Permissive stimulation of Ca^{2+} -induced phospholipase A_2 by an adenosine receptor agonist in a pertussis toxin-sensitive manner in FRTL-5 thyroid cells: a new 'cross-talk' mechanism in Ca^{2+} signalling

Satoshi SHIMEGI, Fumikazu OKAJIMA and Yoichi KONDO

Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

We have described the pertussis toxin (PTX)-sensitive potentiation of P_2 -purinergic agonist-induced phospholipase C activation, Ca^{2+} mobilization and arachidonic acid release by an adenosine receptor agonist, N^6 -(L-2-phenylisopropyl)adenosine (PIA), which alone cannot influence any of these cellular activities [Okajima, Sato, Nazarea, Sho and Kondo (1989) *J. Biol. Chem.* **264**, 13029–13037]. In the present study we have found that arachidonic acid release was associated with lysophosphatidylcholine production, and conclude that arachidonic acid is produced by phospholipase A_2 in FRTL-5 thyroid cells. This led us to assume that PIA augments P_2 -purinergic arachidonic acid release by increasing $[\text{Ca}^{2+}]_i$ which, in turn, activates Ca^{2+} -sensitive phospholipase A_2 . The arachidonic acid-releasing response to PIA was, however, always considerably higher (3.1-fold increase) than the Ca^{2+} response (1.3-fold increase) to the adenosine derivative. In addition, arachidonic acid release induced by the $[\text{Ca}^{2+}]_i$ increase caused by thapsigargin, an endoplasmic-reticulum Ca^{2+} -ATPase inhibitor, or calcium iono-

phores was also potentiated by PIA without any effect on $[\text{Ca}^{2+}]_i$ and phospholipase C activity. This action of PIA was also PTX-sensitive, but not affected by the forskolin- or cholera toxin-induced increase in the cellular cyclic AMP (cAMP), suggesting that a PTX-sensitive G-protein(s) and not cAMP mediates the PIA-induced potentiation of Ca^{2+} -generated phospholipase A_2 activation. Although acute phorbol ester activation of protein kinase C induced arachidonic acid release, P_2 -purinergic and α_1 -adrenergic stimulation of arachidonic acid release was markedly increased by the protein kinase C down-regulation caused by the phorbol ester. This suggests a suppressive role for protein kinase C in the agonist-induced activation of arachidonic acid release. We conclude that PIA (and perhaps any of the G_i -activating agonists) augments an agonist (maybe any of the Ca^{2+} -mobilizing agents)-induced arachidonic acid release by activation of Ca^{2+} -dependent phospholipase A_2 in addition to enhancement of agonist-induced phospholipase C followed by an increase in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

A variety of extracellular stimuli regulate cellular functions through receptor mechanisms on the plasma membrane. Each stimulus activates its own receptor coupled to an intracellular signal-transduction system and induces specific cellular responses. When more than one signal act on a target cell simultaneously, however, cellular responses to those signals may not be additive if the signal-transduction mechanisms interfere with each other. In previous studies [1,2] on purinergic regulation of FRTL-5 thyroid cells (a continuous line of normal rat thyroid follicular cells), we have found that adenosine or phenylisopropyladenosine (PIA), an unhydrolysable adenosine derivative, augments the P_2 -purinergic agonist-induced activation of phospholipase C followed by intracellular Ca^{2+} mobilization and/or arachidonic acid release, in spite of the fact that both adenosine and PIA are typical inhibitory signals for adenylate cyclase, but not activators of phospholipase C. We have also shown that, in the presence of PIA, the thyrotropin (TSH)-induced cyclic AMP (cAMP) increase is reduced, but the TSH-induced phospholipase C activation and Ca^{2+} mobilization are markedly increased [3]. In a different type of cultured cells, similar permissive augmentation of phospholipase C and Ca^{2+} mobilization induced by one of the Ca^{2+} -mobilizing agonists, bradykinin, has been shown when one of the inhibitory agonists for adenylate cyclase including noradrenaline, muscarinic acetylcholine, enkephalin

and somatostatin is added [4]. In addition, we have found that all these co-operative actions are diminished by prior treatment of the cells with pertussis toxin (PTX), suggesting the involvement of a PTX-sensitive G-protein(s) in these actions [4]. Similar permissive and synergistic actions in cell regulation by adenosine receptor agonists have also been reported by other investigators [5–7].

