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P_2-purinergic agonists activate phospholipase C in a guanine nucleotide- and Ca^{2+}-dependent manner in FRTL-5 thyroid cell membranes

Fumikazu Okajima, Koichi Sato and Yoichi Kondo

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

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Various adenine nucleotides activated phospholipase C of FRTL-5 cell membranes in the following order of activity, ATPγS > ATP > AppNp > AppCp = ADP > MeSATP. This order was well consistent with that observed in intact cells. Such activation occurred only in the presence of appropriate concentrations of GTPγS and Ca^{2+}, in a way similar to the norepinephrine-induced activation. NaF, a non-specific GTP-binding protein (G-protein) activator, also stimulated the enzyme. These adenine nucleotides, norepinephrine and NaF-induced activations were inhibited by GDPβS. We conclude that a G-protein is involved in the adenine nucleotides-induced activation of phospholipase C via P_2-purinergic receptor in FRTL-5 cells.

Purinergic receptor, P_2-; GTP-binding protein; Phospholipase C; (FRTL-5 cell)

1. INTRODUCTION

Recent publications have shown that the receptors for Ca^{2+}-mobilizing hormones are linked to the activation of phospholipase C through a GTP-binding protein (G-protein), based on the fact that GTP or its derivatives synergize those hormones in the activation of the enzyme [1–3]. The G-protein which activates phospholipase C has been termed G_p [2]. Extracellular ATP and related compounds have recently been found, via P_2-purinergic receptor, to stimulate phospholipase C and to cause inositol phosphate formation in many types of cells [4–10] including FRTL-5 thyroid cells [11–14].

In the present paper, we studied the P_2-receptor-mediated activation of phospholipase C in a cell-free membrane preparation of FRTL-5 cells. We used mainly nonhydrolyzable P_2-agonists such as AppNp and AppCp instead of ATP, in order to avoid a possible participation of the agonists in the production of phosphatidylinositol 4,5-bisphosphate (PIP_2), the substrate of phospholipase C, by phosphorylating phosphatidylinositol 4-phosphate (PIP). These P_2-agonists activated phospholipase C in a GTPγS- and Ca^{2+}-dependent manner in the same way as norepinephrine, an α_1-adrenergic receptor agonist [15,16], did, proving that G_p is involved in the P_2-receptor-mediated phospholipase C activation in FRTL-5 thyroid cells.

Correspondence address: F. Okajima, Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

Abbreviations: AppNp, 5′-adenylylimidodiphosphate; AppCp, β,γ-methyleneadenosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); MeSATP, 2-methylthioadenosine 5′-triphosphate

2. MATERIALS AND METHODS

2.1. *Materials*

AppNp, AppCp, ATP and ADP were purchased from Sigma; GTPγS, GDPβS, ATPγS and ADPβS were from Boeh-

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ringer Mannheim; MeSATP was from Research Biochemicals Inc.; *myo*-[2-^{3}H]inositol (23 Ci/mmol) was from American Radiolabeled Chemicals Inc. The sources of all other reagents used were those described in [4,11–13,16].

2.2. *Cell culture*

FRTL-5 thyroid cells, a cell line derived from normal rat thyroid [17] was provided by Interthyr Research Foundation (Baltimore, MD). The cells were grown in 12-well plates (for measuring [^{3}H]inositol phosphates production in intact cells) or 10-cm culture dishes (for preparing membrane fractions) as described in [12,14,16]. The culture medium was changed to Ham's 10 medium containing [^{3}H]inositol (2.5 μCi in 1-ml medium) and 5% calf serum with a six-hormone mixture as described in [16] and maintained for further 2 days.

2.3. *[^{3}H]Inositol phosphates production in intact cells*

In figs 3C and 4B, [^{3}H]inositol phosphates production from intact cells in response to norepinephrine (NE) and various P_2-agonists was measured as described in [11,14,16].

