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Regulation of GH_3-cell function via adenosine A_1 receptors

Inhibition of prolactin release, cyclic AMP production and inositol phosphate generation

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We examined the mechanism by which adenosine inhibits prolactin secretion from GH_3 cells, a rat pituitary tumour line. Prolactin release is enhanced by vasoactive intestinal peptide (VIP), which increases cyclic AMP, and by thyrotropin-releasing hormone (TRH), which increases inositol phosphates (IP_x). Analogues of adenosine decreased prolactin release, VIP-stimulated cyclic AMP accumulation and TRH-stimulated inositol phospholipid hydrolysis and IP_x generation. Inhibition of $InsP_3$ production by *R*-N^6-phenylisopropyladenosine (*R*-PIA) was rapid (15 s) and was not affected by the addition of forskolin or the removal of external Ca^{2+}. Addition of adenosine deaminase or the potent adenosine-receptor antagonist, BW-A1433U, enhanced the accumulation of cyclic AMP by VIP, indicating that endogenously produced adenosine tonically inhibits adenylate cyclase. The potency order of adenosine analogues for inhibition of cyclic AMP and IP_x responses (measured in the presence of adenosine deaminase) was N^6-cyclopentyladenosine > *R*-PIA > 5′-*N*-ethylcarboxamidoadenosine. This rank order indicates that inhibitions of both cyclic AMP and $InsP_3$ production are mediated by adenosine A_1 receptors. Responses to *R*-PIA were blocked by BW-A1433U (1 μM) or by pretreatment of cells with pertussis toxin. A greater amount of toxin was required to eliminate the effect of *R*-PIA on inositol phosphate than on cyclic AMP accumulation. These data indicate that adenosine, in addition to inhibiting cyclic AMP accumulation, decreases IP_x production in GH_3 cells, possibly by directly inhibiting phosphoinositide hydrolysis.

INTRODUCTION

A number of receptor-mediated biological actions of adenosine have been recognized (reviewed by Williams, 1987). Since the discovery of adenosine receptors in brain (Sattin & Rall, 1970), it has been determined that adenosine inhibits neurotransmitter release by binding to presynaptic A_1 receptors (Phillis & Wu, 1981; Gustaffson *et al.*, 1983; Dunwiddie *et al.*, 1986). Adenosine also has been reported to inhibit peptide-hormone secretion and cyclic AMP accumulation in GH_4C1 cells (Dorflinger & Schonbrunn, 1985). GH_4C1 and GH_3 cells are clonal rat pituitary tumour lines which synthesize and release prolactin and growth hormone.

Adenosine in various tissues has been shown to inhibit and to stimulate adenylate cyclase via A_1 and A_2 receptors, respectively (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Receptor subtypes can also be distinguished by a difference in the potency order of various agonists (Londos *et al.*, 1980; Wolff *et al.*, 1981). Adenosine-mediated inhibition of cerebellar glutamate release through an A_1 receptor is prevented by pretreating the tissue with pertussis toxin (PTX) (Dolphin & Prestwich, 1985). This bacterial toxin ADP-ribosylates an inhibitory guanine nucleotide-binding (G) protein, G_i, and prevents coupling of receptors to adenylate cyclase (Murayama & Ui, 1983). Despite the clear involvement of cyclic AMP in many responses, adenosine may produce some of its actions independently of the cyclic nucleotide (Stone, 1985). For instance, in dorsal root ganglion cells adenosine inhibits Ca^{2+} currents directly via a G protein (Scott & Dolphin, 1987). Adenosine and its analogues can potentiate histamine-stimulated inositol phosphate accumulation in guinea-pig cortex (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987) and inhibit the phosphoinositide (PI) pathway in frog paravertebral ganglia (Rubio & Bencherif, 1987), mouse cerebellum (Hill & Kendall, 1988), rat striatum (Petcoff & Cooper, 1987), rat aorta (Long & Stone, 1987) and rat adipocytes (Schimmel, 1986). Inhibition of the PI pathway has been implicated as a regulatory mechanism of agents other than adenosine. In hippocampal neurons, acidic amino acids inhibit carbachol- and histamine-stimulated inositol phosphate formation (Baudry *et al.*, 1986). In pituitary, dopamine was reported to inhibit angiotensin II-stimulated inositol phosphate generation and hormonal secretion (Enjalbert *et al.*, 1986), but in a later study no effect of dopamine on inositol phosphate generation could be demonstrated (Canonico *et al.*, 1986).

The present study was undertaken to investigate the

Abbreviations used: VIP, vasoactive intestinal peptide; TRH, thyrotropin-releasing hormone (thyroliberin); *R*-PIA, *R*-N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; ADA, adenosine deaminase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; PTX, pertussis toxin; PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_2$, inositol bisphosphate; Ins*P*, inositol monophosphate; IP_x, the sum of Ins*P*, $InsP_2$ and $InsP_3$; PI, phosphoinositide.

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mechanisms by which adenosine inhibits prolactin release from GH_3 cells. These cells maintain many responses of the normal mammotroph in culture and have the advantage of being a more homologous group of cells (Gourdji *et al.*, 1982). Prolactin release from GH_3 cells is enhanced by VIP, which increases cyclic AMP (Gourdji *et al.*, 1979), and by TRH, which stimulates the PI pathway (Martin, 1983). Adenosine inhibits prolactin secretion stimulated by either VIP or TRH (Dorflinger & Schonbrunn, 1985), but the mechanism of inhibition of both these secretory processes was unknown. Our results indicate that adenosine, via A_1 receptors and PTX-sensitive pathways, can decrease intracellular concentrations of cyclic AMP and inositol phosphates.

