Relationship between stimulated phosphatidic acid production and inositol lipid hydrolysis in intestinal longitudinal smooth muscle from guinea pig

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Accumulation of [32 P]phosphatidic acid (PA) and total [3 H]inositol phosphates (IPs) was measured in the longitudinal smooth-muscle layer from guinea-pig small intestine. Stimulation with carbachol, histamine and substance P produced increases in accumulation of both [3 H]IPs and [32 P]PA over the same concentration range. The increase in [32 P]PA accumulation in response to carbachol ($1\text{ }\mu\text{M}$ – 0.1 mM) was inhibited in the presence of atropine ($0.5\text{ }\mu\text{M}$). Buffering the external free [Ca^{2+}] to 10 nM did not prevent the carbachol-stimulated increase in [32 P]PA accumulation. Carbachol and Ca^{2+} appear to act synergistically to increase accumulation of [32 P]PA. In contrast, although incubation with noradrenaline also increased accumulation of [3 H]IPs, no increase in accumulation of [32 P]PA could be detected. These results suggest that an increase in formation of IPs is not necessarily accompanied by an increase in PA formation, and imply the existence of receptor-modulated pathways regulating PA concentrations other than by phospholipase-C-catalysed inositol phospholipid hydrolysis.

INTRODUCTION

Much recent evidence implicates inositol lipid metabolism as being involved in cell activation. Receptor occupation activates a phosphodiesterase (phospholipase C), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). Both of these products are putative second messengers. IP_3 releasing Ca^{2+} from an intracellular store, probably the endoplasmic reticulum [1,2], and DG activating protein kinase C [3]. IP_3 can be phosphorylated to form inositol 1,3,4,5-tetrakisphosphate [4], or degraded in a stepwise manner to free inositol [5]. DG may be hydrolysed by DG lipase to form monoacylglycerol, releasing arachidonic acid [6], or it may be phosphorylated by DG kinase to form phosphatidic acid (PA) [7].

However, the regulation of this pathway may differ between tissues; for example, some studies have shown that it is possible to measure changes in accumulation of DG, without the expected corresponding changes in accumulation of PIP_2 or IP_3 [8–11], indicating that DG may be formed from sources other than hydrolysis of PIP_2 . Similarly, changes in accumulation of labelled PA which do not coincide with changes in DG have been observed [12], showing that accumulation of PA may not necessarily reflect production of DG. We have also shown that agonist-induced contraction is related to increases in inositol phosphates (IPs) and PA, but not in DG, in smooth muscle [13].

The receptor-stimulated production of IPs in response to carbachol, histamine, substance P and noradrenaline in the longitudinal smooth muscle of guinea-pig small intestine has been characterized in several studies [14–19]. However, the regulation of PA accumulation has not previously been studied in response to various agonists,

and is investigated in the work presented here. The results suggest a dissociation between PA accumulation and inositol phospholipid hydrolysis in smooth muscle.

MATERIALS AND METHODS

Materials

[32 P] P_i (10 mCi/ml ; aqueous solution, carrier-free, acid-free) and *myo*-[2- 3 H]inositol (14 Ci/mmol ; aqueous solution) were from Amersham International. AG1-X8 resin (200–400 mesh) was from Bio-Rad. *myo*-Inositol, ammonium formate, carbamoylcholine chloride (carbachol) and substance P were from Sigma. Atropine methonitrate was from Wellcome. L-Noradrenaline bitartrate and histamine dihydrochloride were from Koch–Light Laboratories. All other solvents were of analytical grade from BDH Chemicals.

Tissue preparation

For each experiment, one guinea pig (300–500 g, either sex) was killed by a blow to the head followed by exsanguination. The entire length of the small intestine was removed and placed in pre-gassed (100% O_2) physiological salts solution (PSS) of the following composition (mM): NaCl 120, KCl 5.9, Hepes 10.6, MgCl_2 1.2, NaH_2PO_4 1.2, CaCl_2 2.5, glucose 11.5. The pH was adjusted to 7.4 with NaOH solution at room temperature, increasing the [Na^+] by 4.6 mM.

