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Stimulation of adenosine receptor enhances α_1-adrenergic receptor-mediated activation of phospholipase C and Ca^{2+} mobilization in a pertussis toxin-sensitive manner in FRTL-5 thyroid cells

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Norepinephrine (NE) stimulated FRTL-5 thyroid cells via an α_1-adrenergic receptor, resulting in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) mobilization and activation of phospholipase C. Adenosine and its receptor agonist, phenylisopropyladenosine (PIA), although not exerting a direct effect, markedly enhanced the NE-induced changes. Basal NE action was not totally abolished whereas the permissive action of adenosine and PIA was completely abolished by pretreatment of the cells with islet-activating protein (IAP), pertussis toxin. The decrease in cAMP level induced by adenosine or PIA is not the cause of their permissive effect, since this effect was not reversed by the addition of cAMP-increasing agents. We conclude that an IAP substrate GTP-binding protein(s) plays a novel role in forming a stimulatory coupling between an adenosine receptor and an α_1-adrenergic receptor-coupled phospholipase C system.

GTP-binding protein; Islet-activating protein; Phospholipase C; Adrenergic receptor, α_1-; Adenosine receptor; (FRTL-5 cell)

1. INTRODUCTION

GTP-binding proteins (G-proteins) have recently been suggested to be involved in receptor-mediated activation of phospholipase C [1,2]. The postulated G-protein is referred to as G_p [2], being distinct from G_s as stimulatory and G_i as inhibitory G-proteins for adenylate cyclase [3]. Islet-activating protein (IAP), pertussis toxin, ADP-ribosylates G_i and G_i-like proteins resulting in the loss of their regulatory functions for adenylate cyclase [1–3]. The toxin, although not in all cases, also inhibits receptor-mediated phospholipase C activation [1,2], suggesting the presence of at least two species of G_p, i.e. IAP-sensitive and insensitive forms [1,2].

We recently found that extracellular ATP, via a P_2-purinergic receptor, activated phospholipase C and Ca^{2+} mobilization in FRTL-5 thyroid cells [4,5]. This P_2-receptor-mediated enzyme activation was inhibited by IAP, however inhibition was partial even though an approx. 41 kDa IAP substrate G-protein was completely ADP-ribosylated. On the other hand, phospholipase C activation by α_1-adrenergic agonists was hardly influenced by the toxin treatment in the same cells [6]. To gain further insight into the role of the IAP substrate in the regulation of phospholipase C, we investigated here the cooperation between norepinephrine, an α_1-adrenergic agonist, and adenosine receptor agonists which inhibit adenylate cyclase in an IAP-sensitive manner, but do not affect phospholipase C [7]. The adenosine receptor agonists remarkably enhanced the IAP-insensitive action of norepinephrine on FRTL-5 cells. Only the permissive actions of adenosine receptor agonists were totally sensitive to IAP, suggesting the involvement of IAP-substrate G-protein(s) with a novel role.

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2. MATERIALS AND METHODS

2.1. *Materials*

Norepinephrine, prazosin, yohimbine, propranolol, forskolin, N^6-(L-2-phenylisopropyl)adenosine (PIA), adenosine and theophylline were purchased from Sigma; *myo*-[2-^{3}H]inositol (15.6 Ci/mmol) was from New England Nuclear. IAP was generously provided by Dr Michio Ui of Tokyo University (Tokyo, Japan) and 4-(3-butoxy-4-methyoxybenzyl)-2-imidazolidione (RO 20-1724) by Nippon Roche Research Center (Kamakura, Japan). Reagents for cAMP radioimmunoassay were also a gift from Yamasa Shoyu Co. (Chosi, Japan). The sources of all other reagents used were those described in [4,5].

