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Adenosine A_1-receptor stimulation of inositol phospholipid hydrolysis and calcium mobilisation in DDT_1 MF-2 cells

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1. The effect of adenosine receptor-stimulation on inositol phospholipid hydrolysis and calcium mobilization has been investigated in the hamster vas deferens smooth muscle cell line DDT_1 MF-2.

2 Adenosine receptor stimulation increased the accumulation of total [^{3}H]-inositol phosphates in DDT_1 MF-2 cells prelabelled with [^{3}H]-*myo*-inositol. The rank order of agonist potencies was N^6-cyclopentyladenosine > 5'-N-ethylcarboxamidoadenosine > 2-chloroadenosine > adenosine.

3 The response to 2-chloroadenosine was antagonized by the antagonists 8-cyclopentyl-1,3-dipropylxanthine (K_D 1.2 nM), PD 115,199 (K_D 39 nM) and 8-phenyltheophylline (K_D 31 nM).

4 The inositol phosphate response to 2-chloradenosine (10 μM) was not significantly altered when the extracellular Ca^{2+} ion concentration was reduced from 2.4 mM to 1.2 mM or 0.6 mM. Under calcium-free conditions, however, a reduced but still significant response to 2-chloroadenosine was evident (39 ± 10% of the response in calcium-containing medium).

5 The 5-lipoxygenase inhibitor AA861 (10 and 100 μM) inhibited the inositol phosphate response to 2-chloroadenosine by 40 ± 9% and 60 ± 4% respectively. The cyclo-oxygenase inhibitor, indomethacin, however, was without significant effect at 1 μM.

6 2-Chloroadenosine stimulated an increase in intracellular free Ca^{2+} ion concentration in fura-2 loaded DDT_1 MF-2 cells in calcium-free medium containing 0.1 mM EGTA, which could be inhibited by the adenosine A_1-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (0.1 μM).

7 These data suggest that adenosine A_1-receptor stimulation results in inositol phospholipid hydrolysis and calcium mobilization from intracellular stores in DDT_1 MF-2 cells.

Keywords: Adenosine; A_1-receptor; inositol phospholipids; calcium mobilisation; DDT_1 MF-2 cells; smooth muscle

Introduction

Extracellular adenosine receptors have been divided into two major subtypes, A_1- and A_2-, on the basis of agonist structure-activity relationships and the use of the antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and PD 115, 199 (Daly *et al.*, 1986; Bruns *et al.*, 1987a,b; Rakumar *et al.*, 1990). Thus, for the A_1-receptor the rank order of agonist potencies is such that N^6-derivatives of adenosine are more potent than 5'-analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), while the reverse is true for the A_2-receptor (Daly *et al.*, 1986). Furthermore, DPCPX (which is a selective A_1-receptor antagonist) is more potent than PD 115,199 as an antagonist at the A_1-receptor, but less potent at the A_2-receptor (Bruns *et al.*, 1987a,b). A further subdivision of the A_2-receptor into A_{2A}- and A_{2B}- has also been proposed on the basis of the apparent higher affinities of the agonists 2-phenylaminoadenosine and CGS 21680 and the antagonist PD 115,199 for the A_{2A}-receptor compared to those determined for the A_{2B}-receptor (Bruns *et al.*, 1987b; Jarvis *et al.*, 1989; Alexander *et al.*, 1989b; Hargreaves *et al.*, 1991).

Adenosine A_1 and A_2-receptors can also be distinguished functionally on the basis of their differing effect on adenylate cyclase activity (Van Calker *et al.*, 1979; Londos *et al.*, 1980; Stiles, 1986). Thus, A_2-receptors are positively linked to adenylate cyclase via a G_s GTP-binding protein, while A_1-receptors are generally negatively linked to adenylate cyclase via a pertussis toxin-sensitive G_i protein (Stiles, 1986; Ramkumar *et al.*, 1990). However, recent studies have shown that A_1-receptors can also regulate voltage-sensitive calcium channels (Sperelakis, 1987), atrial potassium channels (Kurachi *et al.*, 1986), guanylate cyclase activity (Kurtz, 1987) and agonist-stimulated inositol phospholipid hydrolysis (Linden & Delahunty, 1989; Hill & Kendall, 1989).

In the central nervous system (CNS), adenosine A_1-receptors can selectively augment histamine H_1-receptor-mediated inositol phospholipid hydrolysis in guinea-pig cerebral cortical slices (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987) and selectively inhibit the histamine response in mouse (Kendall & Hill, 1988) and man (Kendall & Firth, 1989) cerebral cortex. In all three species, however, adenosine-receptor stimulation failed to elicit a direct phosphoinositide response on its own. An inhibition of agonist-induced inositol phospholipid hydrolysis has also been demonstrated in the rat pituitary tumour cell line GH_3 (Delahunty *et al.*, 1988) and the human astrocytoma cell line 1321N1 (Nakahata *et al.*, 1991).