On the basis of these findings, we have proposed that, in many types of cell, extracellular signal 'cross-talk' occurs at the step of phospholipase C activation via a PTX-sensitive G-protein(s), when two receptors, one coupled to a stimulatory mechanism for phospholipase C and the other to an inhibitory mechanism for adenylate cyclase, are activated [4]. This does not exclude, however, the possible occurrence of other cross-talk mechanisms in the signalling cascade beyond phospholipase C activation. Such considerations led us to re-examine our previous experiments on the PIA-induced potentiation of agonist-dependent stimulation of arachidonic acid release, which requires further steps beyond the phospholipase C activation [2].

When phospholipase C is activated, arachidonic acid can be produced in the following two ways: (1) hydrolysis of diacylglycerol, a phospholipase C product, by lipases [8,9] and (2) degradation of phosphatidylcholine by phospholipase A_2 which is stimulated by the increase in $[\text{Ca}^{2+}]_i$ induced by inositol trisphosphate, another phospholipase C product. In the present study, we show that Ca^{2+} -activated phospholipase A_2 participates

Abbreviations used: PIA, N^6 -(L-2-phenylisopropyl)adenosine; TSH, thyroid-stimulating hormone (thyrotropin); PTX, pertussis toxin; CTX, cholera toxin; PMA, phorbol 12-myristate 13-acetate; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione; NECA, 5'-N-ethylcarboxamidoadenosine; CHA, N^6 -cyclohexyladenosine; CADO, 2-chloroadenosine; W-5, N -(6-aminoethyl)-1-naphthalenesulphonamide hydrochloride; W-7, N -(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide hydrochloride; LPC, lysophosphatidylcholine; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; cAMP, cyclic AMP.

in P_2 -purinergic agonist-induced arachidonic acid release from FRTL-5 cells. The arachidonic acid release caused by Ca^{2+} -activated phospholipase A_2 is also potentiated by PIA in a PTX-sensitive manner. The results suggest that a PTX-sensitive G-protein(s) mediates the permissive effect of PIA on P_2 -purinergic agonist-induced arachidonic acid release not only at the step of phospholipase C induction but also at the Ca^{2+} -mediated phospholipase A_2 activation.

EXPERIMENTAL

Materials

PTX was generously provided by Dr. Michio Ui of Tokyo University and RO 20-1724 by Nippon Roche Research Center (Kamakura, Japan). Cholera toxin (CTX), noradrenaline, A23187, phorbol 12-myristate 13-acetate (PMA), N^6 -(*L*-2-phenylisopropyl)adenosine (PIA), 5'-*N*-ethylcarboxamidoadenosine (NECA), N^6 -cyclohexyladenosine (CHA), 2-chloradenosine (CADO) and *L*- α -phosphatidylcholine were from Sigma (St. Louis, MO, U.S.A.), and Fura-2 acetomethoxy ester (Fura-2/AM) was from Dojin Laboratories (Kumamoto, Japan). Ionomycin was obtained from Calbiochem (La Jolla, CA, U.S.A.), and lysophosphatidylcholine (egg) (LPC) was from Avanti Polar Lipids (Pelham, AL, U.S.A.). *myo*-[2- 3H]inositol (20.0 Ci/mmol), [5,6,8,9,11,12,14,15- 3H]arachidonic acid (79.9 Ci/mmol) and [*methyl*- 3H]choline chloride (85.1 Ci/mmol) were purchased from Du Pont-New England Nuclear. GTP and reagents for the cAMP assay were obtained from Yamasa Shoyu Co. (Choshi, Japan). *N*-(6-aminohexyl)-1-naphthalenesulphonamide hydrochloride (W-5) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) were from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

FRTL-5 thyroid cells, a continuous line of functional epithelial cells from normal rat thyroid [10], were provided by Interthyr Research Foundation (Baltimore, MD, U.S.A.). The cells were grown in Coon's modified Ham's F-12 medium [10] supplemented with 5% calf serum (Gibco, Grand Island, NY, U.S.A.) and a six-hormone mixture (6H) containing crude whale TSH (2 munits/ml), transferrin (5 μ g/ml), insulin (10 μ g/ml), somatostatin (10 ng/ml), hydrocortisone (5 ng/ml) and Gly-His-Lys acetate (10 ng/ml) in 10 cm tissue culture dishes (Costar, Cambridge, MA, U.S.A.) as previously described [10]. For measurements of [3H]arachidonic acid release, [3H]LPC and [3H]inositol phosphates production, cells were subcultured for 3–4 days to 60–70% confluency in 12-well plates (Costar), and for [Ca^{2+}]_i measurement, to 70–80% confluency in 10 cm culture dishes. At 1–2 days before experiments, the culture medium was changed in Ham's 10 medium supplemented only with 5% calf serum, except in the experiments in which noradrenaline was used as the agonist. In the latter case, Ham's 10 medium was supplemented with 6H in addition to 5% calf serum to avoid α -receptor down-regulation in FRTL-5 cells. The toxin treatment was performed by incubating the cells with PTX (100 ng/ml) or CTX (1 μ g/ml) for 24 or 6 h respectively as previously reported [2].

