PERMEATION OF DIBUTYRYL cAMP INTO HELA CELLS AND ITS CONVERSION TO MONOBUTYRYL cAMP

E. Kaukel and H. Hilz

(Institut für Physiologische Chemie, Universität Hamburg, Germany)

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SUMMARY

^{3}H-Dibutyryl cAMP (DBcAMP) when exposed to HeLa cultures proved rather resistant to extracellular degradation. It was taken up by the cells and led to an accumulation of monobutyryl cAMP (MBcAMP), which - in contrast to DBcAMP - showed a high affinity to Gilman's (1) cAMP-binding protein. cAMP and DBcAMP were not accumulated in the cells to a comparable degree under these conditions.

Other than DBcAMP, cAMP was rapidly degraded extracellulary to various metabolites including adenosine which was taken up by the cells and converted to purine nucleotides. Only at high extracellular concentrations cAMP could permeate the cells and lead to a transient rise in intracellular cAMP levels. These levels, however, never reached the steadily increasing concentrations of protein kinase-binding substances (MBcAMP + cAMP) when similar concentrations of DBcAMP were added to cultures.

These results indicate that the sustained hormone-like actions of DBcAMP come about mainly by a high resistance to extracellular and intracellular phosphodiesterase as well as by the enzymic conversion to MBcAMP accumulating in the cells.

INTRODUCTION

It is generally assumed that the higher efficiency of DBcAMP in many cAMP-responding systems is due to the better permeation into cells of the less polar dibutyryl derivative compared to cAMP itself, or to a higher resistance to phosphodiesterase (2, 3). To our knowledge there exists no comparative study on the permeation of the two substances and their eventual accumulation within the cells. We therefore synthesized ^{3}H-DBcAMP and analyzed the kinetics of the uptake of ^{3}H-label-

ed cAMP and DBcAMP by HeLa S3 cells. During these studies it was observed that pronounced metabolic alterations of the compounds inside and outside the cells occurred which, however, were quite characteristic for each nucleotide, and which may explain the opposite effects of cAMP and its dibutyryl derivative on various metabolic parameters in HeLa cells (4, 5).

MATERIAL AND METHODS

HeLa S3 cells were propagated in modified Joklik medium (F-13, Grand Island Biological Co.) as described previously (6). Cyclic nucleotides were obtained from Boehringer, Mannheim. ^{3}H-cAMP was purchased from the Radiochemical Center, Amersham, England. ^{3}H-DBcAMP and ^{3}H-MBcAMP were synthesized from labeled cAMP by slight modifications of the method of Posternak et al. (3). Cyclic nucleotides, eventually after alkaline hydrolysis, were determined acc. to Gilman (1).

RESULTS

Extracellular degradation of ^{3}H-labeled cAMP, MBcAMP and DBcAMP in HeLa cultures.

When ^{3}H-labeled cAMP and its butyryl derivatives were exposed to HeLa cells in suspension culture (fig. 1), by far the most radioactivity was taken up by the cells which were exposed to ^{3}H-cAMP, while ^{3}H-DBcAMP led to very low cell-associated radioactivity. ^{3}H-MBcAMP showed intermediate uptake. At first sight, these results were surprising. They do, however, not demonstrate better cell permeability for cAMP compared to DBcAMP:

As shown in fig. 2A, ^{3}H-cAMP (10^{-5} M) is rapidly and nearly completely degraded in the medium via AMP and IMP to adenosine, inosine, and the corresponding bases (mainly hypoxanthine). DBcAMP (fig. 2B) proved to be quite resistant to the extracellular and membrane-accociated enzymes. Only 13% were degraded to MBcAMP, 1-2% to cAMP, and 5% to further degradation products during a 2 hour incubation period. The high tritium activity in the cells after exposure to ^{3}H-cAMP (fig. 1) is probably taken up in the form of ^{3}H-adenosine (fig. 3): Cold AMP

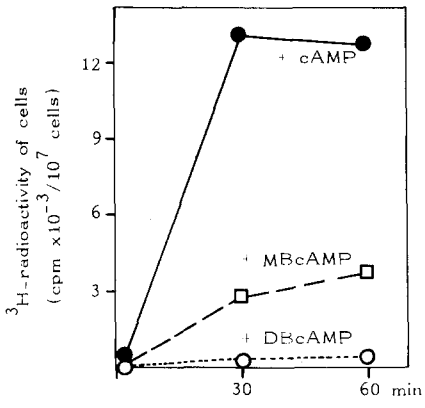

fig. 1: Uptake of ^{3}H-radioactivity from labeled cyclic nucleotides into HeLa cells

