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Effect of adenosine receptor agonists on release of the nucleoside analogue [^{3}H]formycin B from cultured smooth muscle DDT_1 MF-2 cells

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Abstract

Adenosine has receptor-mediated effects in a variety of cell types and is predominantly formed from ATP by a series of nucleotidase reactions. Adenosine formed intracellularly can be released by bidirectional nucleoside transport processes to activate cell surface receptors. We examined whether stimulation of adenosine receptors has a regulatory effect on transporter-mediated nucleoside release. DDT_1 MF-2 smooth muscle cells, which possess nitrobenzylthioinosine-sensitive (ES) transporters as well as both adenosine A_1 and A_2 receptors, were loaded with the metabolically stable nucleoside analogue [^{3}H]formycin B. N^6-cyclohexyladenosine (CHA), a selective adenosine A_1 receptor agonist, produced a concentration-dependent inhibition of [^{3}H]formycin B release with an IC_{50} value of 2.7 μM. Further investigation revealed CHA interacts directly with nucleoside transporters with a K_i value of 3.3 μM. Neither 5′-*N*-ethylcarboxamidoadenosine (NECA), a mixed adenosine A_1 and A_2 receptor agonist, nor CGS 21680, a selective adenosine A_{2A} receptor agonist, affected nucleoside release. We conclude that release of the nucleoside formycin B from DDT_1 MF-2 cells is not regulated by adenosine A_1 or A_2 receptor activation. © 1998 Elsevier Science B.V.

Keywords: Adenosine; Nucleoside transport; Formycin B; Adenosine A_1 receptor; Adenosine A_2 receptor; CHA (N^6-cyclohexyladenosine); MECA (5′-*N*-ethylcarboxamidoadenosine); CGS 21680; 8-cyclopentyl-1,3-dipropylxanthine; Nitrobenzylthioinosine; DDT_1 MF-2 smooth muscle cell

1. Introduction

Adenosine, an endogenous nucleoside with autocrine and paracrine regulatory actions, is formed from the dephosphorylation of ATP. The concentration of this nucleoside is tightly regulated by purine enzymes as well as by transport processes. Intracellular adenosine concentrations are kept at nanomolar concentrations by the enzymes adenosine kinase, which phosphorylates adenosine to adenosine monophosphate, and adenosine deaminase, which deaminates adenosine to inosine (Geiger et al., 1997).

Nucleoside transporters catalyze the movement of nucleosides across biological membranes. There are two classes of transport proteins (Griffith and Jarvis, 1996) which facilitate the movement of adenosine across cellular membranes. Sodium/nucleoside cotransporters move adenosine unidirectionally into cells by utilizing the sodium gradient while equilibrative (sodium-independent) transporters can move adenosine bidirectionally across plasma membranes by facilitated diffusion. Two subtypes of equilibrative transporters have been characterized, equilibrative-sensitive (ES) and equilibrative-insensitive (EI), based on their sensitivity to inhibition by nanomolar concentrations of nitrobenzylthioinosine (Vijayalakshmi and Belt, 1988). Cellular release of nucleosides via equilibrative transporters has been previously demonstrated with human erythrocytes (Plagemann and Woffendin, 1989) and hamster DDT_1 MF-2 cells (Foga et al., 1996).

Four adenosine receptor subtypes have been characterized and cloned and are termed A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 1994). Activation of adenosine A_1 and A_3 receptors is generally inhibitory as adenylyl cyclase activity is inhibited. Adenosine A_1 receptors can also enhance K^+ conductance and inhibit Ca^{2+} conductance.

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Adenosine A_{2A} and A_{2B} receptors are G_s protein linked stimulatory receptors which enhance cAMP formation.

