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Release of Adenosine by C1300 Neuroblastoma Cells in Tissue Culture

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Previous work in our laboratory led us to postulate that N2a cells release adenosine into growth medium, where it acts at the extracellular adenosine receptors to modulate the sensitivity of the cells to the cyclic AMP-elevating effect of adenosine [Green, RD, J Pharmacol Exp Ther 201:610, 1977]. We have now devised a high-performance liquid chromatographic (HPLC) procedure capable of quantitating the concentrations of adenosine in cells and tissue culture media. Growth media of N2a cells and a variant of N2a cells deficient in hypoxanthine-guanine phosphoribosyltransferase ($HGPRT^-$) contain 10–20 nM adenosine, while that of a variant deficient in adenosine kinase (AK^-) is elevated severalfold. It appears that the concentration of adenosine in growth media is determined by both the rate at which it is released by cells into the medium and the rate at which it is metabolized by adenosine deaminase present in the serum in the growth medium. Both N2a and AK^- cells release considerable amounts of adenosine into serum-free medium (SFM) over a short period. Adenosine release is greater from AK^- cells and is accelerated by erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), a potent adenosine deaminase inhibitor. This accelerated release is retarded by dipyridamole and homocysteine. Surprisingly, dipyridamole and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20 1724), a potent phosphodiesterase inhibitor, stimulate basal adenosine release from N2a but not from AK^- cells. It remains to be determined if this is due to an effect of these compounds on adenosine kinase. These results give further support for the hypothesis that adenosine in growth medium modulates the sensitivity of the cells to the cyclic AMP-elevating affect of adenosine, and furthermore they suggest that adenosine in growth media may tonically stimulate adenylate cyclase and affect processes controlled by the cyclic AMP:cyclic AMP-dependent protein kinase system.

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It is well established that many tissues and cell lines in culture possess plasma membrane adenosine receptors that modulate the activity of adenylate cyclase [1]. Adenosine, in the presence of the nonxanthine phosphodiesterase inhibitor Ro 20 1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], elevates the cyclic AMP content of intact C1300 murine neuroblastoma cells [2, 3] and stimulates the adenylate cyclase activity in broken cell preparations of the same cells [4, 5]. This effect of adenosine in neuroblastoma cells, as well as in other tissues or cells, is antagonized by methylxanthines such as theophylline [2, 3]. Previous work in this laboratory showed that the maximal elevation in cyclic AMP in neuroblastoma cells (clone N2a) in response to adenosine + Ro 20 1724 is increased in cultures pretreated overnight with theophylline or adenosine deaminase (the pretreatment agents being removed before the experiment proper) [6]. This prompted us to postulate that the cells release adenosine into the growth medium, where it acts at the extracellular adenosine receptors to modulate the sensitivity of the cells to adenosine. As the effect of theophylline pretreatment was specific for adenosine (responses to PGE_1 were unaffected) and was blocked by cycloheximide, we proposed that adenosine was acting to modulate the number of adenosine receptors present. We recently reported experiments in which we tested the effects of various conditions and treatments on cyclic AMP levels and interpreted the cyclic AMP levels as reflections of adenosine release by the cells [7]. We now report studies that use a sensitive assay for adenosine and show directly that the growth media of N2a cells contains a significant concentration of adenosine. The effects of various agents of interest on adenosine release are also presented.

METHODS

C1300 neuroblastoma cells (clone N2a) were grown and maintained in culture as previously described [3]. The isolation of a variant of N2a cells deficient in adenosine kinase (AK^-) has also been described [8]. Cells deficient in hypoxanthine-guanine phosphoribosyltransferase ($HGPRT^-$) were selected in media containing 6-thioguanine. Cells were assayed for cyclic AMP by the method of Gilman [9]. Protein determinations were performed with the technique of Lowry et al [10]. The amount of cell protein expressed as $\mu g/cm^2$ of growing surface is given in some instances as an index of the culture density.

