BASAL cAMP IN HELA CELLS IS PROTECTED FROM PHOSPHODIESTERASE AND DOES NOT TURN OVER

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Received March 24,1975

SUMMARY

In HeLa S3 cultures, theophylline even after 24 hours scarcely increased basal cAMP levels although it is an effective inhibitor of HeLa phosphodiesterases in vitro as well as in intact cells. The persistance of cAMP in concentrated homogenates incubated at 0° or 25°, and its resistance to charcoal adsorption suggest that it is protected from PDE by being strongly bound to specific proteins. In contrast to HeLa cultures, Ehrlich ascites carcinoma (EAC) cells do respond to theophylline by a rapid increase in cAMP. On incubation of EAC homogenates, free cAMP is degraded while the bound cAMP fraction again is protected. The existence of two cAMP pools - free cAMP and protein-bound cAMP - in tissues is postulated. They are not in (rapid) equilibrium with each other, and they differ in turnover rates.

INTRODUCTION

During analysis of the dibutyryl cAMP (DBcAMP) action mechanism in HeLa S3 cells (cf. 1) it became apparent that neither DBcAMP nor theophylline treatment resulted in elevated cAMP levels. As both compounds were inhibitors of cAMP phosphodiesterases in HeLa homogenates (1), basal cAMP in these cells may not be available to the degrading enzymes. This would indicate that basal cAMP in these cultures is not subject to turnover. The general importance of this question led us to analyze in more detail the inability of theophylline to increase cAMP levels in HeLa cells and to gain information on the nature and metabolism of the basal cAMP in these cultures.

MATERIAL AND METHODS

HeLa S3 cultures were propagated either as monolayers or in suspension culture in modified Joklik medium (F 13, Grand Island Biol. Co.) containing 5% non-inactivated calf serum. Rinsed monolayers (2×10^7 cells/Roux flask) were extracted directly with 2 ml cold 10% trichloracetic acid (1).
EAC cells were propagated in Balb/c mice by serial i.p. transplant-

ation. Ascites was drawn on the 6th day after transplantation, filtered through glass wool and extracted with trichloracetic acid either directly or after sedimentation of the cells.
cAMP determination was performed acc. to Gilman (2). In most cases a $Ba(OH)_2/ZnSO_4$ precipitation of nucleotides at 4° was included which removed 0.1 - 0.2 pmoles unspecific cAMP equivalents / 10^6 cells. Suspension cultures were centrifuged (5 min at 3000 rpm), the cell pellet was either washed once with isotonic saline, or with fresh medium, or extracted directly with 10% trichloracetic acid ($\geq$ 1.0 ml/10^8 cells). Phosphodiesterase activity was determined as described previously (1). Charcoal ('Aktivkohle') was obtained from E. Merck, Darmstadt, cAMP Phosphodiesterase from Boehringer u. Soehne, Mannheim.

RESULTS

Theophylline inhibits phosphodiesterase but does not elevate significantly cAMP levels in intact HeLa S3 cells.

When HeLa S3 monolayer cultures were incubated with 3×10^{-3} M theophylline, only a small increase in intracellular cAMP level occurred after 24 hours of treatment (table 1). Cell proliferation was inhibited only to a small extent (20-30%) under these conditions. The inefficacy of theophylline to increase cAMP levels in these cultures is not due to an inefficiency as an inhibitor of cAMP phosphodiesterase (PDE): In HeLa homogenates, the presence of two phosphodiesterases could be derived from kinetic experiments (1), one with a $K_m = 4$ µM, the other with a $K_m = 330$ µm. Similar findings, though with different K_m values, have been reported (3). The high affinity enzyme exhibited a K_i value for theophylline of 4×10^{-4} M. 3×10^{-3} M theophylline led to a nearly complete inhibition of the PDE activity (not shown).