The longitudinal smooth muscle was stripped from the small intestine as described by Rang [20]. About 1 g of muscle (wet wt.) was obtained from each guinea pig. The muscle was finely chopped with scissors, washed with fresh PSS, and then incubated with radioisotope. All incubations were at $37\text{ }^\circ\text{C}$.

Abbreviations used: PIP_2 , phosphatidylinositol 4,5-bisphosphate; IPs, inositol phosphates; IP_3 , inositol 1,4,5-trisphosphate; DG, diacylglycerol; PA, phosphatidic acid; PSS, physiological salts solution.

Measurement of [^3H]inositol phosphates

The tissue was incubated for 3 h with 30 μCi of *myo*-[2- ^3H]inositol, which had previously been purified by application to a column containing 0.5 ml of AG1-X8 resin (formate form) and elution with 2.5 ml of water. At the end of this period, the tissue was washed with 10 ml of PSS containing no radioactivity, and then resuspended in PSS containing 1 mM unlabelled *myo*-inositol and 10 mM-LiCl. Samples (200 μl) of tissue fragments (about 30 mg of wet wt. of tissue) were transferred to glass tubes containing 700 μl of pregassed PSS with 10 mM-LiCl and 1 mM unlabelled *myo*-inositol. Incubations were started by addition of 100 μl of PSS containing the various test substances and were stopped by addition of 3 ml of chloroform/methanol/35% (v/v) HCl (200:100:1, by vol.) and left for 10 min. The tubes were then vortex-mixed for 15 s and the two phases separated by centrifugation (1000 *g* for 5 min). The upper aqueous phase was removed, added to 2.5 ml of water, the pH adjusted to 7.0 by addition of NaOH solution, and then the sample was applied to a column containing 1 ml of AG1-X8 resin. The lower phase was discarded, and the remaining tissue dissolved in 5 ml of 0.1% SDS/0.1 M-NaOH solution by heating at 60 °C for 30 min and the tissue protein was measured with the Folin phenol reagent [21].

Free inositol and IPs were eluted from the resin, either sequentially with 4 $\times$ 2 ml of increasing concentrations of formate as described by Berridge *et al.* [22], or the inositol phosphates were eluted together, as an index of inositol phospholipid hydrolysis, by 4 $\times$ 4 ml of a single high concentration of formate (0.1 M-formic acid/1.0 M-ammonium formate). The ^3H content of the fractions was determined by scintillation counting of 1 ml samples in 10 ml Scintran (Packard). Results are expressed as d.p.m./mg of protein.

Measurement of [^{32}P]PA

The tissue was incubated with 250 μCi of [^{32}P]P_i for 1 h in phosphate-free PSS. At the end of this period, the tissue fragments were resuspended in PSS containing non-radioactive phosphate, and 200 μl portions of tissue fragments (about 30 mg wet wt. of tissue) were transferred to glass tubes containing 700 μl of pregassed PSS. Incubations were started by addition of 100 μl of PSS containing the various test substances. In an experiment measuring the effects of altering free [Ca^{2+}] on carbachol-induced accumulation of [^{32}P]PA, the 200 μl tissue sample and the 100 μl drug dilution were made up in PSS with no added CaCl_2 , and the [Ca^{2+}] of the 700 μl of PSS to which these were added was varied by buffering the PSS with 1.43 mM-EGTA and altering the added [CaCl_2] as follows: 0.01 μM - Ca^{2+} (no added CaCl_2), 0.1 μM - Ca^{2+} (0.52 mM- CaCl_2), 1 μM - Ca^{2+} (1.21 mM- CaCl_2), 10 μM - Ca^{2+} (1.41 mM- CaCl_2), 0.1 mM- Ca^{2+} (1.57 mM- CaCl_2), 1 mM- Ca^{2+} (1 mM- CaCl_2 , no added EGTA). This assumes a contaminating presence of 10 μM - Ca^{2+} in the absence of added CaCl_2 .

