Adenosine Stimulation of DNA Synthesis in Mammary Epithelial Cells (44302)

IN-SUH YUH[1] AND LEWIS G. SHEFFIELD[2]

Endocrinology-Reproductive Physiology Program and Department of Dairy Science, University of Wisconsin, Madison, Wisconsin 53706

Abstract. **Adenosine increased the DNA synthesis rate and the percentage of S-phase cells 2–3-fold in mouse mammary epithelial cells (NMuMG), with an optimum concentration of 10–100 μM. This effect was not mimicked by adenosine metabolites adenine, hypoxanthine, or inosine. N-ethylcarboxamidoadenosine (NECA, a relatively nonselective adenosine receptor agonist) and 2-p-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarbox-amidoadenosine (CGS-21680, an A_2 selective agonist) also increased DNA synthesis by mammary epithelial cells. However, N^6-cyclohexyladenosine (CHA, an agonist for A_1 type receptors) decreased DNA synthesis. The A_1 selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) had no effect on basal or adenosine-induced DNA synthesis, whereas the A_2 selective antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) decreased adenosine-induced DNA synthesis. Similar effects were observed in another nontumorigenic mouse mammary epithelial line, HC11, as well as the nontumorigenic human lines MCF-10A and 184.A1. Binding studies indicated that NMuMG cells contained approximately 3200 A_1 receptors and about 5300 A_2 receptors per cell. Both CGS-21680 and CHA increased GTPase activity in isolated cell membranes, whereas only CGS-21680 increased activity of adenylyl cyclase. Adenosine and CGS-21680 increased expression of a cyclic AMP responsive reporter gene. In addition, the protein kinase A inhibitor H-89 blocked the ability of adenosine and CGS-21680 to induce DNA synthesis, but did not affect EGF-induced DNA synthesis. These results indicate that adenosine appears to be a possible growth promoting agent in mammary tissue, and this effect may be mediated by extracellular receptors of the A_2 type.** [P.S.E.B.M. 1998 Vol 218]

Nucleosides regulate a variety of cellular functions (1–3). Adenosine is well established as a regulator of hypoxic ischemia, lymphocyte function, neural function, blood flow, adipose tissue metabolism, cell growth, and differentiation of a variety of tissues (2, 4–7). Adenosine and its analogs have a variety of receptors (3, 8). Extracellular A_1 type receptors are generally thought to be linked to adenylyl cyclase *via* G_i proteins, and thus mediate inhibition of cyclic AMP formation. The other major class of extracellular receptor, A_2, is thought to be G_s linked, and to stimulate adenylyl cyclase activity. In addition to these extracellular receptors, intracellular adenosine binding sites are present, but their significance is unclear. Although well described in some tissues, such as vascular and neural, the receptors for adenosine have not been studied extensively in the mammary gland, although they are thought to exist there (9).

Elevated intracellular cyclic AMP levels are correlated with mammary growth (10–13), and agents that increase intracellular cyclic AMP increase mammary growth *in vivo* and *in vitro* (14–18). However, the hormonal control of intracellular cyclic AMP is uncertain. Current hypotheses focus largely on possible roles of adrenergic systems, since β-adrenergic receptors have been found in rat mammary epithelial cells (19). The hypothesis that an adenosine pathway might be involved in mammary development has received little attention. Therefore, the objective of this study was to determine if adenosine alters growth of mammary epithelium.

[1] Present address: Department of Dairy Science, College of Animal Agriculture, Kangwon National University, Chuncheon, Korea.
[2] To whom requests for reprints should be addressed at Endocrinology-Reproductive Physiology Program, University of Wisconsin, 1675 Observatory Drive, Madison, WI 53706-1284.
Supported by University of Wisconsin College of Agricultural and Life Sciences, USDA project WIS 3769 and NRICP/USDA grant 9202994.

Received November 12, 1997. [P.S.E.B.M. 1998, Vol 218]
Accepted March 31, 1998.

0037-9727/98/2184-0341$10.50/0

Materials and Methods

Cells and Treatments. Normal mouse mammary epithelial cells (NMuMG line, 20) were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPML-1640 supplemented with 5% fetal calf serum. HC11 cells were obtained from Dr. B. Groner (Institute for Experimental Cancer, Freiburg, Germany, 21) and cultured in RPMI 1640 supplemented with insulin (1 μg/ml), EGF (10 ng/ml), and 10% FBS. MCF-10A (22) and 184.A1 (23) cells were obtained from ATCC and cultured in Ham's F12:DMEM (1:1 mixture) containing 10% FBS and 10 ng/ml EGF.

