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INVESTIGATION OF MITOCHONDRIAL PERMEABILITY FOR LABELED CYCLIC AMP

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The effect of cyclic AMP on various mitochondrial processes has recently been discovered [3] and has made the elucidation of the mechanisms of this effect an urgent task. One fact supporting the view that cyclic AMP can penetrate into mitochondria was activation of the matrix enzyme NAD-dependent isocitrate dehydrogenase by this nucleotide in the mitochondrion [4]. Data indicating that on incubation with mitochondria cyclic AMP binds with the mitochondrial transcription complex [7] have also been obtained. However, no adequate quantitative description of this phenomenon has yet been given and the distribution of labeled cyclic AMP within mitochondria has not been studied. The present investigation was devoted to an examination of these matters.

EXPERIMENTAL METHOD

Experiments were carried out on 16 Wistar rats. Mitochondria were isolated from the rat liver by the usual method of centrifugation of the postnuclear supernatant at 6000g in a sucrose medium of the following composition: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer, pH 7.4 (with one rinsing). The suspension of mitochondria (60-80 mg protein/ml) was incubated for 10 min at 30°C with cyclic AMP in a final concentration of $0.4 \cdot 10^{-6}$ M in the presence of 10 mM theophylline. These conditions are optimal for activation of mitochondrial

TABLE 1. Specific Activity of Submitochondrial Fraction (pmoles cyclic AMP/mg protein) after Incubation of Mitochondria and Mitoplasts with Cyclic AMP (M ± m)

Labeled compound	Incubation with mitochondria						Incubation with mitoplasts	
	whole mitochondria	fraction of mitochondria		Digitonin supernatant	mitoplasts of fraction		fraction	
		solubilized	membranes		solubilized	membranes	solubilized	membranes
^{14}C-cyclic AMP	1,0±0,36	2,7±0,65	0,69±0,08	1,4±0,12	0,40±0,11	0,36±0,12	0,91±0,50	0,12±0,04
^{3}H-cyclic AMP·10^{-1}	0,64±0,22	1,6±0,38	0,39±0,07	0,92±0,08	0,25±0,07	0,26±0,08	0,67±0,31	0.16±0,04

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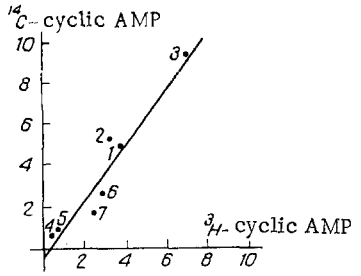

Fig. 1

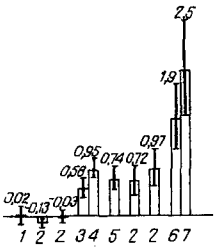

Fig. 2

Fig. 1. Correlation of accumulation of ^{3}H- and ^{14}C-cyclic AMP (in percent of total quantity of label in incubation medium) in different fractions of mitochondria. Abscissa, ^{3}H-cyclic AMP; ordinate, ^{14}C-cyclic AMP. 1, 2) Solubilized and membranous fractions of mitochondria, respectively; 3) digitonin supernatant; 4, 5) solubilized and membranous fractions, respectively, of digitonin residue after incubation with mitochondria; 6, 7) analogous fractions after incubation with mitoplasts. Regression line corresponds to equation: $y = -0.26 + 1.29X$.

Fig. 2. Comparison of theoretical and experimental dilution of radioactive label. Data shown as $\log_{10}$ of ratio of experimentally discovered label to theoretically calculated on the basis of dilution. Horizontal line corresponds to complete equality of these values (log of ratio = 0). 1) Incubation medium; 2) rinsing; 3, 4) solubilized and membranous fractions of mitochondria, respectively; 5) digitonin supernatant; 6, 7) solubilized and membranous fractions of digitonin residue, respectively.