2.2. *Cell culture*

FRTL-5 cells, a cell line derived from normal rat thyroid [8], was provided by Interthyr Research Foundation (Baltimore, MD). Cells were grown as in [5]. After cells had reached 60–70% confluence in 12-well plates (for measuring cAMP content and [^{3}H]inositol phosphate production) or 10-cm culture dishes (for measuring cytosolic Ca^{2+} ($[Ca^{2+}]_i$)), the culture medium was changed to Ham's 10 medium containing 5% calf serum with a six-hormone mixture of thyrotropin, insulin, hydrocortisone, transferrin, somatostatin and Gly-His-Lys acetate as described [5] and maintained for a further 2 days. Where indicated, the medium was supplemented with 10 ng/ml IAP, [^{3}H]inositol (3 μCi in 1 ml medium) or both as shown in [4].

2.3. *Measurement of $[Ca^{2+}]_i$*

$[Ca^{2+}]_i$ was estimated from the change in fluorescence of fura 2-loaded cells in the presence of 2 mM extracellular Ca^{2+} as described [4,5].

2.4. *Measurement of [^{3}H]inositol phosphate production and cAMP content*

After preincubation at 37°C for 10 min in Hepes-buffered medium (composition as described in [4]), [^{3}H]inositol-loaded cells were incubated first for 10 min with 10 mM LiCl, and further for the indicated times with agents to be tested. Reactions were terminated by aspirating off the medium and addition of 1 ml of 0.1 N HCl, followed by the cells being frozen. The supernatant (acid extract) of the thawed cells was used for separation of [^{3}H]inositol phosphates as described [4]. Data were normalized to 10^5 dpm of the radioactivity (not extracted with acid) remaining in the control (unstimulated) cells, which corresponded to the total inositol lipid. In the experiments shown in fig.4, part of the acid extract (0.1 ml) was also used for measurement of cAMP by radioimmunoassay as in [9].

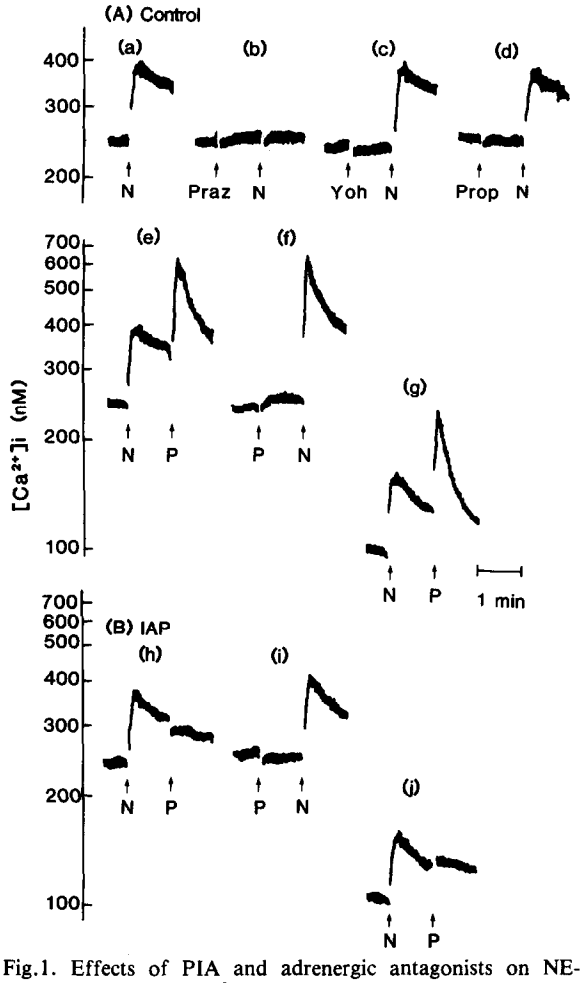

Fig.1. Effects of PIA and adrenergic antagonists on NE-induced increase in $[Ca^{2+}]_i$. FRTL-5 cells treated with (B) and without (A) IAP were used in suspension and loaded with fura 2 as described in section 2. In (g) and (j), the loaded cells were washed twice with Ca^{2+}-free Hepes-buffered medium containing 0.1 mM EGTA, and the change in fluorescence intensity was monitored in the same Ca^{2+}-free medium. At the times indicated by arrows, norepinephrine (N) (1 μM), prazosin (Praz) (10 nM), yohimbine (Yoh) (100 nM), propranolol (Prop) (10 μM) or PIA (P) (100 nM) was added to the incubation medium. Data are representative of 3–10 separate experiments.

