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Stimulation of Nucleoside Efflux and Inhibition of Adenosine Kinase by A_1 Adenosine Receptor Activation

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ABSTRACT. Adenosine is produced intracellularly during conditions of metabolic stress and is an endogenous agonist for four subtypes of G-protein linked receptors. Nucleoside transporters are membrane-bound carrier proteins that transfer adenosine, and other nucleosides, across biological membranes. We investigated whether adenosine receptor activation could modulate transporter-mediated adenosine efflux from metabolically stressed cells. DDT_1 MF-2 smooth muscle cells were incubated with 10 μM [^{3}H]adenine to label adenine nucleotide pools. Metabolic stress with the glycolytic inhibitor iodoacetic acid (IAA, 5 mM) increased tritium release by 63% ($P < 0.01$), relative to cells treated with buffer alone. The IAA-induced increase was blocked by the nucleoside transport inhibitor nitrobenzylthioinosine (1 μM), indicating that the increased tritium release was primarily a purine nucleoside. HPLC verified this to be [^{3}H]adenosine. The adenosine A_1 receptor selective agonist N^6-cyclohexyladenosine (CHA, 300 nM) increased the release of [^{3}H]purine nucleoside induced by IAA treatment by 39% ($P < 0.05$). This increase was blocked by the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (10 μM). Treatment of cells with UTP (100 μM), histamine (100 μM), or phorbol-12-myristate-13-acetate (PMA, 10 μM) also increased [^{3}H]purine nucleoside release. The protein kinase C inhibitor chelerythrine chloride (500 nM) inhibited the increase in [^{3}H]purine nucleoside efflux induced by CHA or PMA treatment. The adenosine kinase activity of cells treated with CHA or PMA was found to be decreased significantly compared with buffer-treated cells. These data indicated that adenosine A_1 receptor activation increased nucleoside efflux from metabolically stressed DDT_1 MF-2 cells by a PKC-dependent inhibition of adenosine kinase activity. BIOCHEM PHARMACOL **59**;5:477–483, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. chemical hypoxia; adenosine efflux; adenosine kinase activity; protein kinase C; adenosine A_1 receptor

Adenosine, through the stimulation of membrane-bound receptors, has many biological functions; it is an inhibitory neuromodulator, a potent vasodilator, an inhibitor of lipolysis, and an anti-inflammatory agent [1]. There are four subtypes of adenosine receptors; A_1 and A_3 activate G-proteins of the G_i/G_o family to inhibit adenylyl cyclase activity, inhibit Ca^{2+} influx, enhance K^+ efflux, and/or alter PLC† activity, whereas A_{2A} and A_{2B} couple to G_s proteins to stimulate adenylyl cyclase activity. Under normoxic conditions, adenosine levels are maintained at low basal concentrations by three metabolic enzymes: *S*-adenosylhomocysteine hydrolase, adenosine deaminase, and adenosine kinase. Hypoxia or ischemia increases intracellular adenosine levels due to faster rates of ATP hydrolysis than synthesis.

Nucleoside transporters are carrier proteins that transfer nucleosides, including adenosine, across plasma membranes. Two subtypes of equilibrative nucleoside transporters, termed *es* and *ei* [2], can transport adenosine in either direction according to its concentration gradient. Na^+-dependent nucleoside transporters are symporters that couple the movement of nucleosides to the inward movement of Na^+. However, when transmembrane Na^+ gradients are disrupted, Na^+-dependent nucleoside transporters also can mediate cellular release of nucleosides [3].

A number of physiological systems have been reported to have autoregulatory feedback mechanisms. For example, stimulation of presynaptic α_2-adrenoceptors inhibits noradrenaline release. Evidence for positive or negative autoregulation of release also has been reported for the neurotransmitters glutamate [4, 5], serotonin [6], and γ-aminobutyric acid [7]. We hypothesized that an autocrine mechanism exists through which adenosine recep-

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† *Abbreviations*: PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; IAA, iodoacetic acid; NBMPR, nitrobenzylmercaptopurine riboside (nitrobenzylthioinosine); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CHA, N^6-cyclohexyladenosine; cAMP, cyclic AMP; and PKC, protein kinase C.

