HORMONE-LIKE ACTION OF ADENOSINE IN MOUSE THYMOCYTES AND SPLENOCYTES

Evidence for the existence of membrane adenosine receptors coupled to adenylate cyclase

Jean-Claude BONNAFOUS, Jacques DORNAND and Jean-Claude MANI

Laboratoire de Biochimie des Membranes, ER CNRS no. 228, Ecole Nationale Supérieure de Chimie, 8 rue de l'Ecole Normale, F-34075 Montpellier, France

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1. Introduction

Adenosine has been reported to regulate adenylate cyclase (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1.) in various cell types [1–11]. This nucleoside and structural analogues induce cyclic AMP (cAMP) accumulation in rat thymocytes [12] and in human lymphocytes [13,14]. They inhibit cytotoxic T lymphocyte-mediated cytolysis by increasing cAMP level [15,16]. In [17,18] we showed that the early increases in cAMP content induced by 2-chloroadenosine (an adenosine deaminase-resistent analogue) in pig lymph node lymphocytes could not be correlated to similar effects on the adenylate cyclase activity of disrupted cell preparations, but rather to the competitive inhibition of the low K_m cAMP phosphodiesterase. This points out the difficulty to draw definitive conclusions from experiments carried out on intact cells or on homogenates with incomplete blockade of phosphodiesterases. In spite of the data for mouse thymocytes [19] direct stimulation of lymphocyte adenylate cyclase by adenosine or analogues has never been clearly established.

Here we demonstrate that these nucleosides at submicromolar concentrations directly stimulate adenylate cyclase in cell-free systems from mouse thymocytes and, to a lesser extent, of mouse splenocytes.

2. Materials and methods

Creatine phosphate and creatine phosphokinase were obtained from Boehringer, 2-chloroadenosine, isoproterenol, prostaglandin E_1, calf intestinal mucosa adenosine deaminase from Sigma, [α-^{32}P]ATP from Amersham Radiochemical Centre, c[^{3}H]AMP from CEN Saclay.

Thymus were removed from week 4–5 male Swiss mice, the cells were teased apart in Hanks medium, filtered through nylon screen and washed twice with Hanks medium. Splenocytes were obtained by homogenizing mouse spleens in a glass Potter homogenizer. Contaminating erythrocytes were selectively killed by 0.83% ammonium chloride.

The cells were washed with 0.9% NaCl, then resuspended in a hypotonic solution consisting of 10 mM Tris–HCl (pH 7.5) (~1 ml/2–3 × 10^8 cells) using a teflon–glass homogenizer (4 gentle strokes with hand). Aggregated material was removed by filtration through a very small layer of glass wool and the filtrate immediately used for adenylate cyclase determinations.

Determination of intracellular cAMP levels was carried out as in [18] with a protein binding assay, using the Amersham Radiochemical Centre kit, after purification of the cell extracts on Dowex-resin columns.

Adenylate cyclase was determined as in [20] in 25 mM Tris–HCl (pH 7.5) containing 25 U creatine phosphokinase/ml, 15 mM creatine phosphate 2.5 mM $MgCl_2$, 1 mM cAMP, 0.5 mM [α-^{32}P]ATP (1.5–3 × 10^6 cpm/assay) and in some experiments 0.6 U adenosine deaminase/ml. ATP was pre-purified on Dowex resin as in [1]. The reaction (100 μl) lasted 15 min at 30°C and was stopped by adding 150 μl 10 mM Tris,HCl (pH 7.5) containing 5 mM ATP, 50 μM c[^{3}H]AMP

(~20 000 cpm/assay) and by heating the tubes for 3 min. c[^{32}P]AMP was purified as in [1] using two successive chromatographies on Dowex resin and alumina columns.