Cells were plated (7.5×10^4 per well for 24 well plates, 1×10^4 per chamber for culture slides and 3×10^5 per well for 12 well plates), attached for 24 hr and serum deprived 18–24 hr prior to treatment. For HC11, MCF-10A and 184.A1 cells, growth arrest also included removal of EGF from culture media.

Nucleosides were from Sigma Chemical Co. (St. Louis, MO). NECA (N-ethylcarboxamidoadenosine), CGS-21680 (2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride), CHA (N^6-cyclohexyladenosine), DMPX (3,7-dimethyl-1-propargylxanthine) and DPCPX (8-cyclopentyl-1,3-dipropylxanthine) were from Research Biochemicals Inc. (Natick, MA). H-89 was obtained from Calbiochem (San Diego, CA).

DNA Synthesis. Adenosine and various agonists and antagonists were added at the indicated concentrations. For these studies, no adenosine deaminase or adenosine deaminase inhibitors were added to cells. At 18 hr after treatment, 1 μCi/ml ^{3}H-thymidine (DuPont, Wilmington, DE) was added to cells on 24-well culture plates. Cells were incubated for 1 hr, then rinsed three times with Tris-buffered saline (TBS) (150 m*M* NaCl, 50 m*M* Tris, pH 7.5), three times with ice-cold 10% trichloroacetic acid (TCA), and two times with ethanol. Cells were dried, dissolved in 0.5 *M* NaOH containing 0.1% Triton X-100, neutralized with HCl, and counted by liquid scintillation.

S-Phase Cells. Cells on eight-chambered culture slides (Lab-Tek, Miles Scientific, Naperville, IL) were pulsed for 1 hr with 1 μCi/ml ^{3}H-thymidine, rinsed with TBS, fixed with 10% phosphate-buffered formalin for 30 min, washed with ethanol, and dried. Cells were then dipped in photographic emulsion (Illford K-5, Polysciences, Warrington, PA), dried, and exposed for 1 week at 4°C. Slides were developed in Kodak D-19 developer and counterstained with hematoxylin. Cells with 10 or more silver grains over the nucleus were counted.

Binding Studies. Membranes from NMuMG cells were prepared from cells grown and serum deprived as described above. Briefly, cells were lysed with lysis buffer (20 m*M* HEPES, pH 7.0 containing 5 m*M* EDTA, 10 m*M* $MgCl_2$ and 1 m*M* PMSF). Lysate was centrifuged at 1000*g* for 5 min, and the resulting supernatant centrifuged for 1 hr at 50,000*g*. Membranes were then suspended in lysis buffer containing 1 unit/ml adenosine deaminase. Approximately 200 μg of membrane protein (determined by dye binding, BioRad, Hercules, CA) was added to 96-well filter-bottom plates (Millipore, Bedford, MA). Ligand (^{3}H-CGS-21680 or ^{3}H-DPCPX, DuPont, Boston, MA) was added in various concentrations, with or without 100-fold excess unlabeled ligand, and plates were incubated for 2 hr at 37°C. Assay was stopped by vacuum filtration, and membranes were washed four times with assay buffer. Filter bottoms were then punched into vials and counted by liquid scintillation. Receptor numbers and affinity were determined by the Ligand program (Biosoft, Fergeson, MO).

GTPase Activity. Membranes were prepared from NMuMG cells grown to near-confluency on 100-mm petri dishes and serum deprived for 24 hr. Cells were incubated with lysis buffer (20 m*M* HEPES, pH 7.0, 1 m*M* dithiothreitol, 1 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride), scraped into a tube, allowed to sit on ice for 10 min with occasional gentle mixing, and then were centrifuged at 1000*g* for 5 min. Supernatant was then centrifuged for 1 hr at 50,000*g*, and membrane protein content was determined by BCA assay (Pierce, Rockford, IL). GTPase assays were conducted by suspending membranes in assay buffer (20 m*M* HEPES, pH 7.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 1 m*M* adenylylimidodiphosphate, 1 m*M* ATP, 10 m*M* creatine phosphate, 50 units/ml creatine phosphokinase and 1 μ*M* of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenyl hydrochloride) (EHNA) to a concentration of 50 μg membrane protein/50 μl reaction. The same buffer system and additives were used for all agonists. Agonists were added, then γ^{32}P-GTP (2 μ*M*, 10^4 dpm/pmole; DuPont) was added. Assays were continued at 37°C for 10 min, then stopped by 200 μl 5% TCA and 200 μl of activated charcoal (100 mg/ml). Samples were centrifuged at 10,000*g* for 5 min and supernatant was counted by liquid scintillation. In other assays, adenosine deaminase inhibitor was eliminated and adenosine deaminase (1 unit/ml) was added to assays to assess possible effects of endogenous adenosine production.