MATERIALS AND METHODS

Materials

Cell culture media, sera and antibiotics were from GIBCO (Grand Island, NY, U.S.A.). Drugs and other materials were from the following sources: VIP and TRH, Peninsula Laboratories (San Carlos, CA, U.S.A.); forskolin, Calbiochem (La Jolla, CA, U.S.A.); *R*-PIA and ADA, Boehringer Mannheim (Mannheim, Germany); adenosine, Aldrich Chemicals (Milwaukee, WI, U.S.A.); NECA, Warner Lambert (Ann Arbor, MI, U.S.A.); CPA, Research Biochemicals Inc. (Natick, MA, U.S.A.); EHNA, Burroughs Wellcome (Research Triangle Park, NC, U.S.A.); *myo*-[^{3}H]inositol, New England Nuclear (Boston, MA, U.S.A.); Dowex 1-X8 (formate form), Bio-Rad (Richmond, CA, U.S.A.); bovine serum albumin (fraction V), Sigma (St. Louis, MO, U.S.A.). The following were generously given: BW-A1433U, from Dr. S. Daluge, Burroughs Wellcome; Ro7-2956, from Dr. P. F. Sorter, Hoffman–LaRoche (Nutley, NJ, U.S.A.); maitotoxin, from Dr. T. Yasumoto, Tohuko University, Japan; PTX, from Dr. Eric Hewlett, University of Virginia; [^{3}H]inositol phosphates (Ins*P*, InsP_2 and InsP_3) from Dr. James Garrison, University of Virginia. Reagents for the prolactin radioimmunoassay, obtained from the National Hormone and Pituitary Program, were from Dr. Michael Thorner, University of Virginia.

Methods

Cell culture. GH_3 cells were grown in tissue-culture flasks in Ham's F-10 medium with 15% (v/v) horse serum and 2.5% (v/v) fetal-calf serum at 37 °C in air/CO_2 (19:1). The medium was changed three times a week and the cells were passaged every 2 weeks. Cells were detached with 1 mM-EGTA, counted with a Coulter counter, and plated on Falcon Primaria 24-well plates at a density of 1×10^5 cells/well. Experiments were performed 1 week later, at which time the cell density was $(2.5–3) \times 10^5$ cells per well.

Prolactin release and cyclic AMP accumulation. Cells were washed for 30 min with serum- and bicarbonate-free RPMI medium containing 20 mM-NaHepes, pH 7.4, at 37 °C before the application of drugs, which were made up in the same medium. To determine basal cyclic AMP accumulation, cells were incubated with 1 mM-Ro 7-2956, a phosphodiesterase inhibitor which does not act as an antagonist at adenosine receptors (Linden *et al.*, 1982). The cyclic AMP, extracted from cells with 0.1 M-HCl, was acetylated and assayed by automated radioimmunoassay (Brooker *et al.*, 1976). Prolactin released into the tissue-culture medium was collected after a 30 min incubation at 37 °C and assayed by radioimmunoassay. Pretreatment of cells with 50 ng of PTX/ml for 6 h was sufficient to block completely the effect of adenosine on cyclic AMP accumulation.

Extraction and assay of inositol phosphates. GH_3 cells were incubated in serum-free Ham's F-10 medium with 0.1% bovine serum albumin and 1 μCi of *myo*-[^{3}H]-inositol/ml at 37 °C for 48 h. Longer incubation did not significantly increase incorporation of label into phospholipids (results not shown). The incubation medium was removed and cells were rapidly washed twice with Krebs bicarbonate-buffered medium (Krebs & Henseleit, 1932) at 37 °C, pH 7.4, containing 119 mM-NaCl, 4 mM-KCl, 1 mM-KH_2PO_4, 2 mM-$MgSO_4$, 2 mM-$CaCl_2$, 10 mM-$NaHCO_3$, 5 mM-NaHepes, 0.2% glucose and 0.1% bovine serum albumin. Drugs were made up and applied in Krebs medium with 10 mM-LiCl. In some experiments 1 mM-EGTA was added to Ca^{2+}-free Krebs medium and applied to cells. Reactions were terminated by adding 0.5 M-$HClO_4$ with 5 mM-EDTA and 1 mM-diethylenetriaminepenta-acetic acid to prevent the precipitation of Mg^{2+} salts of the inositol phosphates. Plates were kept on ice for 30 min, and then cell extracts were transferred to centrifuge tubes containing sufficient K_2CO_3 (5 M) to raise the pH to 8–9. Protein and perchlorate precipitates were pelleted by centrifugation, and the supernatants were applied to Dowex AG 1X8 (formate form) anion-exchange columns and eluted as described by Berridge *et al.* (1983). An elution profile with tritiated standards was performed to verify separation. Quantification of [^{3}H]inositol phosphates recovered was performed by liquid-scintillation spectrometry and corrected for quenching. For both cyclic AMP and IP_x, each value represents the mean of four determinations, and each experiment was repeated independently at least twice.

Extraction and separation of *myo*-[^{3}H]inositol-labelled phosphoinositides. GH_3 cells were labelled to equilibrium by incubating them for 48 h in inositol-free Ham's F10 medium supplemented with dialysed serum to which *myo*-[^{3}H]inositol (1 μCi/ml) was added. Cells were detached with EGTA and washed, and then added to test tubes in 90 μl portions of 5×10^5 cells per tube. Phosphoinositides were extracted and separated by the method of Martin (1986). Reactions were terminated by adding chloroform/methanol/HCl (10:10:1, by vol.). Separation into two phases was promoted by adding 0.25 ml of 10 mM-EDTA and 0.1 mM-sodium orthovanadate. The organic phase was washed twice with 0.1 M-KCl/methanol (1:1, v/v) and dried under a stream of N_2. Lipids were deacylated by incubation for 20 min at room temperature in 1 ml of chloroform, 0.2 ml of methanol and 0.2 ml of 1 M-KOH (in methanol/water, 19:1, v/v). the reaction was stopped by adding 1 ml of chloroform, 0.6 ml of methanol and 0.6 ml of water. After phase separation the aqueous phase was removed and diluted with 6 ml of 6 mM-$Na_2B_4O_7$ and applied to Dowex AG 1X8 columns (formate form). Sequential washes eluted the glycerophospholipids as follows: 5 mM-$Na_2B_4O_7$/0.18 M-ammonium formate, glycerophosphoinositol; 0.5 M-ammonium formate/0.1 M-formic acid, glycerophosphoinositol phosphate; 1 M-ammonium

Table 1. Effect of *R*-PIA on prolactin release from GH_3 cells

Cells were incubated for 30 min in medium alone, 100 nM-VIP or 100 nM-TRH in the absence and presence of 10 μM-*R*-PIA alone, or with 1 μM-BW-A1433U. All incubations were carried out in the presence of ADA (1 unit/ml). Each point represents the mean ± S.E.M. for 12 wells assayed in duplicate: * significant decrease ($P < 0.01$) compared with the other two groups.