Since adenosine that is released from cells can activate its cell surface receptors, this study was designed to test whether adenosine receptor activation affects transporter-mediated release of nucleosides. For this study we chose DDT_1 MF-2 smooth muscle cells, which appear to possess only nucleoside transporters of the ES subtype (Parkinson et al., 1996) as well as A_1 and A_2 (A_{2A} and/or A_{2B}) adenosine receptors (Ramkumar et al., 1989). We investigated cellular release of [^{3}H]formycin B, a poorly metabolized nucleoside analogue (Plagemann and Woffendin, 1989; Dagnino and Paterson, 1990; Wu et al., 1993) that can permeate ES transporters in DDT_1 MF-2 cells (Parkinson and Geiger, 1996).

2. Materials and Methods

2.1. Materials

[^{3}H]Formycin B was purchased from Moravek (Brea, CA) and [^{3}H]nitrobenzylthioinosine was obtained from DuPont (Mississauga, Ontario). N^6-Cyclohexyladenosine (CHA), nitrobenzylthioinosine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), CGS 21680, and 5′-*N*-ethylcarboxamidoadenosine (NECA) were purchased from Research Biochemicals (Natick, MA). Formycin B, Triton X-100, trypan blue and HEPES were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco (Burlington, Ontario). Dilazep was provided by Hoffmann-LaRoche (Basel, Switzerland).

2.2. Cell Culture

DDT_1 MF-2 smooth muscle cells, originally isolated from steroid-induced leiomyosarcoma of Syrian hamster vas deferens (Norris et al., 1974), were obtained from American Type Culture Collection. Cells were grown in suspension and maintained as exponentially proliferating cultures in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 5% qualified fetal bovine serum and 2 mM L-glutamine as previously described (Parkinson et al., 1996).

2.3. [^{3}H]Formycin B efflux measurements

Cells were harvested by centrifugation ($100 \times g$ for 10 min), washed twice ($100 \times g$ for 5 min) and resuspended (3×10^6 cells/ml) in physiological buffer containing 120 mM NaCl, 1 mM $MgCl_2$, 3 mM K_2HPO_4, 1.2 mM $CaCl_2$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mM glucose. Osmolarity of the buffer was adjusted as necessary to 300 ± 10 mosM and pH was adjusted to 7.4 with NaOH.

Cells were loaded for 30 min at 37°C with 10 μM [^{3}H]formycin B, a metabolically stable nucleoside analogue (Plagemann and Woffendin, 1989; Dagnino and Paterson, 1990; Wu et al., 1993) that is a permeant for nucleoside transporters in DDT_1 MF-2 cells (Parkinson and Geiger, 1996). Cells were pelleted (5 s, $11\,000 \times g$), extracellular [^{3}H]formycin B was removed and pellets were placed on ice. Release was stimulated by resuspending cells in 500 μl buffer alone or buffer containing nitrobenzylthioinosine, an inhibitor of ES nucleoside transporters; CHA, a selective adenosine A_1 receptor agonist; NECA, an A_1/A_2 mixed agonist; DPCPX, an adenosine A_1 receptor antagonist; CGS 21680, a selective adenosine A_{2A} receptor agonist; or forskolin, an adenylyl cyclase activator. Cells were incubated for 90 s at 22°C and then a 400 μl aliquot was centrifuged (30 s; $16\,000 \times g$) over oil (85 parts silicon oil: 15 parts paraffin oil; 200 μl) to separate cells from the extracellular medium. Samples of the supernatants were taken for radioactive determination by liquid scintillation spectroscopy. The microcentrifuge tubes were washed three times with distilled water, the oil was removed, and the pellets were dissolved in 10% Triton X-100 for determination of radioactivity.

Cell viability was assayed by trypan blue exclusion at the end of each experiment and was routinely greater than 95%.

