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POTENTIATION BY HOMOCYSTEINE OF ADENOSINE-STIMULATED ELEVATION OF CELLULAR ADENOSINE 3′,5′-MONOPHOSPHATE

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Abstract—The novel ability of L-homocysteine (Hcy) to potentiate the cellular elevation of adenosine 3′,5′-monophosphate (cAMP) caused by adenosine (Ado) is described. This effect of Hcy is highly selective in that it is not mimicked by L-cysteine, and Hcy does not potentiate the elevation of cellular cAMP caused by either 2-chloroadenosine or prostaglandin E_1. Hcy also augments the Ado-stimulated increase in 2-fluoroadenosine 3′,5′-monophosphate in cells preloaded with nucleotides of 2-fluoroadenosine. Addition of Hcy to cells during their incubation with radioactive Ado results in a decrease in the cellular content of radioactive Ado and a concomitant buildup of *S*-adenosylhomocysteine. The enhancive effect of Hcy on the Ado-stimulated elevation of cAMP may be due to this associated reduction in the intracellular pool of Ado (due to condensation of Ado with Hcy via *S*-adenosylhomocysteinase) and to a resultant reduction in inhibition of adenylate cyclase by intracellular Ado, thereby allowing greater net stimulation of the cyclase by extracellular Ado.

The ability of adenosine (Ado)* to stimulate an increase in cellular adenosine 3′,5′-monophosphate (cAMP) was first recognized in studies with brain slices [1, 2]. Subsequently, a wide variety of tissues, including lymphocytes [3–10], bone cells [11], skin [12], neutrophils[+], platelets [13–15], vagus nerve [16], coronary artery [17], ventricular myocardium [18], lung cells [19–21] and tumor cells of neural [21–29], adrenal and testicular [30] origin, has been shown to respond to Ado (and certain of its structural analogues) with a rapid buildup of cAMP. This cAMP-elevating activity of Ado-like compounds appears to be due to their interaction with a specific receptor which is located on the exterior of cell membranes [4, 5, 9, 15, 18–20, 24, 27, 31, 32] and to the resultant stimulation of adenylate cyclase which is situated on the cytosol side of the plasma membrane [33].

Another biochemical activity of Ado of physiological importance concerns its ability to serve as both a substrate and an inhibitor of *S*-adenosylhomocysteinase, the enzyme which catalyzes reversibly the hydrolysis of *S*-adenosylhomocysteine (AdoHcy) to Ado and L-homocysteine (Hcy) [34]. Cells treated with Ado accumulate AdoHcy, and the co-addition of exogenous Hcy greatly enhances this cellular buildup of AdoHcy by Ado [35–37]. Cell growth [35], human monocyte chemotaxis [36] and lymphocyte-mediated cytolysis [37] are inhibited by Ado and this inhibition is potentiated by Hcy in all three cases. While investigating the potentiation by Hcy of Ado inhibition of lymphocyte-mediated cytolysis, we found that, in addition to its effect on AdoHcy levels, Hcy also potentiated the cellular buildup of cAMP caused by Ado. This communication documents the ability of Hcy to potentiate the Ado-stimulated elevation of cellular cAMP and suggests a possible explanation for this novel effect.

MATERIALS AND METHODS

[2-^{3}H]Ado (5.2 Ci/m-mole) and [8-^{3}H]cAMP (26 Ci/m-mole) were products of the Amersham Corp., Arlington Heights, IL. Prostaglandin E_1 (PGE_1), AdoHcy, L-homocysteine thiolactone (Hcy), L-cysteine, calf intestine Ado deaminase and 3′,5′-cyclic nucleotide phosphodiesterase were from the Sigma Chemical Co., St. Louis, MO. Other materials were from sources identified elsewhere [5].

Cytolytic lymphocytes were obtained from CD-1 mice as reported [5]. Dulbecco's phosphate-buffered saline supplemented with 10% fetal calf serum (heat-inactivated) was used as the medium for the lymphocytes. *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 7.9 μM), an inhibitor of Ado deaminase [38], was included in the medium for all cellular incubations reported here.

cAMP and 2-fluoroadenosine 3′,5′-monophosphate (F-cAMP), present in acid-soluble extracts of lymphocytes, were determined by radioimmunoassay after purification of the extracts on sequential columns of aluminum oxide and Dowex 1-X8 and subsequent 2′-*O*-succinylation of the resultant samples [5].