Incubations were stopped by addition of 3 ml of chloroform/methanol/35% HCl (200:100:1, by vol.) and left for 10 min. The tubes were vortex-mixed for 15 s, 1 ml of water was added, the tubes were again vortex-mixed, and then the two phases were separated by centrifugation. The upper aqueous phase was discarded and the lower phase washed with 1 ml of methanol/water

(1:2, v/v). The upper phase was again discarded, and the lower phase removed to clean glass tubes and evaporated to dryness under vacuum.

The lower phase was redissolved in 200 μl of chloroform/methanol/35% HCl (200:100:1, by vol.) and [^{32}P]PA was separated by one-dimensional t.l.c. on 0.25 mm-thick silica-gel 60 plates (Merck). Before use, t.l.c. plates were sprayed with methanol/water (2:2, v/v) containing 1% potassium oxalate, dried at room temperature, and then activated at 115 °C for 15 min [23]. After application of the lipid extracts, the t.l.c. plates were developed in ethyl acetate/acetic acid/2,2,4-trimethylpentane (9:2:5, by vol.), which isolates [^{32}P]PA as a single spot, the other phospholipids remaining at the origin [24]. The resolved lipids were localized by autoradiography on Kodak X-Omat S film (24 h exposure). The phospholipid spots were scraped from the t.l.c. plates and the radioactivity was determined by scintillation counting in 10 ml of Scintran. The total radioactivity in the chloroform extract varied between samples by less than 10%, so results are expressed as c.p.m. in PA as a percentage of the total lipid c.p.m. in the extract. This method of expressing the results gives a similar concentration-response relationship and time course of response to carbachol to that obtained by using c.p.m./mg of protein.

Measurement of [^{32}P]ATP

The specific radioactivity of [^{32}P]ATP in tissue samples incubated for 1 h with [^{32}P]P_i and then resuspended in PSS (containing no non-radioactive phosphate) was determined before and after 10 min incubation in PSS in the absence or presence of carbachol (0.1 mM) in a total volume of 1 ml as described above. The incubations were stopped by addition of 0.3 ml of 50% (w/v) trichloroacetic acid and standing the tubes on ice. The protein precipitate was centrifuged down and discarded, and trichloroacetic acid was extracted from the supernatant by vigorous washes with 5 $\times$ 5 ml of diethyl ether. The samples were then diluted by addition of water to a final volume of 5 ml.

The specific radioactivity of [^{32}P]ATP in the samples was determined by h.p.l.c. on a TSK-DEAE 5PW anion-exchange column (formate form). The flow rate was set at 1 ml/min. Water was run through the column for 10 min before application of the sample, and then the 5 ml sample was pumped on to the column. Water was then run through the column for a further 5 min, and then the sample was eluted at 1 ml/min by a linear gradient increasing from water to 100% 0.35 M-ammonium formate (pH adjusted to 3.5 with formic acid) in 20 min, and fractions were collected every 20 s. The eluent was then returned linearly to water over 1 min. U.v. absorbance was monitored at 254 nm in order to detect ATP. The total c.p.m. (determined by Čerenkov counting in the absence of scintillant) in the fractions corresponding to the ATP peak minus the basal rate of counts (taken as the c.p.m. in the five fractions eluted before and after the elution of ATP) were divided by the concentration of ATP (determined by comparison of the area under the eluted peak with standards) to give the specific radioactivity of [^{32}P]ATP in c.p.m./nmol.

Presentation of results

The results are presented as means $\pm$ S.E.M. These values are derived from at least three separate tissue-

Fig. 1. Effect of carbachol on accumulation of $[^{32}\text{P}]\text{PA}$ in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with $[^{32}\text{P}]\text{P}_i$ were incubated for 5 min in PSS with carbachol in the absence (●) or presence (■) of atropine ($0.5 \mu\text{M}$). Each point represents the mean $\pm$ S.E.M. for at least three separate incubations.

