Turnover of Adenosine 3':5'-Cyclic Monophosphate in Chicken Erythrocytes

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Turnover of cyclic AMP was studied in intact chicken erythrocytes. Production of cyclic AMP was stimulated by adrenaline and then blocked by propranolol. The decline in the cyclic AMP concentration under these conditions is solely due to its intracellular degradation, whereas efflux of the nucleotide, although existing in these cells, does not contribute significantly to the change in its concentration. Intracellular degradation of cyclic AMP follows a first-order kinetics with a half-life of about 6 min. Similar half-lives were obtained at widely different adrenaline concentrations or when the ratio of propranolol to adrenaline was varied by 25-fold. Theoretical equations were applied to calculate the rates of cyclic AMP synthesis and degradation in the intact cells under different experimental conditions. Maximal adrenaline concentrations raise the rate of cyclic AMP synthesis and its steady-state concentration by about 10-fold. The addition of caffeine causes a further 33% increase in intracellular concentration of the nucleotide, which is in good agreement with the theoretical increase computed from its slowed-down degradation.

The intracellular concentration of cyclic AMP is determined by the rates of its synthesis, degradation and efflux from the cells to the surrounding medium. The steady-state concentration within the cells can be regulated by modulating each one of these parameters.

Numerous reports have demonstrated changes in cyclic AMP steady-state concentration in different animal tissues in response to various hormonal stimuli as well as to drug administration (Robison *et al.*, 1971). Despite the intensive investigations on factors influencing cyclic AMP synthesis and degradation, very little is known at present about its turnover in intact cells. Measurements of adenylate cyclase activity or phosphodiesterase activity in cell-free preparations probably do not reflect the exact situation within the cell.

In the present study an attempt was made to determine the turnover of cyclic AMP in adrenaline-stimulated intact chicken erythrocytes. Kinetics of breakdown and efflux of cyclic AMP *in situ* were followed and the half-life of the nucleotide within the cell was determined.

Methods

Preparation of chicken erythrocytes

Heparinized chicken blood was diluted with Krebs–Ringer phosphate buffer (pH 7.4) (Umbreit *et al.*, 1959), containing 10 mM-glucose, and was centrifuged at 1000g for 5 min at 5°C. The supernatant fraction and the upper buffy coat of the

precipitate were removed by aspiration. The erythrocytes were then resuspended in the buffer and centrifuged as above. After two similar washes, the cells were resuspended to give a 40% suspension.

Incubation procedure

Cells were incubated in the presence of various adrenaline concentrations, as detailed in the Results section, in a New Brunswick Gyrotory Water Bath Shaker at 37°C. At various times during the incubation, duplicate portions of 1.0 ml were withdrawn, added to 1.5 ml of boiling water and boiled for 5 min. The samples were then centrifuged at 1000g for 30 min, and the supernatant was used for determination of total cyclic AMP content and ATP concentration. For determination of extracellular cyclic AMP content, duplicate portions of 1.0 ml of cell suspension were withdrawn at various times and centrifuged for 1.5 min at 3000g. The separated medium was then immediately boiled for 5 min and used for cyclic AMP measurement. Intracellular cyclic AMP content was calculated by subtracting the extracellular cyclic AMP from total nucleotide content. All values are results of a representative experiment. Six to eight replicate experiments yielded very similar results. The error associated with duplicate determinations in each experiment was equal to or less than 5%.

Determination of cyclic AMP

Cyclic AMP concentration was measured by the method of Brown *et al.* (1971) by using the cyclic

AMP assay kit obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Determination of ATP

ATP was measured by the luciferin-luciferase assay adapted to liquid-scintillation spectrometry as described by Stanley & Williams (1969).

Materials

L-Adrenaline, propranolol and caffeine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Chicken erythrocytes respond to L-adrenaline with an elevation in the intracellular cyclic AMP concentration accompanied by appearance of increased amounts of the cyclic nucleotide in the outer medium. As shown in Fig. 1, the presence of $10\mu\text{M}$ -adrenaline in the incubation medium caused maximum intracellular accumulation of cyclic AMP within 30 min, and this was maintained for at least an additional 30 min.

Under these conditions, cyclic AMP was released to the outer medium at a constant linear rate. The amount of cyclic AMP accumulated in the medium

after 60 min of incubation exceeded 50% of its intracellular concentration at the same period.

Introduction of the β -adrenergic-blocking agent, propranolol, 45 min after adrenaline was added and a new enhanced steady-state concentration of cyclic AMP had been reached resulted in an immediate fall in the intracellular concentration of the nucleotide.