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tor activation can modulate adenosine release. To investigate this, we used DDT_1 MF-2 smooth muscle cells that have adenosine A_1 and A_2 receptors as well as *es* nucleoside transporters [8, 9].

MATERIALS AND METHODS

Materials

[^{3}H]Adenine was purchased from NEN Life Sciences. PMA, adenine, adenosine, UTP, histamine, and IAA were purchased from the Sigma Chemical Co. CGS 21680 (2-[*p*-(2-carboxyethyl) phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine), DPCPX, NECA, CHA, and NBMPR were purchased from Research Biochemicals International. Chelerythrine chloride was purchased from Calbiochem. Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco BRL.

Cell Culture

DDT_1 MF-2 smooth muscle cells were obtained from the American Type Culture Collection and were cultured as previously described [9]. Cells were harvested, washed twice (100 *g*, 5 min), and resuspended in physiological buffer (NaCl, 118 mM; HEPES, 25 mM; KCl, 4.9 mM; K_2HPO_4, 1.4 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 1 mM; glucose, 11 mM; adjusted to pH 7.4 with NaOH) to a concentration of 1 x 10^6 cells/mL. At the end of each experiment, cell viability was evaluated by trypan blue exclusion staining and was found to be greater than 90%.

Measurement of [^{3}H]Purine Release

DDT_1 MF-2 smooth muscle cells (1×10^6 cells/mL) were incubated with 10 μM [^{3}H]adenine for 30 min at 37°. At the end of the 30-min loading period, 500-μL aliquots of cell suspensions were centrifuged for 5 sec (16,000 *g*), and the supernatants were aspirated. Release was initiated by resuspending the cell pellets in 500 μL of buffer (37°) containing drug additions as indicated below. Release was terminated after 10 min by layering aliquots (400 μL) of each cell mixture over 200 μL of oil (85% silicone oil:15% mineral oil) and centrifuging at 16,000 *g* for 30 sec. Supernatants (250 μL) were added to 5 mL of scintillation fluid and were assayed for radioactivity.

The effects of glycolytic inhibition on tritium release were determined through replacement of glucose in the buffer with 5 mM IAA. The selective inhibitor of the *es* subtype of equilibrative nucleoside transporters, NBMPR (1 μM), was used to block efflux of [^{3}H]purine nucleosides through *es* transporters.

To determine the effects of adenosine receptor activation or blockade on IAA-induced release of tritiated nucleosides, the A_1 adenosine receptor antagonist DPCPX (1 μM), the A_1 and A_2 nonselective agonist NECA (1 μM), the A_{2A} selective agonist CGS 21680 (1 μM), or the A_1 selective agonist CHA (300 nM) was placed into the resuspension buffer along with 5 mM IAA. DPCPX (10 μM) in combination with CHA (300 nM) also was tested. These concentrations of agonists and antagonists ensured maximal activation or blockade of the respective receptors without directly inhibiting *es* transporter function.* Activation of the histamine H_1 receptor with histamine (100 μM) or the purinergic P2Y receptor with UTP (100 μM) was investigated in the presence or absence of CHA. PMA (10 μM) and chelerythrine chloride (500 nM), a stimulator and an inhibitor of PKC, respectively, were tested separately, together, and in combination with CHA for effects on [^{3}H]purine release.

HPLC Analysis of Adenosine

The release of adenosine *per se* from cells treated with IAA was determined by HPLC. Cells were pretreated with adenine (10 μM), and release assays were performed as described above. Supernatants were analyzed for adenosine using a slight modification of the method described by Delaney and Geiger [10]. Briefly, equal volumes of supernatant, 0.3 M $ZnSO_4$, and 0.3 M $BaOH_2$ were added sequentially, vortexed, and centrifuged for 4 min at 16,000 *g*. Supernatant (150 μL) was derivatized by adding 25 μL of 5% chloracetaldehyde and incubating for 1 hr at 80°. Samples were injected into a μBondapak C_{18} column (3.9 × 150 mm) and eluted using a mobile phase of 0.01 M KH_2PO_4 with 12% methanol (pH 5) and run isocratically at 1.5 mL/min. The adenosine derivative 1′-N^6-ethenoadenosine was measured by fluorescent detection with excitation at 275 nm and emission at 407 nm.