	Prolactin released (ng/ml)		
	Basal	VIP	TRH
Control	76±8	151±15	127±9
R-PIA	42±5*	76±8*	60±5*
R-PIA + BW-A1433U	84±8	191±22	144±22

formate/0.1 M-formic acid, glycerophosphoinositol bisphosphate. The separated lipids were quantified by liquid-scintillation spectrometry of the eluted fractions.

Data analysis

ED_{50} concentrations were calculated by Marquadt's (1963) least-squares non-linear regression, as modified by Tabata & Ito (1975), to the equation:

$$R = B + (M - B)[D]/(ED_{50} + [D])$$

where $[D]$ = drug concentration, R = response, B = basal value and M = maximum response. Statistical significance was determined by analysis of variance and Duncan's multiple-range or Newman–Keuls tests.

RESULTS

Release of prolactin from GH_3 cells is inhibited by adenosine

GH_3 cells secrete prolactin continually into the surrounding medium. The addition of 10 μM-*R*-PIA decreased the basal rate of secretion by 44%. The secretagogues VIP and TRH enhanced prolactin release by 100% and 66% above control values ($P < 0.01$) respectively. The stimulatory effects of both of these hormones were decreased by more than 50% in the presence of 10 μM-*R*-PIA (Table 1). The adenosine A_1-receptor antagonist BW-A1433U at a concentration of 1 μM, which has no effect on cyclic AMP phosphodiesterase activity (Daluge & Leighton, 1986; Clemo *et al.*, 1987), completely blocked the effects of *R*-PIA, confirming that its actions are mediated by adenosine receptors.

Adenosine and cyclic AMP accumulation

Adenosine and its analogues inhibited basal, forskolin- and VIP-stimulated increases in the cyclic AMP content of GH_3 cells. The rank order of potency was CPA > *R*-PIA > NECA > adenosine (Table 2), typical of A_1 receptors (Wolff *et al.*, 1981). The inhibition of VIP-stimulated cyclic AMP accumulation by 10 μM-*R*-PIA was abolished by 1 μM-BW-A1433U (Table 3), or by pretreatment of cells with PTX (50 ng/ml) for 6 h (results not shown) and for 24 h (Table 4). Adenosine or its analogues did not induce an increase in cyclic AMP content in cells pretreated with PTX, suggesting that these cells do not contain stimulatory adenosine A_2 receptors linked to adenylate cyclase. In the absence of ADA, cyclic AMP accumulation in response to VIP alone was enhanced 2-fold by PTX, and the toxin alone also caused a 3-fold increase in basal cyclic AMP (Table 4). ADA and BW-A1433U also elevated basal and VIP-stimulated cyclic AMP accumulation, suggesting that endogenous adenosine is released by GH_3 cells in quantities sufficient to activate partially A_1 receptors (Fig. 1). Basal cyclic AMP contents increased from 11.6 to 15.8 pmol/well in the presence of ADA (1 unit/ml) ($P < 0.001$), but the maximal effect of the enzyme required in excess of 3 units/ml. Both the efficacy and the potency of VIP were significantly enhanced by both ADA and BW-A1433U (Fig. 1).

Table 2. Inhibition of VIP-stimulated cyclic AMP accumulation by adenosine and its analogues

Adenosine, *R*-PIA and NECA effects on cyclic AMP accumulation produced by VIP (100 nM) were evaluated by using eight concentrations in the range 10 pM–0.1 mM. Cells were incubated for 5 min in the presence of 3 units of ADA/ml (except for adenosine). Identical potency orders were obtained in two additional experiments. All ED_{50} values differed significantly from each other ($P < 0.01$). Maximal inhibition was defined as the percentage decrease in the VIP-stimulated response by 100 μM of each agonist. Results are means ± S.E.M.

Drug	ED_{50} (nM)	Maximal inhibition (%)
CPA	0.4±0.1	84.0±3.0
R-PIA	12.0±4.0	81.0±7.0
NECA	48.0±9.0	86.0±10.0
Adenosine	74.0±10.0	80.0±2.0

Adenosine and IP_x accumulation

In order to examine the effects of drugs on PI metabolism, it was desirable to achieve a constant specific radioactivity of the radioactive inositol incorporated into lipids. Then changes in inositol phospholipid contents could be directly monitored by measuring the radioactive content of phosphoinositides and inositol phosphates. In the course of this study, GH_3 cells were labelled to equilibrium in all experiments. The addition of 10 μM-adenosine or -*R*-PIA decreased the IP_x accumulation after a 10 min incubation with 100 nM-TRH in the presence of 10 mM-LiCl by 60–65% ($P < 0.0001$; data pooled from six experiments consisting of four replicates with each drug). This inhibition was abolished by 1 μM-BW-A1433U (Table 3). No significant effect of BW-A1433U or ADA alone on basal or TRH-stimulated IP_x accumulation could be demonstrated, suggesting little or no role for endogenous adenosine in the regulation of IP_x production. In some experiments *R*-PIA was found to decrease significantly basal IP_x production (Table 5). Li^+ impedes breakdown of inositol sugars to free inositol (Drummond *et al.*, 1984). In the presence of LiCl, basal IP_x accumulation and TRH-stimulated IP_x accumulation were linear with time up to 10 min. *R*-PIA also inhibited IP_x accumulation in the absence of LiCl (results not shown), indicating that *R*-PIA does not act by interfering with the action of LiCl. Adenosine and its analogues had no effect on the elution of $[^3H]IP_x$ standards from the Dowex columns (results not shown).

Table 3. Effect of various drugs on the IP_x and cyclic AMP content of GH_3 cells

Drugs (100 nM-VIP, 100 nM-TRH, 10 μM-*R*-PIA, 10 μM-adenosine, 1 μM-carbachol, 100 nM-somatostatin and 1 μM-forskolin) were made up in buffer with 3 units of ADA/ml (except adenosine). For IP_x determinations, 10 mM-LiCl was included. Results are means ± S.E.M. for the numbers of replicates indicated in parentheses; * inhibition significant compared with respective control ($P < 0.01$); ND, not determined.