Adenylyl Cyclase Activity. Membranes were prepared as described above. Membranes were suspended in assay buffer (HEPES, 20 m*M*, pH 7.5, 5 m*M* $MgCl_2$, 1 m*M* cyclic AMP, 2.5 m*M* phosphoenolpyruvate, 1.5 units pyruvate kinase, 100 μg/ml BSA, 30 m*M* KCl, 100 m*M* sucrose, 1 μ*M* of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenyl hydrochloride, and 0.5 m*M* 1-methyl-3-isobutylxanthine (IBMX) to a concentration of 50 μg protein/50 μl solution. The same buffer system and additives were used for all agonists. Agonists were added, and then α^{32}P-ATP (5×10^4 dpm/pmole, DuPont) added. Reactions were continued for 5 min at 37°C, then cyclic AMP was separated by chromatography as previously described (24). Cyclic AMP was measured by liquid scintillation counting. In other assays, adenosine deaminase inhibitor was eliminated and adenosine deaminase (1 unit/ml)

was added to assays to assess possible effects of endogenous adenosine production.

Reporter Gene Expression. Two tandem repeats of an oligonucleotide containing a consensus cyclic AMP response element (TGCAGTCTCTGCAGT) was inserted into the Sma I site of pGL2 promoter vector (Promega, Madison, WI). Plasmids were cloned in *E. coli* DH5α. Cells were transiently transfected by electroporation using a 2-kV/cm field strength. Cells were plated onto 12-well culture dishes (3×10^5 cells per well), allowed to attach for 12–18 hr, then serum deprived for 20–24 hr. They were then treated with adenosine agonists and luciferase activity determined 4 hr later using a commercially available kit (Promega).

Statistical Analysis. Each experiment was replicated on at least three occasions (exact replication given with results). Data were analyzed by analysis of variance. Means were compared using Dunnett's test to compare treatments with control, and orthogonal comparisons for other comparisons (25). Unless otherwise stated, significance was $P < .05$. For calculation of maximum responses and ED_{50} concentrations, nonlinear regression was used (JMP Statistics, SAS Institute, Cary, NC). The model used was:

$$y = \frac{\alpha}{1 + \beta e^{-\gamma x}}$$

where Y was the response variable, X was the dose of hormone used and α, β, and γ were regression parameters.

Results

After 18 hr, 1 μ*M* added adenosine gave a small (50%) increase in DNA synthesis, whereas 10 or 100 μ*M* resulted in DNA synthesis approximately 2.5 times control levels. Higher concentrations of added adenosine (1 m*M*) decreased DNA synthesis to control values (Fig. 1). The decreased DNA synthesis with high concentrations of adenosine did not appear to be due to toxic effects. Cell viability was between 85% and 90% for all treatments, and the number of attached cells did not vary among adenosine concentrations. Autoradiographic estimation of the percentage of cells undergoing DNA synthesis showed a dose response curve similar to total DNA synthesis (Fig. 1), indicating that the increased DNA synthesis was likely due to increased S-phase cells. Time course studies (not shown) also indicated that maximum DNA synthesis was observed 15–18 hr after adding adenosine, with no significant effect prior to 9 hr after treatment. Metabolites of adenosine, adenine, hypoxanthine, inosine, and xanthine were incapable of mimicking the effect of adenosine in concentrations from 0.1 μ*M* to 1 m*M* (not shown).