The DDT_1 MF-2 smooth muscle cell line, derived from a steroid-induced leimyosarcoma tumour of the vas deferens of an adult Syrian hamster, has recently been shown to possess high levels of both adenosine A_1- and A_2-receptors that are respectively coupled negatively and positively to adenylate cyclase (Gerwins *et al.*, 1990; Ramkumar *et al.*, 1991). These cells also possess glucocorticoid receptors which following activation can up-regulate adenosine A_1-receptors and down-regulate A_2-receptors (Gerwins & Fredholm, 1991a). We now show that adenosine A_1-receptors can also stimulate inositol phospholipid hydrolysis and intracellular calcium mobilization in this cell line. A preliminary account of some of this work has been presented to the British Pharmacological Society (White & Hill, 1992).

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Methods

Cell culture

DDT_1 MF-2 cells (European Cell Collection, Porton Down) were cultured at 37°C in a humidified air/CO_2 (90:10) atmosphere in 75 cm^3 flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged twice a week (1:5 split ratio) by vigorous shaking of the flask and fed with fresh growth medium every 48 h. Cells for inositol phospholipid hydrolysis were finally grown in 24 well cluster dishes and cells for $[Ca^{2+}]_i$ determination were grown on 24 mm × 10 mm glass cover slips in 90 mm Petri dishes. All experiments were performed on confluent monolayers (passages 4–12, number assigned after receiving the cell line).

Inositol phospholipid hydrolysis

At confluence, monolayer cultures were loaded with [^{3}H]-*myo*-inositol (37 kBq/well) for 24 h in 24 well cluster dishes in inositol-free DMEM containing 2 mM glutamine and 10% FCS. Prelabelled cells were then washed twice with 1 ml/well Hanks/HEPES (20 mM) buffer pH 7.4 and incubated at 37°C for 30 min in the presence of 20 mM LiCl. Where appropriate, antagonists were added at the beginning of this incubation period. Agonists were then added in 10 µl medium and the incubation continued for 45 min. Incubations were terminated by aspiration of the incubation medium and the addition of 1 ml cold (−20°C) methanol/0.12 M HCl (1:1 v/v). Cells were left a minimum of 2 h at −20°C before isolation of total ^{3}H-inositol phosphates in the supernatant of the disrupted cell monolayers by anion exchange chromatography (Alexander *et al.*, 1989a).

Measurement of intracellular free Ca^{2+}

Intracellular free calcium was measured by loading confluent cell monolayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm Petri dishes with 1 ml of physiological buffer (composition, mM: NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1, $CaCl_2$ 2, pH 7.45) containing 10% FCS (v/v), 3 µM fura-2/AM and incubated for 30 min at 37°C. After this loading period, the fura-2 containing buffer was replaced with fresh buffer that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37°C for a further 15 min. Where appropriate, antagonist drugs were added at the beginning of this 15 min period. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (containing antagonist drug where appropriate) and agonists were added in 100 µl of medium. Fluorescent measurements were made at 37°C with a Perkin Elmer LS 50 spectrophotometer. The excitation wavelengths were 340 and 380 nm, with emission measured at 500 nm. The slit widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between excitation wavelengths was 0.8 s. Intracellular free $[Ca^{2+}]$ was calculated every 1.6 s from the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = \frac{(R - R_{min})}{(R_{max} - R)} \times \beta \times K_d$$

where R is the 340 nm/380 nm ratio of fluorescence values and K_d is the dissociation constant of fura-2 for Ca^{2+} ions (224 nM at 37°C) and β is the ratio of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values (R_{max} and R_{min}) were determined on separate cover slips under saturating $[Ca^{2+}]_i$ (achieved by increasing extracellular $[Ca^{2+}]$ to 20 mM followeed by 10 µM ionomycin, pH 7.45) and calcium-free (achieved with 8.3 mM EGTA immediately followed by 25 µl 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 µM ionomycin) conditions respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where calcium-free conditions were required, experiments were performed in nominally calcium-free buffer containing 0.1 mM EGTA.