	[^{3}H]Inositol phosphates (c.p.m./well)	Cyclic AMP (pmol/well)
Basal	144±7 (24)	ND
TRH	825±34 (24)	ND
TRH + *R*-PIA	471±13* (24)	ND
TRH + adenosine	493±17* (12)	ND
TRH + adenosine + BW-A1433U	810±42 (12)	ND
TRH + forskolin	820±36 (8)	ND
TRH + *R*-PIA + forskolin	474±15* (8)	ND
Basal	109±13 (8)	ND
TRH	716±33 (8)	ND
TRH + somatostatin	715±1 (8)	ND
TRH + carbachol	748±29 (8)	ND
Basal		4.0±0.1 (9)
Forskolin	ND	322±10 (9)
Forskolin + *R*-PIA	ND	148±4* (9)
VIP	ND	80±4 (9)
VIP + *R*-PIA	ND	36±3* (9)
VIP + *R*-PIA + BW-A1433U	ND	85±3 (9)
VIP + somatostatin	ND	59±1* (9)
VIP + carbachol	ND	66±2* (9)

Table 4. Effect of PTX treatment on *R*-PIA-induced inhibition of TRH-stimulated IP_x production and VIP-stimulated cyclic AMP production

Cells were pretreated with the toxin for 24 h. Results represent means ± S.E.M. for four values from two or more independent experiments; * indicates significant inhibition by *R*-PIA ($P < 0.01$); ND, not determined.

		Concn. of PTX		
		0	50 ng/ml	100 ng/ml
[^{3}H]IP_x (c.p.m./well)	Basal	144±7	145±5	123±8
	TRH	825±34	836±33	745±35
	TRH + *R*-PIA	471±13*	579±20*	739±35
Cyclic AMP (pmol/well)	Basal	2±1	6±2	ND
	VIP	80±4	89±5	ND
	VIP + *R*-PIA	36±3*	83±5	ND

Since adenosine and its analogues are inhibitors of adenylate cyclase activity in GH_3 cells, we considered the possibility that inhibition of PI degradation might be an indirect result of this action. However, the ability of *R*-PIA to inhibit TRH-stimulated IP_x accumulation was not changed by addition of 1 μM-forskolin (Table 3). Even in the presence of 1 μM-*R*-PIA, forskolin increased cyclic AMP by more than 40-fold over the basal value. Moreover, carbachol and somatostatin decreased VIP-stimulated cyclic AMP accumulation, but had no effect on TRH-stimulated IP_x production (Table 3).

The effect of PTX on adenosine-induced inhibition of TRH-stimulated IP_x accumulation was also investigated. Although pretreating cells with PTX (50 ng/ml) for as little as 6 h abolished adenosine-mediated effects on cyclic AMP accumulation, such treatment did not remove the inhibitory action of adenosine on IP_x production. Increasing the time of incubation to 24 h had a partial effect, but it was necessary to increase the concentration of PTX to 100 ng/ml for 24 h in order to abolish completely the effect of *R*-PIA on IP_x accumulation (Table 4). IP_x accumulation in response to TRH decreased slightly when cells were pretreated with 100 ng of PTX/ml for 24 h, an observation also reported by Martin *et al.* (1986). This may be a trophic effect, as serum-stimulated proliferation is inhibited in the presence of PTX (Murayama & Ui, 1987). Additional support for this proposal is the finding that PTX treatment at this concentration decreased basal IP_x concentrations by 15%. Incubation with PTX for shorter times at lower

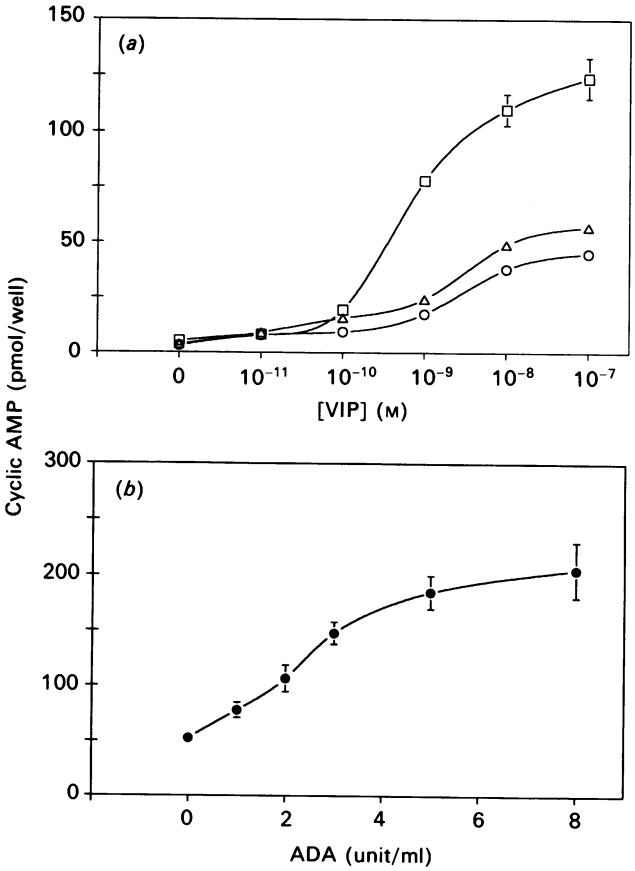

Fig. 1. Effect of BW-A1433U and ADA on VIP-stimulated cyclic AMP accumulation in GH_3 cells

(*a*) VIP dose/response alone (○) and in the presence of 1 unit of ADA/ml (△) or 1 μM-BW-A1433U (□). In the presence of ADA (1 unit/ml) the ED_{50} of VIP decreased from 2.4 to 1.8 nM (not significant) and the maximum stimulation increased by 26% ($P < 0.001$). In the presence of BW-A1433U the ED_{50} of VIP decreased to 0.6 nM ($P < 0.001$) and the maximum stimulation increased by 170% ($P < 0.001$). (*b*) Effect of ADA on VIP (100 nM)-stimulated cyclic AMP production. Incubations were for 5 min, and each point represents the mean ± S.E.M. for eight wells assayed in duplicate.

doses did not decrease the IP_x or cyclic AMP responses to TRH and VIP respectively.