Adenosine was assayed by reverse-phase HPLC with a fluorescent detector. Samples were processed and assayed as follows: 1) protein-containing samples (growth medium or cells) were made 10% with respect to trichloroacetic acid (TCA), tubercidin was added as an internal standard, and the protein precipitates were removed by centrifugation (the addition of TCA was not necessary when assaying adenosine in serum-free medium); 2) samples were passed over small Dowex formate columns and the eluates and 4 consecutive 2 ml water washes were collected; 3) 1/10 volume of 2.5 M ammonium acetate (NH_4Ac) buffer (pH 9.5) was added, and the samples were put on small Bio-Gel P-2 phenylboronate resin columns equilibrated with 0.25 M NH_4Ac buffer, pH 8.8; 4) after a 10 ml wash with NH_4Ac buffer, the samples were eluted with 10 ml of 0.1 M formic acid and lyophylized; 5) the samples were redissolved in 400 μl of a solution containing 20 mM sodium acetate buffer, pH 5.0, and 67 mM chloroacetaldehyde and incubated in a water bath at 80°C for 40 min to derivitize adenosine and tubercidin to their $1,N^6$ ethenoderivatives, which are highly fluorescent [11, 12]; 6) 20 μl aliquots were

chromatographed on a μBondapak C_{18} reverse-phase column with a mobile phase of 18–25% methanol:82–75% 5 mM potassium phosphate buffer pH 7.5 [12]. Fluorescence was detected with an Aminco Fluoromonitor equipped with a germicidal lamp, a Corning 7-51 primary filter, and Wratten 2A and 47B secondary filters. The mobile phase was pumped at a rate of 60 ml/h.

Tissue culture supplies were obtained from GIBCO. Ro 20 1724 and EHNA [erythro-9-(2-hydroxy-3-nonyl) adenine] were kindly supplied by Hoffman La Roche and Burroughs Wellcome, respectively. A 1–1.6 M solution of chloroacetaldehyde was prepared according to the method of Secrist et al [11]. This was assumed to be 1 M for the purpose of the experiments. Adenosine and tubercidin were quantitatively derivatized the $1,N^6$-ethenoderivatives under the conditions employed. Bio Gel P-2 phenylboronate resin was prepared as described by Olsson [13]. A similar resin is now commercially available from Bio Rad Laboratories.

RESULTS

Figure 1A shows representative chromatograms of growth medium and cells processed to assay adenosine. The chromatograms shown were processed with a mobile phase containing 25% methanol. In later experiments we switched to a mobile phase with 18% methanol, which increased the retention time slightly and thereby allowed better separation of the adenosine peak from the preceding variable fluorescence. While similar chromatograms of media samples can be obtained when the Dowex formate step is eliminated, this step is necessary when cell samples are assayed, because large peaks, probably adenine nucleotides, precede and obscure the adenosine peak. Adenosine was quantitated from standard curves such as those shown in Figure 1B, which were constructed by processing samples of TCA-precipitated unused growth medium containing different concentrations of adenosine and a constant concentration of tubercidin. Such standard curves were constructed periodically to ensure the validity of the assays. They

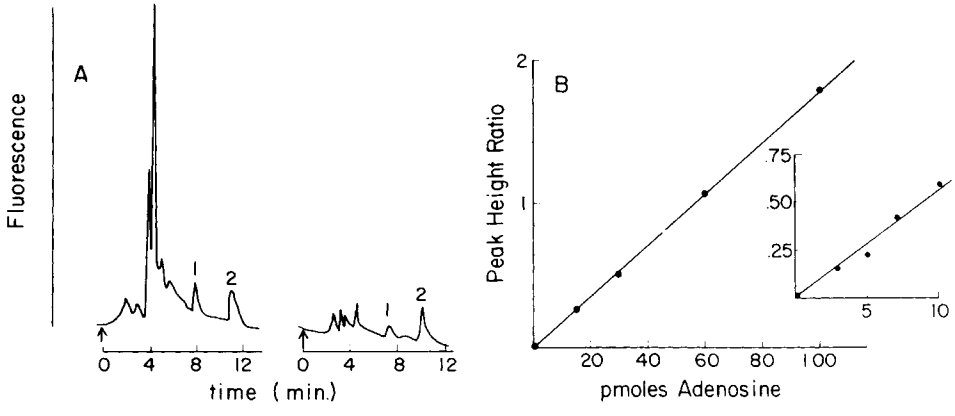

Fig. 1. A. Chromatograms of medium (left) and cell extracts (right). Peak 1 is adenosine, Peak 2 is tubercidin. B. Standard curves for determination of adenosine. Ordinate: peak height of adenosine/peak height of tubercidin. The aliquot of the sample contained either 15 pmoles (main figure) or 5 pmoles (inset) tubercidin. Abscissa = pmoles adenosine.

did not vary appreciably. We have not attempted to maximize the sensitivity of the assay. Under the conditions we have used 2 pmoles of adenosine (a peak height ratio of ~ 0.12 with 5 pmoles tubercidin) may be unambiguously assayed.