3. RESULTS AND DISCUSSION

Fig.1A shows time courses of $[Ca^{2+}]_i$ in the presence and absence of norepinephrine (NE) as well as PIA, an adenosine derivative. $[Ca^{2+}]_i$ was increased about 1.5-fold by addition of NE (a). This NE-induced increase was inhibited by prazosin, an α_1-adrenergic antagonist (b), but not by yohimbine, an α_2-antagonist (c) and propranolol, a β-antagonist (d), suggesting α_1-receptor-mediated Ca^{2+} mobilization. When PIA was added 1 min after NE addition, $[Ca^{2+}]_i$ was further increased (e). Although addition of PIA induced no change in $[Ca^{2+}]_i$, the subsequent addition of NE caused a marked increase in $[Ca^{2+}]_i$ (f) which reached the same level as that obtained when PIA was added after NE. Removal of extracellular Ca^{2+} (g) resulted in essentially no

change in the action of either NE or PIA, except for a decrease in both basal and stimulated level of $[Ca^{2+}]_i$. This suggests that at least part of the Ca^{2+} was mobilized from an intracellular pool in response to NE and PIA. When cells were treated with IAP, the PIA effect was completely lost regardless of extracellular Ca^{2+} (fig.1B,h–j), whereas the basal response to NE was not appreciably affected by the toxin treatment.

As shown in fig.2A, theophylline, an adenosine receptor antagonist, shifted the PIA dose-response curve to the right and increased the EC_{50} from about 50 nM to 1 μM, without substantial change in the basal Ca^{2+} response to NE. Such a competitive nature of the theophylline effect suggests that the PIA response is mediated by an adenosine receptor. The action of adenosine was similar to that of PIA, as also shown in fig.2A. The potency of adenosine was one order lower than that of PIA. The action of adenosine was also completely abolished by IAP pretreatment (fig.2B).

The NE-induced $[Ca^{2+}]_i$ increase has been shown to be associated with phospholipase C activation [6,10]. We therefore examined the effect of PIA on the NE-induced activation of this enzyme by determining the increase in inositol phosphates, as demonstrated in fig.3. As expected, PIA, which had no effect by itself, caused a significant enhancement in NE action. IAP pretreatment of cells again completely inhibited the permissive action of PIA, with no influence on the basal NE action (fig.3B).

When we consider the mechanism of this novel permissive effect of adenosine receptor agonists, it should be noted that PIA [7] and NE [11] have been shown to inhibit cAMP accumulation in FRTL-5 cells. These facts provide support for the possibility that the decrease in cAMP content may be related to the mechanism of permissive action. This led us to investigate whether the increase in cAMP level induced by forskolin, an activator of adenylate cyclase, and Ro 20-1724, a phosphodiesterase inhibitor without antagonistic action on adenosine receptors, would reverse the permissive action of PIA (fig.4). In accordance with the previous study [7], PIA inhibited cAMP accumulation either stimulated by Ro 20-1724 plus forskolin or unstimulated, and this PIA action completely disappeared on IAP pretreatment (fig.4B), suggesting G_i-mediated inhibition. However, when