Cellular pools of [^{3}H]Ado and [^{3}H]AdoHcy were determined after rapid separation of the lymphocytes from their incubation medium by a modification of the method of Strauss *et al.* [39]. Cellular incubations were carried out for 10 min at 37° and contained in 300 μl:

* The abbreviations used are: Ado, adenosine; AdoHcy, *S*-adenosylhomocysteine; cAMP, adenosine 3′,5′-monophosphate; Cl-Ado, 2-chloroadenosine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; F-cAMP, 2-fluoroadenosine 3′,5′-monophosphate; Hcy, L-homocysteine (thiolactone); PGE_1, prostaglandin E_1; and Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

[+] C. R. Stopford and G. Wolberg, unpublished observations.

Table 1. Effects of adenosine, 2-chloroadenosine, prostaglandin E_1, Ro 20-1724 and L-homocysteine thiolactone on cAMP levels of lymphocytes*

Additive(s)	cAMP (pmoles/10^7 cells)
None (saline)	1.18 ± 0.13
Ro 20-1724 (50 μM)	1.80 ± 0.12
Hcy (200 μM)	1.27 ± 0.05
Ado (2.0 μM)	4.69 ± 0.19
Ado (2.0 μM) + Ro 20-1724 (50 μM)	9.19 ± 1.01
Ado (2.0 μM) + Hcy (200 μM)	9.12 ± 0.43
Cl-Ado (9.4 μM)	6.97 ± 0.78
Cl-Ado (9.4 μM) + Ro 20-1724 (50 μM)	10.52 ± 0.76
Cl-Ado (9.4 μM) + Hcy (200 μM)	6.72 ± 0.67
PGE_1 (2.5 μM)	10.10 ± 0.32
PGE_1 (2.5 μM) + Ro 20-1724 (50 μM)	15.79 ± 0.34
PGE_1 (2.5 μM) + Hcy (200 μM)	7.21 ± 0.53

* Cytolytic lymphocytes (7.1×10^6 cells/5.0 ml of medium) were incubated for 10 min at 37° prior to the addition of drug(s) or saline. After 30-min incubation times with or without drug(s), the cell suspensions were acid-extracted for cAMP determinations, as described previously [5]. All incubations were performed in duplicate and each column-purified extract was radioimmunoassayed in duplicate for cAMP. Each value represents the mean ± S.E.M. for four determinations. Abbreviations: Ado, adenosine; Cl-Ado, 2-chloroadenosine; PGE_1, prostaglandin E_1; and Hcy, L-homocysteine thiolactone.

2×10^6 lymphocytes, 7.9 μM EHNA, 2.4 μM [^{3}H]Ado plus the specified additive. Incubations were terminated by layering 200 μl of each cellular incubation into a 400-μl microfuge tube containing 40 μl of 1.5 M perchloric acid/250 μM Ado/250 μM AdoHcy as the bottom phase and a 150-μl layer of dibutyl phthalate. The tubes were spun at 10,000 *g* for 2 min in a Beckman model B microfuge. Only radioactive material associated with the lymphocytes was found in the acid layer, since centrifugation of radioactive medium lacking cells gave negative results. The tip of each tube was cut off above the acid-organic meniscus and the contents thereof were transferred to tubes containing 1.0 ml of cold 1.0 M perchloric acid. These 1.0-ml samples were extracted one time with 5.0 ml of ether to remove dibutyl phthalate. The resultant acidic samples were neutralized with KOH, clarified by filtration through plugs of glasswool, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus) and reconstituted in 300 μl of 25 mM ammonium phosphate buffer, pH 7.0. These samples were fractionated by reversed-phase high performance liquid chromatography, as described in detail elsewhere [37]. Fifty-μl samples were injected into the chromatograph and the effluent was monitored both for ultraviolet absorbance (254 and 280 nm) and for radioactivity (by collecting 0.5-min fractions). Inosine, AdoHcy and Ado were eluted with retention times of 19, 28 and 31 min respectively. The ultraviolet peak areas of the Ado and AdoHcy recovery markers were used to normalize the corresponding radioactivity measurements to the original cell count.

For assay of cAMP phosphodiesterase, an appropriate dilution of beef heart 3′,5′-cyclic nucleotide phosphodiesterase was incubated at 30° in 50 mM Tris/HCl, pH 7.5, 4.0 mM magnesium sulfate, and 1.0 μM [8-^{3}H]cAMP, in a total volume of 100 μl. The enzyme reaction was stopped after 20 min by boiling, the reaction mixtures were fractionated on thin layers of polyethyleneimine cellulose [5], and the radioactivity distribution was determined after elution of appropriate regions of the sheets with 50 mM NaOH. Under the conditions of this assay, the [^{3}H]AMP product was not dephosphorylated significantly.