The efficacy rather than the potency (ED_{50} 10 nM) of TRH was decreased by *R*-PIA (Fig. 2*a*). Adenosine analogues inhibited TRH-stimulated IP_x generation in a concentration-dependent manner, with the potency order CPA > PIA > NECA (Fig. 2*b*), identical with that observed for inhibition of VIP-stimulated cyclic AMP accumulation (Table 2). The responses differed, however, in their IC_{50} values (concn. giving 50% inhibition) and maximal effects. The IC_{50} values for inhibition of IP_x generation were higher than for cyclic AMP, and maximal inhibition was achieved in the micromolar range (Fig. 2*b*). Increasing drug concentrations further resulted in a lessening of inhibition and produced a U-shaped dose–response curve.

There was a lag of over 2 min before significant inhibition of TRH-stimulated IP_x production was observed in the presence of *R*-PIA (Fig. 3). In measuring

Table 5. Effect of $[Ca^{2+}]$ on inhibition of IP_x accumulation by *R*-PIA

Extracellular and intracellular Ca^{2+} concentrations were manipulated by preincubating cells with EGTA, and by adding KCl and maitotoxin (MTX; 50 ng/ml). Inositol phosphates and cyclic AMP were extracted and measured as described under 'Methods'. Each point represents the mean ± S.E.M. for 12 wells; * inhibition by *R*-PIA significant compared with respective control ($P < 0.01$); † greater than basal ($P < 0.001$).

	[^{3}H]Inositol phosphates (c.p.m./well)		
	1.5 mM-Ca^{2+}	140 mM-KCl	Ca^{2+}-free + 1 mM-EGTA
Basal	186 ± 12	225 ± 20	179 ± 5
R-PIA	130 ± 17*	190 ± 33	153 ± 9*
TRH	895 ± 19†	944 ± 39†	577 ± 12†
TRH + *R*-PIA	453 ± 18*	619 ± 19*	386 ± 17*
MTX	595 ± 35†	–	–
MTX + *R*-PIA	402 ± 13*	–	–

IP_x formation in GH_3 cells, in most experiments we evaluated the sum of all metabolites, as opposed to analysing individual IP_x species. In light of the large number of inositol phosphate metabolites formed, with variable kinetics, true resolution of cycle dynamics in GH_3 cells will require further study. Since production of $InsP_3$ is still considered to precede production of all other metabolites, we examined its appearance over time. In GH_3 cells two isomers of $InsP_3$ can be measured by h.p.l.c. TRH-stimulated Ins(1,4,5)P_3 accumulation has been shown to reach a peak in 1 s and to decrease to half-maximal values in 30 s, whereas Ins(1,3,4)P_3 begins to increase by 30 s and reaches a maximum at 1 min (Dean & Moyer, 1987). Thus $InsP_3$ measured at early time points should primarily reflect the initial product of PtdIns(4,5)P_2 hydrolysis, Ins(1,4,5)P_3. *R*-PIA inhibited $InsP_3$ generation at the earliest time point measured, 15 s (Fig. 3*b*), at which time no significant decrease in the sum of all inositol phosphates eluted together could be detected. At times in excess of 1 min, *R*-PIA produced much more pronounced inhibition of IP_x than of $InsP_3$ production.

We investigated whether the inhibitory effect of adenosine on IP_x accumulation is influenced by the internal and external Ca^{2+} concentrations ($[Ca^{2+}]_i$ and $[Ca^{2+}]_o$). Near-zero $[Ca^{2+}]_o$ was achieved by preincubating cells in Ca^{2+}-free buffer containing 1 mM-EGTA. Since GH_3 cells contain voltage-dependent Ca^{2+} channels (Dufy *et al.*, 1986; Schlegel *et al.*, 1984), $[Ca^{2+}]_i$ was elevated by depolarizing the plasma membrane with 140 mM-KCl or by adding maitotoxin, which activates Ca^{2+} channels (Takahashi *et al.*, 1983) and elevates $[Ca^{2+}]_i$ in pituitary cells (Anderson & Cronin, 1987). The effects of these agents are shown in Table 5. IP_x production measured in the presence of KCl and maitotoxin was still significantly decreased by *R*-PIA (Table 5). In cells incubated in 1.5 mM-Ca^{2+} or pre-incubated for 10 min in Ca^{2+}-free buffer containing 1 mM-EGTA, 10 μM-*R*-PIA inhibited TRH-stimulated IP_x generation by 52% ($P < 0.001$) and 49% ($P < 0.01$), respectively. After addition of 140 mM-KCl, inhibition of

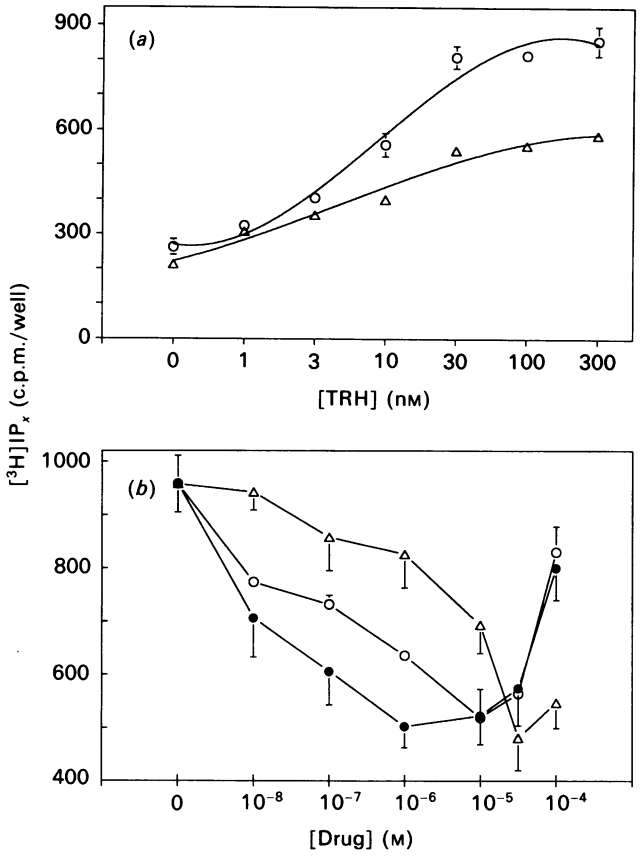

Fig. 2. Dose/response curves for (*a*) TRH-stimulated IP_x accumulation in the presence and absence of 10 μM-*R*-PIA and (*b*) for inhibition of TRH-stimulated IP_x generation by various adenosine analogues