The decrease in the pre-existing cyclic AMP within the cell seems to be exclusively due to intracellular degradation of the nucleotide, since cyclic AMP efflux from the cells was cut off immediately after the administration of the drug (Fig. 1). This is in accordance with our earlier observations that the efflux of cyclic AMP from chicken erythrocytes is related to the rate of the synthesis of the nucleotide, rather than to its intracellular concentration (E. Gorin & S. Dickbuch, unpublished work). Hence, the employment of propranolol enables direct measurement of the rate of cyclic AMP degradation in intact erythrocytes. Furthermore, the exact kinetics of cyclic AMP degradation can be precisely determined: as shown in Fig. 2, intracellular cyclic AMP decayed exponentially towards basal values and yielded a straight line on a semilogarithmic plot. Degradation within the intact cell is therefore a first-order reaction. Assuming that the rate of cyclic AMP synthesis is a zero-order reaction, the change in cellular cyclic AMP content can be expressed by eqn.

$$(1): \quad dP/dt = S - K_dP \quad (1)$$

Fig. 1. Effect of adrenaline and propranolol on intracellular and extracellular concentrations of cyclic AMP in chicken erythrocytes

Erythrocytes were incubated in the presence of $10\mu\text{M}$ -L-adrenaline. After 45 min the incubation was continued for an additional 15 min in the presence (●) or absence (○) of $50\mu\text{M}$ -propranolol. At different times, portions were withdrawn for determination of intracellular cyclic AMP (—) and for determination of extracellular cyclic AMP (---).

Fig. 2. Decay of intracellular cyclic AMP after the addition of propranolol to adrenaline-stimulated erythrocytes

Erythrocytes were incubated with $10\mu\text{M}$ -adrenaline. At 45 min incubation, propranolol was added to a final concentration of $50\mu\text{M}$. Portions were withdrawn for cyclic AMP determination at 2 min intervals. The concentration of cyclic AMP before the addition of propranolol at time 0 was 400 pmol/ml of packed cells.

where P is the concentration of cyclic AMP, S the rate constant for synthesis expressed as cyclic AMP formed/time and K_d the first-order rate constant for cyclic AMP degradation expressed as time^{-1} . A general solution of eqn. (1) can be represented by eqn. (2):

$$P/P_0 = \frac{S}{K_d P_0} - \left(\frac{S}{K_d P_0} - 1 \right) e^{-K_d t} \quad (2)$$

where P_0 is the content of cyclic AMP at $t = 0$ [for details see Berlin & Schimke (1965) for a similar situation in the case of protein degradation].

Under conditions in which cyclic AMP synthesis is inhibited, the degradation constant (K_d) can therefore be determined by eqn. (3):

$$\ln(P/P_0) = -K_d t \quad (3)$$

This evaluation was used to compute the degradation constant of cyclic AMP (K_d) from the data shown in Fig. 2. The calculated value of K_d is 0.125. The rate of degradation can also be expressed as the half-life ($t_{1/2}$), which is defined as:

$$t_{1/2} = \frac{\ln 2}{K_d} \quad (4)$$

where $\ln 2$ is the natural logarithm of 2 and K_d is the rate constant of degradation (Reiner, 1953; Tarver, 1954). The data shown in Fig. 2 yield a half-life for intracellular cyclic AMP of about 5.5 min.

The above measurements of the degradation rate constant and half-lives of cyclic AMP might be valid only if propranolol totally inhibited the adrenaline-induced adenylate cyclase activity. A series of experiments were therefore carried out in which the degradation constant and half-life were determined under various propranolol/adrenaline ratios. If the blocking of the β -receptor by propranolol were insufficient, shorter apparent half-lives would be expected at lower adrenaline or higher propranolol concentrations. However, as can be seen in Table 1, similar K_d and $t_{1/2}$ values are obtained at different adrenaline concentrations and with the propranolol/

adrenaline ratio ranging between 1- and 25-fold. These data therefore indicate that, under the experimental conditions employed, propranolol indeed totally inhibited the adrenaline-induced adenylate cyclase activity. The means $\pm$ s.e.m. computed from these experiments are 0.114 ± 0.01 for the degradation rate constant and $6 \text{ min } 5 \text{ s} \pm 32 \text{ s}$ for the $t_{1/2}$ of cyclic AMP *in situ*.

As a further control, the integrity of the cells during the incubation was examined by determining the ATP content of the cells. No change in ATP concentration was observed during incubation with different concentrations of adrenaline or propranolol (Fig. 3b).