Data analysis

Agonist concentration-response curves and antagonist inhibition curves were fitted to a logistic equation by use of the non-linear regression programme GraphPad (ISI) as described previously (Ruck *et al.*, 1990). Antagonist dissociation constants (K_d) were estimated by a modification of the null method described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to 2-chloroadenosine was generated and a concentration (C; usually 10 µM) of 2-chloroadenosine was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of 2-chloroadenosine by 50% was then determined. The 2-chloroadenosine concentration-response curve was fitted to a logistic equation as described above and a concentration of 2-chloroadenosine (C') identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_d was then determined from the relationship:

$$C/C' = IC_{50}/K_d + 1$$

Statistical analysis was performed by paired and unpaired *t* tests; *n* in the text refers to the number of separate individual experiments.

Chemicals

Adenosine, 2-chloroadenosine, 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine, indomethacin and 8-phenyltheophylline were purchased from Sigma and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) from Research Biochemicals Incorporated. [2-^{3}H]-myo-inositol (529 GBq $mmol^{-1}$) was obtained from New England Nuclear. PD 115,199 (N-[2-(dimethylamino)ethyl]N-methyl-4-(1,3-dipropylaxanthine)benzene sulphonamide) and AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10- dodecadiynyl)1,4 - benzoquinone) were generous gifts from Warner Lambert (Ann Arbor, U.S.A.) and Takeda Chemical Industries (Osaka, Japan) respectively. Ionomycin and fura-2/AM were purchased from Calbiochem.

Results

Adenosine receptor-stimulated [^{3}H]-inositol phosphate accumulation

Adenosine (1 mM, 7.7 ± 0.8 fold over basal levels, $P < 0.05$, $n = 14$) and 2-chloroadenosine (10 µM, 6.8 ± 0.5 fold over basal levels, $P < 0.05$, $n = 26$) produced marked and significant accumulations of total [^{3}H]-inositol phosphates in the DDT_1 MF-2 cell line (Figure 1). Both agents produced a rapid increase in [^{3}H]-inositol phosphate accumulation over the first 10 min of agonist stimulation which began to plateau between 20 and 40 min (Figure 1b). Similar responses were obtained with N^6-cyclopentyladenosine (CPA; 1 µM, 8.5 ± 0.9 fold over basal levels, $n = 7$) and 5'-N-ethylcarboxamidoadenosine (NECA; 100 µM, 7.0 ± 0.8 fold over basal levels, $n = 4$) (Figure 2). Analysis of agonist concentration-response curves gave a rank order of potency which was typical of adenosine A_1-receptors, i.e. CPA > NECA > 2-chloroadenosine > adenosine (Figure 2; Table 1). The

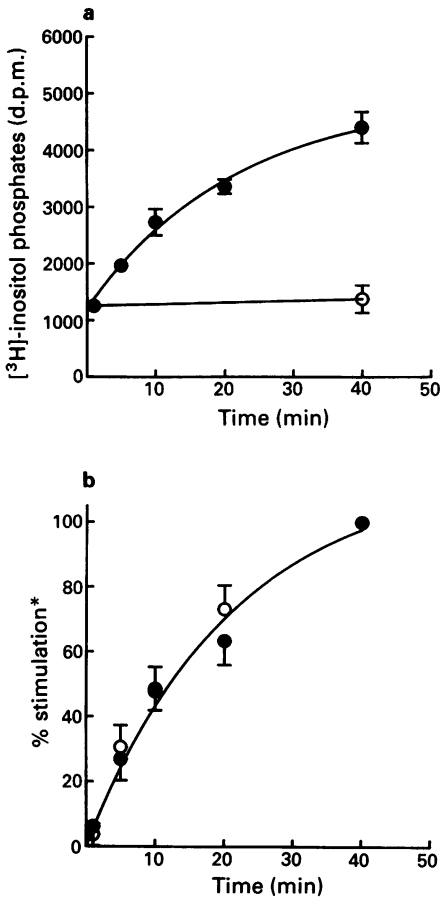

Figure 1 Time course of adenosine- and 2-chloroadenosine-stimulated [^{3}H]-inositol phosphate accumulation in DDT_1 MF-2 cells. (a) Response to 10 μM 2-chloroadenosine (●) added at time zero; (○) unstimulated control. Values represent mean (s.e.mean shown by vertical bars) from quadruplicate determinations in a single experiment. Similar data were obtained in three other experiments. (b) Mean stimulation produced by 10 μM 2-chloroadenosine (○) or adenosine (0.1 mM; ●). *Values represent mean ± s.e.mean of the percentage stimulation (response at 40 min = 100%) of total [^{3}H]-inositol phosphate accumulation (after subtraction of basal levels) obtained in four separate experiments performed in quadruplicate.