2.4. [^{3}H]Nitrobenzylthioinosine binding

Cells were harvested, washed twice and resuspended in buffer as above. Cells, 25 000 per milliliter assay volume, were incubated (22°C) with 0.5 nM [^{3}H]nitrobenzylthioinosine and CHA (10 nM to 100 μM) for 1 h and reactions were terminated by filtration through Whatman GF/B filters using a Brandel cell harvester. Total binding was measured in the presence of [^{3}H]nitrobenzylthioinosine alone and nonspecific binding was measured in the added presence of 100 μM dilazep or 1 μM nitrobenzylthioinosine. [^{3}H]Nitrobenzylthioinosine concentrations were corrected for ligand depletion. The K_i value for CHA was determined with the equation of Cheng and Prusoff (1973) using a K_d value for nitrobenzylthioinosine of 0.26 nM (Parkinson et al., 1996).

2.5. Data Analysis

[^{3}H]Formycin B release measurements were in triplicate and [^{3}H]nitrobenzylthioinosine binding measurements were in duplicate. Each experiment was performed at least three times and all values are reported as mean ± S.E.M. Nonlinear regression was performed using the software package GraphPad PRISM version 2. To test for significant differences between three or more means, data were analyzed using a one-way ANOVA with Tukey's multiple

Table 1
Effect of adenosine receptor agonists on ES transporter-mediated release of [^{3}H]formycin B from smooth muscle DDT$_1$ MF-2 cells

	[^{3}H]Formycin B release (pmol/10^6 cells)
Control	51.2 ± 3.3 (14)
Nitrobenzylthioinosine (10 μM)	9.1 ± 4.8 (3)***
CHA (30 μM)	5.1 ± 3.6 (6)***
NECA (30 μM)	67.6 ± 3.3 (3)
CGS 21680 (10 μM)	35.2 + 7.4 (3)
Forskolin (10 μM)	53.6 ± 13.6 (3)
Forskolin (1 μM)	58.1 ± 1.0 (3)
DPCPX (10 μM)	51.2 ± 5.3 (7)
CHA (30 μM) + DPCPX (10 μM)	15.7 ± 4.7 (4)***

Cells were loaded in the presence of 10 μM [^{3}H]formycin B for 30 min, extracellular tritium was removed, cells were exposed to indicated compounds for 90 s, and release of [^{3}H]formycin B into supernatants was determined. The number of experiments is shown in parentheses.
*** $P < 0.001$ relative to control; ANOVA with Tukey's multiple comparison post-tests.

comparisons post-hoc test. A significance level of $P \leq 0.05$ was chosen a priori.

3. Results

DDT$_1$ MF-2 cells possess nucleoside transport processes of the ES subtype. By imposing an inwardly directed concentration gradient, cells were loaded with [^{3}H]formycin B. Cells were stimulated to release [^{3}H]formycin B by removing extracellular tritium and thereby providing an outwardly directed concentration gradient. Release of [^{3}H]formycin B was inhibited 82% by the transport inhibitor nitrobenzylthioinosine (Table 1), confirming that release was mediated by ES transporters.

To test whether adenosine receptor stimulation affects ES transporter-mediated nucleoside release, cells loaded with [^{3}H]formycin B were treated with the adenosine A_1 receptor agonist CHA, the mixed adenosine A_1/A_2 receptor agonist NECA or the adenosine A_{2A} receptor selective agonist CGS 21680. CHA (30 μM) significantly inhibited [^{3}H]formycin B release by 90%. [^{3}H]Formycin B release was not significantly altered by the adenosine A_1/A_2 mixed receptor agonist NECA (30 μM) or the selective adenosine A_{2A} receptor agonist, CGS 21680 (10 μM) (Table 1). During the brief 90 s time intervals required for release assays, the concentrations of CHA and CGS 21680 used were effective at inhibiting and stimulating cAMP levels, respectively (Tables 2 and 3). In contrast, the mixed agonist NECA had no significant effect on cAMP production (Table 3).