Fig. 3. Effect of various concentrations of adrenaline on the intracellular concentration of cyclic AMP and ATP. Erythrocytes were incubated for 65 min in the presence of 50 μM (●), 10 μM (▲) and 2 μM (■) adrenaline. At different times 1.0 ml portions were withdrawn and treated as described in the Methods section. (a) cyclic AMP content (—); (b) ATP content (----).

Table 1. Cyclic AMP-degradation rate constant and half-lives in the presence of different propranolol/adrenalin ratios. Erythrocytes were incubated in the presence of adrenaline. After 45 min of incubation, propranolol was added. K_d and $t_{1/2}$ were computed from the slopes of semilogarithmic decay plots as in Fig. 2.

[Adrenaline] (μM)	[Propranolol] (μM)	[Propranolol]/ [adrenaline]	K_d (min^{-1})	$t_{1/2}$ (min)
2	50	25	0.127	5.45
10	10	1	0.108	6.37
10	50	5	0.126	5.50
10	100	10	0.114	6.08
50	50	1	0.101	6.80
50	100	2	0.110	6.30
Mean $\pm$ s.e.m.			0.114 ± 0.010	6.08 ± 0.52

The data presented here furthermore allow the estimation of the rates of cyclic AMP synthesis in various experimental conditions. Under a steady-state situation, where $dP/dt = 0$, the rate of cyclic AMP synthesis is equal to the rate of its degradation:

$$S = K_d P \quad (5)$$

Fig. 3(a) demonstrates that the intracellular cyclic AMP concentrations indeed achieved steady-state values at various adrenaline concentrations. Maximal activation of cyclic AMP synthesis was obtained at $10 \mu\text{M}$ -adrenaline. No further increase in the concentration of the nucleotide was observed at $50 \mu\text{M}$ -adrenaline. In the presence of $2 \mu\text{M}$ -adrenaline, a lower temporary steady-state concentration of intracellular cyclic AMP was reached and the time course of the hormone effect was shorter than with the optimum concentration of adrenaline.

Table 2 summarizes the calculated rates of cyclic AMP synthesis in the presence of adrenaline at different concentrations. At optimal concentrations of adrenaline the rate of cyclic AMP synthesis corresponds to about $47.0 \text{ pmol/min per ml}$ of packed cells. Under basal unstimulated conditions the calculated value for the rate of cyclic AMP synthesis is about $4.2 \text{ pmol/min per ml}$ of packed cells. It should be stressed, however, that under basal conditions, intracellular cyclic AMP may be partially bound to cellular proteins and not all accessible to phosphodiesterase.

The method employed in this work could be further utilized to estimate the effects of a phosphodiesterase inhibitor on cyclic AMP degradation in intact cells. Addition of caffeine (in the presence of adrenaline) slowed down the breakdown of cyclic AMP (Fig. 4) from a half-life of 7.3 min ($K_d = 0.094$) to a half-life of 9.3 min ($K_d = 0.071$). Assuming that the rate of cyclic AMP synthesis is unaffected by the phosphodiesterase inhibitor, it can be calculated

Table 2. Rates of cyclic AMP synthesis in intact cells stimulated by adrenaline

Erythrocytes were incubated with adrenaline at various concentrations. At 45 min incubation, propranolol was added to a final concentration of $50 \mu\text{M}$. Portions were withdrawn for cyclic AMP determination at 2 min intervals. K_d values are those shown in Table 1. The symbols of rate constants are described in the text.

[Adrenaline] (μM)	K_d (min^{-1})	P_0 (pmol/ml of packed cells)	S (pmol/min per ml of packed cells)
—	0.114*	37.5	4.25
2	0.127	237.5	30.10
10	0.126	395.0	49.80
50	0.114	400.0	45.60

* Mean value obtained with adrenaline.

Fig. 4. Decay of intracellular cyclic AMP after the addition of propranolol to erythrocytes incubated with adrenaline and caffeine

Erythrocytes were incubated with $10 \mu\text{M}$ -adrenaline in the absence (▲) or presence (●) of $5 \mu\text{M}$ -caffeine. At 45 min incubation, propranolol was added to a final concentration of $50 \mu\text{M}$. The concentration of cyclic AMP before the addition of propranolol was 387 pmol/ml of packed cells in the absence of caffeine and 515 pmol/ml of packed cells in the presence of caffeine.

from eqn. (5) that the steady-state concentration of the nucleotide should increase by 32% . This agrees well with the observed 33% increase in the concentration of cyclic AMP in erythrocytes incubated with caffeine.

Discussion

In the present study, the rate of cyclic AMP turnover in intact chicken erythrocytes was quantitatively measured. Turnover rate was calculated on the basis of measurements of intracellular cyclic AMP degradation and efflux during return of its concentration from an adrenaline-stimulated value toward the basal (*i.e.* without hormone) value.