Table 1 Agonist potencies of adenosine analogues for stimulation of [^{3}H]-inositol phosphate accumulation in DDT_1 MF-2 cells

Agonist	*Inositol phosphate* (EC_{50} nM)		*A_1-binding** (K_D nM)
CPA	26 ± 4	(6)	58
NECA	290 ± 80	(4)	445
2-Chloroadenosine	480 ± 140	(6)	801
Adenosine	38,000 ± 9,000	(6)	1630

CPA = N^6-cyclopentyladenosine; NECA = 5'-N-ethylcarboxamidoadenosine. Values represent mean ± s.e.mean of the EC_{50} values for total [^{3}H]-inositol phosphate accumulation obtained in 4–6 individual experiments. The actual number of experiments is given in parentheses.
*Values reported (Gerwins *et al.*, 1990) for agonist K_D values from inhibition of [^{3}H]-DPCPX binding to adenosine A_1-receptors in intact DDT_1 MF-2 cells.

involvement of the A_1-class of adenosine receptor was further tested through the use of the selective A_1-receptor antagonist DPCPX (Bruns *et al.*, 1987a) and the high affinity mixed A_1/A_2 receptor antagonist, PD 115,199 (Bruns *et al.*, 1987b) (Figure 3). The apparent K_D values determined from individual inhibition curves for these two antagonists and for 8-phenyltheophylline were in close agreement with the values reported for the binding of these antagonists to the adenosine A_1-receptor in rat brain (Table 2).

Role of extracellular Ca^{2+} and arachidonic acid metabolites

To investigate whether adenosine receptor-stimulation was indirectly linked to inositol phospholipid hydrolysis via an effect on Ca^{2+} ion influx from the extracellular medium or mediated by liberation of cyclo-oxygenase or lipoxygenase products of arachidonic acid metabolism, experiments were performed in the presence of varying extracellular calcium concentrations (Figure 4) or the presence of indomethacin (an inhibitor of cyclo-oxygenase) and AA861 (an inhibitor of lipoxygenase) (Figure 5). In the presence of 0.6 mM and 1.2 mM extracellular calcium, the inositol phospholipid response to 10 μM 2-chloroadenosine was 97 ± 9% and 96 ± 10% of that obtained in normal calcium-containing medium (2.4 mM; $n = 4$, Figure 4). Furthermore, in calcium-free media, a significant response to 2-chloroadenosine

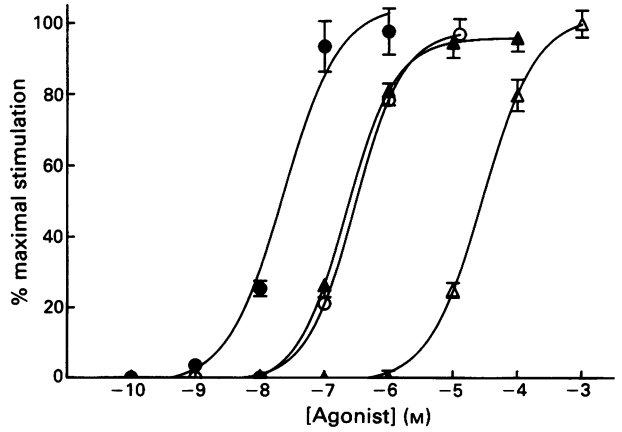

Figure 2 Effect of adenosine-receptor agonists on the accumulation of total [^{3}H]-inositol phosphates in DDT_1 MF-2 cells. Responses to N^6-cyclopentyladenosine (CPA; ●), N-ethylcarboxamidoadenosine (NECA; ▲), 2-chloroadenosine (○) and adenosine (△) are expressed as a percentage (mean with s.e.mean shown by vertical bars) of the maximal stimulation (after subtraction of basal values) from quadruplicate determinations in four (NECA) or six (all others) separate experiments.

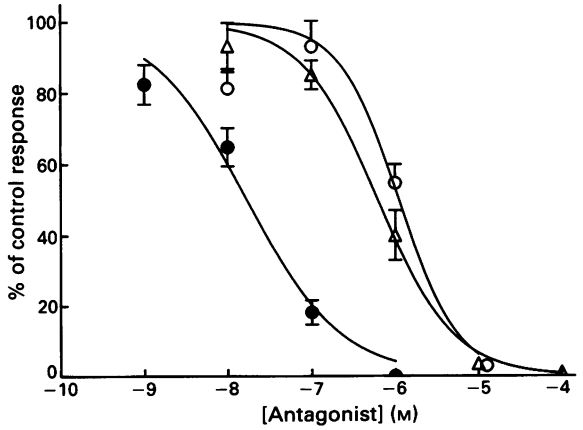

Figure 3 Effect of adenosine-receptor antagonists on 2-chloroadenosine-stimulated inositol phospholipid hydrolysis in DDT_1 MF-2 cells. Antagonism produced by DPCPX (●), 8-phenyltheophylline (△) or PD 115,199 (○) of the response to 10 μM 2-chloroadenosine. Values represent the combined mean (s.e.mean, shown by vertical bars) from quadruplicate determinations in six (8-phenyltheophylline) or four (DPCPX & PD 115,199) separate experiments.