3. Results

3.1. *Effects of adenosine and 2-chloroadenosine on cAMP accumulation in mouse thymocytes and splenocytes*

Short incubations (12 min) of mouse thymocytes or splenocytes with adenosine or 2-chloroadenosine induced large increases in their cAMP content (table 1). The effects were more pronounced for thymocytes than for splenocytes, and 2-chloroadenosine was found much more efficient than adenosine, probably because it is resistant to adenosine deaminase. Up to 15-fold enhancements of cAMP content were induced by 5 μM 2-chloroadenosine in mouse thymocytes. It is possible that part of these stimulations were due to a nucleoside-induced inhibition of cAMP-phosphodiesterases [18]. The fact that sub-μM doses of nucleosides are sufficient to produce significant effects is consistent with a direct stimulation of adenylate cyclase, reminiscent of the action of hormones. Moreover, to determine whether adenosine must enter the cell to produce its effect, we checked for the action of 6-chloropurine riboside, an inhibitor of adenosine transport already used in guinea pig heart [22]. In our assay conditions 100 μM 6-chloropurine riboside inhibited adenosine transport in thymocytes by 90% (see [23] for measurement technique) and yet did not antagonize the stimulation effect of 2-chloroadenosine (table 2), which suggests that this latter compound acts through extracellular receptor sites. However the decisive proof of the existence of adenosine receptors coupled to adenylate cyclase can only be given by directly measuring the adenylate cyclase activities of disrupted cell preparations.

Table 1
Effects of adenosine and 2-chloroadenosine on cAMP accumulation in mouse thymocytes and splenocytes

	cAMP content (% control)
1. Thymocytes	
Control	100 ± 10
5×10^{-7} M adenosine	130 ± 10
2×10^{-6} M adenosine	280 ± 30
5×10^{-6} M adenosine	610 ± 30
10^{-5} M adenosine	740 ± 50
10^{-7} M 2-chloroadenosine	120 ± 10
10^{-6} M 2-chloroadenosine	520 ± 30
5×10^{-6} M 2-chloroadenosine	1500 ± 200
2. Splenocytes	
Control	100 ± 10
10^{-6} M adenosine	150 ± 20
10^{-5} M adenosine	270 ± 20
10^{-4} M adenosine	300 ± 30
10^{-6} M 2-chloroadenosine	210 ± 20
10^{-5} M 2-chloroadenosine	800 ± 50

After 30 min equilibration at 37°C, 1 ml aliquots of thymocytes or splenocytes in Hanks' medium (15×10^6 cells/ml) were incubated 12 min at 37°C in the presence of indicated nucleoside doses. The cAMP contents of the controls were 7.5 ± 2.5 pmol/10^7 thymocytes and 10 ± 2 pmol/10^7 splenocytes. Results are the mean of 3 separate determinations for each assay

3.2. *Effects of 2-chloroadenosine on the adenylate cyclase activity of mouse thymocytes and splenocytes*

Figure 1 represents the dose–response curve relative to the modulation of the adenylate cyclase activity of homogenates from mouse thymocytes and splenocytes, under experimental conditions where cAMP-phosphodiesterases were saturated with 1 mM

Table 2
Effects of 6-chloropurine riboside on 2-chloroadenosine-induced cAMP accumulation in mouse thymocytes

Treatment of the cells	cAMP content (pmol/10^7 cells)
Control	11.6 ± 0.7
1 μM 2-chloroadenosine	37.6 ± 3.1
100 μM 6-chloropurine riboside	12.3 ± 1.6
100 μM 6-chloropurine riboside + 1 μM 2-chloroadenosine	38.7 ± 2.9

After 30 min equilibration at 37°C, thymocytes (1.3×10^7 cells/ml Hanks' medium) were incubated for 6 min with or without 100 μM 6-chloropurine riboside, then for 12 min with or without 1 μM 2-chloroadenosine. Results are the mean of 3 separate determinations for each assay