The nonmetabolizable adenosine agonist NECA was capable of inducing DNA synthesis in NMuMG cells, although to a somewhat lesser extent than adenosine alone (Fig. 2, top). Similarly, the somewhat more A_2-receptor–

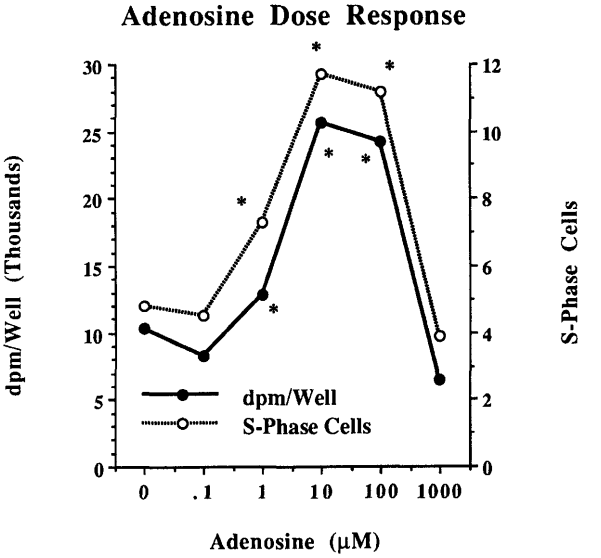

Figure 1. Effect of adenosine on DNA synthesis on NMuMG cells. Cells were serum starved for 24 hr, adenosine was added, and DNA synthesis was estimated 18 hr later as ^{3}H-thymidine incorporated into acid insoluble material (DNA Synthesis, dpm) or as percent S-phase cells determined by autoradiography. Pooled SEM was ± 1.7 for DNA synthesis; ± 0.6 for autoradiography. * = significantly different from control (0 adenosine), $P < .05$. $n = 6$ for DNA synthesis, 8 for autoradiography.

selective analog CGS-21680 had a stimulatory effect on DNA synthesis, whereas the A_1 agonist CHA failed to increase DNA synthesis, and in fact decreased basal DNA synthesis. Maximum DNA synthesis was induced by 10 μ*M* adenosine, whereas 10 μ*M* NECA or CGS-21680 induced a near-maximum increase in DNA synthesis.

The A_1 receptor antagonist DPCPX had little effect on either basal or adenosine-induced DNA synthesis (Fig. 2, bottom). However, the A_2 selective antagonist DMPX resulted in significant inhibition of adenosine-induced DNA synthesis at as little as 0.1 μ*M*, a near-complete inhibition of DNA synthesis at 1 μ*M*, and complete inhibition of adenosine-induced DNA synthesis when present in concentrations of 10 or 100 μ*M*.

The above results did not appear to be confined to NMuMG cells. Other nontumorigenic mammary epithelial cell lines, including the mouse line HC11 and the human lines MCF-10A and 184.A1 also increased DNA synthesis in response to adenosine, NECA, and CGS-21680. However, the A_1 receptor agonist CHA caused a decline in DNA synthesis in three of the four cell lines (Table I).

Presence of A_1 and A_2 receptors on NMuMG cell membranes was further indicated by ligand binding studies. Using the A_1 selective ligand DPCPX, a single affinity class of receptors with an apparant K_d of 1.5 ± 0.3 n*M* was found. The estimated number of receptors per cell was $3.2 \pm 0.9 \times 10^3$. Nonspecific binding, estimated by adding a 100-fold excess of unlabeled DPCPX, averaged 18%. CGS-21680 in 100-fold excess inhibited binding by less than 10%. Using the A_2 selective ligand CGS-21680, a receptor with a K_d of 28 ± 6 n*M* was found. Cells appeared to contain 5.3 ± 1.7 ×