Adenosine Kinase Activity

Cells were harvested, washed twice (100 *g*, 5 min), and resuspended at a concentration of 1×10^6 cells/mL in physiological buffer alone or containing CGS 21680 (1 μM), CHA (300 nM), DPCPX (1 μM), or CHA + DPCPX at 37° for 10 min. The PKC inhibitor chelerythrine chloride (500 nM) also was tested in the presence or absence of PMA (10 μM) or CHA. For co-incubations, chelerythrine chloride or DPCPX was added 2 min prior to CHA or PMA. As IAA (5 mM) was found to have no significant effect on adenosine kinase activity (data not shown), IAA was not included in the assay. Adenosine kinase activity was determined as previously described [11]. Briefly, cells were harvested, homogenized in ice-cold 50 mM Tris–HCl (pH 7.4), and then centrifuged at 38,000 *g* (1 hr, 4°). Assay reaction mixtures (100 μL) contained 50 mM Tris–HCl (pH 7.4), 0.1% (w/v) BSA, 500 nM erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA), 50% (v/v) glycerol, 1.6 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 50 mM KCl, 1.2 mM ATP, 2 μM (0.25 μCi) [^{3}H]adenosine, and 2 μg of cytosolic protein. After incubation at 37° for 5 min, reactions were terminated by heating to 90°. Reaction products (20 μL) were spotted, in triplicate, on DE81 ion

* Parkinson *et al.*, unpublished work.

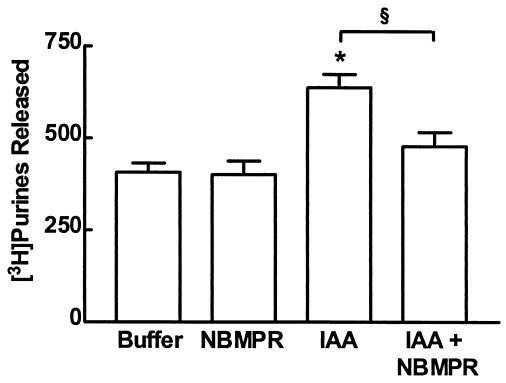

FIG. 1. Effect of glycolytic inhibition with IAA on release of [^{3}H]purines from DDT$_1$ MF-2 cells loaded with [^{3}H]adenine. Cells were loaded for 30 min with 10 μM [^{3}H]adenine, and then were pelleted and resuspended in buffer alone or in buffer containing 5 mM IAA for 10 min in the absence or presence of the nucleoside transport inhibitor NBMPR (1 μM). Values represent means ± SEM (N = 3) and are expressed as picomoles per 10^6 cells. Statistical significance was determined by ANOVA followed by Bonferroni's post-hoc test. Key: (§) $P < 0.05$ between IAA and IAA + NBMPR; and (*) $P < 0.01$ between buffer and IAA.

exchange filters, dried, and washed sequentially with 1 mM NH_4COOH, distilled deionized water, and 100% ethanol. Then HCl (0.25 mL, 0.2 M) and KCl (0.25 mL, 0.8 M) were added to the filters to elute [^{3}H]adenine nucleotides, and the tritium content was determined by scintillation spectroscopy [12].

Data Analysis

Tritium release measurements were performed in triplicate, and each experiment was performed at least three times. All values are expressed as means ± SEM, and statistical significance was determined by ANOVA followed by Bonferroni's post-hoc test. Statistical analyses were performed using the software package GraphPad PRISM Version 2.0.

RESULTS

DDT$_1$ MF-2 smooth muscle cells, loaded with [^{3}H]adenine, were subjected to glycolytic inhibition with IAA (5 mM) (Fig. 1). IAA increased tritium efflux by 63% ($P < 0.01$) over release into buffer alone. The *es* transport inhibitor NBMPR (1 μM) was used to determine whether the increase in tritium release was due to increased efflux of [^{3}H]purine nucleosides. NBMPR significantly blocked the IAA-induced increase ($P < 0.05$). As both adenosine and its metabolite, inosine, are permeants of *es* nucleoside transporters, we treated cells with adenine and then measured, by HPLC, adenosine release induced by buffer alone or by IAA (Table 1). Release into buffer alone showed undetectable levels of adenosine in the extracellular fluid; however, IAA treatment produced extracellular adenosine concentrations of 72 ± 4 nM. By scintillation spectroscopy, [^{3}H]purines were detected in the supernatants of cells