Measurement of the release of [3H]arachidonic acid and its metabolites

Cells labelled with [3H]arachidonic acid (1 μ Ci/ml) for 24 h in Ham's 10 medium supplemented with 5% calf serum were preincubated for 10 min at 37 °C in a Hepes-buffered medium

composed of 10 mM Hepes (pH 7.4), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose and 0.1% BSA (fraction V). After being washed three times with the medium, the cells were then incubated at 37 °C with various agents for the time indicated in the same buffer in a final volume of 750 μ l. After the incubation, the medium in each well that contained released [3H]arachidonic acid and its metabolites was each transferred to a counting vial and counted in a liquid-scintillation spectrometer (Beckman LS 7500). Data are expressed as percentages of total radioactivities incorporated into the cells.

To examine the effect of protein kinase C on [3H]arachidonic acid release, the enzyme was activated by the addition of PMA (10 ng/ml) 5 min before the addition of agonist, or down-regulated by the addition of a high dose of PMA (1 μ g/ml) 24 h before the experiment. Neither PMA activation nor down-regulation of protein kinase C changed the rate of incorporation of [3H]arachidonic acid into the cells.

Measurement of LPC production

Cells were labelled with [*methyl*- 3H]choline chloride (3 μ Ci/ml) for 24 h. The labelled cells were incubated with various agonists under the same conditions as those for the arachidonic acid release. After termination of cellular activities by the addition of methanol (0.75 ml), the cells were scraped off and phospholipids were extracted with 1.5 ml of chloroform containing 200 mg each of *L*- α -phosphatidylcholine and LPC. The extract was dried in a vacuum evaporator. The lipid residue was redissolved in 50 μ l of chloroform. LPC was isolated by one-dimensional t.l.c. using a mixture composed of chloroform/methanol/acetic acid/water (50:25:8:4, by vol.) by the method described by Currie et al. [11]. The spots corresponding to LPC and phosphatidylcholine were stained with iodine vapour, collected separately and counted in a liquid-scintillation spectrometer (Beckman LS 7500). Data are expressed as percentages of total radioactivities incorporated into phosphatidylcholine in the unstimulated cells.

Measurement of [Ca^{2+}]_i

The amount of cytosolic Ca^{2+} was estimated from the change in fluorescence in the Fura-2-loaded cells in the presence of 2 mM extracellular Ca^{2+} as described previously [12].

Measurement of [3H]inositol phosphate production

Cells were labelled with *myo*-[2- 3H]inositol (2.5 μ Ci/ml) for 48 h in Ham's 10 medium supplemented with 5% calf serum. After preincubation under the same conditions as those for the arachidonic acid-release experiments, except that the Hepes-buffered medium was supplemented with 10 mM LiCl, the cells were incubated with 750 μ l of various agents for the time indicated. Termination of the reaction and separation of various inositol phosphates were performed as described previously [12,13] except that various inositol phosphates were eluted together from the column with a buffer containing 1 M ammonium formate and 0.1 M formate. Data were normalized to 10⁵ d.p.m. of the radioactivity (not extracted with acid) remaining in the control (unstimulated) cells, which correspond to the total inositol lipid in the cells.

Measurement of cAMP accumulation

[3H]Arachidonic acid (1 μ Ci/ml)-labelled cells were preincubated in the same manner as in the experiments on arachidonic acid

release and then incubated for a further 5 min with the agents to be tested. After the medium was collected to measure arachidonic acid release, the cells were extracted with 500 μ l of 0.2 M HCl. The cAMP content in the acid extract was measured by a specific radioimmunoassay as described previously [14]. Results are expressed as nmol/100 mg of cellular protein.