Table 2 Antagonism of 2-chloroadenosine-mediated inositol phosphate accumulation in DDT_1 MF-2 cells

Antagonist	*Inositol phospholipid hydrolysis* IC_{50} (nM)	Apparent K_D (nM)		*Adenosine receptor* A_1*	A_{2A}#	K_D (nM) A_{2B}
DPCPX	37 ± 12	1.2 ± 0.4	(4)	0.5	340	234[a], 163[b]
8-PT	970 ± 350	31 ± 11	(6)	86	850	400[c]
PD 115,199	1200 ± 200	39 ± 7	(4)	14	16	275[a], 395[b]

8-PT = 8-phenyltheophylline. Values for inositol phospholipid hydrolysis represent mean ± s.e.mean of 4–6 determinations (actual number of experiments given in parentheses) of the antagonist IC_{50} values obtained from inhibition of 2-chloroadenosine-stimulated (10 μM) total [^{3}H]-inositol phosphate accumulation in DDT_1 MF-2 cells. Apparent K_D values were calculated from these IC_{50} values as described under Methods. *,#K_D values for inhibition of [^{3}H]-cyclohexyladenosine binding to A_1-receptor (*) or [^{3}H]-NECA binding to A_{2A} receptors (#) in rat brain membranes (Bruns *et al.*, 1987c); [a]K_D values obtained from antagonism of A_{2B}-receptor-mediated relaxation of guinea-pig aorta (Hargreaves *et al.*, 1991) or stimulation of cyclic AMP accumulation in guinea-pig cerebral cortex ([b]Alexander *et al.*, 1989b; [c]Alexander *et al.*, 1989a).

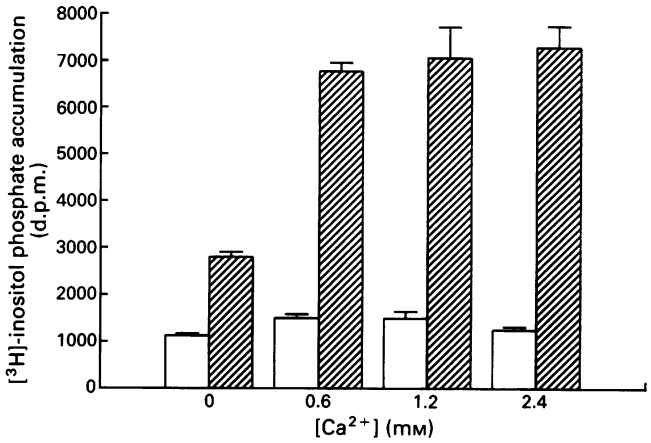

Figure 4 Influence of extracellular Ca^{2+} concentration on A_1-receptor-mediated inositol phospholipid hydrolysis in DDT_1-MF-2 cells. Measurements were made in the absence (open columns) or presence (hatched columns) of 10 μM 2-chloroadenosine. Data represent mean (s.e.mean shown by vertical bars) of quadruplicate determinations in a single experiment. Similar data were obtained in three other experiments and the combined data are summarized in the text.

(39 ± 10% of that obtained in 2.4 mM calcium-containing medium, $P<0.05$, $n=4$) was still evident (Figure 4).

Indomethacin (100 μM; $P<0.05$, $n=4$) and AA861 (100 μM; $P<0.05$, $n=4$) both produced an inhibition of the inositol phosphate response to 10 μM 2-chloroadenosine (Figure 5a), but significant effects with indomethacin were only evident at relatively high concentrations (Figure 5b). AA861 was, however, more potent that indomethacin (Figure 5b) and inhibited inositol phosphate accumulation at concentrations within the same range as that required for inhibition of 5-lipoxygenase (Yoshimoto *et al.*, 1982).