respiration and of isocitrate dehydrogenase by cyclic AMP [2-4]. A double radioactive label was used in the experiments: 8-^{3}H cyclic AMP and adenine-U-^{14}C cyclic AMP (from the Radiochemical Centre, Amersham, England). The nucleotides were added in doses of 0.9 and 0.1 μCi, respectively; the final concentrations were 34 and 360 nM. The mitochondria, washed after incubation, were fractionated with digitonin as described in [8] to separate the outer membranes with the intermembranous space (digitonin supernatant) from the mitoplasts (digitonin residue). In another version of the experiment mitoplasts isolated from intact mitochondria were incubated with cyclic AMP under the same conditions as the mitochondria. Next, after preliminary washing, the mitoplasts were treated with 0.1% solution of Triton X-100 in sucrose medium for 10 min at 0°C and centrifuged for 30 min at 6000g. This supernatant is hereafter referred to as the solubilized fraction and the residue as the membrane fraction. Aliquots of incubated mitochondria were subjected to similar treatment. The membrane fractions were dissolved in 0.1% NaOH with heating. The radioactivity of the samples was measured on a Beckman (USA) LS-230 radiometer with ZhS-8 scintillation fluid.

EXPERIMENTAL RESULTS

As a result of incubation with radioactive cyclic AMP of both mitochondria and isolated mitoplasts, the label penetrated into these particles and was found in all the fractions tested (Table 1). The specific activities of both the digitonin and the Triton supernatants as a rule were significantly higher than those of the corresponding residues. This was evidently due to the fact that the label was much more readily solubilized than protein on treatment with detergents. Probably cyclic AMP does not bind firmly with protein.

Accumulation of ^{14}C-cyclic AMP was on average 14.5 times higher than that of ^{3}H-cyclic AMP. It must be remembered, however, that the molar concentration of the first label was also an order of magnitude higher. Consequently, the two labels accumulated almost equally. This is confirmed also by correlation analysis: $r = +1.0$ for specific activities and $r = +0.97$ for fractions of incorporated label ($P < 0.001$) (Fig. 1). Moreover, the regression line passes through the origin of coordinates ($a = -0.26 \pm 0.54$; $P > 0.5$) and at an angle of 52° ($b = 1.29 \pm 0.15$), which does not differ significatnly from the theoretical value of 45° ($b = 1.0$; $P < 0.1$), which ought to be found in the case of absolutely equal accumulation of the two labels. Such close correlation and the shape of the regression equation are evidence

the cyclic AMP molecule can penetrate into the mitochondrion. However, since both labels used are located in the adenine ring, all that these experiments proved directly was that this ring is penetrated. Although the mitochondria were carefully washed and the phosodiesterase inhibitor theophylline was added, further experiments are needed to identify the label as cyclic AMP.

To verify whether the label actually accumulates inside the mitochondria, the following approach was used. Expected concentrations of radioactivity were calculated, allowing for the dilutions used, and these were compared with those determined experimentally. The results are shown in Fig. 2 as the logarithm of the ratio of the experimental and expected concentrations. It will be clear that the logarithm of this ratio in the incubation medium and the first two washings did not differ from 0 ($P > 0.2$–0.4), i.e., that the ratio itself was 1. By contrast, in all other fractions the ratio significantly exceeded 1 ($P < 0.05$): in whole mitochondria and the digitonin supernatant by 0.6–1 order and in the digitonin residue by about 2 orders of magnitude. This shows that it is not simple dilution of the label but its actual accumulation which takes place in the mitochondria and, in particular, in the digitonin residue (in both the solubilized and the membranous fractions). Probably cyclic AMP is actively transported into the mitochondria, including into the inner membrane and the matrix. In this case it may directly or indirectly affect all the mitochondrial processes.

This provides the simplest explanation of our data on stimulation by cyclic AMP of the activity of the matrix enzyme isocitrate dehydrogenase [3, 4], of the internal membrane enzymes of the succinate and cytochrome oxidase systems [1], and of mitochondrial respiration [1–3]. It may also be supposed that extramitochondrial cyclic AMP is the physiological activator of the recently described cyclic AMP-dependent mitochondrial protein kinase [5, 6].

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