RESULTS

Phospholipase A₂ participation in arachidonic acid release induced by GTP, a P₂-purinergic agonist, and its permissive stimulation by PIA, a P₁-purinergic agonist

Figure 1 shows the time courses of purinergic agonist-induced metabolic changes. In agreement with our previous report [2], GTP, a P₂-purinergic agonist, but not PIA, a P₁-purinergic agonist, caused inositol phosphate production as a result of phospholipase C activation (Figure 1d), intracellular Ca²⁺ increase (Δ [Ca²⁺]_i) (Figure 1c) and arachidonic acid release (Figure 1a). When PIA was added together with GTP, these effects of GTP, especially arachidonic acid release, were markedly increased, although PIA alone had no appreciable stimulatory action. As shown in Figure 1(b), GTP alone induced the immediate increase in lysophosphatidyl[³H]choline which should be produced in parallel with arachidonic acid by phospholipase A₂, and PIA greatly enhanced this GTP action. The lower increase in arachidonic acid release than that in the lyso compound production at 1 min might be explained by the additional releasing process in the case of arachidonic acid [11]. We conclude therefore that arachidonic acid is produced mainly by the action of phospholipase A₂ on phospholipids, and not by the combination of phospholipase C and acylglycerol lipases [8,9], at least under the present conditions.

However, the fact that the permissive stimulation by PIA was more obvious in phospholipase A₂ products (3.1-fold increase) than in phospholipase C products (1.3-fold increase) suggests that additional mechanisms are involved in the PIA stimulation of arachidonic acid release.

Role of [Ca²⁺]_i increase and protein kinase C activation in agonist-induced arachidonic acid release

In several types of cell, phospholipase A₂ has been shown to be sensitive to increases in [Ca²⁺]_i and protein kinase C activation [15–17]. As the P₂-purinergic stimulation of phospholipase C and its potentiation by PIA causes a substantial [Ca²⁺]_i increase and probably protein kinase C activation, it is possible that purinergic agonists primarily stimulate phospholipase C, and the resultant increase in [Ca²⁺]_i and protein kinase C activity, in turn, induces phospholipase A₂ activation. To explore this possibility, we examined the effect of an increase in [Ca²⁺]_i or protein kinase C activity by means other than agonist stimulation of the phospholipase C cascade. As shown in Table 1 (Experiment 1A), 300 nM ionomycin, a potent Ca²⁺ ionophore, markedly increased arachidonic acid release, which reached 15 times the control value. Although the addition of PMA, an activator of protein kinase C, alone induced only a 172% increase in arachidonic acid release, the combination of ionomycin and PMA synergistically stimulated arachidonic acid release to a level almost 60 times that of the control. All the results support the idea that phospholipase A₂ is regulated by P₂ receptor agonists via the phospholipase C cascade, particularly Ca²⁺-dependent mechanisms.

As several papers have reported phospholipase A₂ stimulation by protein kinase C activation [7,18–20], the role of protein

Figure 1 Time courses of P₁- and/or P₂-purinergic agonist-induced actions

In all the experiments, labelled or non-labelled FRTL-5 cells were incubated for the indicated times without (○) or with 300 μ M GTP (□), 1 μ M PIA (●) or 300 μ M GTP + 1 μ M PIA (■). In (a), the cells were preincubated with [³H]arachidonic acid to label phospholipids. After the incubation of the labelled cells with or without agonists, the radioactivity of the medium derived from the released arachidonic acid and its metabolites was measured as the released [³H]arachidonic acid. The values are expressed as percentages of total radioactivity of the cells. In (b), the cells were preincubated with [³H]choline to label phosphatidylcholine. The labelled cells were incubated with agonists as described in (a). The LPC produced was measured by the procedure given in the Experimental section. The values are expressed as percentages of total radioactivity incorporated into cellular phosphatidylcholine. In (c), the cells were detached from culture dishes and suspended in the medium and loaded with Fura-2 as described in the Experimental section. The increase above the basal [Ca²⁺]_i was plotted. The basal value (nM) was 149 ± 1.4 . In (d), the cells were incubated with [³H]inositol to label phosphatidylinositol. The labelled cells were incubated with agonists as described in (a). The inositol phosphates produced were analysed as described in the Experimental section. Radioactivity was normalized to 10^5 d.p.m. of total radioactivity incorporated into the phospholipid fraction. The data represent means $\pm$ S.E.M. of three (in the Ca²⁺ measurements) or four values from two separate experiments.

Table 1 Effect of PIA on ionomycin-, PMA-, GTP- and noradrenaline-induced arachidonic acid release in the presence of active or down-regulated protein kinase C

FRTL-5 cells were prelabelled with [3 H]arachidonic acid for 24 h in the presence or absence of 1 μ g/ml PMA and then incubated for 5 min in the presence of agents as indicated. Data are presented as the value relative to the value for the control without any addition (None), taken as 100. Each value is the mean $\pm$ S.E.M. for three separate experiments performed in triplicate.