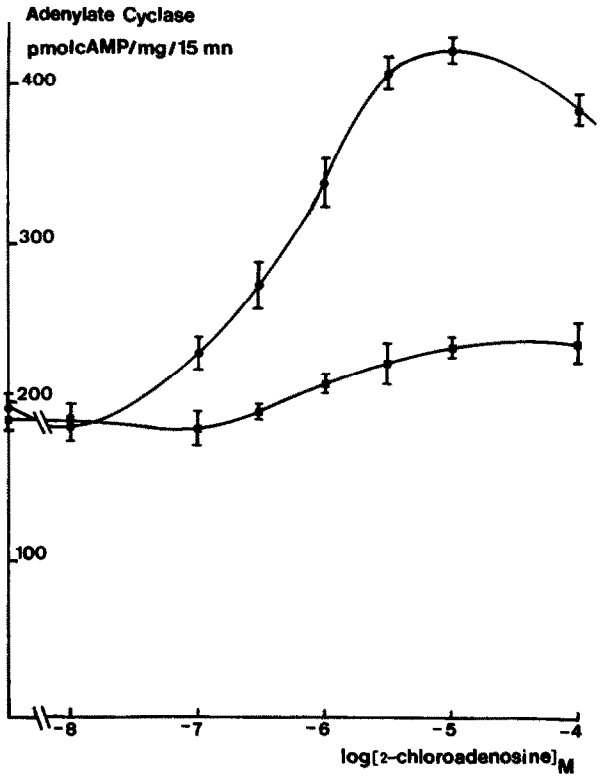

Fig. 1. Stimulation of mouse thymocyte and splenocyte adenylate cyclase by 2-chloroadenosine. The effects of various doses of 2-chloroadenosine were tested on the adenylate cyclase activity of freshly prepared homogenates from mouse thymocytes (• - - •) and splenocytes (▪ - - ▪), as in section 2. Each point represents the mean of 3 separate determinations.

cAMP. The extent of stimulation was much greater for thymocytes than for splenocytes, in agreement with the determinations of cAMP content of intact cells (table 1); the doses of 2-chloroadenosine necessary for half-maximal stimulation were about 0.55 μM and 0.9 μM for thymocytes and splenocytes, respectively.

The addition of GTP ($\leqslant$100 μM) in the assay medium did not improve the responsiveness of adenylate cyclase to 2-chloroadenosine.

The presence of exogenous adenosine deaminase during the preincubation period and during the reaction time, as described [4] neither affected the basal adenylate cyclase activity nor the 2-chloroadenosine-stimulated activity; this means that the contribution of endogenous adenosine was negligible, probably because its concentration is maintained to a low level by endogenous adenosine deaminase activity (and perhaps by other enzymatic activities) of the homogenates. This inactivation of adenosine (which is expected to be more important in homogenates than in intact cells) was confirmed when we tested adenosine itself on thymocyte adenylate cyclase; the sensitivity of the enzyme to adenosine was considerably reduced as compared to the response to 2-chloroadenosine and was shifted towards higher concentrations. Only adenosine doses at >10 μM induced significant stimulations (30% stimulation with 100 μM adenosine, table 3).

The reversal of adenosine or analogue effects on adenylate cyclase by theophylline has often been considered as a criterion for the existence of adenosine receptors [3,4,9,13,14]; we did find that theophylline almost completely reversed the 2-chloroadenosine stimulation (table 3). The data presented in table 3 also show that classical stimulators of adenylate cyclase (prostaglandin E_1, isoproterenol and fluoride ions) strongly enhanced this enzyme activity in mouse thymocytes (3.1-, 3.9- and 5-fold stimulation, respectively). The splenocyte adenylate cyclase was stimulated by prostaglandin E_1 (2.4-fold) and by fluoride (5-fold).

4. Discussion

Adenosine-sensitive adenylate cyclase activities have been reported for a certain number of cell types; adenosine or structural analogues produced stimulation in neuroblastoma cells [3,9], striatum [4], human glioma cell and fibroblast lines [1], erythrocytes [8], adrenal and Leydig tumor cells [10], inhibition in liver [5], in fat cells [7] and thyroid [11], or biphasic response in platelets [2,6]. These nucleosides are able to increase cAMP accumulation in mouse cytotoxic T lymphocytes [15,16], rat thymic lymphocytes [12] and human peripheral lymphocytes [13,14]. However direct stimulation of adenylate cyclase by adenosine had never been clearly established for a lymphocyte cell free system. Among the above mentioned works, the most conclusive would be that in [14] where adenosine doses at 10^{-7} M were found to enhance the cAMP content of human lymphocytes, but their lymphocyte preparations from blood were contami-