A suspension of HeLa cells (6×10^7 cells/ml medium) was incubated at 37°C with tritium labeled cyclic nucleotides at a final concentration of 1×10^{-5} M and 0.6 µCi/ml. After incubation cells were centrifuged and washed twice with medium. The pellet was extracted with 500 µl of ice-cold 4% $HClO_4$. Radioactivity of the extract was quantified by liquid scintillation counting.

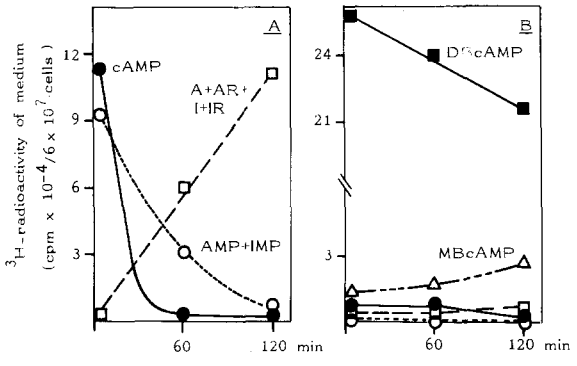

fig. 2: Extracellular degradation of cAMP (A) and DBcAMP (B) in HeLa suspension cultures

Conditions are identical to those described in the legend to fig. 1. TCA was extracted from the supernatant by water-saturated diethyl-ether, residual ether removed by heating samples to 95°C for 3 min. Separation of radioactive split products was performed by paper chromatography (paper: Schleicher & Schüll, 2043 bM; solvent system(8): 1 M ammonium acetate pH=7.5/ethanol=3/7.5 (v/v)) after addition of carrier cAMP, MBcAMP, DBcAMP, AMP and AR. UV-absorbing spots were cut out, eluted with 0.1 N HCl, and analyzed for radioactivity (500 µl + 15 ml dioxane-omnifluor scintillation mixture). The graphic symbols in fig. 2B correspond to those in fig. 2A. (A = adenine; AR = adenosine; I = hypoxanthine; IR = inosine).

and adenosine in equimolar amounts, but not adenine, suppressed completely the uptake of tritium by the cells due to trapping the isotope. Adenosine, which is taken up by the cells rather rapidly, shows a 'break-through' of the isotope after 3 hours of incubation. Partial suppression of ^{3}H-uptake also occurred in the presence of IMP and - to a smaller degree - of inosine, which may incidate that part of cAMP was degraded via AMP - IMP - inosine.

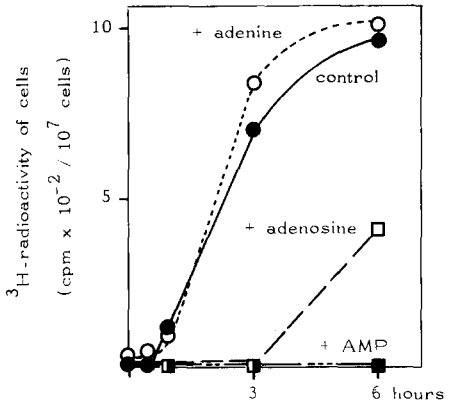

fig. 3: Suppression of tritium uptake by cells from exogenous ^{3}H-cAMP by exogenous unlabeled AMP and adenosine

Monolayer cultures (2×10^{6} cells/flask; 24 h after seeding) were incubated with ^{3}H-cAMP (1×10^{-4} M; 680 nCi / 10 ml medium). AMP, adenosine, and adenine were added at equimolar concentrations. After incubation, monolayers were rinsed twice with ice-cold medium, and cells harvested with the aid of a policeman. Cell number was determined with a Coulter-counter. The cell suspension was centrifuged for 5 min at 3000 rpm. The pellet was extracted with 500 µl 4% $HClO_4$ and the acid-soluble fraction was analyzed for radioactivity as described in the legend to fig. 2.