	Arachidonic acid release			
	None	PIA (1 μ M)	PMA (1 μ g/ml) long treatment	PIA (1 μ M)
Experiment 1A				
None	100	121 $\pm$ 4	122 $\pm$ 9	133 $\pm$ 10
Ionomycin (300 nM)	1589 $\pm$ 324	3584 $\pm$ 482	1268 $\pm$ 263	2888 $\pm$ 363
PMA (10 ng/ml)	272 $\pm$ 28	386 $\pm$ 45	131 $\pm$ 11	164 $\pm$ 10
Ionomycin (300 nM) + PMA (10 ng/ml)	5815 $\pm$ 940	7102 $\pm$ 1063	1673 $\pm$ 248	3928 $\pm$ 602
Experiment 1B				
GTP (300 μ M)	368 $\pm$ 50	1841 $\pm$ 152	1476 $\pm$ 149	5579 $\pm$ 483
Experiment 2				
None	100	128 $\pm$ 3	128 $\pm$ 8	125 $\pm$ 9
Noradrenaline (10 μ M)	143 $\pm$ 6	575 $\pm$ 38	462 $\pm$ 53	6799 $\pm$ 247

kinase C in the agonist-induced release of arachidonic acid was further investigated by using cells pretreated for 24 h with PMA (1 μ g/ml), a situation in which protein kinase C should be down-regulated. In fact, in these cells, the acute effect of PMA alone was diminished to less than 10% above the control from 172% in the non-treated cells. The ionomycin effect was not reduced in the PMA-pretreated cells, indicating that the increase in $[Ca^{2+}]_i$ induced phospholipase A_2 activation independently of the action of protein kinase C. These results show that long-term pretreatment with PMA can be used to evaluate the roles of protein kinase C activation in signalling pathways when purinergic agonists stimulate the arachidonic acid release. As shown in Table 1 (Experiment 1B), long-term PMA treatment did not inhibit, but even enhanced, GTP-induced arachidonic acid release. In a separate experiment, noradrenaline-induced arachidonic acid release was also increased by the down-regulation of protein kinase C (Table 1, Experiment 2). These results suggest that, when receptor-mediated phospholipase C activation induces arachidonic acid release, the endogenously activated protein kinase C seems to play a negative rather than a positive role in arachidonic acid release, as shown for the α_1 -adrenergic receptor [21].

Effects of PIA on ionomycin- or thapsigargin-induced arachidonic acid release, LPC production and Ca^{2+} mobilization

Table 1 shows effects of PIA on arachidonic acid release under various conditions. The co-operation of PIA with P_2 -purinergic agonists in the stimulation of arachidonic acid release has already been reported [2] and is reproduced in the present experiments (Table 1, Experiments 1B and 2). Of particular importance is the

Figure 2 Time courses of thapsigargin- and/or P_2 -purinergic agonist-induced actions

In all the experiments, the procedures and data presentation are the same as those described in the legend to Figure 1 except that 300 μ M GTP was replaced with 30 nM thapsigargin. The cells were incubated without (○) or with 30 nM thapsigargin (□), 1 μ M PIA (●) or both (■). The inset in (a) shows dose-response curves of thapsigargin-induced arachidonic acid release for 5 min incubation in the presence (●) or absence (○) of 1 μ M PIA. In (c), the basal value was 155 ± 5.2 . The data represent means $\pm$ S.E.M. of three (in the Ca^{2+} measurements) or four values from two separate experiments.

fact that PIA potentiated the ionomycin-induced release of arachidonic acid (Table 1, Experiment 1A). As ionomycin-induced increase in $[Ca^{2+}]_i$ is thought to be due to influx of extracellular Ca^{2+} , and independent of agonist-induced phospholipase C activation, this permissive effect of PIA on arachidonic acid release is not due to its potentiation of agonist-dependent phospholipase C activation. This result suggests the presence of

Figure 3 Effects of P₁-purineric agonists on Ca²⁺-induced arachidonic acid release

The prelabelling procedure and incubation conditions were the same as described in the legend to Figure 1. Cells were incubated for 5 min with increasing concentrations of PIA (○), CHA (●), NECA (□) and CADO (■). Experiments are representative of at least two or more duplicate determinations.