(*a*) Results represent means ± S.E.M. for four determinations. In the presence of *R*-PIA (△) the ED_{50} value did not change significantly and the efficacy decreased by 35% ($P < 0.001$). (*b*) The IC_{50} values for inhibition are as follows: CPA (●), 12 ± 2 nM; *R*-PIA (○), 19 ± 9 nM; NECA (△), 700 ± 43 nM. Each point represents the mean ± S.E.M. for four wells determined in the presence of 10 mM-LiCl and 3 units of ADA/ml. Similar results were obtained in three independent experiments. IP_x measurements were as described under 'Methods'.

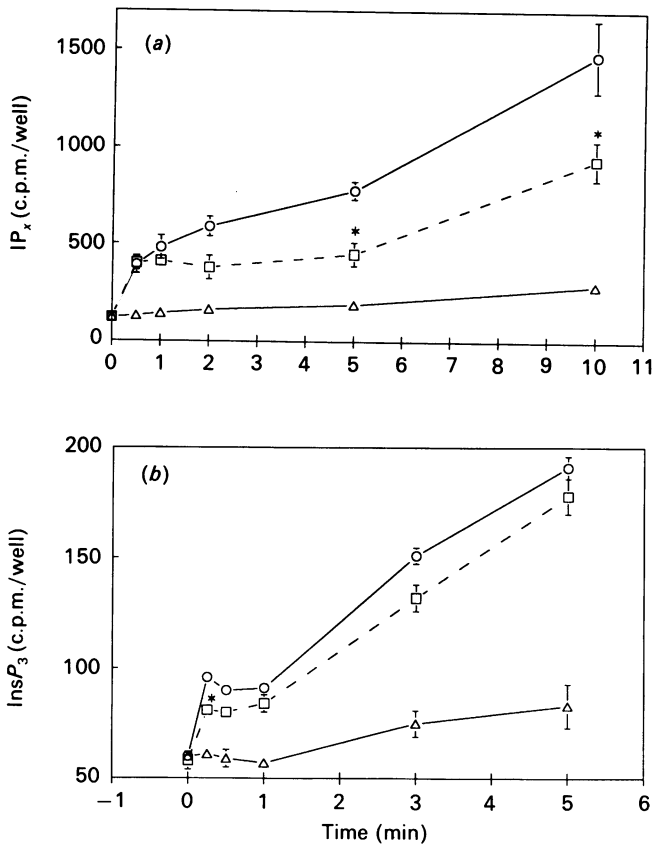

Fig. 3. Effect of *R*-PIA on TRH-stimulated IP_x accumulation (*a*) and $InsP_3$ production (*b*)

IP_x production was stimulated by 100 nM-TRH in the presence (□) or absence (○) of 10 μM-*R*-PIA. Each point represents the means ± S.E.M. for four wells repeated independently three times and measured in the presence of 10 mM-LiCl. *R*-PIA had no effect on basal IP_x contents (△). Error bars smaller than the symbols are not shown; * indicates significant inhibition by *R*-PIA ($P < 0.01$).

the TRH response was decreased to 28%, but was still significant ($P < 0.005$) (Table 5). In an additional experiment (results not shown), KCl produced a significant ($P < 0.05$) increase in basal IP_x, as has been previously reported (Biden *et al.*, 1987), but did not significantly change the response to TRH. When cells were pre-exposed to EGTA for 30 min before application of drugs, the response to TRH was further decreased, but the ability of *R*-PIA to inhibit TRH-stimulated IP_x accumulation was not affected (results not shown). Exposure of the cells to EGTA for more than 1 h abolished the response to TRH [$InsP_3$ accumulation measured after 15 s in the presence of TRH (59 ± 3 c.p.m.) and TRH + *R*-PIA (60 ± 5 c.p.m.) did not differ significantly from basal values (61 ± 2 c.p.m.)]. The effect of a prolonged decrease in $[Ca^{2+}]_i$ to decrease hormone-stimulated IP_x generation has been observed in a variety of cell types (Abdel-Latif, 1986).

Effect of *R*-PIA on TRH-stimulated polyphosphoinositide hydrolysis

TRH caused a modest decrease in PtdIns (Fig. 4*a*) and a rapid and transient decrease in PtdIns4*P* (Fig. 4*b*) and PtdIns(4,5)P_2 (Fig. 4*c*). In the presence of *R*-PIA (10 μM), the TRH-stimulated hydrolysis of these phospholipids was almost completely prevented (Figs. 4*a*, 4*b* and 4*c*). In two experiments each consisting of six replicates, basal concentrations of each of the three phospholipids were 6–10% higher in the presence of *R*-PIA. The effects of *R*-PIA on the hydrolysis of phospholipids were not prevented by the addition of 1 μM-forskolin (results not shown).