Does theophylline inhibit PDE also in intact HeLa cells ? If assumed that in intact cells the cAMP present was not available for degradation by PDE, inhibition of this enzyme by theophylline would not lead to an increase in intracellular cAMP. If, however, the substrate level could be increased artificially, theophylline should now produce an effect. Available substrate could be increased in HeLa cells by incubation of suspension cultures with dibutyryl cAMP, which was taken up by the cells and converted mainly to N^6-monobutyryl cAMP, and to a small extent also to cAMP (4). Both cyclic nucleotides are biologically active (4),

theophylline	cAMP content (pmoles/10^6 cells)
-	0.92 ± 0.06
+	1.47 ± 0.40

Table 1: cAMP levels in HeLa S3 monolayer cultures treated for 24 hours with theophylline. - Monolayer cultures were incubated $\pm$ 3 x 10^{-3} M theophylline for 24 h, then extracted with cold TCA, and cAMP was determined as described (1). Mean values $\pm$ SEM from 3 separate experiments, each run in triplicates.

and both are susceptible to enzymic hydrolysis by PDE (5), though to a different degree. When theophylline was added under these conditions (fig. 1), it strongly increased the accumulation of the two cyclic nucleotides (= $\sum$ cAMP equivalents) derived from intracellular deacylation of DBcAMP indicating effective inhibition of PDE in intact cells, too. The inefficiency of theophylline in elevating basal cAMP levels in proliferating HeLa cells as shown in table 1, therefore, must be due to a protection of the endogenous, basal cAMP from PDE action. This at the same time, indicates that basal cAMP does not exhibit significant turnover in proliferating HeLa S3 cells.

In HeLa cells, most cAMP is protected from PDE action by being bound to protein.

There are several ways in which the protection of cAMP from PDE action could come about: If cAMP was protected by compartmentalization, homogenization of cells should result in rapid degradation of the endogenous cyclic nucleotide. Similarly, the dilution of an eventual PDE inhibitor in the homogenate should bring about at least a partial de-inhibition of the enzyme with a concomitant decrease in cAMP. Only when protected by being bound to specific proteins (e.g. to regulatory subunits (R) of protein kinases (6-11)), homogenates should have similar cAMP levels as intact cells, and this cAMP level should persist during incub-

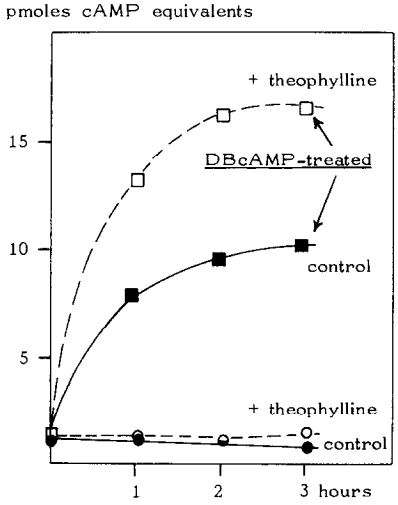

Fig. 1: Cyclic nucleotides (binding to protein kinase) in controls and in dibutyryl cAMP-treated cultures under the influence of theophylline. - HeLa suspension cultures were treated ± 3 x 10^{-4} M DBcAMP and ± 2 x 10^{-3} M theophylline. At the times indicated, aliquots were taken, the cells sedimented by centrifugation, and analyzed for cAMP equivalents in the Gilman test. While cAMP and N^6-monobutyryl cAMP bind nearly equally well, DBcAMP and $O^{2'}$-monobutyryl cAMP do not bind (4).

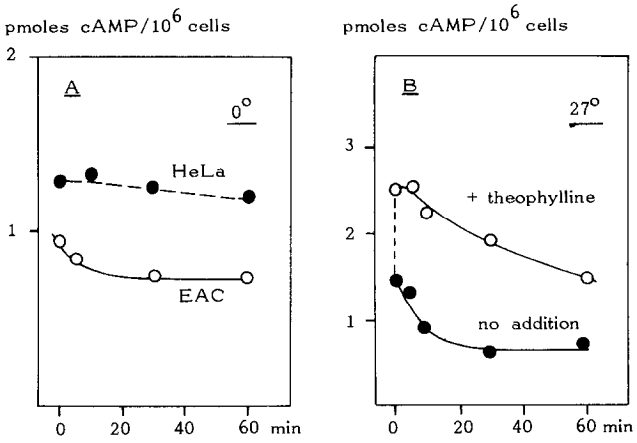

Fig. 2: Stability of basal cAMP in HeLa and EAC cell homogenates. - HeLa suspension cultures (5 x 10^8 cells) were centrifuged and the cell pellet immediately sonified (Branson sonifier) in 3 ml cold 50 mM Tris buffer pH 7.4. The homogenate was incubated at 0° (A), and aliquots were taken at the times indicated and analyzed for cAMP. EAC cells (4 x 10^8 cells) were suspended in 2.5 ml buffer as above, or in 2.5 ml buffer containing 30 mM theophylline (fig. 2B), and sonified. The homogenates were incubated as indicated, and aliquots were analyzed at appropriate times for cAMP.