an additional site of action of PIA beyond the Ca²⁺-mobilization step. This idea is consistent with the finding that the effect of PIA on the action of phospholipase A₂ is substantially greater than that on phospholipase C action (see Figure 1). The ionomycin-induced [Ca²⁺]_i increase is due to Ca²⁺ influx through the plasma membrane. In order to stimulate Ca²⁺ mobilization from an intracellular pool by purinergic or adrenergic stimulation, we examined events induced by thapsigargin, as thapsigargin is an inhibitor of endoplasmic-reticulum Ca²⁺-ATPase and capable of increasing [Ca²⁺]_i by inhibiting the re-entry of Ca²⁺ once mobilized from the intracellular pool [22]. As shown in Figures 2(a) and 2(b), thapsigargin stimulated arachidonic acid release and LPC production in a dose- and time-dependent manner. In an

early phase of the reaction, the thapsigargin-induced production of lyso compound, which seems to represent phospholipase A₂ activity, was rather low (Figure 2b), probably reflecting the pattern of [Ca²⁺]_i increase (Figure 2c). This thapsigargin-evoked [Ca²⁺]_i increase was not accompanied by inositol phosphate production (Figure 2d). PIA, nevertheless, enhanced the action of thapsigargin on phospholipase A₂ without causing any further change in [Ca²⁺]_i (Figure 2c). These results clearly confirm the presence of a new site of action of PIA 'cross-talk' beyond the intracellular Ca²⁺-mobilization step in the phospholipase C cascade.

Identification of the receptor responsible for the effect of PIA on Ca²⁺-induced arachidonic acid release

To characterize the P₁-purinergic receptor responsible for the PIA-evoked permissive action on Ca²⁺-induced arachidonic acid release, we investigated the order of potency of four adenosine derivatives (Figure 3). All the adenosine derivatives tested enhanced Ca²⁺-induced release of arachidonic acid in the order of potency PIA = CHA > CADO = NECA. This rank order is in accordance with that of agonist potencies for the A₁ receptor [23]. The results were practically the same as those obtained in the ionomycin- and thapsigargin-provoked events (results not shown). Therefore the A₁ receptor appears to mediate the action of PIA in potentiating Ca²⁺-induced arachidonic acid release.

PIA augmentation of Ca²⁺-induced arachidonic acid release is PTX-sensitive

Figure 4(a) shows the dose-response curves of arachidonic acid release induced by PIA in the presence and absence of 100 nM ionomycin. Arachidonic acid release increased at concentrations above 0.1 nM PIA and reached a plateau at 0.1 μM, although, in the absence of ionomycin, no effect of PIA was observed over the whole PIA concentration range tested. The highest level of arachidonic acid released was almost 8-fold that induced by ionomycin alone. This PIA effect was virtually absent in cells pretreated with PTX (Figure 4b). Thus PTX-sensitive G-protein appears to mediate the PIA-induced permissive action.

Figure 4 Effect of PTX treatment on PIA potentiation of Ca²⁺-induced arachidonic acid release

FRTL-5 cells were treated with (b) or without (a) PTX as described in the Experimental section during prelabelling with [³H]arachidonic acid. The labelled cells were incubated for 5 min with increased concentrations of PIA in the absence (○) or presence (●) of 100 nM ionomycin. The data represent means ± S.E.M. from two separate experiments performed in triplicate.

Table 2 PIA potentiation of Ca^{2+} -induced arachidonic acid release at various cAMP levels

FRTL-5 cells were prelabelled for 24 h with [^3H]arachidonic acid in the presence or absence of 1 $\mu\text{g/ml}$ CTX and then incubated for 5 min in the presence of agents as indicated. Data are presented as means $\pm$ S.E.M. of four values from two separate experiments performed in duplicate. ^a $P < 0.01$ compared with the value obtained for cells treated with thapsigargin alone; ^b $P < 0.05$, ^c $P < 0.01$ compared with the value obtained for cells treated with the indicated agents and thapsigargin; ^d $P < 0.01$ compared with the value obtained for cells treated with thapsigargin + PIA only.