TABLE 1. IAA-induced release of adenosine or total purines from DDT$_1$ MF-2 cells

Treatment	Adenosine (nM)	[^{3}H]Purines (nM)
Buffer	ND*(5)	201 ± 8 (21)
IAA	72 ± 4 (4)	329 ± 24 (21)

Cells were preincubated with 10 μM adenine or 10 μM [^{3}H]adenine for 30 min at 37°C. Cells were then pelleted and resuspended in buffer alone or in 5 mM IAA. After 10 min, cells were pelleted through oil, and supernatants were analyzed by HPLC for adenosine content or by scintillation spectroscopy for [^{3}H]purine content. Data are mean concentrations ±SEM; the number of determinations is given in parentheses.

*Not detected.

treated with buffer alone; however, following treatment with IAA, released [^{3}H]purines increased by approximately 130 nM (Table 1). Thus, about half of the increase in tritium efflux seen following treatment of cells with IAA was due to release of [^{3}H]adenosine.

The effect of adenosine receptor stimulation or inhibition was determined by resuspending the [^{3}H]adenine-loaded cells into buffer containing IAA with or without the A_1 selective agonist CHA, the nonselective agonist NECA, the A_{2A} agonist CGS 21680, or the A_1 antagonist DPCPX (Fig. 2A). Of the compounds tested, only CHA significantly changed release; it produced a 39% increase ($P < 0.05$) over IAA alone. None of the agonists or the antagonist had any significant effects on tritium release in the absence of IAA (data not shown). To indicate whether this increase by CHA was mediated by the A_1 receptor, the experiments were repeated in the presence of DPCPX (Fig. 2B). DPCPX completely inhibited the CHA-mediated increase in [^{3}H]purine nucleoside efflux ($P < 0.05$). As A_1 adenosine receptor stimulation is known to inhibit adenylyl cyclase activity, we investigated the role of cAMP in altering purine nucleoside release. Forskolin (10 μM) as well as CGS 21680 (1 μM), both of which will activate adenylyl cyclase in these cells [13, *], failed to alter IAA-induced nucleoside release (data not shown; Fig. 2A). Thus, we found no evidence that cAMP formation affects adenosine efflux.

As A_1 receptors in DDT$_1$ MF-2 cells are known to activate PLC directly and to enhance the effects of other PLC activators [14–16], we examined the effect of PLC and PKC activation on IAA stimulation of [^{3}H]purine release. Both nucleotide P2Y [17] and histamine H_1 [14] receptor stimulation have been shown to increase PLC activity in these cells. We investigated whether UTP (100 μM) or histamine (100 μM) could enhance IAA-stimulated efflux of [^{3}H]purines and found that each compound caused a 35% increase relative to that of IAA ($P < 0.05$) (Fig. 3, A and B). When UTP or histamine was combined with CHA, there appeared to be an additive effect, and release was increased by 58 and 52%, respectively ($P < 0.01$), relative to IAA alone. The role of PKC in these increases in

* Parkinson *et al.*, unpublished work.