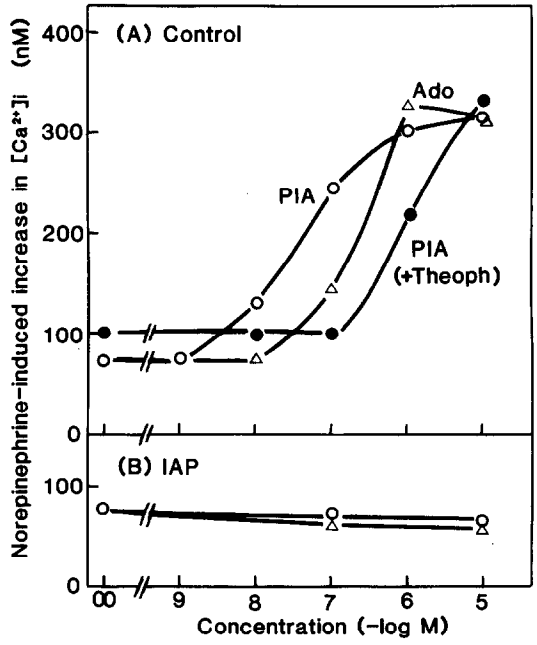

Fig.2. Dose-dependent enhancement of NE (1 μM)-induced increase in $[Ca^{2+}]_i$ by PIA in the presence (●) or absence (○) of theophylline (Theoph), and by adenosine (Ado) (△) in control cells (A) and IAP-treated cells (B). Theophylline (100 μM) and PIA (or adenosine) were added 1 min before and at the same time as NE addition, respectively. The difference between maximal $[Ca^{2+}]_i$ estimated from the peak height at around 10 s after NE addition and basal $[Ca^{2+}]_i$ just before NE addition is shown. PIA, adenosine or theophylline alone had no significant effect on $[Ca^{2+}]_i$ at the concentrations employed. Data are means of two separate experiments.

PIA was added to Ro 20-1724 plus forskolin-stimulated cells, the cAMP level was still much higher than that of unstimulated cells regardless of the presence of NE. Under such conditions of high cAMP levels, PIA definitely enhanced the NE-induced production of inositol phosphates, although the rates of NE and PIA actions slightly decreased (fig.4C). In addition, NE inhibited Ro 20-1724 plus forskolin-induced cAMP accumulation in an IAP-insensitive manner (fig.4A,B), probably by activating Ro 20-1724-insensitive Ca^{2+}-dependent phosphodiesterase [11,12]. This G_i-independent decrease in cAMP level also was not accompanied by permissive action in IAP-treated cells (fig.4A,B). These results suggest that a change in cAMP content is not responsible for the permissive action of PIA and its reversal by IAP.

The present findings indicate the involvement of

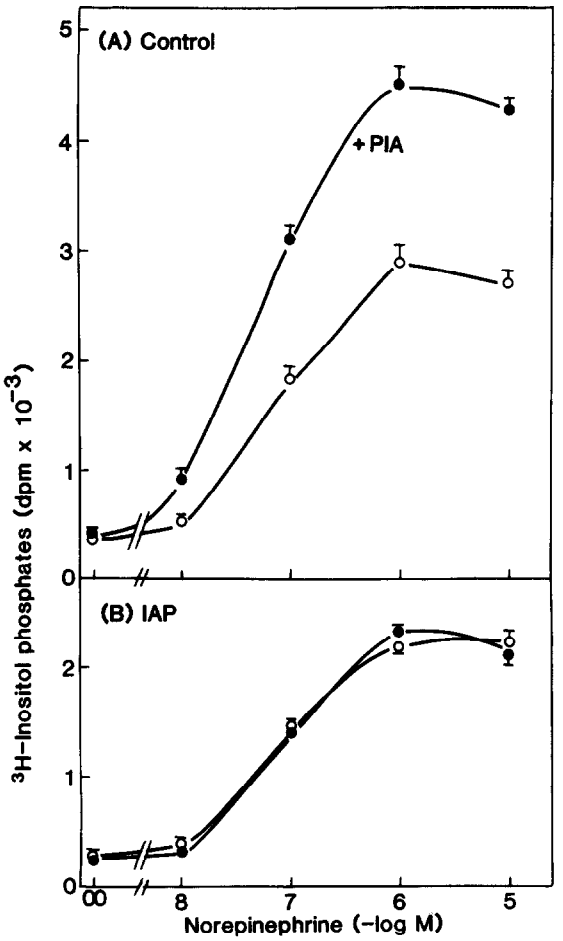

Fig.3. Effect of PIA on NE-induced inositol phosphate production. Cells were treated with (B) or without (A) IAP in the presence of [^{3}H]inositol as described in section 2. Cells were incubated for 30 min with the indicated concentrations of NE in the presence (●) or absence (○) of 1 μM PIA. Data are means ± SE of three determinations for a representative experiment. Two other experiments gave similar results.