The experiments presented in fig. 4 also point to split products of ^{3}H-cAMP as the form in which ^{3}H from labeled cAMP is taken up by the cells. Theophylline as an inhibitor of the extracellular phosphodiesterase strongly retarded tritium uptake from ^{3}H-cAMP. It had, however, no influence on the uptake of ^{3}H-DBcAMP by the HeLa suspension cultures.

In contrast to DBcAMP the monobutyryl derivative seemed to be considerably less resistant to extracellular splitting.

Accumulation of MBcAMP in cells exposed to DBcAMP

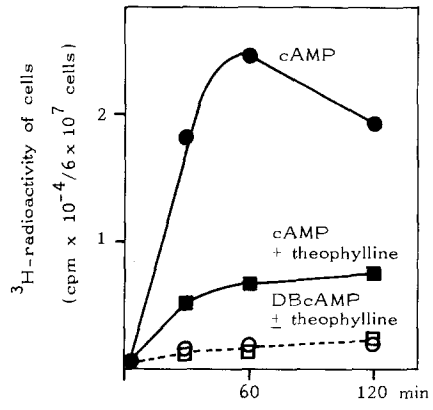

fig. 4: Influence of theophylline on the uptake of tritium by HeLa cells from labeled cyclic nucleotides

Conditions were the same as in fig. 1. The concentrations of cAMP and DBcAMP were 1×10^{-3} M, and of theophylline 2×10^{-3} M. Zero values from the non-washed cells (identical in all four cases) were subtracted. Other details are described in the legend to fig. 1.

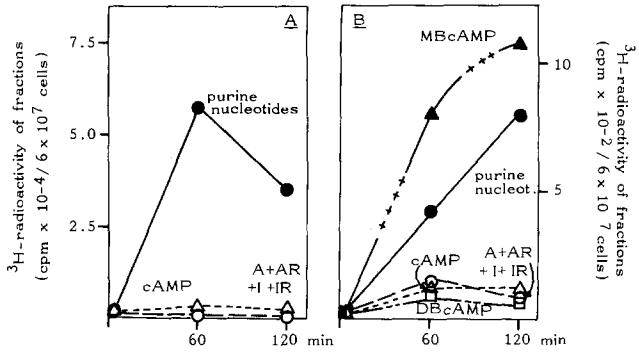

fig. 5: Labeling pattern of intracellular derivatives after incubation with ^{3}H-cAMP (A) and ^{3}H-DBcAMP (B)

Incubation conditions and extraction of acid-soluble material are described in the legend to fig. 1, with a final cAMP or DBcAMP concentration of 1×10^{-5} M and 0.6 μCi/ml. An aliquot of the acid-soluble fraction was neutralized with $KHCO_3$, and chromatographed (see fig. 2).

The extracellular degradation of ^{3}H-cAMP to ^{3}H-adenosine and the uptake of ^{3}H in this form followed by intracellular phosphorylation is also reflected by the intracellular distribution of the label (fig. 5A). When exposed to 10^{-5} M cyclic nucleotide, tritium was found during the first 2 hours nearly exclusively in purine nucleotides. The cAMP fraction contained less than 0.5% of total acid-soluble radioactivity taken

up by the cells. Quite different distribution was observed in the case of ^{3}H-DBcAMP (which yielded a much lower level of total tritium) (fig. 5B). ^{3}H-adenosine phosphates comprised less than half of the acid-soluble cellular radioactivity. The interesting point, however, is the fact that neither DBcAMP nor cAMP showed a significant increase whereas MBcAMP accumulated inside the cells to substantial values, even at these low (10^{-5} M) exogenous DBcAMP concentrations.

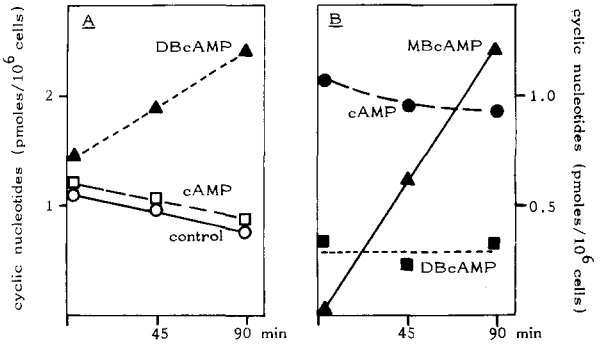

fig. 6: Alterations of intracellular levels of cyclic nucleotides in the presence of 1 x 10^{-5} M cAMP or DBcAMP
A) Levels of protein kinase-binding material
B) Intracellular distribution of cAMP derivatives during incubation with DBcAMP

The experiments in fig. 6A were performed under conditions described in fig. 1. Determination of protein kinase-binding material was performed in TCA extracts (cf. fig. 2) acc. to Gilman (1).
Fig. 6B. Intracellular distribution of cyclic nucleotides was analyzed by chromatographic separation of labeled DBcAMP and derivatives as described in the legend to fig. 2.