ation of the homogenate. As shown in fig. 2A, HeLa homogenates incubated at 0° lost their cAMP at a very slow rate. The total cAMP in the homogenates corresponded to the values in intact cells. Addition of extra cAMP to homogenates (final concentration 1×10^{-5} M) resulted in a rapid degradation of the free, non-protected cyclic nucleotide until the basal level was reached again, and addition of extra phosphodiesterase did not lower significantly the endogenous cAMP level (not shown). These finding are consistent with the thesis that most cAMP in proliferating HeLa cells is 'bound cAMP' not available to PDE.

In contrast to HeLa cells, Ehrlich ascites carcinoma (EAC) cells grown in mice contained cAMP which was only partially protected. This could be shown by the partial degradation of endogenous cAMP on incubation of homogenates leveling off after 20-30 min at 0° or 27° (fig. 2A and 2B)[1]. These results, then, indicate the existence of free cAMP in EAC cells besides a fraction of protected, protein-bound cAMP. Since free cAMP in homogenates, as well as in intact cells, is not protected from PDE it must be subject to rapid turnover being continuously degraded and resynthesized. Hence, it can be postulated that - unlike the situation in HeLa cells - addition of the PDE inhibitor theophylline to EAC cells will increase their cAMP levels. Indeed, theophylline led to a threefold increase in intracellular cAMP within 5 min at 37° (table 2). In some experiments, even a sevenfold increase of the basal cAMP level could be achieved after 20 min of incubation. At 0°, theophylline did not increase cAMP.

If the 'protected cAMP' in these two cell types is indeed protein-bound cAMP, it should also be resistant to charcoal treatment, which removes free cAMP but not the protein-bound form (12). Furthermore, charcoal should have no effect on the cAMP levels in HeLa homogenates because

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1) Although addition of 2 mM theophylline to fresh EAC homogenates (presumably still containing sufficient ATP) rapidly increased total cAMP (fig. 2B), it could not completely prevent a slow degradation of free cyclic nucleotide in later stages of the incubation period. - The variation in total cAMP as well as in the ratio of resistant to non-resistant cAMP is due to differences in time of harvest after tumor transplantation and on cell density of the ascites.

treatment	pmoles cAMP/10^6 cells after incubation for		
	0 min	5 min	20 min
0°, + saline	0.77	0.70	0.72
37°, + saline	0.69	0.60	-
0°, + theophylline	0.80	0.54	-
37°, + theophylline	0.82	2.34	2.05

Table 2: Rapid effects of theophylline on EAC cells. - Ascites was drawn from mice 6 days after transplantation and immediately mixed with an equal volume of cold isotonic saline, or 50 mM theophylline in cold saline. The cell suspensions ($\sim 10^7$ cells) were incubated in microtubes at 0° or 37°, centrifuged (0.5 min at 12 000 rpm, Eppendorf minifuge), the cell pellet extracted with 10% TCA and analyzed for cAMP. Mean values from triple determinations.

all cAMP is protected cAMP, while EAC homogenates should lose about 1/4 - 1/2 of their cAMP (cf. fig. 2). When such experiments were carried out at 0° and in the presence of theophylline (table 3), no significant alterations were seen in the case of HeLa homogenates, while EAC homogenates lost 1/3 of their cAMP.