2.4. *Membrane preparation and assay of phospholipase C*

The cells cultured with [^{3}H]inositol were washed twice with PBS containing 1 mM EGTA and scraped with a rubber policeman. The cells were washed once more with PBS, suspended in 50 mM Hepes (pH 7.4) containing 50 mM sucrose, 1 mM EGTA and 100 U/ml aprotinin, then homogenized in a Physcotron homogenizer (NS-310E, Niti-on, Tokyo, Japan) for 20 s, and centrifuged at 500 × *g* for 10 min. The supernatant was recentrifuged at 10000 × *g* for 15 min and the resultant pellet was used as crude plasma membranes. These membranes (~100 μg protein), containing 5 × 10^5 dpm in 100 μl, were incubated at 37°C for 10 min (in a final volume of 200 μl) with 500 μM $CaCl_2$ (unless otherwise specified), 1 mM EGTA, 50 mM sucrose, 100 U/ml of aprotinin, 2.5 mM $MgCl_2$, 100 mM KCl, 10 mM LiCl, 0.1 mg/ml of bovine serum albumin, 50 mM Hepes (pH 7.4) and various agents tested. Free Ca^{2+} concentration was measured by Quin 2 fluorescence and was approximately 50 nM under these conditions. The reaction was terminated by adding first 1 ml of $CHCl_3$/MeOH/HCl (100:100:1) and afterwards 0.3 ml of H_2O. ^{3}H-labeled inositol phosphate (IP), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) were separated as described in [11]. The IP_3 fraction contains both IP_3(1,4,5) and IP_3(1,3,4). Data were normalized to 5 × 10^5 dpm of the radioactivity in membranes.

3. RESULTS AND DISCUSSION

Fig.1 shows the formation of IP, IP_2 and IP_3 by ^{3}H-labeled membranes incubated with GTPγS, AppNp, NE or their combinations in the presence of the indicated concentration of free Ca^{2+}. The inositol phosphate formation, reflecting phospholipase C activation, was increased with the increase in Ca^{2+} concentration either in the presence or absence of additives (fig.1A–F). A half-maximal effective concentration (EC_{50}) of Ca^{2+} was about 10 μM in all the cases without any addition. 10 μM GTPγS activated the enzyme only when the Ca^{2+} level was physiological or lower (10–100 nM), but did not at the higher Ca^{2+} level (100 μM–1 mM). Thus, GTPγS lowered the effective Ca^{2+} concentration for enzyme activation. The results were essentially similar to those reported with other types of cells (see for reviews [1,2]) as well as FRTL-5 cells [18,19], in terms of a guanine nucleotide-dependent activation. This suggests the involvement of a G-protein, G_p, in the regulation of phospholipase C activity in such a way that G_p increases the sensitivity of the enzyme to Ca^{2+}.