A_1-receptor-mediated release of intracellular Ca^{2+}

Monitoring of intracellular free calcium levels by use of the calcium-sensitive dye, fura-2, allowed the visualisation of an adenosine receptor-mediated increase in intracellular calcium levels in DDT_1 MF-2 cells (Figure 6). The addition of 10 μM 2-chloroadenosine to monolayers of DDT_1 MF-2 cells caused a rapid increase in intracellular free calcium concentrations from 86 ± 8 nM to 306 ± 25 nM (Figure 6a, $n=6$). The response was fairly well maintained and only declined slowly towards basal levels in the presence of extracellular calcium ions. Pretreatment of cells (15 min) with the adenosine A_1-selective antagonist DPCPX (100 nM) completely inhibited

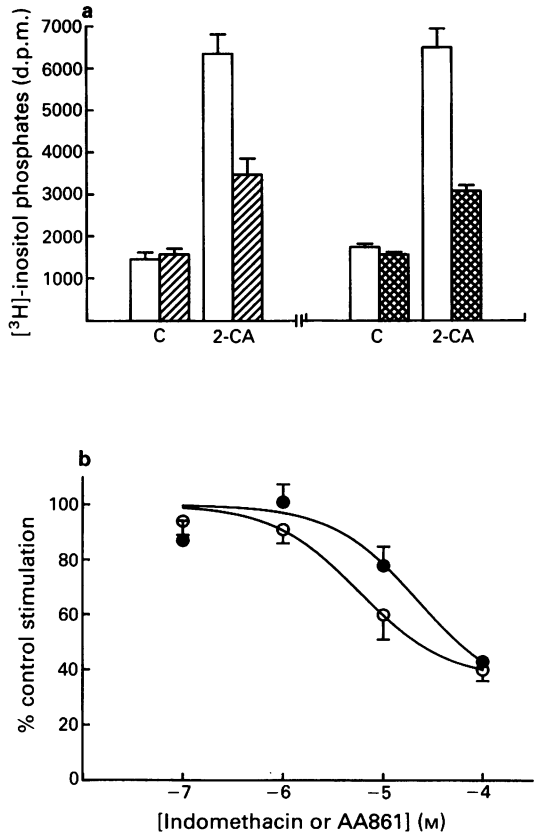

Figure 5 Effect of indomethacin and AA861 on 2-chloroadenosine-stimulated [^{3}H]-inositol phosphate accumulation in DDT_1 MF-2 cells. (a) Control (C) and 2-chloroadenosine (2-CA) responses were measured in the absence (open columns) or presence of 100 μM indomethacin (hatched columns) or 100 μM AA861 (cross-hatched columns). Values represent mean (s.e.mean shown by vertical bars) obtained from quadruplicate determinations in a single experiment. Similar data were obtained in two other experiments. (b) Concentration-response curves for the inhibition of 2-chloroadenosine-stimulated inositol phospholipid hydrolysis in DDT_1 MF-2 cells; (●) indomethacin; (○) AA861. Values represent combined mean (± s.e.mean) of quadruplicate determinations in four separate experiments. Data are expressed as a percentage of the control response to 10 μM 2-chloroadenosine (after subtraction of basal levels). The line through the AA861 data points was fitted to the equation: % control stimulation = $100-[A \times I_{max}/(IC_{50}+A)]$, where A is the concentration of AA861 and I_{max} is the maximal inhibitory effect. The fitted values for log IC_{50} and maximal inhibitory effect of AA861 were -5.23 ± 0.12 and 63.4 ± 4.2% inhibition respectively. The curve for indomethacin was insufficiently well-defined for least squares analysis and was drawn by inspection.

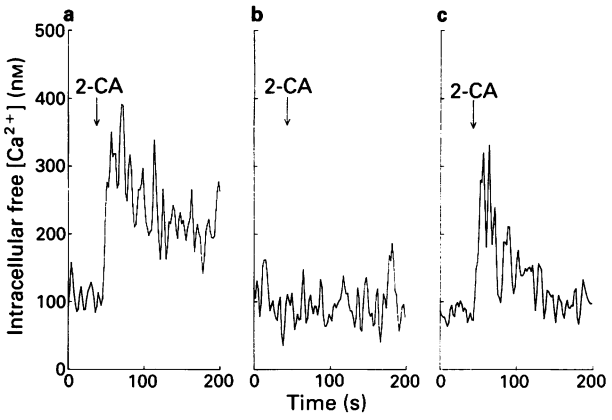

Figure 6 Effect of adenosine-receptor stimulation on intracellular Ca^{2+} concentration in fura-2 loaded DDT_1 MF-2 cells in the presence (a) of extracellular Ca^{2+} (2 mM) and in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA (c). (b) As (a) but cells were incubated with 100 nM DPCPX for 15 min before the addition of 2-chloroadenosine. 2-Chloroadenosine (2-CA, 10 μM) was added where indicated. Similar data were obtained in three other experiments.

the response to a subsequent addition of 2-chloroadenosine (10 μM; Figure 6b). The maintenance of the response to 2-chloroadenosine appeared to be dependent upon influx of extracellular calcium since experiments performed in nominally calcium-free medium containing 0.1 mM EGTA (Figure 6c) resulted in a loss of the maintained phase, leaving the initial transient peak (presumably due to intracellular calcium release) relatively unaffected (90 ± 16% of the response in Ca^{2+}-containing media, $n = 3$).