Apparently, DBcAMP was hydrolyzed - presumably by an intracellular esterase (7) - to MBcAMP at an appreciable rate.

When intracellular levels of cyclic nucleotides were analyzed by the Gilman method (binding to muscle protein kinase (1)) (fig. 6A), again no significant increase in intracellular cAMP occurred in the presence of 10^{-5} M exogenous cAMP. In contrast, a definite rise (3-fold) in kinase-binding material was detected in the presence of DBcAMP. As DBcAMP is practically not bound by the cAMP-binding protein while MBcAMP and cAMP exhibit nearly identical affinities (7), the increase in the presence of DBcAMP must be due to cAMP or

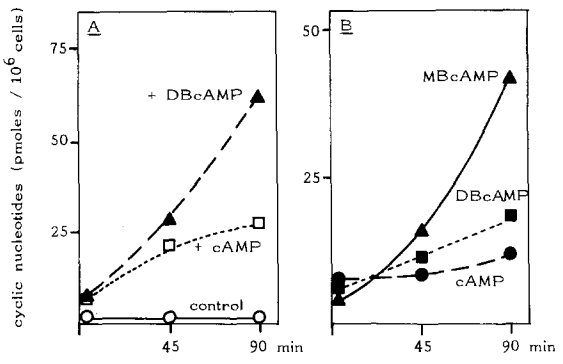

fig. 7: Increase in intracellular cyclic nucleotides in cultures exposed to high cAMP and DBcAMP concentrations :
A) Level of protein kinase-binding material
B) Intracellular distribution of cAMP derivatives during incubation with DBcAMP

Conditions and analytical details were the same as described in the legend to fig. 6, the concentrations and radioactivities of added cAMP and DBcAMP were 1×10^{-3} M and 0.6 μCi/ml in both cases. Distribution of intracellular cyclic nucleotides was analyzed after chromatographic separation and elution by the protein kinase-binding test (1)

MBcAMP formation. Fig. 6B shows again that this time-dependent increase in protein kinase-binding material is solely caused by a rise in MBcAMP under these conditions.

At 10^{-5} M concentrations of cyclic nucleotides HeLa cells show very slight, if any metabolic alterations. When added in 10^{-3} M concentrations, strong deviations from normal of various parameters could be observed (4, 5). When cells were exposed to these higher concentrations (fig. 7A), a rapid initial rise in intracellular cAMP was found with exogenous cAMP. In the case of exogenous DBcAMP, the increase in kinase-binding material in the cells is 60 times higher than normal cAMP levels. Under these high doses of extracellular DBcAMP too, MBcAMP was the derivative with the highest increase inside the cells (fig. 7B). It seems important to point out, that in the case of exogenous cAMP the intracellular cyclic nucleotide concentration passed through an optimum and then levelled off. An increase and early decline of intracellular cAMP occurred also in monolayer cultures in spite of a persistence of high (2×10^{-4} M) extracellular cAMP concentration under these conditions (67% left after 24 hours).

The data presented do not support the view that the sustained hormone-like actions of DBcAMP come about by a better penetration compared to cAMP. Rather, the superiority of DBcAMP in HeLa cultures is the consequence of two metabolic characteristics:
High resistance of DBcAMP to extracellular and intracellular phosphodiesterase(s) --
Deacylation with concomitant intracellular accumulation of MBcAMP having an affinity to protein kinase(s) comparable to cAMP --

MBcAMP seems to be the true imitator of intracellular cAMP, when DBcAMP is added to tissues as judged from its accumulation within the cells, its high affinity to Gilman's cAMP-binding protein and from the early observations (3) that MBcAMP, but not DBcAMP, stimulated phosphorylase b → a conversion in cell homogenates.

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