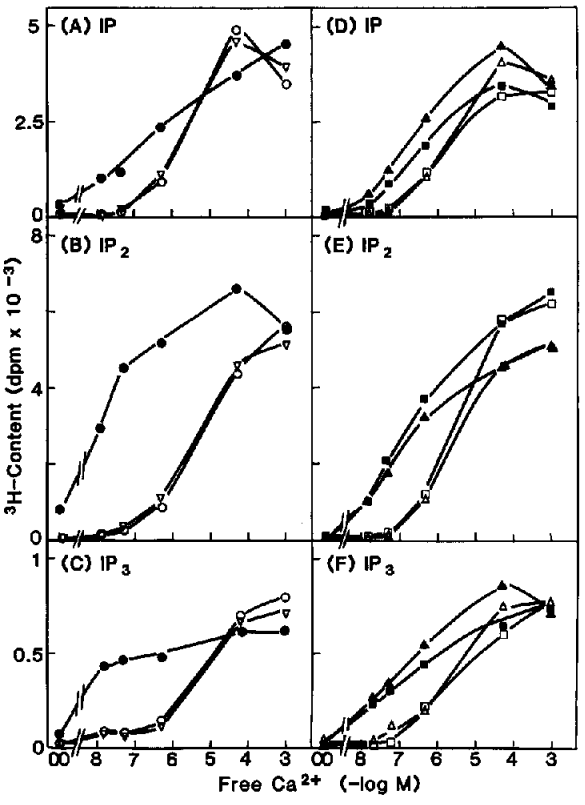

Fig.1. Ca^{2+}-dependent phospholipase C activity. ^{3}H-labeled membranes were incubated with no addition (○), 0.1 μM GTPγS (▽), 10 μM GTPγS (●), 1 μM norepinephrine (NE) (△), 1 μM NE plus 0.1 μM GTPγS (▲), 100 μM AppNp (□), and 100 μM AppNp plus 0.1 μM GTPγS (■) in the presence of the indicated free Ca^{2+} concentration. Results were the mean of two separate experiments. The difference in the value between two experiments was less than 10% of the mean value.

As shown in fig.1D–F, this cell-free system was not activated by the addition of 100 μM AppNp, a

P_2-agonist nor by 1 μM NE in the absence of the GTP derivative. Moreover, the GTPγS-induced activation was not appreciable at 0.1 μM of the nucleotide (fig.1A–C). However, in the presence of this low dose (0.1 μM) of the nucleotide, these agonists induced a phospholipase C activation, in a manner similar to the high GTPγS concentration; the rate of activation was very high at a physiological level of free Ca^{2+} (fig.1D–F).

In fig.2, we examined in detail the AppNp-induced activation of phospholipase C at a sub-physiological level of Ca^{2+} (50 nM) where no appreciable Ca^{2+} effect was observed. GTPγS alone increased the enzyme activity in a dose-dependent manner. In consistency with the results shown in fig.1, 100 μM AppNp potentiated the GTPγS action in the low dose range of GTPγS (3 nM–1 μM), although AppNp never changed the maximum level of GTPγS-induced phospholipase C, presumably because the enzyme activity was already fully activated at such high concentrations of GTPγS. Thus, AppNp decreased EC_{50} for the GTPγS-induced activation from around 1 μM to around 50 nM. This AppNp action was quite similar to the NE action on GTPγS-induced phospholipase C (data not shown). These results support the idea that the AppNp stimulation of P_2-receptors as well as the NE stimulation of α_1-receptors of FRTL-5 cell membranes induce phospholipase C activation via G_p in a manner analogous to the G_s-dependent activation of receptor-coupled adenylate cyclase [3].

The involvement of G-protein (G_p) in the P_2-agonists' activation of phospholipase C was further supported by the experiments shown in fig.3. A non-specific G-protein activator, NaF (in the presence of $AlCl_3$) activated the enzyme in the absence of guanine nucleotide. This NaF-induced activation was significantly inhibited by a GDP derivative, GDPβS (fig.3A). On the other hand, the inhibition rate was decreased with the increase in NaF concentration; the percentage inhibition in the presence of 1 mM GDPβS was 88%, 59%, and 37% when 0.5 mM, 1 mM, and 5 mM NaF was added, respectively. The results suggest that GDPβS competitively inhibited the NaF-induced action. The GDP derivative also inhibited the enzyme activation in response to either AppNp or NE (fig.3B). In these cases, GDPβS probably acts at a transducer (G_p) level rather than a receptor level,