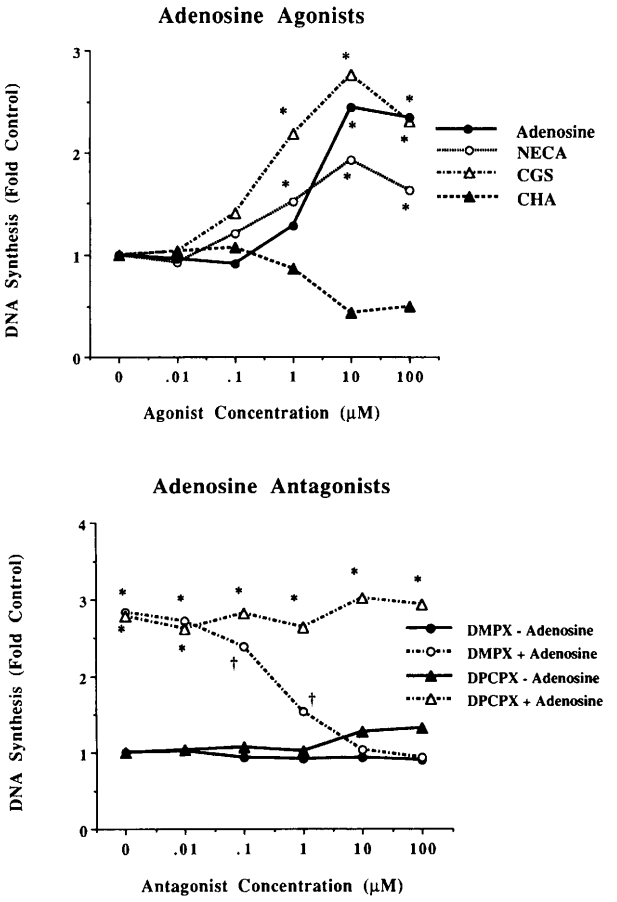

Figure 2. Effect of adenosine, NECA, CGS-21680, and CHA on DNA synthesis in NMuMG cells. Cells were serum starved for 24 hr, adenosine analogs were added at the indicated concentration, and DNA synthesis was measured 18 hr later as dpm ^{3}H-thymidine incorporated into acid insoluble material. DNA synthesis was reported as DNA synthesis in the indicated group/DNA synthesis in the absence of adenoseine or analogs (Fold Control). Pooled SEM = ± 0.24. $n = 6$. * = significantly different from control (no analogs) ($P < 0.05$). (bottom) Effect of DMPX (A_2 selective antagonist) and DPCPX (A_1 selective antagonist) on basal and adenosine (10 μ*M*)-induced DNA synthesis in NMuMG cells. Cells were serum starved for 24 hr, adenosine analogs were added at the indicated concentration with or without 10 μ*M* adenosine, and DNA synthesis was measured 18 hr later as dpm ^{3}H-thymidine incorporated into acid insoluble material. DNA synthesis was reported as DNA synthesis in the indicated group/DNA synthesis in the absence of adenosine or analogs (Fold Control). Pooled SEM = ± 0.19. $n = 6$. * = significantly different from control (no adenosine or analogs); * = significantly different from control and from + adenosine with no antagonist ($P < 0.05$).

10^3 A_2 receptors per cell (based on mean ± SEM of four experiments). Nonspecific binding, estimated in the presence of 100-fold excess CGS-21680, was 21%. Excess DPCPX reduced CGS-21680 by less than 10%.

In additional studies to ascertain the possible mechanisms by which adenosine might influence mammary epithelial cell growth, exogenous adenosine, CGS-21680, and CHA all increased GTPase activity in cell membranes in the presence of the adenosine deaminase inhibitor EHNA (Fig. 3, top). Adenylyl cyclase activity of isolated cell membranes in the presence of EHNA was also increased significantly by NECA, CGS-21680, and exogenous adenosine, but not by CHA (Fig. 3, bottom). In both assays, omission of EHNA reduced the response to adenosine to less than half that observed in the presence of EHNA, but did not affect the response to CGS-21680 or CHA. Furthermore, addition of 1 Unit/ml adenosine deaminase abolished adenosine-stimulated GTPase and adenylyl cyclase activity. However, deaminase had a modest effect (less than a 30% increase on any dose tested) on CGS-21680 or CHA-induced GTPase or CGS-21680-induced adenylyl cyclase activity (not shown), suggesting only a modest effect of endogenous adenosine production.

In further studies, added adenosine and CGS-21680, but not CHA, increased expression of a cyclic AMP responsive reporter gene in intact cells (cultured in the absence of adenosine deaminase or EHNA) (Fig. 4). Luciferase activity was unaffected by any of the treatments if the cyclic AMP response element was omitted from the plasmid (not shown).

In a final series of studies, the protein kinase A inhibitor H-89 was found to eliminate the ability of cholera toxin, adenosine, and CGS-21680 to induce DNA synthesis in NMuMG cells (Fig. 5). However, the growth response to EGF, which presumably does not require cyclic AMP for mitogenesis, was unaffected by H-89, suggesting that the observed effect was not due to a general inhibition of DNA synthesis.

Discussion

Previously, a variety of roles have been proposed for adenosine and related nucleotides. Among these roles are regulation of cell growth, vascular tone, cardiac ischemia, lymphocyte function, and neurotransmission (4–7). The present report indicates that adenosine also increases DNA synthesis in mammary epithelium. This response was first noted in a mammary epithelial cell line (NMuMG), but was also confirmed in other nontumorigenic mammary cell lines, suggesting that the response is not an artifact of a particular cell line.