RESULTS

Ado, 2-chloroadenosine (Cl-Ado) and PGE_1 all caused the expected elevation of cAMP in the mouse lymphocytes, and each was potentiated in this activity by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), an inhibitor of cAMP phosphodiesterase [40] (Table 1). However, quite unexpectedly, Hcy was also found to potentiate the cellular buildup of cAMP caused by Ado. This effect of Hcy was quite selective, in that Hcy did not augment the elevation of cAMP caused by either Cl-Ado or PGE_1 (Table 1) and equimolar L-cysteine was unable to mimic this activity of Hcy (results not shown). The cAMP-potentiating activity of Hcy was evident over the range of Ado concentrations examined (Fig. 1A). With 2.0 μM Ado, the effect of Hcy on cAMP levels was nearly maximal with 100 μM amino acid (Fig. 1B). Mouse neutrophils also exhibited enhancement by Hcy of the Ado-stimulated elevation of cAMP.* AdoHcy was shown not to cross-react significantly in the cAMP radioimmunoassay.

It has been shown previously [5] that 2-fluoroadenosine is metabolized rapidly and extensively to both 2-fluoroadenosine 5′-triphosphate and 2-fluoroadenosine 3′,5′-monophosphate (F-cAMP) in these lymphocytes

* C. R. Stopford and G. Wolberg, unpublished observations.

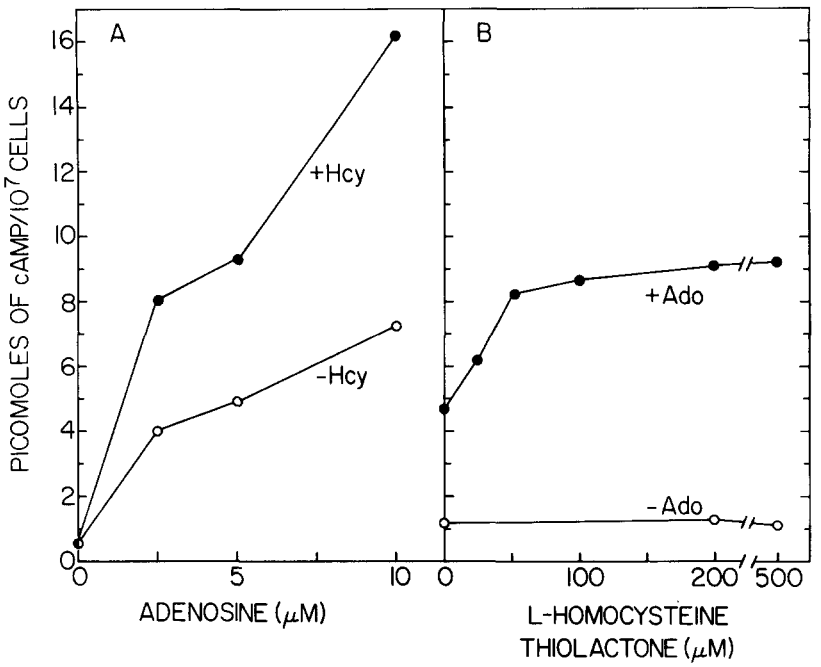

Fig. 1. Effects of varying Ado and Hcy concentrations on cAMP levels in mouse lymphocytes. Cytolytic lymphocytes (0.7–1.0 × 10^7 cells/5.0 ml of medium) were incubated for 30 min at 37° (A) with different concentrations of Ado in the presence of 200 μM Hcy (●) or saline (○) and (B) with different concentrations of Hcy in the presence of 2.0 μM Ado (●) or saline (○). All incubations were performed in duplicate and each column-purified extract was radioimmunoassayed in duplicate for cAMP. Each point represents the mean of four determinations.

and that Ado can stimulate the buildup of F-cAMP in cells preloaded with nucleotides of 2-fluoroadenosine. As shown in Table 2, Hcy was also capable of potentiating this latter effect of Ado on cellular levels of F-cAMP.