Table I summarizes several experiments in which the adenosine content of growth media of cultures of N2a cells and variants of N2a deficient in adenosine kinase (AK^-) or hypoxanthine-guanine phosphoribosyltransferase ($HGPRT^-$) was determined. All cultures in these experiments were quite dense and contained 80–130 μg cell protein/cm^2 growing surface. Experiment A shows that unused growth medium (Dulbecco's Modified Eagle's Medium containing 10% newborn calf serum) contains no adenosine, whereas the growth medium of N2a cultures contains approximately 15 nM adenosine. Adenosine was not detectable in equal aliquots of the same media that were incubated with adenosine deaminase, which verifies the validity of the assay. The results of other experiments summarized in Table I show that this concentration of adenosine was relatively constant. We could not establish a clear relationship between cell densities and the content of adenosine in the growth medium, as adenosine was not detectable in the medium of very "light" cultures. The concentration of adenosine in the growth medium of cultures of AK^- cells, but not that of $HGPRT^-$ cells, was elevated as compared to that of N2a cells.

We recently reported that the basal (+Ro 20 1724) level of cyclic AMP in dense theophylline-pretreated cultures incubated in 1 ml of theophylline-free, serum-free medium (SFM) was greater than that in identically treated cultures incubated in 5 ml SFM and that EHNA elevated cyclic AMP to a greater extent in cells incubated in 1 ml SFM than in cells incubated in 5 ml SFM [7]. We interpreted the levels of cyclic AMP present to be reflections of the adenosine concentrations in the SFM. Table II summarizes a similar experiment in which the cyclic AMP in the cells and the adenosine in the SFM were determined. It is clear from this experiment that our earlier interpretations were correct. The adenosine concentrations in the SFM of the 1 ml control and 5 ml EHNA groups are similar, as are the cyclic AMP levels in the cells. Similarly, the 5 ml control and 1 ml EHNA groups show the extremes of both values. In a separate experiment we determined the effect of 30 nM adenosine (15-min treatment) on the cyclic AMP content of cultures of N2a cells bathed in SFM containing 100 μM dipyridamole and 0.7 mM Ro 20 1724. The basal (dipyridamole and Ro 20 1724) cyclic AMP concentration was 35 ± 5 pmoles/mg protein (N = 3), and that of adenosine treated cultures was 176 ± 10 pmoles/mg protein (N = 3) $P < 0.001$.

TABLE I. Adenosine Concentrations (nM) in Growth Medium of C1300 Murine Neuroblastoma Cells

A. Experiment in which 10 ml of growth medium was removed from dense 100 mm N2a cultures and one-half were incubated with adenosine deaminase before processing.	
Untreated control	15.7 ± 1.1 (5)
Adenosine deaminase treated	0.0 (5)
Unused growth medium	0.0
B. Summary of adenosine concentrations in the growth medium in 4 separate experiments.	
	12.3 ± 0.9 (10)
C. Comparison of adenosine concentrations in the growth medium of N2a, AK^-, and $HGPRT^-$ neuroblastoma cells.	
N2a	12.8 ± 0.8 (5)
AK^-	125.0 ± 6.3 (5)
$HGPRT^-$	17.6 ± 0.7 (4)

The experiment on N2a cells summarized in Table III was performed in the presence of Ro 20 1724 to make it directly comparable with earlier experiments in which cyclic AMP levels were determined. An identical experiment of AK^- cells is also shown. EHNA increased the release of adenosine from both types of cells. In both cases the EHNA stimulated release was decreased by the simultaneous presence of dipyridamole or homocycteine. The only clear increase in intracellular adenosine occurred when AK^- cells were incubated with both EHNA and dipyridamole. This no doubt did not occur in N2a cells because of the presence of adenosine kinase in these cells. Surprisingly, in the absence of EHNA the release of adenosine by N2a cells, but not by AK^- cells, was increased by dipyridamole.