Few reports exist on the nucleotide content of milk. Adenosine and hypoxanthine total about 1 μ*M* in bovine milk (26). Little additional information is available on adenosine production by mammary tissue. Adenosine is produced by adipocytes (7, 27) and other cell types (28). The local concentration of adenosine is difficult to estimate from published studies, since adenosine deaminase activity and adenosine stability in milk and mammary tissue are unknown. Thus, local concentrations of adenosine may well be higher than values reported in milk. The present report observed effects of adenosine on DNA synthesis at concentrations of 10 μ*M*. Thus, the concentrations used in this study may be within physiological ranges of local adenosine concentrations.

In the present study, high concentrations of adenosine (1 m*M*) resulted in decreased DNA synthesis. This biphasic effect of adenosine might suggest toxicity of high concentrations, since adenosine is known to induce death in other

Table I. Effect of Adenosine, NECA, CGS-21680, and CHA on DNA Synthesis in Various Mammary-Derived Cell Lines

Line	Adenosine		NECA		CGS		CHA	
	ED_{50} (μM)	Max (fold)	ED_{50} (μM)	Max (fold)	ED_{50} (μM)	Max (fold)	ED_{50} (μM)	Max (fold)
NMuMG	3.1	2.6	0.4	1.6	0.3	2.2	1.1	0.4
HC11	3.4	2.4	0.3	1.8	0.2	2.5	0.9	0.4
MCF-10A	2.9	2.5	0.4	1.9	0.4	2.1	—	NS
184.A1	3.2	3.1	0.4	1.8	0.2	2.2	0.8	0.5

Note. ED_{50} (in μM) and asymptotic maximum effect (expressed as fold increase relative to control) were calculated from dose response studies using nonlinear regression analysis. Reported increases are significant at $P > 0.05$. NS = no significant effect ($P > 0.05$). Based on $n = 8$.

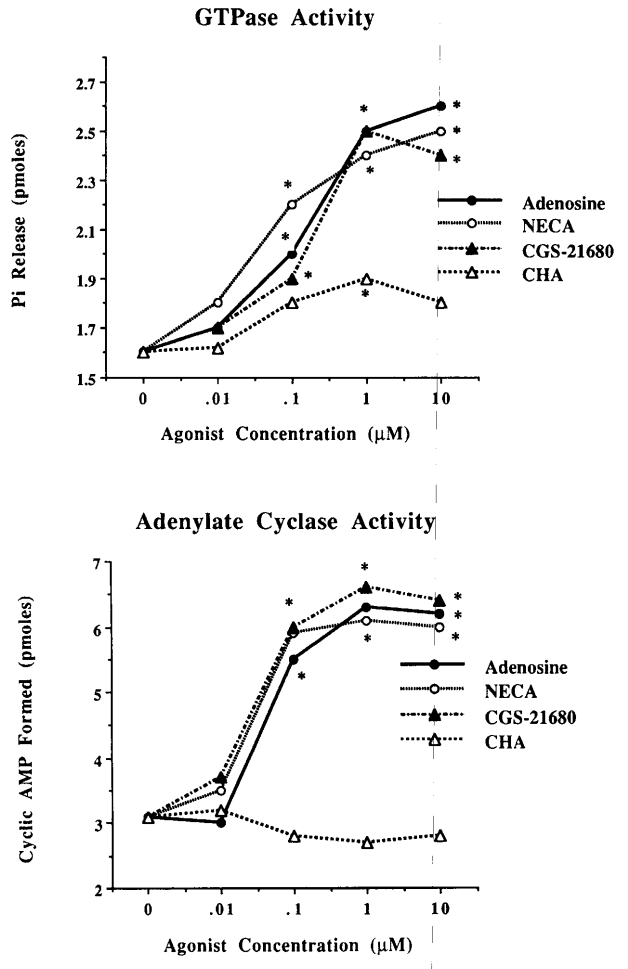

Figure 3. (VOP) GTPase activity in NMuMG cell membranes treated with adenosine agonists. Mean of four determinations. Pooled SEM = 0.21. * = significantly different from control (no analogs) ($P < 0.05$). (bottom) Adenylyl cyclase activity in NMuMG cell membranes treated with adenosine agonists. Mean of four determinations. Pooled SEM = 0.36. * = significantly different from control (no analogs) ($P < 0.05$).