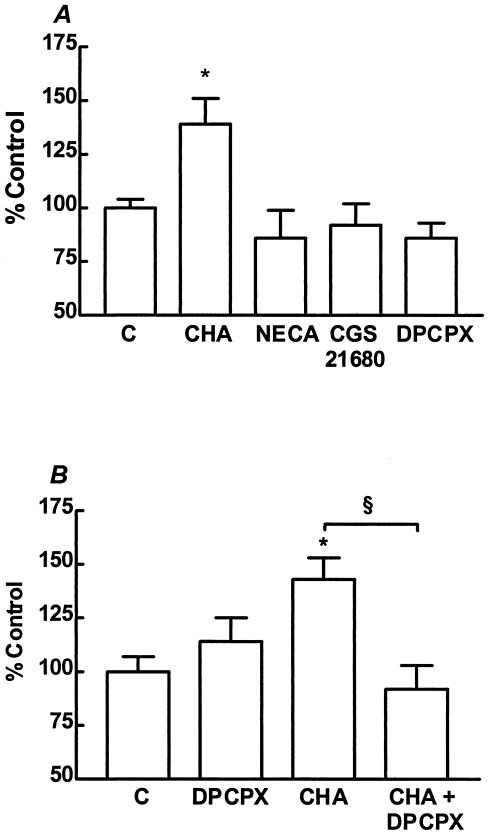

FIG. 2. Effects of adenosine receptor agonists and an antagonist on IAA-stimulated efflux of [^{3}H]purines from DDT$_1$ MF-2 cells loaded with [^{3}H]adenine. (A) Cells were loaded for 30 min with 10 μM [^{3}H]adenine, and then were pelleted and resuspended into buffer containing IAA alone (C; control) or with the A_1 agonist CHA (300 nM), the nonselective agonist NECA (1 μM), the A_{2A} agonist CGS 21680 (1 μM), or the A_1 antagonist DPCPX (1 μM). (B) Cells were resuspended into buffer containing IAA alone (C; control) or with DPCPX (10 μM), CHA (300 nM), or DPCPX and CHA. Data for [^{3}H]purines released during 10 min (37°) are means ± SEM (N = 4), expressed as a percentage of purines evoked by IAA treatment. In panel A, the control value was 120 ± 20 pmol/10^6 cells; in panel B, it was 118 ± 29 pmol/10^6 cells. Statistical significance was determined using ANOVA, and Bonferroni's post-hoc test was used to compare treatments. Key: (§) $P < 0.05$ between CHA and CHA + DPCPX; and (*) $P < 0.05$ between C and CHA.

[^{3}H]purine release was investigated with the phorbol ester PMA or chelerythrine chloride to activate or inhibit PKC, respectively. PMA (10 μM) caused a significant increase in tritium efflux over that of IAA alone ($P < 0.01$); however, unlike the findings with UTP or histamine, there was no additional effect with CHA (Fig. 3C). A concentration of chelerythrine chloride (500 nM) that did not modify basal efflux inhibited both CHA- and PMA-mediated increases in [^{3}H]purine release ($P < 0.05$) completely (Fig. 4).

Previous work in our laboratory showed that CHA at concentrations of less than 5 μM did not affect nucleoside transporter function directly in DDT$_1$ MF-2 cells [13]. We examined whether the increased [^{3}H]purine release induced