Fig. 2. Time course of carbachol-induced increases in accumulation of $[^{32}\text{P}]\text{PA}$ in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with $[^{32}\text{P}]\text{P}_i$ were incubated in PSS in the absence (○) or presence (●) of carbachol (0.1 mM) added at zero time. Each point represents the mean $\pm$ S.E.M. for at least three separate incubations.

sample incubations. The significance of differences between values was assessed by the two-tailed Student's *t* test. Where the calculated value for *t* corresponded to values of $P < 0.05$, differences were considered to be significant.

Fig. 3. Influence of Ca^{2+} on carbachol-induced increases in accumulation of $[^{32}\text{P}]\text{PA}$ in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with $[^{32}\text{P}]\text{P}_i$ were incubated for 5 min in PSS in the absence (○) or presence (●) of carbachol (0.1 mM) at various buffered free Ca^{2+} concentrations. Each point represents the mean $\pm$ S.E.M. for three separate incubations.

RESULTS

Production of $[^{32}\text{P}]\text{PA}$

A concentration-dependent stimulation of $[^{32}\text{P}]\text{PA}$ accumulation in $[^{32}\text{P}]\text{P}_i$ -labelled longitudinal-smooth-muscle fragments was produced over the range $0.1 \mu\text{M}$ – 1 mM -carbachol, maximal stimulation being approached at 0.1 mM -carbachol. The increase in $[^{32}\text{P}]\text{PA}$ accumulation stimulated in response to carbachol (1 – $100 \mu\text{M}$) was inhibited in the presence of $0.5 \mu\text{M}$ -atropine (Fig. 1).

The time course of accumulation of $[^{32}\text{P}]\text{PA}$ was investigated in response to 0.1 mM -carbachol over a 10 min incubation period (Fig. 2). No increase in accumulation of $[^{32}\text{P}]\text{PA}$ was detected in the absence of carbachol; indeed, there was a very small but significant decrease in basal accumulation of $[^{32}\text{P}]\text{PA}$ after 5 min incubation in PS, which may reflect a decrease in $[^{32}\text{P}]\text{ATP}$ labelling, although no significant change in this was detected (see below). However, the basal values detected after 5 min and 10 min incubation are not significantly different. In contrast, in the presence of 0.1 mM -carbachol, a significant increase ($P < 0.05$) in accumulation of $[^{32}\text{P}]\text{PA}$ (from basal values of 10.8 ± 0.6 to $12.6 \pm 0.4\%$ of total lipid c.p.m.) was detected after 5 s incubation. This reached a maximal 2-fold increase over basal values (at $20.8 \pm 1.1\%$ of total lipid c.p.m.) 1 min after stimulation, and this was sustained over the remainder of the 10 min period.

This result suggests that carbachol increases the amount of phosphatidic acid in the muscle. However, this would be true only if the specific radioactivity of $[^{32}\text{P}]\text{ATP}$ did not change during the incubation period (10 min) or with carbachol. Values for the specific

Fig. 4. Time course of accumulation of $[^{32}\text{P}]\text{PA}$ in response to (a) histamine, (b) substance P and (c) noradrenaline in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with $[^{32}\text{P}]\text{P}_i$ were incubated in PSS in the absence (○) or presence (●) of 0.1 mM agonist added at zero time. Each point represents the mean $\pm$ S.E.M. for at least three separate incubations.

radioactivity of $[^{32}\text{P}]\text{ATP}$ were $1.48(\pm 0.10) \times 10^5$ c.p.m./nmol after resuspension in non-radioactive PSS but before further incubation, and $1.19(\pm 0.11) \times 10^5$ and $1.55(\pm 0.13) \times 10^5$ c.p.m./nmol after 10 min incubation in the absence and in the presence of 0.1 mM-carbachol respectively. These values are not significantly different. This result suggests that, notwithstanding the use of a pulse-chase technique, changes in $[^{32}\text{P}]\text{PA}$ accumulation do not reflect changes in ^{32}P labelling of ATP, but reflect an increase in the accumulation of PA, as observed by others in this tissue [25] on stimulation with carbachol.