Table 2
Adenosine A_1 receptor-mediated inhibition of cAMP production by CHA

	cAMP (pmol)
Isoproterenol (1 μM)	48.8 ± 9.2
+ CHA (30 μM)	10.5 ± 1.0*
+ DPCPX (10 μM)	41.6 ± 6.2
+ CHA (0.3 μM)	10.1 ± 0.8*
+ DPCPX (10 μM)	50.7 ± 9.5

Cells were exposed to the indicated compounds and to the phosphodiesterase inhibitor rolipram for 90 s at 37°C. Cyclic AMP levels were measured as described in the text.
* $P < 0.05$ relative to isoproterenol alone; ANOVA with Tukey's multiple comparison post-test.

Table 3
Effect of NECA and CGS 21680 on cAMP production in DDT$_1$ MF-2 cells

	cAMP (pmol)
Control	4.8 ± 0.4
NECA (30 μM)	3.9 ± 0.3
CGS 21680 (0.1 μM)	6.8 ± 0.8
CGS 21680 (1 μM)	11.9 ± 2.0*
CGS 21680 (10 μM)	10.8 ± 1.2*

Cells were exposed to the indicated compounds and rolipram for 90 s at 37°C. Cyclic AMP levels were measured as described in the text.
* $P < 0.05$ relative to control; ANOVA with Tukey's multiple comparison post-tests.

Forskolin is an activator of adenylyl cyclase that has previously been shown to inhibit adenosine uptake into bovine chromaffin cells or pig kidney cells (Sen et al., 1990; Sayos et al., 1994). Treatment of DDT$_1$ MF-2 cells with forskolin for 90 s resulted in a concentration-dependent increase in cAMP production; 1 μM, 10 μM and 100 μM forskolin elevated cAMP values by 4.3-, 8.3- and 9.7-fold over basal, respectively ($n = 3$). However, no effect of forskolin (1 or 10 μM) on [^{3}H]formycin B release was detected (Table 1).

To test whether the inhibition of [^{3}H]formycin B release by CHA was due to stimulation of adenosine A_1 receptors, the effect of the selective adenosine A_1 receptor antagonist, DPCPX (10 μM), was investigated (Table 1). DPCPX

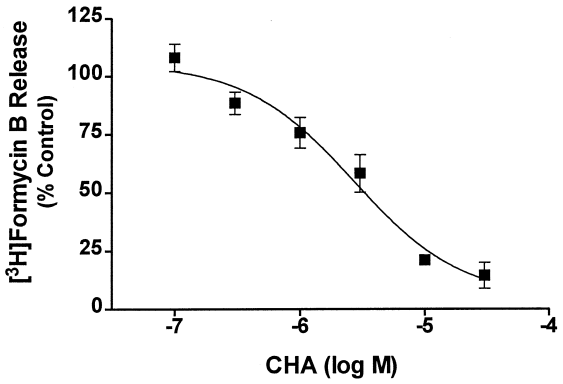

Fig. 1. Concentration-dependent inhibition of [^{3}H]formycin B release by CHA. Release was stimulated by resuspending cells, loaded with [^{3}H]formycin B, in buffer containing 0.1 μM to 30 μM CHA. Supernatants were collected after 90 s and assayed for tritium content. Symbols represent mean ± S.E.M. of at least three experiments. Control represents release in the absence of CHA.

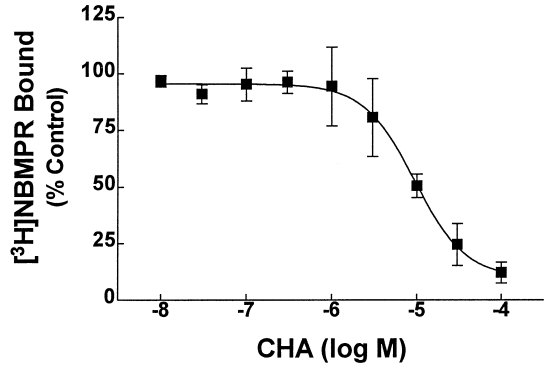

Fig. 2. Concentration-dependent inhibition of site-specific [^{3}H]nitrobenzylthioinosine (0.5 nM) binding by CHA (10 nM to 100 μM). Cells (25000/ml assay volume) were incubated for 1 h with radioligand and graded concentrations of CHA. Site-specific binding (control) of [^{3}H]nitrobenzylthioinosine was the difference between binding in the absence and presence of dilazep (100 μM) or unlabeled nitrobenzylthioinosine (1 μM). Symbols represent mean $\pm$ S.E.M. of three experiments performed in duplicate.

