P_2. It may be that the present assay system for PI-P_2 is not sensitive enough to pick up the effects of histamine on the concentration of PI-P_2 in the tracheal tissue. In the experiments with ileum smooth muscle, however, the concentration of histamine used was 10^{-4} M, and incubations were for a total of 60 min, with histamine present during the final 30 min (Jafferji & Michell, 1976; Jones *et al.*, 1979) while in the present experiments 10^{-5} M histamine was applied for 2 min. Thus the difference observed in the action of histamine on PI-P_2 concentrations in ileal and tracheal smooth muscles might be due to different experimental procedures. However, it should be kept in mind that similar treatment with 5-HT (10^{-5} M) or with ACh (10^{-8} M) evoked the hydrolysis of PI-P_2 resulting in the synthesis of Ins-P_3, in the present experiments, It is known that airway smooth muscle cells contain H_2-receptors which mediate airway relaxation, in addition to H_1-receptors that mediate airway constriction (Okpako *et al.*, 1978; Drazen *et al.*, 1978; Maengwyn-Davies, 1980). However, the hydrolysis of PI-P_2 in response to histamine was not observed in the presence of cimetidine (H_2-antagonist). On the other hand, it was reported that histamine constricts isolated dog trachealis muscle through H_1-receptors (Stephens & Kroeger, 1980). Thus, in the initiation of histamine-induced contraction, an intracellular second messenger other than Ins-P_3 should be considered.

$PGF_{2\alpha}$ was also without effect on the contents of PI-P_2 or PA in the lipid fraction of the dog smooth muscle cells. However, in the dog trachea, thromboxane or leukotriene C_4 failed to evoke a contraction in Ca^{2+}-free solution containing 2 mM EGTA, whereas ACh did evoke contraction (Inoue & Ito, 1985). These observations indicate that thromboxane or leukotriene C_4, products of the cyclo-oxygenase or lipoxygenase pathway of arachidonic acid, may act as a calcium ionophore at the membrane of the smooth muscle cells. Thus, $PGF_{2\alpha}$, one of the products of cyclo-oxygenase activity, may act as a calcium ionophore, as in the case of thromboxane or leukotriene C_4.

The present results show that intracellular mechanisms involved in the agonist-induced contractions in the airway smooth muscle are complex, and that Ins-P_3 may play a physiological role in the initiation of ACh- or 5-HT-induced contraction, but not in histamine- or $PGF_{2\alpha}$-induced contraction.

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