5′-NUCLEOTIDASE–ADENYLATE CYCLASE RELATIONSHIPS IN MOUSE THYMOCYTES

A re-evaluation of the effects of concanavalin A on cyclic AMP levels

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1. Introduction

We reported the existence of membrane adenosine receptors coupled to adenylate cyclase in mouse thymocytes and splenocytes [1], while our attempts to evidence such receptors in pig lymph node lymphocytes were unsuccessful [2]. This nucleoside and structural analogues had been claimed to induce cyclic AMP (cAMP) accumulation in rat lymphocytes [3], human lymphocytes [4,5] and pig lymphocytes [2], even if direct stimulation of adenylate cyclase was not clearly established. So one can predict that any mechanism which regulates the adenosine level may affect the intracellular level of cAMP.

5′-Nucleotidase (EC 3.1.3.5) is an ectoenzyme [6,7] which specifically hydrolyses nucleoside 5′-monophosphates to nucleosides and P_i; we demonstrated that lymphocyte 5′-nucleotidase, which is inhibited by concanavalin A (Con A) is one of the lectin receptors [8,9]. Since the concentration of 5′-AMP in serum is relatively high (~40 μM [10]) we checked the hypothesis that adenosine resulting from 5′-AMP hydrolysis by 5′-nucleotidase might control the level of cAMP in thymocytes. Here we show a 5′-nucleotidase–adenylate cyclase correlation through adenosine receptor sites and showed that Con A might indirectly control cAMP level through the level of adenosine.

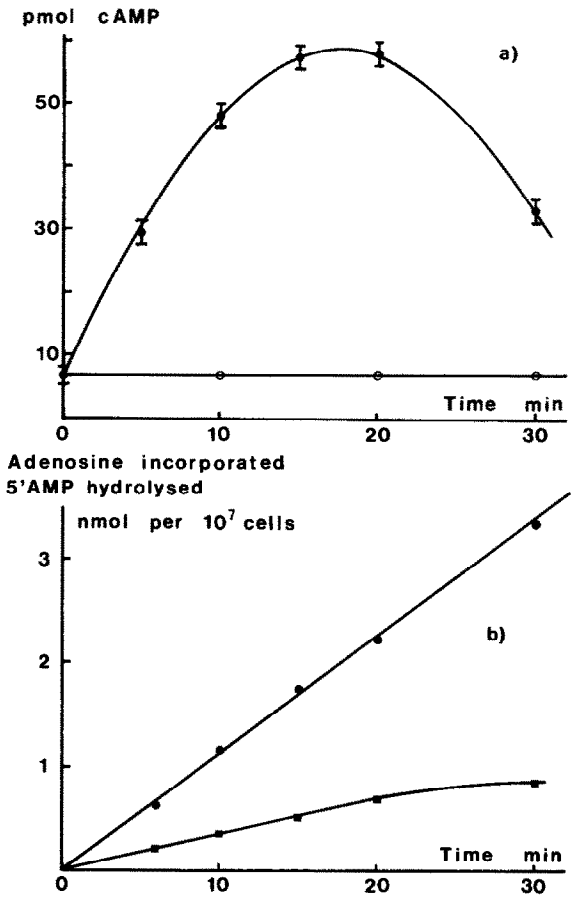
2. Materials and methods

Thymocytes were prepared from week 4–5 Swiss mice as in [1]. Determination of intracellular cAMP levels was carried out using the Amersham Radiochemical Centre kit after purification of cell extracts on Dowex resin column as in [2]. 5′-Nucleotidase activity was determined using 5′-[^{32}P]AMP as reported, except that incubation medium was Hank's medium [9]; increases in P_i up to 0.1 M had no effect on the enzyme activity (ourselves and [6]). Adenosine uptake from 5′-[^{3}H]AMP was determined after rapid centrifugation of lymphocytes from the incubation medium through an oil cushion [11].