Table IV summarizes experiments similar to those in Table III, except in this case the effect of Ro 20 1724 alone was tested. The effects of Ro 20 1724 in these experiments

TABLE II. Effect of EHNA on Adenosine Release and Intracellular Levels of Cyclic AMP in Cells Incubated in 1 ml or 5 ml of Serum-Free Medium*

	Control		EHNA	
	1 ml	5 ml	1 ml	5 ml
Intracellular cyclic AMP (pmoles/mg protein)	230 ± 7	98 ± 10	430 ± 52	297 ± 12
Adenosine in SFM				
Total pmoles	37.5 ± 2.5	20 ± 0	162 ± 11	155 ± 9
Concentration (nM)	37.5	4	162	31

*Cultures were pretreated overnight with 1 mM theophylline. All cultures were rinsed with SFM and incubated with 5 ml SFM for 45 min prior to the experiment. At zero time the SFM was aspirated and replaced with either 1 ml or 5 ml of SFM containing 0.7 mM Ro 20 1724. EHNA (10 μM) was added 15 min later. All incubations were terminated at 45 min. The values represent the mean ± SEM (n = 4).

TABLE III. Adenosine in C1300 Neuroblastoma Cells and in Serum-Free Medium

	N2a[a]		AK−[b]	
Treatment[c]	Intracellular pmole/mg protein	SFM (total pmoles)	Intracellular pmoles/mg protein	SFM (total pmoles)
Control	192.2 ± 11.5	386.7 ± 43.3	423.7 ± 8.1	720.0 ± 0.0
Dipyridamole 100 μM	202.1 ± 25.7	673.3 ± 44.8	504.0 ± 76.2	790.0 ± 80.8
EHNA, 10 μM	236.7 ± 41.7	1,703.7 ± 36.9	569.4 ± 34.1	2,826.7 ± 225.2
Dipyridamole + EHNA	278.7 ± 31.3	690.0 ± 85.1	1,163.8 ± 57.0	1,036.7 ± 62.3
Homocysteine 200 μM + EHNA	224.4 ± 8.4	926.7 ± 76.9	567.8 ± 24.5	980.0 ± 30.5

[a]130 μg protein/cm²
[b]66.3 μg protein/cm²
[c]The growth medium was aspirated, the cells rinsed 2× with SFM, and 5 ml SFM added to each culture. After 30 min Ro 20 1724 (0.7 μM) was added, and 15 min later the 30-min treatment commenced. All incubations were terminated 75 min after the addition of SFM. All values are mean ± SEM (n = 3).

TABLE IV. Adenosine in C1300 Neuroblastoma Cells and in Serum-Free Medium

	N2a[a]		AK$^-$[b]	
Treatment[c]	Intracellular pmole/mg protein	SFM (total pmoles)	Intracellular pmoles/mg protein	SFM (total pmoles)
Control	56.3 ± 1.6	83.3 ± 3.3	107.8 ± 29.4	725.0 ± 45.0
Ro 20 1724, 0.7mM	55.4 ± 1.1	136.7 ± 3.3	141.2 ± 12.5	513.3 ± 23.3
EHNA, 10 μM	63.9 ± 5.1	303.3 ± 49.8	157.5 ± 10.3	2,073.0 ± 69.6
Ro + EHNA	65.7 ± 4.5	733.3 ± 67.4	151.0 ± 7.1	1,253.0 ± 37.1

[a]140.3 μg protein/cm^2
[b]78.5 μg/protein/cm^2
[c]The growth medium was aspirated, the cultures were washed twice with SFM, and 5 ml of SFM was added to each culture. The total periods for the treatments were 1) Ro 20 1724, 45 min; 2) EHNA, 30 min; 3) Ro 20 1724, 45 min; EHNA, 30 min (ie, Ro was added 15 min before EHNA). All incubations were terminated in 75 min after the addition of SFM. All values are mean ± SEM (n = 3).

are interesting in that in N2a cells it increased adenosine release both by itself and in combination with EHNA, whereas in AK$^-$ cells it retarded both basal and EHNA-stimulated release.

DISCUSSION

C1300 murine neuroblastoma cells in tissue culture clearly are capable of releasing adenosine into their growth medium to achieve concentrations in the nanomolar range. Similar concentrations are present in the media of cultures of N2a and HGPRT$^-$ cells, while that present in the media of AK$^-$ cultures is somewhat elevated. This is not surprising, even though newborn calf serum is known to contain adenosine deaminase activity [14], because the K_M of this enzyme is not very favorable. As significant amounts of adenosine are released from cells into SFM over a short period, it would seem that the adenosine concentration in growth medium is determined by the rate at which adenosine is released by cells and the rate at which it is metabolized by the adenosine deaminase in the growth medium. In the case of AK$^-$ cells, the cells release adenosine at an accelerated rate, and the concentration in growth medium is elevated as compared to that in media of N2a cultures.