DISCUSSION

The only reaction known to terminate the action of cAMP in mammalian cells is the enzymic hydrolysis to AMP by cAMP phosphodiesterase. However, it has been shown that cAMP is at least partially protected from phosphodiesterase when bound to the regulatory subunit of protein kinases. The rate limiting factor in the hydrolysis of the bound form appeared to be the dissociation of cAMP from the binding protein (13,8). However, the question arises whether a special mechanism is required in order to release bound cAMP into a degradable form. The affinity of cAMP to binding proteins is high enough to prevent significant equilibration with free cAMP. This is the basis for the efficient separation of protein-bound cAMP by membranes filtration in the Gilman-Test (2)

Charcoal treatment	cAMP content (pmoles/ml homogenate)
HeLa cells:	
-	75.0 ± 9.2
+	85.5 ± 6.2
EAC cells:	
-	282 ± 50
+	196 ± 26

Table 3: Charcoal treatment of HeLa and EAC homogenates. - A HeLa suspension culture (1000 ml, 4.6 x 10^8 cells) was treated for 10 min with 5 mM theophylline, the cells centrifuged off, washed in 3 mM theophylline-isotonic saline, and the pellet sonified in 6 ml 50 mM tris buffer pH 7.4 - 20 mM theophylline (0°). After centrifugation (15 min, 18 000 rpm) 200 µl aliquots of the supernatant were mixed with 200 µl of a charcoal suspension (100 mg charcoal in 1 ml containing 50 mM tris buffer pH 7.4 - 20 mM theophylline - 2 mg bovine serum albumin), or with 200 µl of the suspension buffer without charcoal (control). The samples were centrifuged (4 min, 12 000 rpm, Eppendorf minifuge), an aliquot of the supernatant extracted with TCA and analyzed for cAMP. No significant differences between homogenate and 45 000 g supernatant cAMP levels were found. EAC cells were obtained by centrifugation (7 min, 3000 rpm, 4°) of ascites. The pellet (5 x 10^8 cells) was immediately sonified in 2.5 ml cold 50 mM tris buffer pH = 7.4 - 20 mM theophylline. 300 µl aliquots were mixed with 300 µl of charcoal suspension or suspension buffer, and analyzed as above. Control experiments were performed by treating aliquots of the homogenate, to which 20 µl 0.01 M (^{3}H)cAMP (10^4 cpm) had been added, ± charcoal as above. Charcoal removed > 99% of the added excess cAMP.

and of the removal of free cAMP by charcoal treatment in the Brown Test (12). Three consequences arise from these findings:

- In EAC cells, in liver (14) and probably in most other tissues too (cf.9), some of the basal cAMP is free cAMP showing high turnover as indicated by the rapid accumulation after PDE inhibition. Besides this, a fraction of cAMP exists which is bound to proteins and is thus protected from PDE in vitro as well as in vivo.

- In HeLa cells, practically all the basal cAMP consists of bound cAMP,

which is not in (rapid?) equilibrium with free cAMP, not available for PDE and therefore not subject to (rapid) turnover. This interpretation is supported by the finding, that in HeLa S3 cells no transient decrease in cAMP levels during late G2 phase occurs (15), although such a decrease was observed in normal 3T3 fibroblasts (16).

- As bound cAMP is at least in part equivalent to cAMP bound to the regulatory subunits of protein kinase (3), it must be also equivalent to a corresponding amount of catalytically active protein kinase acc. to the general formulation: $R \cdot C + cAMP \longrightarrow R \cdot cAMP + C$ (11). In order to allow a return of the protein kinase from the activated C to the non-activated $R \cdot C$ status, bound cAMP has to be released into a PDE susceptible form identical with free cAMP. Since the free and the bound form of cAMP are not in equilibrium as shown in this paper, a special factor must be postulated which effects the release of bound cAMP into the free form. Such a factor could be an allosteric effector acting on $R \cdot cAMP$ to effect the release of cAMP into a free form. This cAMP-releasing factor may be low or absent in rapidly proliferating tumor cells, but present in normal, hormon responsive tissues.

Addendum: Recently, Cheung arrived at a similar conclusion on the basis of experiments with the cAMP-binding subunit of kidney protein kinase (8), and Beavo et al. discussed the possible existence of parameters affecting the equilibrium between free cAMP and cAMP bound to protein kinase (17).

ACKNOWLEDGEMENTS

We would like to thank G. Jarmers for propagating HeLa cell cultures, and C. Winkens for transplantation of EAC cells. This work was supported by the Deutsche Forschungsgemeinschaft - SFB 34.

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