3. Results

3.1. *Effect of 5′-AMP on cAMP accumulation in mouse thymocytes*

Short incubations of mouse thymocytes with 30 μM 5′-AMP induced large increases in their cAMP level (fig.1a). The intracellular concentration of cAMP (8.0 ± 2.0 pmol/10^7 cells) increased within the first minutes of exposure to 5′-AMP. The cAMP level was maximum (7–10-fold enhancement, depending on experiments) over 15–20 min and remained high at 30 min. We measured in the same experiment the rate of 5′-AMP hydrolysis, the uptake of adenosine from 5′-[^{3}H]AMP (fig.1b) and the increase of cAMP content in thymocytes. With 30 μM 5′-AMP the hydrolysis rate remained constant during 30 min; 1800 pmol adenosine 10^7 cells were produced in the medium, in 15 min. Part of this nucleoside was incorporated into the cells and metabolized [11]. The rate of adenosine uptake remained constant during 15 min (490 pmol/10^7 cells in 15 min), then slightly decreased between 15 and 30 min. Identical results were reported for mouse splenocytes [11].



3.2. *Effect of 6-chloropurine riboside and α,β-methylene ADP on 5'-AMP-induced cAMP accumulation*

The 5'-AMP-induced increase of thymocyte cAMP level could be explained by the fact that adenosine which enter the cell through a facilitated process [11] might serve as cAMP precursor, but this seems rather unlikely because:

(1) The peak of cAMP accumulation was observed at 15–20 min while adenosine continued to enter the cell with an unchanged metabolism [11];

(2) If 200 μM 6-chloropurine riboside, an inhibitor of adenosine transport [1], was added to the incubation medium before 5'-AMP, cAMP accumulation and 5'-AMP hydrolysis were unchanged

Fig.1.(a) Time course of the effect of 3×10^{-5} M 5'-AMP on cAMP levels in mouse thymocytes. After 20 min equilibration 1 ml aliquots of thymocytes in Hank's medium (10^7 cells) were incubated with (●—●) or without (○—○) 3×10^{-5} M 5'-AMP. Trichloroacetic acid (1 ml) was added and cAMP levels determined. Results are the mean of 3 expt. (b) Time courses of 5'-AMP hydrolysis and adenosine uptake by mouse thymocytes. At the same time and under the same conditions as for (a) 3×10^{-5} M 5'-AMP hydrolysis [7] (●—●) by mouse thymocytes and adenosine uptake from 5'-AMP [11] (■—■) were determined. Results are expressed in nmol 5'-AMP hydrolysed and nmol adenosine incorporated/10^7 cells.

Table 1
Effects of AOPC and 6-chloropurine riboside on 5'-AMP-induced cAMP accumulation, on 5'-AMP hydrolysis and on adenosine incorporation in mouse thymocytes

Cell treatment	cAMP accumulation (% of control)	5'-AMP hydrolysed (pmol/10^7 cells) in 15 min	Adenosine incorporated (pmol/10^7 cells) in 15 min
Control	100 ± 10	0	0
200 μM AOPC	120 ± 20	0	0
200 μM 6-Chloropurine riboside	110 ± 10	0	0
30 μM 5'AMP	800 ± 200	1800 ± 200	490
200 μM AOPC + 30 μM 5'AMP	250 ± 20	400 ± 100	42
200 μM 6-Chloropurine riboside + 30 μM 5'AMP	900 ± 150	1620 ± 100	48

After 20 min equilibration at 37°C, 10^7 cells in 1 ml Hank's medium were incubated for 10 min with or without 200 μM AOPC or 200 μM 6-chloropurine riboside, then for 15 min with 3×10^{-5} M 5'-AMP. cAMP levels were then determined (control contents were 8.0 ± 2.0 pmol/10^7 cells). In parallel the concentration of 5'-AMP hydrolysed and that of adenosine incorporated into the cells, under the same conditions, were determined

while adenosine incorporation was inhibited by 90% (table 1).

These results suggest that the cAMP accumulation induced by 5′-AMP does not occur through an intracellular process.

When 200 μM α,β-methylene ADP (AOPC), a well-known 5′nucleotidase inhibitor [12], was added to the incubation medium, adenosine production was inhibited by 80% and cAMP accumulation was only 22% of that observed in the absence of AOPC (table 1). This experiment and the fact that 5′-AMP did not directly stimulate adenylate cyclase of thymocyte homogenates (data not shown) rule out the possibility that the stimulation occurs through 5′-AMP extracellular sites acting exactly as the adenosine sites [1].