Table 2. Effects of adenosine and L-homocysteine thiolactone on the level of 2-fluoroadenosine 3′,5′-monophosphate in lymphocytes which had been preloaded with nucleotides of 2-fluoroadenosine *

Additive(s)	F-cAMP (pmoles/10^7 cells)
None (saline)	0.56 ± 0.03
Hcy (200 μM)	0.32 ± 0.06
Ado (10 μM)	2.48 ± 0.12
	(P < 0.001)
Ado (10 μM) + Hcy (200 μM)	3.85 ± 0.21

* Cytolytic lymphocytes (1.78 × 10^8 cells in 120 ml of medium) were incubated for 60 min at 37° with 9.4 μM 2-fluoroadenosine. These cells were then harvested by centrifugation, washed one time, resuspended in fresh medium (lacking 2-fluoroadenosine) at a density of 6.4 × 10^6 cells/5.0 ml of medium, and incubated for 15 min at 37°. At this time the cell suspensions (5.0 ml each) were supplemented with 7.9 μM EHNA plus the specified additives and incubated further at 37° for 30 min prior to their acid-extraction for F-cAMP determination, as described previously [5]. All incubations were performed in triplicate and each column-purified extract was radioimmunoassayed in duplicate for F-cAMP. Each value represents the mean ± S.E.M. for six determinations. The statistical comparison between the last two sets of data in the table was calculated by means of the two-tailed Student's *t*-test. Abbreviations: Ado, adenosine; Hcy, L-homocysteine thiolactone; F-cAMP, 2-fluoroadenosine 3′,5′-monophosphate.

In an attempt to elucidate the mechanism by which Hcy potentiates the elevation of cAMP caused by Ado, commercial beef heart 3′,5′-cyclic nucleotide phosphodiesterase was examined for inhibition by several agents. At the highest concentration of each agent tested (1.0 mM), Ado, Hcy, AdoHcy and Ado plus Hcy had no effect on the hydrolysis of 1.0 μM [^{3}H]cAMP.

The facile cellular metabolism of Ado to AdoHcy in the presence of exogenous Hcy [35–37] prompted us to determine the effect of Hcy on the pool size of Ado in lymphocytes during their incubation with Ado. Hcy was found to cause a dose-dependent reduction in the cellular pool of [^{3}H]Ado in lymphocytes incubated for 10 min with 2.4 μM [^{3}H]Ado (Fig. 2). At a concentration of 200 μM, Hcy caused a 50–60 percent decrease in cellular [^{3}H]Ado in several different experiments of this type. Concomitantly, Hcy caused a large increase in cellular [^{3}H]AdoHcy. In contrast to Hcy, Ro 20-1724 (50 μM) had no effect on the lymphocyte pools of [^{3}H]Ado and [^{3}H]AdoHcy under these same experimental conditions (data not shown). The identity of the peak of [^{3}H]Ado in these chromatograms was verified by its shift to the retention time of inosine after treatment of a portion of a cell extract with Ado deaminase.

Treatment of lymphocytes with 18.8 μM Cl-Ado plus 200 μM Hcy for 30 min and subsequent analysis of acid-soluble extracts of these cells by reversed-phase high performance liquid chromatography failed to detect evidence of metabolic formation of *S*-2-chloroadenosylhomocysteine. Under these same experimental conditions, Ado metabolism to AdoHcy and 3-deazaa-

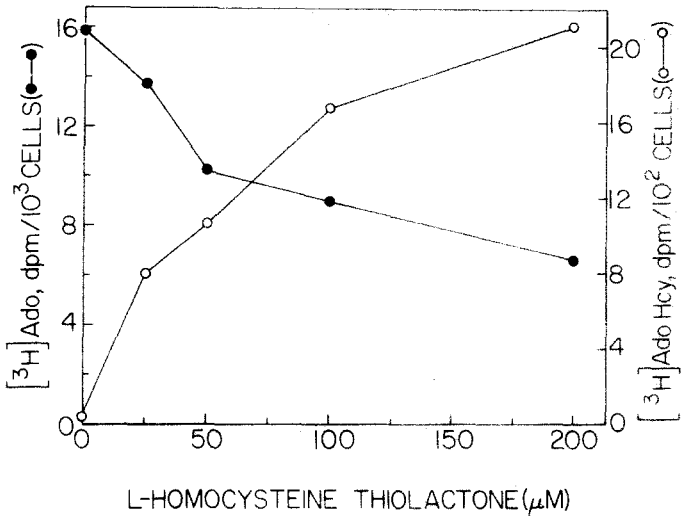

Fig. 2. Effects of varying Hcy concentrations on pool sizes of Ado (●) and AdoHcy (○) in lymphocytes incubated with Ado. After a 10-min incubation with 2.4 μM [^{3}H]Ado plus the specified concentration of Hcy, cells were separated rapidly from their medium and acid-extracted. Other experimental details were described under Materials and Methods. This experiment was performed in duplicate and each point represents the mean of the two analyses.

denosine metabolism to *S*-3-deazaadenosylhomocysteine are readily observable [37].