alone had no effect on [^{3}H]formycin B release. While DPCPX blocked the effect of CHA on cAMP production (Table 2), it produced only a partial reversal of the inhibitory effect of CHA on [^{3}H]formycin B release (Table 1).

To examine further the effect of CHA on release of [^{3}H]formycin B, we measured release in the presence of 0.1 μM to 30 μM CHA. Concentration-dependent inhibition of [^{3}H]formycin B release by CHA was observed with a half-maximal inhibition constant (IC_{50}) of 2.7 $\pm$ 1.5 μM ($n = 3$) (Fig. 1).

Because several adenosine analogues have been shown to interact directly with nucleoside transporters (Geiger et al., 1988; Hammond, 1991), we performed competition binding assays with [^{3}H]nitrobenzylthioinosine and CHA (Fig. 2). CHA produced a concentration-dependent inhibition of [^{3}H]nitrobenzylthioinosine binding to DDT_1 MF-2 cells and an IC_{50} value of 9.6 μM, corresponding to a calculated K_i value of 3.3 μM, was obtained.

A concentration of CHA (300 nM), which did not inhibit [^{3}H]nitrobenzylthioinosine binding (Fig. 2) but was able to cause significant activation of adenosine A_1 receptors in these cells during 90 s exposure (Table 2), was examined for inhibition of [^{3}H]formycin B release. In the presence of 300 nM CHA, [^{3}H]formycin B release was 43.4 $\pm$ 3.2 pmol/10^6 cells ($n = 3$), and was not significantly different from control values.

4. Discussion

Formycin B is a poorly metabolized inosine analogue which is a permeant for ES transporters in DDT_1 MF-2 cells (Parkinson and Geiger, 1996). We used formycin B for this study because adenosine is rapidly metabolized by intracellular, and possibly extracellular (Ciruela et al., 1996) enzymes, thus, it is difficult to achieve a stable releasable pool of intracellular adenosine. While it is possible to load cells with [^{3}H]adenosine in the presence of inhibitors of adenosine metabolism, several inhibitors of adenosine metabolism, such as the adenosine kinase inhibitor iodotubercidin, can block nucleoside transport processes and interfere with uptake or release assays (Parkinson and Geiger, 1996). Formycin B is a poorly metabolized nucleoside that is a permeant for ES nucleoside transporters and equilibrates across cell membranes. These properties make formycin B a useful probe for assaying cellular release of nucleosides.

Release of [^{3}H]formycin B was initiated by imposing an outwardly directed concentration gradient. Release was inhibited by the transport inhibitor nitrobenzylthioinosine, indicating that release occurred through ES transporters. [^{3}H]Formycin B release through ES transporters in human erythrocytes has been observed previously (Plagemann and Woffendin, 1989).

To investigate the effect of adenosine receptor stimulation on nucleoside release processes, high concentrations of adenosine A_1 and A_2 receptor agonists were chosen so that significant receptor activation would occur within a minimum release interval. Release intervals had to be kept to a minimum to avoid depletion of intracellular [^{3}H]formycin B. Significant inhibition of [^{3}H]formycin B release by 30 μM CHA was observed. This inhibition was concentration-dependent and an IC_{50} value of 2.7 μM was obtained. However, the adenosine A_1 receptor antagonist DPCPX did not completely reverse the inhibition of [^{3}H]formycin B release by CHA, indicating that inhibition of release by CHA was not mediated solely by adenosine A_1 receptor activation. We tested whether CHA could interact directly with ES transporters and found that CHA inhibited [^{3}H]nitrobenzylthioinosine binding with a K_i value similar to the IC_{50} value for inhibition of [^{3}H]formycin B release. Several structural analogs of adenosine, including receptor agonists and antagonists, have been found to inhibit nucleoside uptake (Geiger et al., 1988; Hammond, 1991) but effects on nucleoside release have not been reported previously. A concentration of CHA that had no effect in [^{3}H]nitrobenzylthioinosine binding assays but was effective in adenosine A_1 receptor assays had no effect on [^{3}H]formycin B release, indicating that inhibition of [^{3}H]formycin B release from DDT_1 MF-2 cells by CHA was due to direct inhibition of ES transporters.