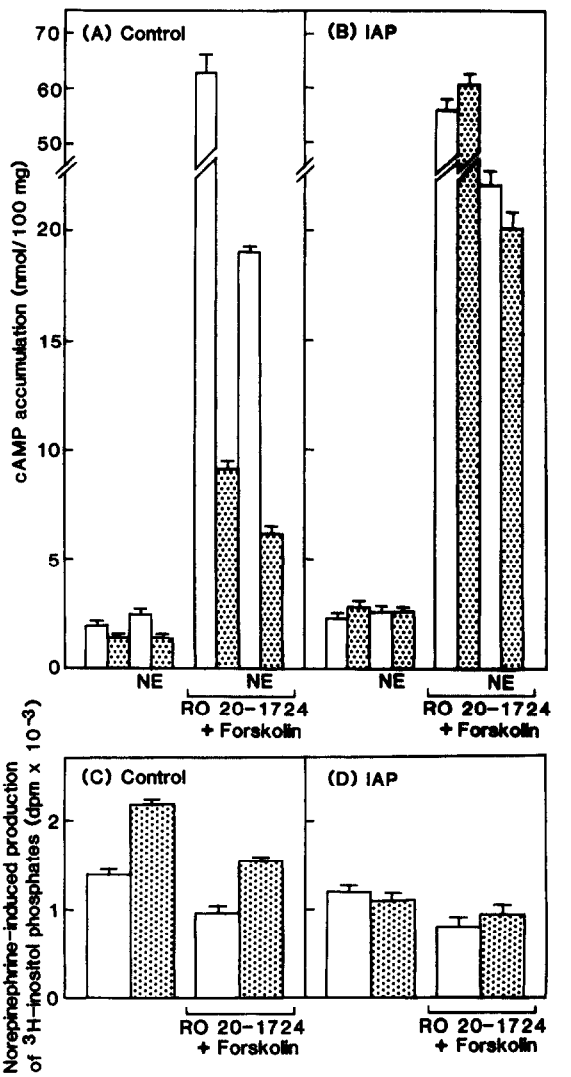

Fig.4. Effect of PIA on cAMP accumulation (A,B) and NE-induced inositol phosphate production (C,D). Cells were treated with (B,D) or without (A,C) IAP in the presence of [^{3}H]inositol as described in section 2. The cells were incubated for 10 min with (stippled bars) or without (open bars) PIA (1 μM) in the presence or absence of 1 μM norepinephrine (NE). The incubation medium, where indicated, was supplemented with Ro 20-1724 (200 μM) plus forskolin (10 μM). Data are means ± SE of three separate experiments.

adenosine receptors and an IAP substrate G-protein(s) in the permissive effect of adenosine and PIA on norepinephrine-induced phospholipase C activation and Ca^{2+} mobilization. Since either adenosine or PIA alone cannot exert these metabolic effects, the IAP substrate G-protein may not directly link the adenosine receptor to the phospholipase C-Ca^{2+} system. This occurred only when the system was activated by an agonist-coupled receptor in an IAP-insensitive manner. We recently observed similar permissive effects of adenosine receptor agonists on P_2-purinergic agonist (such as GTP and bromo-ATP)-induced phospholipase C activation and Ca^{2+} mobilization also in FRTL-5 thyroid cells (submitted). Furthermore, adenosine has been reported to modulate permissively a histamine-receptor-linked phospholipase C system in the guinea-pig cerebral cortex [13]. Thus, the permissive action of adenosine

receptor agonists may be a general signal transduction mechanism rather than a tissue-specific or α_1-receptor-specific form. An IAP substrate G-protein plays a key role in the permissive actions. On the other hand, it is not clear whether the same G-protein mediates adenosine receptor-linked adenylate cyclase inhibition (fig.4). This is a novel mechanism by which an IAP substrate G-protein regulates receptor-linked phospholipase C activity, although the mechanism of action of this G-protein in the permissive effect also remains to be elucidated.

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