Table 3
Responses of thymocyte adenylate cyclase to adenosine, 2-chloroadenosine, prostaglandin E_1, isoproterenol and fluoride. Reversal of 2-chloroadenosine effect by theophylline

Effectors	Adenylate cyclase (% basal activity)
None	100 ± 3
100 μM adenosine	130 ± 8
5 μM 2-chloroadenosine	195 ± 2
100 μM theophylline	87 ± 4
100 μM theophylline	89 ± 1
5 μM 2-chloroadenosine + 100 μM theophylline	110 ± 8
5 μM 2-chloroadenosine + 200 μM theophylline	103 ± 4
10 μM prostaglandin E_1	312 ± 11
20 μM isoproterenol	391 ± 31
10 mM NaF	494 ± 30

Adenylate cyclase of freshly prepared homogenates was determined as in section 2. The adenosine assays were carried out without exogenous adenosine deaminase. The addition of 0.6 U exogenous adenosine deaminase/ml did not change any of the other activities. We have checked that 100 μM 6-chloropurine riboside did not affect basal or 2-chloroadenosine-stimulated adenylate cyclase activity. Results are the mean of 3 separate determinations

nated with platelets. Zenser claimed to have observed direct stimulation of the adenylate cyclase of mouse thymocyte homogenates [19], but his enzyme determinations were carried out in the presence of 0.5 mM RO-20-1724 which does not completely inhibit the cAMP phosphodiesterases [18]; in our work on pig lymph node lymphocytes we demonstrated that when cAMP phosphodiesterases were not completely blocked or saturated their inhibition by adenosine analogues could account for the measured stimulations of cAMP production by cell homogenates [18].

These data unambiguously demonstrate that mouse thymocyte (and to a lesser extent mouse splenocyte) homogenate adenylate cyclase can be directly stimulated by 2-chloroadenosine, under experimental conditions where cAMP phosphodiesterases were saturated with cAMP. The low doses at which stimulation occured (half-maximal stimulation of thymocyte adenylated cyclase with 0.55 μM nucleoside) together with the shape of the dose–response curve which is similar to those obtained for hormone stimulation, ascertain the existence of adenosine receptors coupled to adenylate cyclase; our mouse thymocyte system fulfils classical criteria for the presence of such receptors: the 2-chloroadenosine action in disrupted cell preparations was reversed by theophylline and the nucleoside-induced cAMP accumulation in intact cells was not reversed by 6-chloropurine riboside, a potent inhibitor of adenosine transport.

We have no definite explanation for the difference of responsiveness between thymocytes and splenocytes; it is not due to a complete lack of sensitivity of B lymphocytes since we verified that splenocytes from homozygous (nu/nu) mice displayed the same response as those from heterozygous (nu/+) mice. Apart from possible differences in maturation or functions, the observed differences might also be the result of variable lability of adenosine receptors or slight variations in the methods of cell isolation. Our negative results reported for pig lymph node lymphocytes [18] might have been due to the lability of adenosine receptors, as a consequence of the more drastic method of tissue homogenization.

The presence in the membrane of adenosine receptors coupled to lymphocyte adenylate cyclase confers to adenosine a hormone-like role, and strenghtens the hypothesis formulated by several authors that cAMP (which inhibits many lymphocyte functions [24]) is involved in the immunosuppressive action of adenosine and particularly in the association of severe combined immunodeficiency with adenosine deaminase deficiency [12–16]. One can predict that

any mechanism which regulates the level of adenosine may influence the intracellular level of cAMP; we have obtained evidence for a 5′-nucleotidase–adenylate cyclase correlation and tested the hypothesis that concanavalin A, which inhibits 5′-nucleotidase [25,26], might indirectly control cAMP through the level of adenosine (in preparation).

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