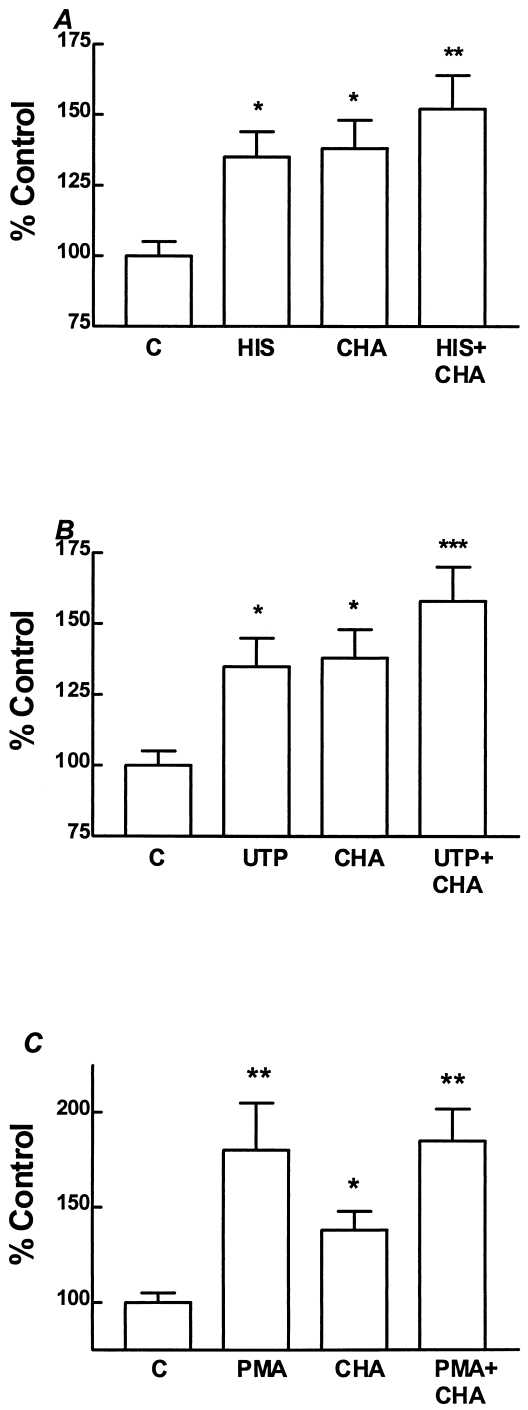

FIG. 3. Effects of PLC and PKC activation on purine efflux induced by IAA with or without CHA. Cells were loaded for 30 min with 10 μM [^{3}H]adenine, and then were pelleted and resuspended into buffer containing IAA alone (C; control) or with histamine (100 μM; panel A), UTP (100 μM; panel B), or PMA (10 μM; panel C) in the presence or absence of CHA (300 nM) at 37° for 10 min. Values represent means ± SEM (N = 5) and are expressed as percentages of [^{3}H]purine nucleosides evoked by IAA treatment. In panels A and B, the control value was 121 ± 23 pmol/10^6 cells; in panel C, it was 65 ± 19 pmol/10^6 cells. Statistical significance was determined using ANOVA, and Bonferroni's post-hoc test was used to compare treatments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ relative to C.

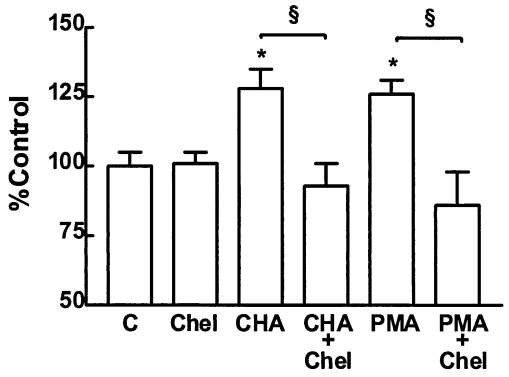

FIG. 4. Effects of PKC inhibition on CHA- or PMA-mediated increases in [^{3}H]purine nucleoside efflux. Cells were loaded for 30 min with 10 μM [^{3}H]adenine, and then were pelleted and resuspended into buffer containing IAA alone (C; control) or with PMA (10 μM), CHA (300 nM), or the PKC inhibitor chelerythrine chloride (Chel; 500 nM) alone or in combination with PMA or CHA at 37° for 10 min. Data are expressed as a percentage of [^{3}H]purine nucleosides evoked by IAA treatment; bars represent means ± SEM (N = 4). The control value was 191 ± 29 pmol/10^6 cells. Statistical significance was determined using ANOVA, and Bonferroni's post-hoc test was used to compare treatments. Key: (*) $P < 0.05$ relative to C; and (§) $P < 0.05$ for CHA vs CHA + Chel or PMA vs PMA + Chel.