Discussion

The hamster vas deferens smooth muscle cell line DDT_1 MF-2 has previously been shown to possess a high density of A_1-receptors (Ramkumar *et al.*, 1990; Gerwins *et al.*, 1990). Studies of adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in both intact DDT_1 MF-2 cells (Gerwins *et al.*, 1990) and cell membranes (Ramkumar *et al.*, 1990) have shown that these receptors can negatively couple to adenylate cyclase via a pertussis toxin-sensitive G_i GTP binding protein. The present investigation suggests that A_1-receptors can additionally associate in some way with the activation of phospholipase C leading to inositol phospholipid hydrolysis and mobilization of intracellular calcium.

The A_1-receptor nature of the adenosine-stimulated accumulation of total [^{3}H]-inositol phosphates in DDT_1 MF-2 cells is indicated by both the rank order of agonist potencies and the high affinity of the A_1-receptor selective antagonist DPCPX, deduced from inhibition of 2-chloroadenosine-stimulated inositol phospholipid hydrolysis. Thus, the rank order of agonist potencies is such that the N^6-derivative of adenosine, CPA, is more potent that the 5'-derivative, NECA. Furthermore, the EC_{50} values deduced from inositol phospholipid hydrolysis for the stable analogues of adenosine, namely CPA, NECA and 2-chloroadenosine, agree (within a factor of 2; Table 1) closely with the K_D values obtained for these agonists from inhibition of [^{3}H]-DPCPX binding in intact DDT_1 MF-2 cells (Gerwins *et al.*, 1990).

The most convincing argument for the involvement of an A_1-receptor is, however, provided by the data obtained with antagonists, particularly DPCPX (Table 2). Bruns *et al.* (1987c) have argued that the relative affinities of DPCPX and PD 115,199 provide a useful basis for distinguishing A_1- and A_{2a}-receptor-mediated responses. Thus, DPCPX is 30 times more potent than PD 115,199 at the A_1-receptor, but 20 times less potent than PD 115,199 at the A_{2a} receptor. Furthermore, DPCPX and PD 115,199 are both weak antagonists (K_D *circa* 250 nM) at the A_{2b} receptor in guinea-pig aorta and guinea-pig cerebral cortex (Alexander *et al.*, 1989b; Hargreaves *et al.*, 1991). The data obtained in the present study, where DPCPX is 32.5 fold more potent than PD 115,199, are entirely consistent with the involvement of an A_1-receptor (Table 2). The high affinity of DPCPX (K_D 1.2 nM, Table 2) is similar to that obtained in rat brain membranes (Bruns *et al.*, 1987a) and in good agreement with the K_D (0.95 nM) obtained for A_1-receptor binding in intact DDT_1 MF-2 cells (Gerwins *et al.*, 1990). These data suggest that the receptor involved in the inositol phospholipid response to adenosine in DDT_1 MF-2 cells has the characteristics of a classic A_1-receptor rather than the atypical pharmacology of the adenosine A_1-like receptors involved in modulating histamine-stimulated inositol phospholipid hydrolysis in guinea-pig and mouse brain (Alexander *et al.*, 1989b) or regulating neurotransmission at the frog neuromuscular junction (putative A_3-receptor; Sebastiao & Ribeiro, 1989; 1990), where the K_D values obtained for DPCPX are much higher (10–35 nM).

Stimulation of inositol phospholipid hydrolysis and mobilization of intracellular free calcium by adenosine have also been detected in the rat tumour-derived mast cell line RBL-2H3 (Ali *et al.*, 1990; Hide & Beavan, 1991) and guinea-pig myometrium (Schiemann *et al.*, 1991a,b). In RBL-2H3 cells, the order of adenosine analogue potency indicated an involvement of an A_2-adenosine receptor (Ali *et al.*, 1990). However, adenosine antagonists such as 8-phenyltheophylline and 8-*p*-sulphophenyltheophylline produced only a non-selective antagonism of the NECA-induced inositol phosphate response at high concentrations, indicating the potential involvement of a novel class of adenosine receptor (Ali *et al.*, 1990) which may be related to the atypical adenosine receptor responsible for enhancing mediator secretion from rat peritoneal mast cells (Church *et al.*, 1986).