DISCUSSION

In this study we have shown that adenosine, via A_1 receptors, inhibits not only VIP-stimulated prolactin release and cyclic AMP accumulation but also TRH-stimulated prolactin release and PI breakdown. In agreement with the data of Imai & Gershengorn (1987) and Martin (1986), it was observed that TRH produced a rapid loss of PtdIns4*P* and PtdIn(4,5)P_2, with con-

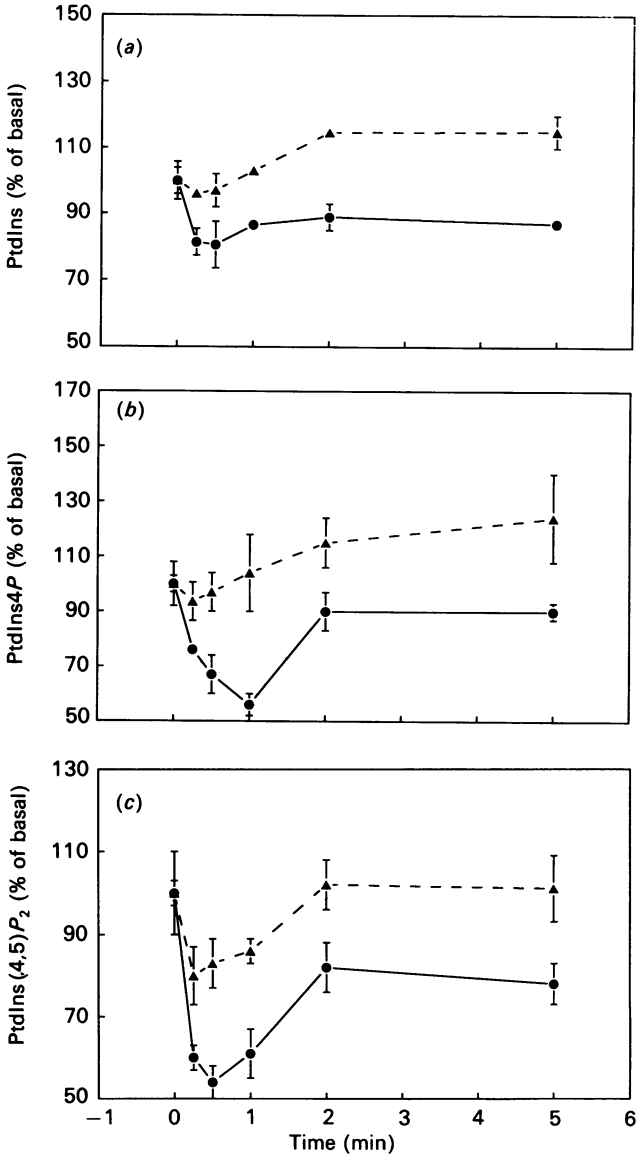

Fig. 4. Time course of inhibition of TRH-stimulated PI breakdown by *R*-PIA

Experiments were carried out in the presence of 3 units of ADA/ml. The effects of TRH (1 μM) were assayed in the absence (●) and in the presence (▲) of 10 μM-*R*-PIA. Values for PI are presented as percentages of initial values with and without *R*-PIA. Initial values for all phosphoinositides were 5–10% higher in the presence of *R*-PIA. (*a*) PtdIns; initial value was 5000 c.p.m./5×10^5 cells. (*b*) PtdIns4*P*; initial value was 900 c.p.m./5×10^5 cells. (*c*) PtdIns(4,5,)P_2; initial value was 300 c.p.m./5×10^5 cells. All measurements represent the means of six determinations measured in duplicate.

comitant appearance of InsP_3 and InsP_2; this implies hydrolysis catalysed by phospolipase C. Thus, our results are consistent with the hypothesis that adenosine A_1 receptors are coupled to inhibition of phospholipase C. The adenosine analogues utilized in this study are not substrates for ADA, and are not actively taken up into the cell, but bind selectively to cell-surface receptors (Westermann & Stock, 1970). The potency order of these analogues indicates that effects on both cyclic AMP and PI metabolism are mediated through adenosine A_1 receptors.

Basal or VIP-stimulated cyclic AMP concentrations were increased by adding ADA, BW-A1433U or PTX, and were decreased by inhibiting ADA with EHNA (results not shown). All of these drugs may act to modify the amount or effect of endogenous adenosine released by GH_3 cells in culture. In contrast with the effect of endogenous adenosine on cyclic AMP metabolism, no effect on basal or TRH-stimulated IP_x production could be detected in this study. This may be a consequence of the fact that adenosine and other agonists are more potent as inhibitors of cyclic AMP production than of IP_x generation. Evidence of autocrine inhibition by adenosine also has been observed in GH_4Cl cells (Dorflinger & Schonbrunn, 1985), adipocytes (Fain & Wieser 1975; Schimmel *et al.*, 1987) and hippocampal nerve terminals (Allgaier *et al.*, 1987).

The efficacy of TRH was decreased in the presence of adenosine agonists, but the potency was unaltered. These findings rule out the possibility that adenosine interferes with TRH binding. Adenosine, *R*-PIA, CPA and NECA all inhibited TRH-stimulated IP_x accumulation by about 50–60%. A further inhibition by any of these drugs could not be elicited by increasing the doses or pretreating the cells with *R*-PIA. A maximal inhibition of 50% may reflect the presence of pools of PtdIns(4,5)P_2 that are accessible to hydrolysis by TRH but differentially regulated by adenosine. Pools could co-exist in the same cell, or could be located in separate cells. GH_3 cells have been recognized as functionally heterogeneous cells, composed of prolactin and growth-hormone secretors (Boockfor *et al.*, 1985).

The curves relating IP_x production to adenosine agonist concentration were U-shaped. This is reminiscent of the dual effect of adenosine on adenylate cyclase when both A_1 and A_2 receptors are present. Since no A_2-receptor-mediated response was observed, the explanation for this biphasic effect might be due to the fact that adenosine and its analogues, at concentrations exceeding 50 μM, selectively inhibit PtdIns kinase in membranes (Doctrow & Lowenstein, 1987; O'Shea *et al.*, 1987). However, PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 all increased in the presence of 10 μM-*R*-PIA, suggesting that adenosine receptors are regulating an enzyme such as phospholipase C, which hydrolyses all three phosphoinositides (Wilson *et al.*, 1984). This inhibition occurs at much lower nucleoside concentrations than are necessary to inhibit PtdIns kinase by a non-receptor-mediated process of dubious physiological significance.