As we clearly established the stimulation of thymocyte adenylate cyclase by extracellular adenosine [1] and as 5′-AMP had to be hydrolysed to induce its effect, it is likely that 5′-AMP controls cAMP accumulation through the level of adenosine produced by 5′-nucleotidase. The fact that adenosine produced by 5′-AMP hydrolysis in thymocyte homogenates did not stimulate directly the adenylate cyclase activity of these homogenates can be explained by the presence of endogenous adenosine deaminase (ADA); effectively significant stimulations of homogenate adenylate cyclase were observed with sub-μM doses of 2-chloroadenosine, an ADA-resistant analogue, but necessitated adenosine at >10 μM (30% stimulation with 100 μM) [1].

3.2. *Comparison of cAMP accumulation induced by exogenous adenosine and by 5′-AMP-derived adenosine*

5′-AMP caused a dose-dependent increase in thymocyte cAMP level with maximal effect near 4×10^{-5} M (table 2). After 15 min incubation, the adenosine resulting from 5′AMP hydrolysis increased from $1.2–3.6 \times 10^{-6}$ M when 5′-AMP increased from $10^{-5}–10^{-4}$ M. At <15 min the amount of adenosine can be calculated from the kinetic equation for 5′-AMP hydrolysis [11]. Moreover part of this produced adenosine enters the cell (fig.1b); so the amount of adenosine able to interact with its extracellular binding sites is much lower than that reported in table 2 (column 2).

Exogenous adenosine gave also a dose-dependent increase of thymocyte cAMP level; this effect was detectable at 5×10^{-7} M adenosine and maximal at 10^{-5} M (table 2 and [1]). With 2×10^{-6} M adenosine this stimulation was 230 ± 10%. If we compare this % stimulation with that obtained by similar concentrations of 5′-AMP-derived adenosine (750 ± 100% for 1.8×10^{-6} M adenosine), it is clear that stimulation was greater with 5′-AMP, especially if we consider that the real amount of adenosine produced from 5′-AMP is $<1.8 \times 10^{-6}$ M, as earlier noticed.

This result may probably be explained by the fact that adenosine produced by 5′-nucleotidase is localized near its extracellular receptor sites, which means that local adenosine concentrations are higher than the bulk concentrations.

Table 2
Effects of 5′-AMP and adenosine on cAMP accumulation in mouse thymocytes

Cell treatment	cAMP content % of control	Concentrations (μM) of 5′-AMP hydrolysed or adenosine produced[a] in 15 min
Control	100 ± 10	0
10^{-5} M 5′AMP	350 ± 10	1.25
3×10^{-5} M 5′AMP	750 ± 100	1.80
4×10^{-5} M 5′AMP	900 ± 120	2.60
10^{-4} M 5′AMP	950 ± 150	3.60
5×10^{-7} M adenosine	130 ± 10	
2×10^{-6} M adenosine	280 ± 10	
5×10^{-6} M adenosine	610 ± 30	
10^{-5} M adenosine	740 ± 50	

[a] These concentrations were calculated by considering no. molecules adenosine produced/ml incubation medium in 15 min

After 20 min equilibration at 37°C, 1 ml aliquots of thymocytes in Hank's medium (10^7 cells) were incubated in the presence of various nucleotide or nucleoside doses. The concentrations of 5′-AMP hydrolysed under the same conditions were also determined

It is more difficult to compare the maxima of stimulation in the two sets of experiments since at high adenosine concentrations phosphodiesterase inhibition is also involved in cAMP accumulation [2]. Nevertheless these results clearly establish a correlation between 5'-nucleotidase and cAMP accumulation in thymocytes.

3.3. *Effect of Con A on cAMP accumulation induced by 5'-AMP*

The fact that Con A inhibits adenosine production by 5'-nucleotidase [8,9] prompted us to check if this lectin could indirectly interact with the cAMP level of thymocytes. At mitogenic doses (ourselves and [13]) we found that Con A alone induced a small increase (120%) of the cAMP level. This effect was maximal at 10–15 min and was no longer detected after 20 min. Con A inhibited the cAMP accumulation induced by 5'-AMP; when the lectin was added 15 min before 5'-AMP (3×10^{-6} M) cAMP accumulation decreased from 660–350% of controls (without 5'-AMP nor Con A), while hydrolysed 5'-AMP decreased from 1.54–0.9 $\times 10^{-6}$ M (table 3).