Addition	cAMP content (nmol/100 mg)		Arachidonic acid release (%)	
	Thapsigargin	Thapsigargin + PIA	Thapsigargin	Thapsigargin + PIA
None	1.00 $\pm$ 0.10	0.71 $\pm$ 0.09 ^c	2.69 $\pm$ 0.46	5.64 $\pm$ 0.53 ^c
RO 20-1724 (200 μM)	1.38 $\pm$ 0.12 ^a	0.83 $\pm$ 0.09 ^{cd}	1.89 $\pm$ 0.25	4.40 $\pm$ 0.63 ^c
CTX (1 $\mu\text{g/ml}$)	1.86 $\pm$ 0.15 ^a	1.57 $\pm$ 0.18 ^{bd}	2.86 $\pm$ 0.64	5.62 $\pm$ 0.42 ^c
CTX + RO 20-1724	19.1 $\pm$ 1.60 ^a	2.64 $\pm$ 0.31 ^{cd}	1.17 $\pm$ 0.11	3.26 $\pm$ 0.28 ^c
Forskolin (10 μM)	1.69 $\pm$ 0.05 ^a	1.16 $\pm$ 0.04 ^{cd}	3.08 $\pm$ 0.22	4.38 $\pm$ 0.19 ^c
Forskolin + RO 20-1724	9.84 $\pm$ 0.49 ^a	1.26 $\pm$ 0.04 ^{cd}	1.35 $\pm$ 0.11	4.52 $\pm$ 0.33 ^c

Protein kinase C is not involved in the PIA potentiation of Ca^{2+} -induced arachidonic acid release

As shown in Table 1 (Experiment 1A), the PIA potentiation of ionomycin-induced arachidonic acid release was not substantially affected by down-regulation of protein kinase C by long-term PMA treatment of the cells. This suggests that protein kinase C is not involved in the action of PIA.

PIA potentiation of Ca^{2+} -induced arachidonic acid release is not due to lowering of the cellular level of cAMP

As PIA inhibits adenylate cyclase via the A_1 receptor in a PTX-sensitive fashion in FRTL-5 cells [2,24], we examined whether such a PIA-dependent decrease in cellular cAMP is a cause of increased arachidonic acid release. The effect of PIA on thapsigargin-induced arachidonic acid release was investigated under conditions in which cAMP concentration was controlled by CTX- or forskolin-induced adenylate cyclase activation in the presence or absence of RO 20-1724, a phosphodiesterase inhibitor. When the cells were treated with CTX or forskolin in the presence of the diesterase inhibitor, the cAMP level was markedly increased (Table 2). Although PIA decreased cAMP levels, they were still significantly higher than the control level. Nevertheless, PIA-dependent arachidonic acid release was not substantially affected by the higher cAMP concentration. Thus it is unlikely that the decrease in cAMP concentration plays a role in the PIA-mediated Ca^{2+} -induced arachidonic acid release. In this experiment, the presence of RO 20-1724 appeared to reduce arachidonic acid release in response to thapsigargin or thapsigargin + PIA (compare Table 2 row 1 with row 2 in columns 3 and 4). Although no adequate explanation can be given for this effect at this stage of the study, it may not be due to the increased cAMP concentration.

Involvement of calmodulin in Ca^{2+} -induced arachidonic acid release

Calmodulin is one of the most important factors in Ca^{2+} -mediated effects. Therefore we investigated the effect of W-5 and W-7, both of which are calmodulin inhibitors with different affinities for calmodulin. As shown in Figure 5, arachidonic acid release was dose-dependently reduced by W-7 and W-5 above 10 μM not only in the presence of PIA but also in its absence. These findings suggest that Ca^{2+} /calmodulin is involved in the Ca^{2+} -evoked arachidonic acid-releasing mechanism.

Figure 5 Effect of calmodulin antagonists on thapsigargin-induced arachidonic acid release in the presence and absence of PIA

FRTL-5 cells were incubated with the indicated doses of W-5 ($\square$, $\blacksquare$) or W-7 ($\circ$, $\bullet$) for 5 min and then thapsigargin (30 nM) was added with ($\blacksquare$, $\bullet$) or without ($\square$, $\circ$) PIA. Incubation was continued for a further 5 min. Arachidonic acid release in the presence of inhibitors is expressed as percentages of arachidonic acid release in the absence of inhibitors, induced by thapsigargin alone or by thapsigargin + PIA. Data are presented as means $\pm$ S.E.M. of quadruplicate experiments. Thapsigargin alone released $4.21 \pm 0.38\%$ of total radioactivity from cells labelled with [^3H]arachidonic acid, and thapsigargin + PIA released $10.19 \pm 0.94\%$. These values were taken as 100% for experiments using thapsigargin alone and those using thapsigargin + PIA respectively.

DISCUSSION

In the present study, we investigated signal-transduction mechanisms for the PIA potentiation of extracellular GTP- and noradrenaline-induced arachidonic acid release from FRTL-5 thyroid cells.