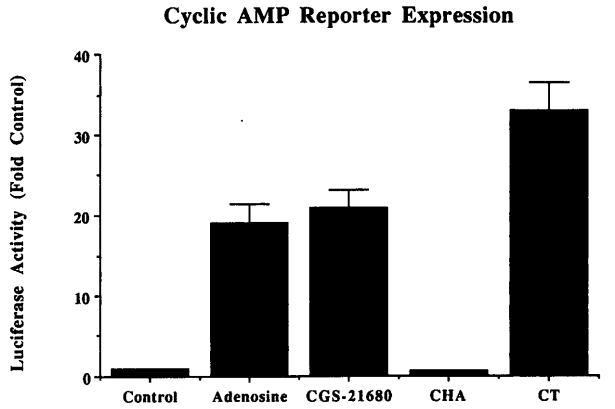

Figure 4. Cyclic AMP responsive reporter gene expression in NMuMG cells. NMuMG cells were transfected with a cAMP responsive reporter vector, serum deprived, and treated as controls or with 10 μM adenosine, 1 μM CGS-12680, 1 μM CHA, or 10 ng/ml cholera toxin Luciferase activity was assessed and reported as activity in the indicated group/activity in the no treatment control group (Fold Control). Mean ± SEM. $n = 4$.

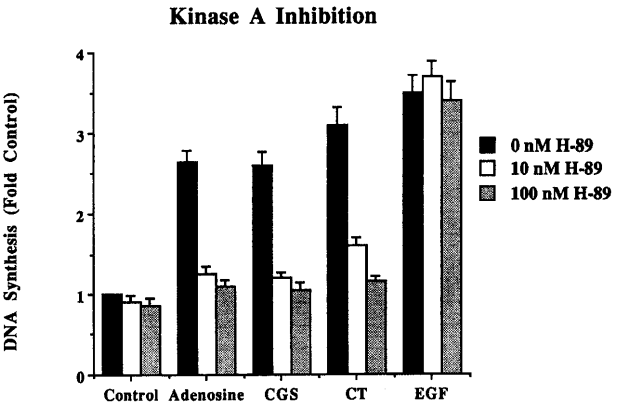

Figure 5. Effect of the kinase A inhibitor H-89 on adenosine-mediated DNA synthesis in NMuMG cells. Cells were treated with H-89 (0, 1, or 10 nM) for 30 min prior to addition of 10 μM adenosine, 1 μM CGS-12680, 10 ng/ml cholera toxin, or 10 ng/ml EGF and DNA synthesis determined as described in Materials and Methods. DNA synthesis was reported as DNA synthesis in the indicated group/DNA synthesis in the absence of adenoseine or other treatments (Fold Control). Mean ± SEM of six determinations.

cell types (1). However, no evidence of toxicity was observed, based on attached cells or viability assessed by trypan blue exclusion. Whether high adenosine concentrations might induce death in mammary epithelial cells over longer periods than used in these studies is not known.

In interpreting dose responses reported in these studies, it should be noted that the indicated concentrations refer to adenosine added to culture media, not to actual adenosine concentrations. Adenosine is produced and degraded by cultured cells, and added adenosine may not indicate actual adenosine concentration. In unpublished studies, we have

observed that NMuMG cells maintained in DMEM without added adenosine accumulate very little adenosine in media, and appear to degrade added adenosine with a half-life of 5–10 min. Thus, the actual concentration of adenosine in DNA synthesis experiments was probably substantially less than the added concentration for much of the treatment period, as adenosine deaminase inhibitors were not added to the cell cultures. However, the alterations in cell physiology occurring in response to adenosine may persist after adenosine is degraded. Previously, we have shown (29) that even transient elevations in cyclic AMP can elicit increased DNA synthesis in NMuMG cells.

Considerations similar to the above paragraph also apply to membrane preparations used for binding studies, GTPase activity studies, and adenylyl cyclase studies. Membranes prepared from NMuMG cells appear to contain sufficient adenosine deaminase to degrade added adenosine. However, the contribution of endogenously produced adenosine appears to be relatively small, as addition of adenosine deaminase had only a modest effect on CGS-21680 or CHA-mediated effects. This appears to be in contrast to studies on brain membranes, in which endogenously produced adenosine interferes with adenosine analog binding in the absence of added adenosine deaminase (30).

Evidence that adenosine acts on specific receptors rather than *via* nonspecific metabolic effects came from the observation that NECA was also able to stimulate DNA synthesis in NMuMG cells. NECA is a nonmetabolizable analog of adenosine that is a potent agonist for both A_1 and A_2 type receptors (31). Additionally, the observation that CGS-21680, an A_2 agonist (32), increased DNA synthesis, whereas CHA, an A_1 agonist (33), decreased DNA synthesis suggests an involvement of A_2 receptors in positive mediation of mammary epithelial cell proliferation. However, the fact that a negative response was observed to CHA may also suggest the presence of A_1 receptors that could function as inhibitors of mammary epithelial proliferation.