DISCUSSION

Hcy can now be added to the list of agents which enhance the cellular elevation of cAMP caused by Ado. Previously, agents which are inhibitors of Ado deaminase [3, 6, 9], cAMP phosphodiesterase [4–6, 9, 15, 18, 24, 27, 29, 31, 41] or Ado uptake [15, 18, 24, 29, 31] have been shown to augment the Ado-stimulated increase in cellular cAMP.

The present results show that exogenous Hcy causes a substantial reduction in the intracellular pool of Ado, and a marked increase in AdoHcy formation, during incubation of cells with Ado. This effect of Hcy is attributed to the rapid condensation of intracellular Ado with Hcy (via *S*-adenosylhomocysteinase) to form AdoHcy.

It appears plausible that the ability of Hcy to potentiate the buildup of cAMP in cells treated with Ado is due to the associated reduction in the intracellular pool of Ado. A number of studies with membrane preparations of adenylate cyclase have shown Ado to have a biphasic effect on this enzyme, stimulating the cyclase at low concentrations of nucleoside and inhibiting its activity at higher concentrations [17, 27, 32, 42–46]. Londos and Wolff [45] have concluded that adenylate cyclase from many sources generally contains two adenosine-reactive sites: one, probably extracellular, whose occupancy by Ado or a suitable structural analogue leads to activation of cyclase; and a second, presumably intracellular, whose occupancy results in lowered cyclase activity. This two-site model suggests that exogenous Ado normally exerts opposing influences on cellular adenylate cyclase, stimulating the enzyme extracellularly (via the Ado receptor) and inhibiting it (upon uptake) intracellularly. Thus, any agent, such as Hcy, which can bring about a reduction in intracellular Ado would be expected to decrease inhibition of adenylate cyclase at the intracellular Ado site and thereby allow greater net stimulation of the cyclase by extracellular Ado. Indeed, inhibitors of Ado uptake may augment the cAMP-elevating activity of Ado for this same reason.

Based upon this proposed mechanism of action of Hcy, it is perhaps not unexpected that Hcy does not potentiate the cellular elevation of cAMP caused by either Cl-Ado or PGE_1. The apparent lack of metabolism of Cl-Ado to *S*-2-chloroadenosylhomocysteine precludes the possibility of a Hcy-associated reduction in the intracellular pool of Cl-Ado similar to that observed with Ado. In order for Hcy to enhance the action of other stimulators of adenylate cyclase, such as PGE_1, it would be necessary that normal intracellular concentrations of Ado be high enough to be inhibitory to the cyclase. However, cellular levels of Ado have been reported to be below 1 μM [47, 48], which may be insufficient to inhibit adenylate cyclase significantly.

An alternative interpretation of the present observations is that AdoHcy formed metabolically from exogenous Ado and Hcy is an intracellular stimulator of adenylate cyclase. Although this possibility has not been explored experimentally, we consider it to be less likely than the above "Ado pool" explanation since, to the best of our knowledge, there is presently no evidence for an intracellular site at which adenine derivatives can stimulate adenylate cyclase.

Regardless of the precise mechanism responsible for this novel effect of Hcy, the present results signal the need for caution in the interpretation of experiments in which Hcy is observed to potentiate a physiological effect of Ado. Previously, the only known biochemical interaction between Ado and Hcy was the ability of the latter to augment the cellular buildup of AdoHcy caused by Ado [35–37]. Since AdoHcy is known to be a potent inhibitor of many different *S*-adenosylmethionine-utilizing methyltransferases [49 and refer-

ences cited therein], the ability of Hcy to potentiate a physiological effect of Ado has been presumed to indicate the critical involvement of one or more methylation reactions in a particular cellular function. However, this newly recognized effect of Hcy on cAMP levels provides an alternative biochemical interpretation for such physiological results. For the purpose of inhibiting cellular methylation reactions in a more selective manner, it is preferable to use an inhibitor of *S*-adenosylhomocysteinase, such as 3-deazaadenosine [50], which does not affect cellular levels of cAMP [37].

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