Carbachol-stimulated $[^{32}\text{P}]\text{PA}$ accumulation persisted in conditions of lowered extracellular $[\text{Ca}^{2+}]$ (Fig. 3). Samples of tissue were suspended in PSS with or without carbachol at different concentrations of free Ca^{2+} buffered with EGTA. The concentration of contaminating Ca^{2+} was assumed to be 0.01 mM, and appropriate

free $[\text{Ca}^{2+}]$ and $[\text{EGTA}]$ were calculated by a computer program designed by Dr. P. Aaronson, based on work by Fabiato & Fabiato [26], which takes pH and $[\text{Mg}^{2+}]$ into account. At each $[\text{Ca}^{2+}]$ investigated, accumulation of $[^{32}\text{P}]\text{PA}$ in response to 0.1 mM-carbachol was significantly increased over basal values of accumulation. In addition, the increase in $[^{32}\text{P}]\text{PA}$ accumulation in response to 0.1 mM-carbachol became greater with increasing $[\text{Ca}^{2+}]$, suggesting a synergistic action between carbachol and Ca^{2+} .

The effects of histamine, substance P and noradrenaline on $[^{32}\text{P}]\text{PA}$ accumulation in guinea-pig longitudinal-smooth-muscle fragments were also investigated over a 20 min period (Fig. 4). Incubation with histamine (0.1 mM) stimulated an approx. 2-fold increase in $[^{32}\text{P}]\text{PA}$ accumulation (from a basal value of 11.5 ± 0.7 to $22.9 \pm 0.5\%$ of total lipid c.p.m.) after 2 min, and this increase was sustained (at $22.4 \pm 1.6\%$ of total lipid c.p.m.) after 5 min (Fig. 4a). However, after 10 min incubation with histamine the accumulation of $[^{32}\text{P}]\text{PA}$ fell back to near-basal values (though remaining slightly above basal at $13.7 \pm 0.5\%$ of total lipid c.p.m.). Incubation for 5 min with 1 mM-histamine also produced an approx. 2-fold increase in $[^{32}\text{P}]\text{PA}$ accumulation (from a basal value of 11.5 ± 0.7 to $22.3 \pm 1.9\%$ of total lipid c.p.m.; not shown).

Incubation with substance P (0.1 mM) produced a small but significant increase ($P < 0.05$) in accumulation of $[^{32}\text{P}]\text{PA}$ (from a basal value of 11.0 ± 0.6 to $13.5 \pm 0.8\%$ of total lipid c.p.m.) after 30 s, and this increase was sustained (at $13.4 \pm 1.0\%$ of total lipid c.p.m.) after 5 min (Fig. 4b). After 20 min incubation with substance P, the accumulation of $[^{32}\text{P}]\text{PA}$ fell back to a value not significantly different from the basal value (at $10.7 \pm 1.0\%$ of total lipid c.p.m.). A high rate of degradation of substance P has been reported in this tissue [27]. Incubation for 5 min with 1 μM - or 10 μM -substance P also stimulated significant increases in accumulation of $[^{32}\text{P}]\text{PA}$ (from a basal value of 10.7 ± 0.5 to 13.3 ± 0.7 and $13.4 \pm 1.1\%$ of total lipid c.p.m. respectively; not shown). Incubation for 5 min with 0.1 μM -substance P did not stimulate any significant increase in accumulation of $[^{32}\text{P}]\text{PA}$ (from a basal value of 10.7 ± 0.5 to $11.8 \pm 0.8\%$ of total lipid c.p.m.).