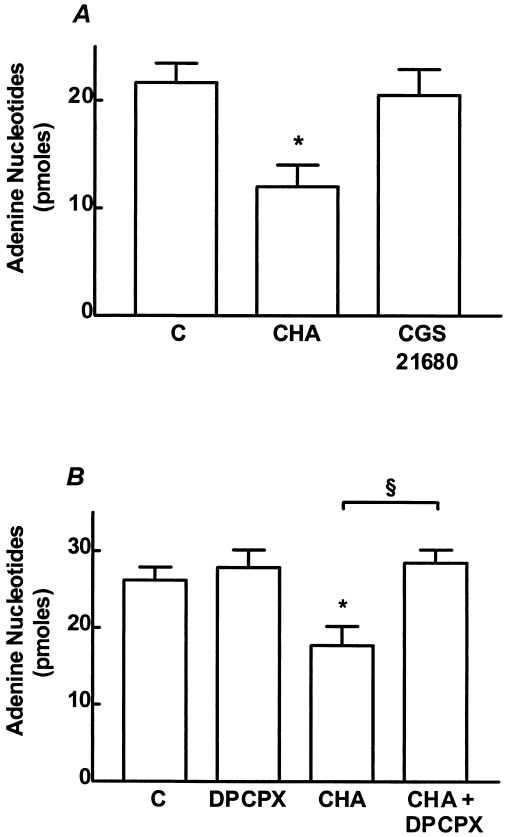

FIG. 5. Effects of adenosine receptor agonist or antagonist treatment of DDT_1 MF-2 cells on cytosolic adenosine kinase activity. (A) Cells were stimulated for 10 min with buffer (C; control), CHA (300 nM), or CGS 21680 (1 μM) at 37°, and then were lysed, and adenosine kinase activity was determined as described in the text. (B) Cells were treated with buffer, CHA (300 nM), DPCPX (1 μM), or CHA + DPCPX for 10 min at 37°. Cytosolic protein was isolated, and adenosine kinase activity was determined. Data for adenine nucleotides are means ± SEM (N = 4), and are expressed as picomoles of adenine nucleotides generated per 2 μg of cytosolic protein during 5 min. Statistical significance was determined using ANOVA, and Bonferroni's post-hoc test was used to compare treatments. Key: (§) $P < 0.05$ between CHA and CHA + DPCPX; and (*) $P < 0.05$ between C and CHA.

by PMA or CHA was associated with inhibition of one of the adenosine-metabolizing pathways. The activity of the enzyme adenosine kinase, which phosphorylates adenosine to AMP, was found to be inhibited significantly by adenosine A_1 receptor activation by CHA but not by activation of A_2 receptors with CGS 21680 (Fig. 5A). These effects were receptor-mediated, as the A_1 receptor antagonist DPCPX was able to block the CHA-mediated effects on adenosine kinase (Fig. 5B). Activation of PKC with the phorbol ester PMA was able to mimic the effects of CHA on adenosine kinase activity, and chelerythrine chloride was able to attenuate the inhibitory effects of CHA and PMA (Table 2).

DISCUSSION

The main findings of this study were that adenosine A_1 receptor activation increased the efflux of [^{3}H]purines from DDT_1 MF-2 cells during glycolytic inhibition. Stimulation of PLC by histamine H_1 and P2Y nucleotide receptor activation as well as direct PKC activation with PMA mimicked this increase in efflux. Adenosine kinase assays showed that CHA or PMA treatment inhibited adenosine metabolism, an effect that could elevate intracellular adenosine levels and enhance adenosine efflux.

Depleting cellular ATP by blocking glycolysis or oxidative phosphorylation is an effective way of enhancing intracellular adenosine production and release [18, 19]. We found that the glycolytic inhibitor IAA was effective at stimulating the release of [^{3}H]purines during a 10-min release period. NBMPR blocked this increase, indicating that IAA treatment induced [^{3}H]purine nucleoside release from cells via *es* nucleoside transporters. Release of [^{3}H]purines also was detected following treatment of cells with buffer alone; as this release was not affected by NBMPR, and adenosine levels were undetectable by HPLC, this basal release could reflect [^{3}H]adenine or its degradation products.

HPLC analysis showed that adenosine accounted for about 50% of the IAA-evoked release of purine nucleosides. Inosine is another *es* transporter permeant that may be released under these conditions. IAA treatment was reported previously to cause the release of similar amounts of adenosine and inosine from neural [20] and cardiac tissue [21]. Inosine can be formed from adenosine by adenosine deaminase or from dephosphorylation of IMP following deamination of AMP. Inosine production may have phys-

TABLE 2. Effects of CHA and PMA on adenosine kinase activity

Treatment	Adenine nucleotides (pmole)	% Control
Buffer	26.2 ± 1.7 (8)	100
CHA	18.3 ± 1.7* (7)	67 ± 4.0*†
PMA	14.9 ± 1.5‡§ (5)	61 ± 4.3‡§
Chel	24.9 ± 1.9 (6)	85 ± 4.5
CHA + Chel	25.0 ± 2.1 (5)	85 ± 2.5
PMA + Chel	27.7 ± 1.4 (3)	97 ± 6.0

Cells were stimulated with CHA (300 nM) or PMA (10 μM) alone or with chelerythrine chloride (Chel; 500 nM) at 37° for 10 min and then were lysed, and adenosine kinase activity was measured as described in the text. Values are means ±SEM; the number of determinations is given in parentheses. Adenosine kinase activity is expressed as picomoles of [^{3}H]adenine nucleotides produced per 2 μg of cytosolic protein during 5 min or as percent control of adenosine kinase activity relative to buffer-treated cells. Statistical analysis was performed by ANOVA followed by Bonferroni's post hoc test.