The permissive effect of PIA on phospholipase C activation in association with [Ca^{2+}]_i increase has been extensively studied by our group [1–3,24]. We have found that, in FRTL-5 thyroid cells, TSH, noradrenaline and extracellular GTP or other P_2 -purinergic agonists activate phospholipase C via their receptors coupled with a PTX-insensitive G-protein, G_p [25]. PIA has been found to potentiate the activation of phospholipase C by all of these agonists in a PTX-sensitive manner, showing that the action of PIA is not receptor-specific. These results suggest that PIA acts, via a PTX-sensitive G-protein, on a mechanism of phospholipase

C activation by Gp. This PIA potentiation of phospholipase C leads to enhancement of Ca²⁺ mobilization via increased inositol phosphate production.

In the present study, we found a new type of PIA action which modulates Ca²⁺-induced phospholipase A₂ activation. As this action does not cause a further increase in [Ca²⁺]_i, but stimulates phospholipase A₂ even under the conditions of high [Ca²⁺]_i, caused by Ca²⁺ ionophores or thapsigargin, PIA appears to enhance the catalytic activity of the enzyme.

We found that reduction of the intracellular cAMP level by PIA is not associated with the effect of PIA on Ca²⁺-induced phospholipase A₂ activation (see Table 2). This is similar to the PIA potentiation of agonist-induced phospholipase C activation which occurs regardless of the intracellular cAMP level [1,2].

Felder et al. [26] have reported that, in CHO cells transfected with any one of the G_i-coupled receptor cDNAs for m₂-muscarinic, D₂-dopaminergic and α₂-adrenergic receptors, extracellular ATP stimulation of arachidonic acid release via endogenous ATP receptor was potentiated by an agonist for the receptor transfected. The potentiation was blocked by protein kinase C inhibition or down-regulation, suggesting protein kinase C involvement in the potentiation mechanism. Discrepancy between this result and ours, which showed no appreciable influence of protein kinase C down-regulation on the PIA potentiation of arachidonic acid release, may be due to differences in the signal-transduction systems, as the protein kinase C action may feedback positively or negatively to the activities of many components in the systems, including phospholipase C and G-proteins.

Two calmodulin inhibitors (W-7 and W-5) completely abolished the thapsigargin-induced arachidonic acid release in both the presence and absence of PIA (Figure 5). This suggests that the elevated [Ca²⁺]_i acts on arachidonic acid release via Ca²⁺/calmodulin as previously shown in human platelet [27] and medullary [28] subcellular preparations. Recently, Ca²⁺ induction of phospholipase A₂ has been suggested to be due to Ca²⁺-induced translocation of the enzyme from cytosol to membranes where the enzyme is facilitated to react with its substrates [29–31]. This idea was supported by the finding of a Ca²⁺-dependent translocation domain with homology to protein kinase C and GTPase-activating protein, in a cytosolic phospholipase A₂ of monocytic leukaemic U937 cells [31]. Although *in vitro* Ca²⁺ has been shown to change the enzyme molecules from hydrophilic to hydrophobic forms [30], calmodulin is likely to be involved in this process in cellular events. If this is the case, the requirement of elevated [Ca²⁺]_i for PIA-dependent stimulation of phospholipase A₂ could be explained by assuming that the Ca²⁺-induced translocation facilitates the interaction of the enzyme with PIA via its receptor coupled to a PTX-sensitive G-protein.

It is likely that phospholipase A₂ interacts with a G-protein in its receptor-mediated activation by PIA, as such G-protein participation has been shown in various cell systems: a PTX-sensitive G-protein(s) mediates agonist-induced phospholipase A₂ activation in platelet and rod segments [32–37], and a PTX-insensitive G-protein(s) in fibroblasts and keratinocytes [38,39]. In analogy with receptor-mediated phospholipase C activation [40], the agonist-coupled receptors seem to activate the G-proteins and produce Gα or βγ subunits [32] which, in turn, modulate phospholipase A₂ activity.

In conclusion, in FRTL-5 thyroid cells, the A₁-purinergic

agonist potentiates P₂-purinergic- or α₁-adrenergic activation of phospholipase A₂ by two distinct mechanisms, one involving potentiation of the agonist-induced phospholipase C activation resulting in [Ca²⁺]_i increase and the other the enhancement of Ca²⁺-induced phospholipase A₂ activation. This shows that the A₁ receptor can couple, via a PTX-sensitive G-protein(s), to three effector enzymes, adenylate cyclase and phospholipases C and A₂.

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