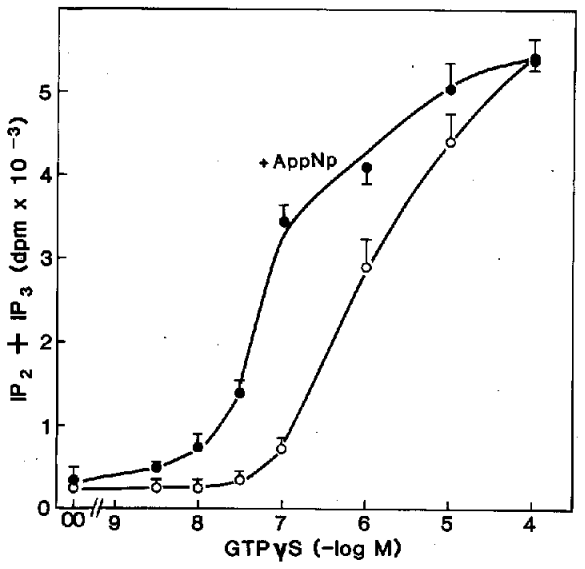

Fig.2. Effect of AppNp on GTPγS-induced phospholipase activity. ^{3}H-labeled membranes were incubated with (●) or without (○) 100 μM AppNp in the presence of the indicated dose of GTPγS. Results were the mean ± SE of three separate experiments.

since this guanine nucleotide failed to affect both AppNp and NE-induced inositol phosphate production in intact cells (fig.3C).

In the foregoing experiments, we used AppNp as a nonhydrolyzable ATP derivative instead of ATP, to analyze P_2-receptor agonist actions in a cell-free system. In all the experiments, AppNp behaved similarly to NE as a receptor agonist which activated phospholipase C, without contradicting the idea that AppNp acts as an agonist for a purinergic, probably a P_2-purinergic receptor. To confirm further the receptor subtype, the effect of various adenine nucleotides known as P_2-agonists on phospholipase C activity was examined in the present cell-free system (fig.4A) and compared with the results obtained in intact FRTL-5 cells (fig.4B). In the cell-free system, the order of magnitude of the responses to these purine nucleotides at concentrations tested was ATPγS > ATP > AppNp > AppCp = ADP > MeSATP > ADPβS, although we could not compare their accurate potency or efficacy because of the unsaturated dose-response curves under the conditions employed (fig.4A). This rank order was well consistent with that obtained in intact cells

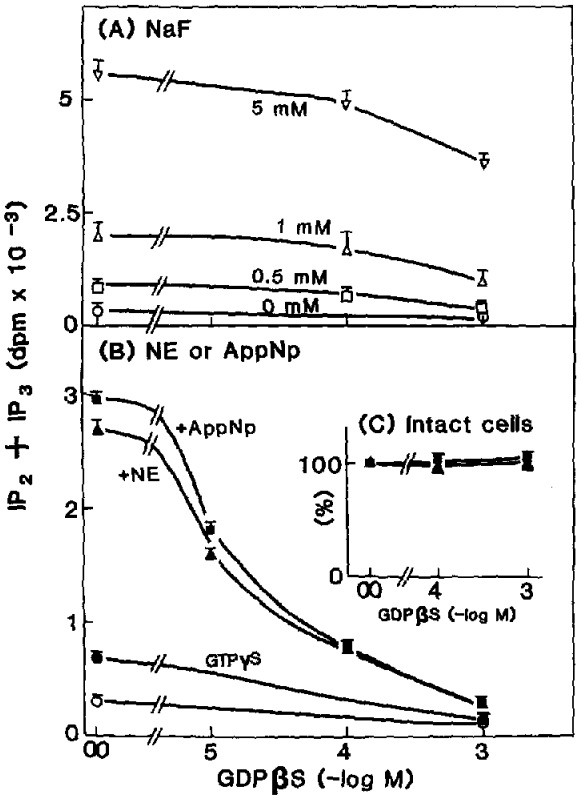

Fig.3. Effect of GDPβS on NaF, NE or AppNp-induced phospholipase C in membrane fractions (A and B) or in intact cells (C). ^{3}H-labeled membranes were incubated with the indicated concentration of NaF plus 10 μM $AlCl_3$ in A, and with no addition (○), 0.1 μM GTP (●), 0.1 μM GTP plus 1 μM NE (▲) or 0.1 μM GTP plus 300 μM AppNp (■) in B, in the presence of the indicated dose of GDPβS. IP_2 + IP_3 were measured. In C, ^{3}H-labeled cells were incubated for 30 min with 1 μM NE (▲) or 300 μM AppNp (■) in the presence of the indicated dose of GDPβS. Total inositol phosphates (IP + IP_2 + IP_3) were measured. In the absence of GDPβS, inositol phosphate formation was increased from 4860 ± 360 (dpm/well) to 35820 ± 1230 and 81690 ± 1893 by NE and AppNp, respectively. Data were expressed as percentage of these activities elicited by these agents. Results were the mean ± SE of at least three separate experiments in each case.