*$P < 0.05$, relative to buffer treatment.

†$P < 0.05$ relative to Chel-treated cells.

‡$P < 0.01$ relative to buffer treatment.

§P 0.01 relative to Chel-treated cells.

iological importance, as a recent study has reported that inosine can stimulate A_3 receptors in mast cells [22].

In this study cAMP-dependent mechanisms did not appear to modulate adenosine release. Both the adenylyl cyclase activator forskolin and the A_{2A} agonist CGS 21680 can stimulate cAMP production in these cells [13]* but did not affect the release of [^{3}H]purine nucleoside. Thus, *es* transporters in DDT_1 MF-2 cells did not appear to be regulated by cAMP-dependent mechanisms, although such regulation may occur in other cell types [23–25].

Stimulation of adenosine A_1, histamine H_1, and nucleotide P2Y receptors enhanced IAA-induced [^{3}H]purine nucleoside release from DDT_1 MF-2 cells. Stimulation of these receptors leads to activation of PLC and PKC [15–17], and A_1 receptor activation can enhance PLC activity induced by other PLC-coupled receptors in these cells [15, 16, 26]. The phorbol ester PMA also enhanced [^{3}H]purine nucleoside release. The PKC inhibitor chelerythrine chloride inhibited both the CHA- and the PMA-induced increases in nucleoside efflux, supporting the role of PKC in this efflux. In contrast to our data, it has been reported previously that PKC activation decreases adenosine uptake and decreases the number of functional transporters in bovine adrenal chromaffin cells [27], although not in bovine endothelial cells [28].

Adenosine kinase is a high-affinity enzyme that has a K_m value for adenosine of 0.2 to 0.5 μM and is subject to substrate inhibition [12, 29]. Inhibition of adenosine kinase can elevate endogenous adenosine levels [30, 31] and has been reported to have neuroprotective [32], anti-inflammatory [33], and anti-nociceptive effects [34, 35]. Treatment of these cells with CHA inhibited the activity of adenosine kinase, whereas the A_{2A} agonist CGS 21680 did not affect adenosine kinase activity. Previously, CHA was reported to inhibit adenosine kinase activity directly with a K_i value of 220 μM [12]; however, the concentration of CHA used in the present study was almost three orders of magnitude lower (300 nM). Inhibition of adenosine kinase by CHA was attenuated by the A_1 selective antagonist DPCPX or the PKC inhibitor chelerythrine chloride, indicating an A_1 receptor-mediated and PKC-dependent mechanism. The finding that PKC activation with PMA or CHA inhibited adenosine kinase activity is novel. DDT_1 MF-2 cells have been reported to contain PKC-α, PKC-ε, and PKC-ζ [36]. As PKC-α or PKC-ε are activated by PMA, one or both of these isoforms may cause direct or indirect inhibition of adenosine kinase activity. Phosphorylation of adenosine kinase by PKC activation is a potential mechanism, as the adenosine kinase sequence has numerous PKC phosphorylation consensus sites [37–39]. Although increased intracellular cAMP levels stimulated by CGS 21680 administration do not modulate adenosine kinase activity directly, it is possible that cAMP may modulate the PKC-mediated inhibition of adenosine kinase in these cells [26]. Whether inhibition of adenosine kinase activity by PKC-dependent mechanisms underlies PMA- or carbachol-induced potentiation of *N*-methyl-d-aspartate-evoked adenosine release from cortical slices [40, 41] has yet to be determined. It is tempting to speculate that inhibition of adenosine kinase may provide a way for local adenosine levels to reach sufficiently high concentrations to activate receptors with relatively low affinity for adenosine, such as the A_{2B} or A_3 subtypes.

In summary, we have shown that adenosine A_1 receptor activation inhibited adenosine kinase activity by a PKC-dependent pathway. In DDT_1 MF-2 cells, this led to enhanced cellular release of purine nucleosides. This may indicate a mechanism by which adenosine can potentiate adenosine levels under conditions of metabolic stress.

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