Chicken erythrocytes are able to release cyclic AMP to outer medium under certain conditions (E. Gorin & S. Dickbuch, unpublished work). However, this mechanism does not contribute significantly to the disposal of the excessive amount of cyclic AMP when a transition from high to low cyclic AMP concentration occurs in the cell (Fig. 1). Therefore the fall in cyclic AMP concentration from hormone-stimulated steady-state value to the basal value is mainly due to its intracellular degradation.

The intracellular breakdown of cyclic AMP under these conditions follows a first-order reaction pattern with an apparent half-life of $6 \text{ min } 5 \text{ s} \pm 32 \text{ s}$. This exponential decay of the intracellular cyclic AMP is probably the sum of the different phosphodiesterase activities present in the cell. The first-order kinetics of cyclic AMP degradation implies that the intracellular concentration is probably below the K_m of phosphodiesterase. Values for the K_m of phos-

phodiesterases in chicken erythrocytes are unfortunately not yet available.

All the measurements of cyclic AMP metabolism in this study were done after the addition of propranolol to the incubation medium, and thus adenylate cyclase activity was inhibited. However, propranolol blocks hormone-stimulated adenylate cyclase activity only and does not affect the basal enzyme activity. In calculating the half-life of cyclic AMP, its basal synthesis was not taken into consideration. Under the experimental conditions used, adrenaline increased the steady-state concentration of cyclic AMP by 10-fold. Since the change in the steady-state concentration of cyclic AMP is proportional to its rate of synthesis, we assumed that propranolol inhibits 90% of the synthetic activity; therefore, the error in the determination of the half-life of cyclic AMP cannot be greater than 10%.

Incubation of erythrocytes with the phosphodiesterase inhibitor caffeine results in a slower cyclic AMP-degradation rate and about 30% higher half-life.

Although quantitative information on cyclic AMP turnover *in situ* might be crucial for better understanding of its physiological role, very little information is available in the literature on this aspect.

A half-life of 1 min 30 s–3 min 45 s was reported for cyclic AMP in thyroid slices (Swillens *et al.*, 1976; Van Sande *et al.*, 1977). This value is somewhat lower than that obtained here. The difference in the half-lives between chicken erythrocytes and thyroid might be due to the different methodology used to obtain the values or to different characteristics of the two tissues. That these two tissues indeed differ with respect to cyclic AMP metabolism is demonstrated by their highly dissimilar steady-state concentrations of the nucleotide under basal conditions, i.e. 0.05 μM and 1 μM for chicken erythrocytes and thyroid tissue respectively.

The rate of cyclic AMP synthesis in chicken erythrocytes, as deduced from its degradation and the steady-state concentration under basal conditions, is 4.25 pmol/min per ml of packed cells. Hence the pool of cyclic AMP under basal conditions should be renewed every 8–9 min.

The quantitative significance of cyclic AMP release under conditions of stimulated synthesis can be evaluated in the light of the turnover of the nucleotide *in situ*. During adrenaline stimulation, cyclic AMP synthesis in intact erythrocytes is about 2850 pmol/60 min per ml of packed cells, whereas cyclic AMP release at the same time is 187 pmol/60 min per ml of packed cells. These data clearly indicate that cyclic AMP released from hormone-stimulated cells constitutes only a small fraction (6%) of the total cyclic AMP synthesized.

It might thus be concluded that, although a significant amount of cyclic AMP appears in the outer

medium during its enhanced production, efflux of the cyclic nucleotide plays a minor role in the regulation of intracellular cyclic AMP. This is in accord with our previous conclusions (E. Gorin & S. Dickbush, unpublished work).

Release of cyclic AMP from intact cells to the surrounding medium under conditions of elevated concentrations of the intracellular cyclic nucleotide has been demonstrated in various eukaryotic cells (Davoren & Sutherland, 1963; Doore *et al.*, 1975; Penit *et al.*, 1974; Kelly *et al.*, 1974; Rindler *et al.*, 1978).

In avian erythrocytes, release of cyclic AMP ceases as soon as the enhanced synthesis of the nucleotide is inhibited. In contrast, in other cell types, such as W138 fibroblasts and glioma cells, concentration of released cyclic AMP exceed the intracellular values and efflux continues when the elevated concentration of intracellular cyclic AMP declines (Rindler *et al.*, 1978).

It might therefore be that in such cells a different mechanism of cyclic AMP release exists; its possible role in regulating intracellular nucleotide concentrations remains to be investigated.

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