No effect on [^{3}H]formycin B release was observed with NECA, a mixed adenosine A_1/A_2 receptor agonist, or CGS 21680, a selective adenosine A_{2A} receptor agonist. At the concentrations used, CGS 21680 stimulated cAMP production, likely by stimulating adenosine A_2 receptors in these cells. In contrast, NECA had no significant effect on cAMP production. In the presence of DPCPX, NECA stimulates cAMP production in these cells (Gerwins and Fredholm, 1991; unpublished observations), therefore the lack of effect on cAMP production in this study is due to

simultaneous activation of both inhibitory A_1 and stimulatory A_2 adenosine receptors. Thus, our data do not provide evidence for regulation of nucleoside release from DDT_1 MF-2 cells by adenosine A_2 receptor activation. In contrast, NECA enhanced adenosine uptake in cultured bovine chromaffin cells (Delicado et al., 1990) that express adenosine A_2 receptors, however, an adenosine A_2 receptor-mediated elevation of cAMP could not explain this effect of NECA since forskolin inhibited adenosine transport in these cells (Sen et al., 1990). Thus, in the same cell type activators of cAMP formation have been shown either to increase or to decrease the activity of nucleoside transporters. In the present study with DDT_1 MF-2 cells, we found no effect on nucleoside release processes by concentrations of forskolin that activate adenylyl cyclase and increase cAMP levels.

Previous studies investigating regulation of nucleoside uptake or release have used direct stimulation of components of second messenger pathways. In cerebellar granule cells, Sweeney (1996) observed that pertussis toxin decreased, while cholera toxin potentiated, adenosine release and she proposed that the activity of equilibrative transporters in cerebellar granular cells was modulated by G-proteins. Phorbol esters, cAMP analogues or forskolin were found to inhibit nucleoside uptake in cultured chromaffin cells (Sen et al., 1990; Delicado et al., 1991) but to have no effect in bovine endothelial cells (Sen et al., 1996). Therefore, regulation of nucleoside transport function by components of signal transduction pathways varies among cell types.

These previous studies which have investigated regulation of adenosine uptake (Sen et al., 1990, 1996; Delicado et al., 1991) or release (Sweeney, 1996) processes have used exogenous or endogenous adenosine, respectively. However, it is difficult to distinguish between the effects of drug treatment on adenosine transport per se and on net adenosine uptake (inward transport + intracellular metabolism) or release (intracellular formation + outward transport). The present study assayed ES transporter function directly and found no evidence for adenosine receptor-mediated regulation. Whether adenosine receptors or other modulators of signal transduction pathways can affect nucleoside transporter function by altering adenosine levels, due to effects on adenosine production and/or metabolism, remains a possibility that could be addressed in a future study.

In summary, the main finding of this study was that [^{3}H]formycin B release from DDT_1 MF-2 cells was inhibited by the adenosine A_1 receptor agonist CHA. This effect was not blocked by DPCPX, and can be attributed to direct interactions with transport processes rather than regulation by receptor activation. The nonselective adenosine receptor agonist NECA, the adenosine A_{2A} receptor agonist CGS 21680, and the adenylyl cyclase activator forskolin did not affect release of [^{3}H]formycin B. These data indicate that adenosine receptor activation in DDT_1 MF-2 cells does not regulate nucleoside transporter function.

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