We found that *R*-PIA inhibits TRH-stimulated InsP_3 accumulation at the earliest point measured, 15 s, and infer that this results from a rapid inhibition of Ins(1,4,5)P_3 production. TRH-stimulated decreases in PtIns(4,5)P_2 were decreased by *R*-PIA at 15 s, implying that hydrolysis is rapidly inhibited. Since adenosine is also an inhibitor of cyclic AMP accumulation in these cells, we considered the possibility that decreased cyclic AMP could be indirectly regulating the PI cycle. This seems unlikely, however, since the action of *R*-PIA of TRH-stimulated IP_x production is unaltered in the presence of 1 μM-forskolin, a concentration which generated high concentrations of cyclic AMP even in the presence of 10 μM-*R*-PIA. Inhibition of neurotransmitter release by *R*-PIA in hippocampal slices also persists in the presence of forskolin (Fredholm *et al.*, 1987). In

addition, PTX abolished the effect of *R*-PIA on cyclic AMP accumulation at a dose which did not completely abolish its effect on PI metabolism. Furthermore, agents known to inhibit cyclic AMP accumulation in GH_3 cells (e.g. somatostatin and carbachol) have no effect on TRH-stimulated IP_x accumulation or phospholipid labelling (Yajima *et al.*, 1986; Sutton & Martin, 1982).

The transduction mechanisms of several receptors mediating PI breakdown appear to involve guanine-nucleotide-regulatory (G) proteins (for review see Abdel-Latif, 1986). The name G_p (p for phospholipid) has been coined to describe this protein (Berridge, 1987), although it appears that there may be more than one G_p with different sensitivities to PTX. In GH_3 cells, $InsP_3$ release can be stimulated by GTP analogues, but TRH-stimulated inositol phosphate accumulation is not sensitive to PTX (Martin *et al.*, 1986; Wojcikiewicz *et al.*, 1986). The inhibition of IP_x metabolism by adenosine was only partially obviated by preincubating the cells with PTX at the same concentration (50 ng/ml for 24 h), which completely blocked inhibition of adenylate cyclase. This result suggests either that there are two G proteins coupled to adenosine receptors, only one of which is sensitive to PTX at this concentration, or that the effect of adenosine on PI metabolism requires less functional G protein than is required to inhibit cyclic AMP production. In adipocytes, adenosine can inhibit both isoprenaline- and phenylephrine-stimulated respiration, but these effects also differ in their sensitivity to PTX treatment (Schimmel *et al.*, 1987). The inhibition of the PI-linked phenylephrine response by adenosine was blocked by PTX treatment only when all G_i present was ADP-ribosylated by the toxin, whereas incomplete ADP-ribosylation of G_i by PTX was sufficient to block adenosine inhibition of the adenylate cyclase-linked isoprenaline effect. A third example of differential sensitivity to PTX was described by Ashkenazi *et al.* (1987), who found that, in Chinese-hamster ovary cells transfected with M_2 muscarinic receptors, a receptor-mediated IP_x response was less sensitive to PTX than was an adenylate cyclase response mediated by the same cholinergic receptor. Although differential sensitivity to PTX could be attributed to more efficient coupling of receptors to phospholipase C than to adenylate cyclase, just the opposite order of coupling efficiency was observed in the transfected cells. These data are consistent with the possibility that different G proteins may mediate the two responses. A G protein which appears to correspond to G_i is ADP-ribosylated in GH_3 cells (Wojcikiewicz *et al.*, 1986; Martin *et al.*, 1986; Yajima *et al.*, 1986). This PTX substrate has been further resolved into two bands, corresponding closely to G_i and G_o (Zysk *et al.*, 1986). Single receptors coupled to multiple effector systems have been noted in several tissues. For instance, in the cardiac atrium, both adenosine and acetylcholine inhibit adenylate cyclase and activate K^+ channels, possibly via the same receptors coupled to different G proteins (Yatani *et al.*, 1987). We have observed that in GH_3 cells adenosine, carbachol and somatostatin all inhibit adenylate cyclase, but only adenosine inhibits IP_x accumulation. These findings imply that components of G_i involved in inhibition of adenylate cyclase (α or β–γ subunits) do not mediate the effect of adenosine on PI metabolism, or are differentially accessible to various receptors. Such observations support the notion that adenosine A_1 receptors communicate with more than one G protein. Furthermore, multiple species of G-protein α subunits are tightly complexed to adenosine A_1 receptors purified from bovine brain by affinity chromatography (R. Munshi & J. Linden, unpublished work).

In a pituitary cell line, Lewis *et al.* (1986) found that somatostatin receptors are coupled to inhibition of adenylate cyclase and are coupled to Ca^{2+} channels by PTX-sensitive guanine nucleotide binding proteins. Studies *in vitro* of PI-hydrolysing phosphodiesterases from numerous sources have indicated that maximal activation of this enzyme requires concentrations of Ca^{2+} well above intracellular values (Irvine, 1982). TRH stimulation of GH_3 cells (Martin *et al.*, 1986) or addition of guanine nucleotide to permeabilized neutrophils (Bradford & Rubin, 1986; Smith *et al.*, 1986) sensitizes the enzyme to Ca^{2+} such that it becomes active at physiological concentrations. In agreement with Martin *et al.* (1986), we found that incubating cells for various times in EGTA decreases and ultimately abolishes the action of TRH. If *R*-PIA was producing its effect by decreasing $[Ca^{2+}]_i$ acutely, then treatment of cells with EGTA should mimic the action of *R*-PIA and prevent a further effect of *R*-PIA on IP_x contents. The ability of *R*-PIA to decrease IP_x content was not diminished by short-term treatment with EGTA. Conversely, when $[Ca^{2+}]_i$ was elevated by K^+ depolarization the inhibition by *R*-PIA persisted, although the magnitude of the response was decreased from 50 % to 28 %. IP_x accumulation measured in the presence of elevated $[Ca^{2+}]_i$ produced by 140 mM-KCl or maitotoxin was significantly decreased in the presence of *R*-PIA. These results suggest that the action of *R*-PIA is independent of both cyclic AMP and $[Ca^{2+}]$. Moreover, preliminary data indicate that adenosine agonists inhibit TRH-stimulated IP_x production in GH_3-cell membranes at concentrations similar to those observed to inhibit IP_x production in whole cells (T. M. Delahunty & J. Linden, unpublished work). The effects of *R*-PIA on PI metabolism could result because *R*-PIA decreases the sensitivity of phospholipase C to available $[Ca^{2+}]_i$, or because *R*-PIA inhibits phospholipase C by a Ca^{2+}-independent mechanism.

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