The addition of 30 nM adenosine to cells treated with dipyridamole to retard the uptake of exogenous adenosine and the efflux of intracellular adenosine results in a significant elevation in intracellular cyclic AMP. It is difficult to make comparisons between elevations in cyclic AMP in response to adenosine in whole cells and the activation of adenylate cyclase by adenosine in broken cell preparations. The EC_{50} for the cyclic AMP-elevating effect of adenosine in whole neuroblastoma cells has been reported to be 1–2 μM [2, 3], whereas the K_A for the stimulatory effect of adenylate cyclase on broken cell preparations of neuroblastoma cells has been reported to be in the range of 50–100 μM [4, 5]. Penit et al [5] regarded their K_A estimate as approximate because of the extensive metabolism of adenosine during the adenylate cyclase assay. The relationship between the stimulation of adenylate cyclase and the concentration of added adenosine shown by Blume and Foster [4] does not appear to be hyperbolic, indicating that their estimate of the K_A may have been similarly affected. In addition, it is now clear that the K_A for the stimulatory effect of adenosine on adenylate cyclase can be severely over-

estimated because of the liberation of adenosine in the adenylate cyclase reaction mixture and the resultant elevation in the "basal" activity [15]. When precautions are taken to circumvent this problem, adenosine can be shown to have a K_A in the range of 0.1 μM in rat liver [15]. Although similar experiments with neuroblastoma cell preparations have not been reported, we have found that the "basal" adenylate cyclase activity in broken cell preparations of neuroblastoma cells is decreased by adenosine deaminase, indicating the presence of adenosine in the reaction mixture (unpublished observations). It is therefore not unreasonable to assume that the elevation in cyclic AMP in response to 30 nM adenosine is due to the stimulation of adenylate cyclase and that the concentrations of adenosine present in growth medium are sufficient to stimulate adenylate cyclase activity.

The release of adenosine is most likely via the nucleoside transport system, as it is antagonized by dipyridamole, a transport inhibitor that has previously been shown to inhibit adenosine transport into AK^- cells [8]. The effect of homocysteine on adenosine release was tested because it was thought that it would react with free intracellular adenosine in the presence of S-adenosylhomocysteine hydrolase to reduce the concentration of adenosine available for transport out of the cells. The results are in accord with this prediction.

We have previously reported that the cyclic AMP content of N2a cells in the presence of Ro 20 1724 is slightly but significantly lowered if adenosine deaminase is added to the growth medium before the Ro 20 1724 [7]. We interpreted this difference to be due to the adenosine normally present in the growth medium. We now find that this interpretation is not entirely correct, as Ro 20 1724 induces a small release of adenosine from N2a cells. It is interesting that dipyridamole, which has previously been reported to inhibit adenosine kinase [16], also increased adenosine release in N2a cells and that neither dipyridamole or Ro 20 1724 had this effect on AK^- cells. Although other explanations are possible, the most direct explanation is that both Ro 1724 and dipyridamole can inhibit adenosine kinase. Ro 20 1724 has been reported to inhibit adenosine uptake by Na2 cells [3] and can be shown to noncompetitively inhibit adenosine transport into AK^- cells (unpublished observation). In the present experiments it lowered both the basal and EHNA-stimulated adenosine release from AK^- cells. We interpret the opposite effects of Ro 20 1724 in N2a cells to be the result of counterproductive effects, the first being to promote adenosine release (perhaps by inhibiting adenosine kinase) and the second being to inhibit adenosine transport.

The present results are supportive of our hypothesis that the sensitivity of C1300 neuroblastoma cells to the cyclic AMP-elevating effect of adenosine is regulated by adenosine released from the cells into the growth medium. One might also expect this adenosine to tonically stimulate adenylate cyclase and thus affect parameters controlled by the cyclic AMP:cyclic AMP-dependent protein kinase system. Nanomolar concentrations of adenosine will elevate the intracellular concentration of cyclic AMP if adenosine transport and phosphodiesterase are inhibited by dipyridamole and Ro 20 1724, respectively. However, elevations in cyclic AMP in response to adenosine alone are minimal [2, 3], and the addition of adenosine deaminase alone to growth medium does not have a detectable effect on intracellular cyclic AMP levels (unpublished observation). It therefore remains uncertain whether an increased rate of intracellular cyclic AMP turnover in response to adenosine in the growth medium is of "physiological" importance. The relative amounts of adenosine released by different types of cells in culture and the importance of variations in the adenosine deaminase activity in different batches of serum used to make culture media remain to be determined.

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