In contrast with the effects of carbachol, substance P and histamine, incubation with 0.1 mM-noradrenaline had no significant effect on $[^{32}\text{P}]\text{PA}$ accumulation over a 20 min period (Fig. 4c). Incubation for 5 min with noradrenaline (1 mM) also produced no significant change in accumulation of $[^{32}\text{P}]\text{PA}$ (from a basal value of 16.0 ± 0.4 to $14.8 \pm 0.3\%$ of total lipid c.p.m.). In other experiments, incubation with noradrenaline (0.1 mM) for 5 min produced an accumulation of $[^{32}\text{P}]\text{PA}$ of $16.2 \pm 0.6\%$ of total lipid c.p.m., a value not significantly different from the control value of $15.4 \pm 1.1\%$ of total lipid c.p.m. Incubation with carbachol (0.1 mM) and noradrenaline (0.1 mM) together for 5 min produced a significant increase in $[^{32}\text{P}]\text{PA}$ accumulation (to $33.6 \pm 1.9\%$ of total lipid c.p.m.). This was not significantly different from the increase in accumulation of $[^{32}\text{P}]\text{PA}$ (to $37.3 \pm 2.6\%$ of total lipid c.p.m.) given in response to 5 min incubation with carbachol (0.1 mM) alone.

It seems from these results that to use PA accumulation as an index of inositol phospholipid hydrolysis [28] may not be appropriate.

Table 1. Effects of carbachol, noradrenaline, histamine and substance P on accumulation of total [3 H]inositol phosphates in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with [3 H]inositol were incubated with the indicated agonists for 20 min in the presence of LiCl (10 mM). The results are means $\pm$ S.E.M. for three separate incubations: * significantly greater than control ($P < 0.01$); (N.S.), not significantly different from control.

Drug addition	Radioactivity in total [3 H]IPs (d.p.m./mg of protein)
Control	4927 $\pm$ 235
Carbachol (10 μ M)	9622 $\pm$ 503*
Carbachol (0.1 mM)	18449 $\pm$ 1585*
Substance P (0.1 μ M)	6215 $\pm$ 1078 (N.S.)
Substance P (1 μ M)	7188 $\pm$ 904 (N.S.)
Substance P (10 μ M)	10801 $\pm$ 1016*
Substance P (0.1 mM)	10671 $\pm$ 1175*
Histamine (1 mM)	10290 $\pm$ 398*
Noradrenaline (1 mM)	10570 $\pm$ 770*

Production of [3 H]inositol phosphates

In confirmation of previous reports [19], carbachol (0.1 mM) in the presence of LiCl (10 mM) produced a significant increase above basal values in the accumulation of [3 H]inositol monophosphate and [3 H]inositol bisphosphate within 1 min of application (results not shown). No change was detected in accumulation of [3 H]IP₃, presumably because it was rapidly converted into the inositol bisphosphate.

Accumulation of total [3 H]IPs was investigated in response to 20 min incubation with carbachol (10 μ M, 0.1 mM), substance P (0.1 μ M–0.1 mM), noradrenaline (1 mM) and histamine (1 mM), all in the presence of LiCl (10 mM) (Table 1). Incubation for 20 min with 10 μ M- or 0.1 mM-carbachol respectively produced approx. 2-fold and 4-fold increases in total [3 H]IPs accumulation. Incubation for 20 min with substance P (10 μ M or 0.1 mM) produced an approx. 2-fold increase in accumulation of total [3 H]IPs. Incubation for 20 min with 1 mM-noradrenaline or with 1 mM-histamine also produced 2-fold increase above basal in accumulation of total [3 H]IPs, in confirmation of a previous report [19].

DISCUSSION

Carbachol, histamine, substance P and noradrenaline have all been shown to increase accumulation of [3 H]IPs in the longitudinal-smooth-muscle layer from guinea-pig small intestine [14–19]. These findings have been confirmed in the present study and are compared with the effects of these agonists on accumulation of [32 P]PA in this tissue.