On the contrary Con A had no effect on the cAMP accumulation induced by adenosine (table 3), which clearly demonstrated that the decrease by Con A of 5'-AMP-induced cAMP accumulation was due to 5'-nucleotidase inhibition.

4. Discussion

We have reported that adenosine directly stimulated adenylate cylase of thymocytes and to a lesser extent that of splenocytes [1], and caused a noticeable increase in their intracellular cAMP content. Present data show that physiological concentrations of 5'-AMP [10] induce a large enhancement of the cAMP content of mouse thymocytes through interaction of 5'-AMP-derived adenosine with its external receptor sites. The stimulation induced by 5'-AMP-derived adenosine appears to be very important if we consider the small amount of nucleoside produced by 5'-AMP hydrolysis and the high stimulation level. This might be explained if 5'-nucleotidase is localized on the plasma membrane near the adenosine receptor sites, which would result in a very high nucleoside concentration near these receptors. Under these conditions it is very difficult to evaluate the part of cAMP stimulation due to adenylate cyclase stimulation [1] and the part due to phosphodiesterase inhibition [2] occurring at high adenosine concentration, but the first mechanism seems to be the most efficient since stimulation occurred even when adenosine transport was inhibited.

The effect of 5'-AMP on cAMP accumulation had already been reported in mouse neuroblastoma [14], but the stimulation observed was less important than in thymocytes.

In spite of the well-documented inhibitory role of cAMP for various lymphocyte functions [15] the effects of Con A on cAMP levels during the first minutes of stimulation have led to controversial hypotheses concerning the role of early cAMP in stimulation triggering [16–18]. Several groups observed a transient peak of cAMP accumulation induced by Con A in human peripheral lymphocytes

Table 3
Effect of Con A on cAMP accumulation induced by either 5'-AMP or adenosine in mouse thymocytes

Cell treatment	cAMP content (% of control)	5'-AMP (μM) hydrolysed in 15 min
Control	100 ± 10	
Con A	120 ± 10	
3×10^{-5} M 5'-AMP	660 ± 50	1.54
Con A + 3×10^{-5} M 5'-AMP	350 ± 50	0.9
5×10^{-6} M adenosine	610 ± 30	
Con A + 5×10^{-6} M adenosine	600 ± 20	

Cells (10^7 cells/ml in Hank's medium) were pre-equilibrated 20 min at 37°C and incubated with or without Con A (10 μg/10^6 cells) for 15 min, then with 3×10^{-5} M 5'-AMP or 5×10^{-6} M adenosine for 15 min. The concentrations of 5'-AMP hydrolysed were determined in parallel as in table 2.

[17,18]; this effect does not fit the antiproliferative properties of cAMP; it was postulated [18] that it was not relevant to the mitogenic action of Con A. The role of serum nucleotides has not been considered; our results show the importance of the presence of 5′-AMP in the incubation medium. ATP (100 μM) also stimulated cAMP accumulation (280%; data not shown) but we do not know yet if this effect is due to adenosine resulting from ATP splitting by external ATPase [6,19] and subsequent ADP hydrolysis by non-specific phosphatases [6,20] leading to 5′-AMP. We established that Con A-induced decrease of cAMP accumulation in the presence of 5′-AMP is related to the inhibition of 5′-nucleotidase, since the lectin had no effect on this accumulation in the presence of adenosine itself. This relationship hypothesized [21] could not be shown in pig lymphocytes [2], probably because a non-negligible amount of 5′-nucleotidase is removed from these cells during their preparation (in preparation).

In vivo or in the presence of serum the determination of basal cAMP level is very difficult for technical reasons, but it is evident that this level strongly depends upon the nucleotide level and upon the activities of the enzymes involved in nucleotide metabolism. 5′-Nucleotidase appears to play a critical role in these regulations; it might exert a negative control on cell proliferation through adenosine production, and this phenomenon could be related to its reduced activity in actively dividing cells [22,23] and in lymphocytes from leukemic patients [24,25]. We showed as others [26] that adenosine inhibits lymphocyte proliferation [2,21,27] only when added during the first 24 h stimulation, exactly as prostaglandin E_1 or theophylline, classical cAMP increasing agents [28].

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