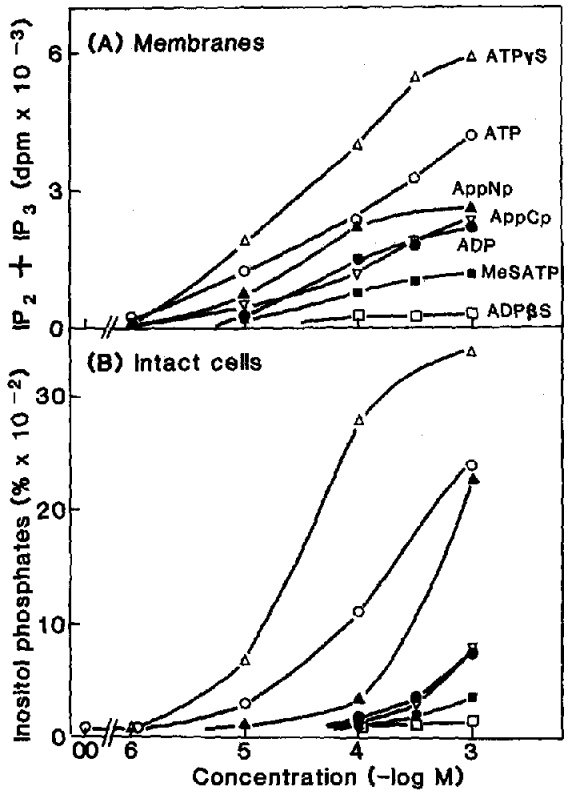

Fig.4. Dose-dependent activation of phospholipase C as induced by P_2-agonists in membranes (A) and in intact cells (B). In A, ^{3}H-labeled membranes were incubated with the indicated concentration of ATPγS (△), ATP (○), AppNp (▲), AppCp (▽), ADP (●), MeSATP (■) or ADPβS (□) in the presence of 0.1 μM GTPγS. The increment in activity as induced by these P_2-agonists was shown. The activity obtained by GTPγS alone was 0.72 (dpm × 10^{-3}). In B, ^{3}H-labeled cells were incubated for 30 min with various P_2-agonists. The formation of total inositol phosphates (IP + IP_2 + IP_3) was measured, and results were expressed as percentage of the control value (without P_2-agonist). The control value was 2973 dpm/well. Symbols were the same as those shown in A. Results in A and B were the mean of two separate experiments. The difference in the value between two experiments was less than 10% of the mean value.

(fig.4B). Adenosine or its derivative, phenylisopropyladenosine known as P_1-agonists was ineffective in the activation of the enzyme both in membranes and in intact cells (data not shown).

Summarizing the above results, in FRTL-5 thyroid cells, their stimulated P_2-purinergic receptors as well as α_1-adrenergic receptors activate phospholipase C with the aid of G_p. An involvement of G_p in the regulation of P_2-receptor-coupled phospholipase C has recently been suggested also in turkey erythrocyte membranes [20]. However, in these avian erythrocyte membranes [20], MeSATP and ADPβS were more potent than ATP, whereas in FRTL-5 cell membranes these nucleotides were very weak agonists, suggesting the presence of two P_2-receptors both linked to phospholipase C via G_p but with different pharmacological specificities, just like β-adrenergic

receptor subtypes, β_1- and β_2-subtype, both of which are linked to adenylate cyclase via the same G-protein, G_s. This would suggest a great variety of P_2-receptor subtypes than those previously proposed by us: two types of P_2-receptors, one coupling to phospholipase C and the other coupling to adenylate cyclase via G_i [4], and by others [21].

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