Accumulation of [32 P]PA reflects both the cellular concentration of PA and the specific radioactivity of [32 P]ATP. As the results presented here show, the increased accumulation of [32 P]PA was not caused by a carbachol-induced increase in the specific radioactivity of [32 P]ATP, as this did not change significantly throughout 10 min incubation in the presence of

Fig. 5. Summary of effects of agonists on accumulation of [32 P]PA and total [3 H]IPs in smooth-muscle fragments from guinea-pig small intestine

Tissue fragments labelled with [3 H]inositol or [32 P]P_i were incubated for 20 min or for 5 min respectively in PSS with carbachol (CCH), substance P (sP), histamine (Hist) or noradrenaline (NA). The percentage increases are calculated from the data given in the text, Fig. 1 or Table 1. All increases are significant ($P < 0.05$) except those marked N.S. (not significant). * Value by a slightly different method from Best *et al.* [19].

carbachol. Since there was also no change in the specific radioactivity of [32 P]ATP after a 10 min incubation period in the absence of agonist, the observed increase in accumulation of [32 P]PA can be taken to reflect the true rise in PA concentration. This extends a previous report of carbachol-induced increases in PA labelling in this tissue by Jafferji & Michell [25], where the specific radioactivity of [32 P]ATP was not determined.

The carbachol-stimulated increase in [32 P]PA had characteristics similar to total [32 P]IPs accumulation in the presence of LiCl (10 mM) reported by Best *et al.* [19], as both were stimulated over the same concentration range and inhibited in the presence of atropine (0.5–1 μ M), and both showed some dependency on free [Ca^{2+}]. Carbachol-induced increases in [32 P]PA were still produced even at free [Ca^{2+}] as low as 0.01 μ M, and carbachol and Ca^{2+} appeared to act synergistically to

increase [32 P]PA accumulation. Histamine and substance P also increased accumulation of [32 P]PA at concentrations which increased accumulation of total [3 H]IPs (the present paper; [15–18]). In contrast, incubation with noradrenaline at concentrations which stimulated increased accumulation of total [3 H]IPs (0.1 mM and 1 mM) (the present paper; [19]) produced no increase in accumulation of [32 P]PA (see Fig. 5 for summary).

Carbachol (10 μ M), substance P (0.1 mM) and histamine (1 mM) all produced increased accumulations of total [3 H]IPs that were the same size as that produced by 1 mM-noradrenaline (from a basal value of 4927 ± 235 to 9622 ± 503 , 10671 ± 1175 , 10290 ± 398 and 10570 ± 770 d.p.m./mg of protein respectively). However, although 10 μ M-carbachol and 1 mM-histamine also produced increases in accumulation of [32 P]PA of similar sizes (from basal values of 10.7 ± 0.6 and 11.5 ± 0.7 to 23.2 ± 1.1 and $22.3 \pm 1.6\%$ of total lipid c.p.m. respectively), 0.1 mM-substance P produced a much smaller increased accumulation of [32 P]PA (from a basal value of 11.0 ± 0.6 to $13.4 \pm 1.0\%$ of total lipid c.p.m.), and 1 mM-noradrenaline produced no significant change in accumulation of [32 P]PA. This suggests that noradrenaline and substance P act on the pathway for inositol phospholipid metabolism at steps different from, or additional to, those at which carbachol and histamine act. A likely possibility is that noradrenaline and substance P act either on DG kinase to suppress PA synthesis, or on PA phosphohydrolase or on CTP:PA cytidyltransferase to stimulate PA hydrolysis. This was investigated by measuring the effect of application of both carbachol and noradrenaline on [32 P]PA accumulation. However, the accumulation of [32 P]PA in response to carbachol (0.1 mM) was not significantly different in the absence or presence of noradrenaline (0.1 mM). Thus an effect on PA synthesis or degradation may be discounted. However, it is also possible that noradrenaline and substance P may act on different cellular pools of PIP₂, DG and/or PA from those affected by carbachol and histamine. The longitudinal-smooth-muscle layer of guinea-pig small intestine responds to noradrenaline and adrenaline with relaxation in proximal ileum via β -adrenoreceptors and contraction in terminal ileum through α_1 -adrenoreceptors, indicating that these two types of receptor are not uniformly distributed along the length of small intestine [29,30]. It is possible that the separate pools proposed will be different pools in different cell populations rather than distinct pools within a homogeneous population of cells. However, our results indicate that phospholipase-C-catalysed inositol phospholipid hydrolysis in smooth muscle does not necessarily result in PA production.

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