The exact mechanism by which adenosine affects mammary epithelial proliferation remains incompletely understood, but appears to involve activation of adenylyl cyclase and protein kinase A *via* an A_2 type receptor. Adenosine is known to act *via* several distinct extracellular and intracellular receptors (3, 8). Our results suggest that the primary mitogenic action is through an extracellular receptor, most likely of the A_2 type, since A_2 selective agonists promote DNA synthesis, whereas A_1 agonists have a negative effect. Results of DNA synthesis assays, as well as ligand binding and membrane GTPase activity, suggest that an A_1 type receptor is also present in mammary epithelial cells, which is consistent with previous studies (9).

Presumably, A_1 adenosine receptors are G_i linked and would be expected to decrease adenylyl cyclase activity, whereas A_2 type receptors are linked to G_s and would increase intracellular cyclic AMP (3, 8). Our results suggest that A_2 receptor agonists are capable of activating adenylyl cyclase in mammary epithelial cell membranes. In these assays (as in GTPase assays), adenosine was effective at lower concentrations than in intact cells, possibly owing to the use of adenosine deaminase inhibitors in the cell-free assays.

An interesting observation in GTPase and adenylyl cyclase assays was that the response of adenylyl cyclase to added adenosine or adenosine analogs had an ED_{50} approximately 10-fold lower than the ED_{50} for adenosine-stimulated GTPase. The reason for this is unclear. One possibility is stimulation of GTPases not involved in regulating adenylyl cyclase. Another is incomplete coupling of GTPase activation to adenylyl cyclase, which could be an artifact of the membrane preparation process.

Reporter gene studies, using a cyclic AMP responsive promoter similar to that used in other studies (34), indicated that the increase in adenylyl cyclase observed in isolated cell membranes appeared capable of inducing a cyclic AMP-mediated biological response in intact cells. Since previous studies have shown that high intracellular cyclic AMP is associated with mammary gland growth (10–13) and agents that increase intracellular cyclic AMP promote mammary growth (14–18), we speculate that adenosine may act *via* an A_2 (G_s-coupled) receptor system in mammary tissue to increase adenylyl cyclase activity. Such cyclic AMP-mediated increases in growth have been demonstrated in a variety of other systems and widely reviewed (35).

The necessity of a cyclic AMP response in adenosine-induced DNA synthesis was confirmed by using the protein kinase A selective inhibitor H-89 (36). This compound blocked the ability of cholera toxin, which presumably acts by increasing adenylyl cyclase (37), as well as adenosine and CGS-21680, to induce DNA synthesis in mammary epithelial cells. However, EGF, which is presumed to act *via* a tyrosine kinase–dependent mechanism (38), was able to induce DNA synthesis in the presence of H-89, suggesting that the inhibition of adenosine-induced DNA synthesis was not due to toxicity, nonspecific inhibition of cell cycle progression, or DNA synthesis.

In addition to adenylyl cyclase activation, heterotrimeric G protein–coupled receptors can modulate a wide variety of other signals, including K^+ channels, phospholipase Cβ, phospholipase A_2, phosphoinositide 3-kinase, β-adrenergic receptor kinase, and possibly *ras* (39–41). Although our results indicate an involvement of cyclic AMP in adenosine-mediated mammary epithelial DNA synthesis, the results do not eliminate possible involvement of other pathways as well. Mammary epithelial cell proliferation is widely acknowledged to depend on the integration of a variety of intracellular signals (42). The results of these studies suggest the possibility that some of these could be regulated by extracellular adenosine.

Physiologically, the role of adenosine in mammary gland development remains unclear. However, a number of hormones important in controlling mammary growth and differentiation interact with adenosine signaling systems in other tissues. For example, adenosine increases the response

of thyroid follicular cells to IGF-I (43). Estrogen and progesterone increase the effects of adenosine on cerebral cortical neurons (44). Glucocorticoids regulate adenosine receptors in brain and smooth muscle (45, 46). Although interactions of adenosine with other factors in mammary tissue have not been studied, the presence of such responses in other tissues suggests a possible role of adenosine in regulating mammary gland development.

Authors would like to thank Linda Kotolski, Josie Lewandowski, Tyler Sisk, Susan Pederson and Sharyl Nass for technical assistance.

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