In oestrogen-primed or pregnant guinea-pig myometrium, adenosine A_1-receptor stimulation can produce smooth muscle contraction and inositol phospholipid hydrolysis via a mechanism which is insensitive to pertussis toxin treatment (Moritoki *et al.*, 1979; Smith *et al.*, 1988; Schiemann *et al.*, 1991a,b). Both of these effects could be completely attenuated by pretreatment of the tissue with cyclo-oxygenase inhibitors suggesting that smooth muscle contraction and inositol-1,4,5-trisphosphate formation may be secondary to the synthesis of prostaglandins from arachidonic acid (Moritoki *et al.*, 1979; Schiemann *et al.*, 1991b). This possibility has also been investigated in DDT_1 MF-2 cells in the present study by use of the cyclo-oxygenase inhibitor indomethacin and the 5-lipoxygenase inhibitor, AA861 (Higgs & Vane, 1983; Yoshimoto *et al.*, 1982; Ashida *et al.*, 1983).

Both agents were able to inhibit the inositol phospholipid response to 2-chloroadenosine by approximately 60% at high concentrations (100 μM) but no significant effect was observed with 1 μM indomethacin, which was sufficient to attenuate completely the A_1-receptor-mediated inositol phosphate response in guinea-pig myometrium (Schiemann *et al.*, 1991b). However, the 5-lipoxygenase inhibitor, AA861, was more potent than indomethacin (Figure 6) and the inhibition of the response to 2-chloroadenosine appeared to plateau at the 60% level. Furthermore, the IC_{50} obtained for AA861-mediated inhibition of 2-chloroadenosine-stimulated inositol phospholipid hydrolysis (5.8 μM) was within the same concentration-range as that required for inhibition of guinea-pig 5-lipoxygenase in cell-free preparations or tissue fragments (Yoshimoto *et al.*, 1982; Ashida *et al.*, 1983). Since indomethacin can inhibit fully cyclo-oxygenase at 1 μM, the inhibitory activity observed at higher concentrations of indomethacin may be accounted for by its effects on lipoxygenase.

These data suggest that lipoxygenase, rather than cyclo-oxygenase, products are involved in the A_1-receptor-mediated inositol phosphate response in DDT_1 MF-2 cells. However, since 40% of the response to 2-chloroadenosine was resistant

to both 100 μM indomethacin and 100 μM AA861, it seems likely that other mechanisms (including the possibility that A_1-receptors are directly coupled to inositol phospholipid hydrolysis via a G-protein) are also involved. Indeed, it is possible that the lipoxygenase products are produced as a consequence of the A_1-receptor-mediated increase in intracellular Ca^{2+} in DDT_1 MF-2 cells (Figure 6) leading to an activation of phospholipase A_2 activity.

Studies in calcium-free media suggest that some 60% of the 2-chloroadenosine-stimulated accumulation of [^{3}H]-inositol phosphates in DDT_1 MF-2 cells is dependent upon an influx of extracellular Ca^{2+} ions. However, experiments in calcium-free medium (containing EGTA) clearly show that adenosine A_1-receptor-stimulation can produce a direct release of Ca^{2+} ions from intracellular stores, presumably as a consequence of the generation of inositol-1,4,5-trisphosphate (Berridge & Irvine, 1989). Interestingly, while this manuscript was in preparation, a short report appeared which demonstrated that the A_1-receptor agonist CPA could increase both inositol-1,4,5-trisphosphate levels and intracellular calcium concentrations in DDT_1 MF-2 via a mechanism which was sensitive to pretreatment with pertussis toxin (Gerwins & Fredholm, 1991b).

In summary, this study provides strong evidence that adenosine A_1-receptors can stimulate inositol phospholipid hydrolysis and release of calcium from intracellular stores in DDT_1 MF-2 cells. It is clear that the A_1-receptor-mediated inositol phospholipid hydrolysis is reduced by either the presence of an inhibitor of lipoxygenase or by removal of extracellular calcium. Consequently, it remains to be established whether A_1-adenosine receptors are directly coupled to phospholipase C via a G-protein or whether inositol phospholipid hydrolysis and calcium mobilization are entirely mediated by indirect mechanisms. However, what is clear is that the DDT_1-MF-2 cell line, in which adenosine A_1-receptors have also been demonstrated to inhibit cyclic AMP accumulation (Gerwins *et al.*, 1991a), will be an important system in which to investigate adenosine receptor-effector coupling mechanisms.

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