INVESTIGATION OF THE MECHANISM OF ACTION OF OUABAIN AND CYCLIC AMP OF THE TRANSPORT OF CALCIUM IONS BY RAT HEART MITOCHONDRIA

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UDC 616.22:547.22].015.4-:612.173.015.31

The action of ouabain and cyclic AMP on the Ca-accumulating capacity and outflow of Ca^{2+} ions from loaded rat heart mitochondria was studied by the tetracycline probe method. In the course of the investigations no effect of ouabain on these processes was found. Cyclic AMP did not act on Ca binding by the mitochondrial membrane but it induced rapid liberation of Ca^{2+} from organelles loaded with these ions.

KEY WORDS: *ouabain*; *cyclic AMP*; *calcium*; *mitochondria*.

Correlation between the positive inotropic effect of the cardiac glycosides and their ability to increase the Ca^{2+} concentration in the myoplasm through an increase in its penetration through the plasma membrane or its outflow from the intracellular membranous structures [9], or through both these processes, has repeatedly been demonstrated. More recently it has been shown that besides the sarcoplasmic reticulum, the mitochondria in the muscle cell may also play an important role in regulating the concentration of Ca^{2+} ions during the coupling of excitation with contraction [5].

The object of this investigation was to study the transport of Ca^{2+} ions by mitochondria of the rat heart under the influence of ouabain and of cyclic AMP, the latter being a possible agent mediating the effect of cardiac glycosides [8].

EXPERIMENTAL METHOD

Mitochondria were isolated from the myocardium of albino rats weighing 150-200 g [11] and their functional state was assessed on the basis of the respiratory control coefficient with succinate as the oxidation substrate. The preparations used in the experiments had a respiratory control coefficient of not less than 3. Ouabain and cyclic AMP were dissolved in the incubation medium and added to a suspension of mitochondria in a final concentration of between 10^{-9} and 10^{-4} M. Binding and liberation of Ca^{2+} ions by the inner mitochondrial membrane were investigated by the tetracycline probe method [6]. Fluorescence of the Ca-tetracycline complex was recorded on a modified ÉF-3MA fluorimeter with constant-temperature cuvette and inbuilt magnetic mixer, with wavelengths of exciting light of 380 nm and fluorescence of 540 nm. The mitochondria were incubated in medium containing 110 mM KCl, 10 mM Tris-HCl, and 10 μM $MgCl_2$, pH 7.2. The temperature in the cuvettes was 25°C. The protein concentration in the sample, determined by Lowry's method, was 2 mg/ml [1].

EXPERIMENTAL RESULTS AND DISCUSSION

Results reflecting the binding of Ca^{2+} ions by mitochondrial membranes of the heart are illustrated in Fig. 1. Addition of tetracycline to the system increased the intensity of fluorescence for 30 min. Increased fluorescence also was observed after the addition of antibiotics to a suspension of mitochondria incubated without succinate. Addition of Ca^{2+} in the absence of the oxidation substrate did not increase the degree of fluorescence, whereas in the presence of succinate the mitochondria bound about 80-100 nmoles Ca^{2+} per milligram of protein.

Department of Molecular Pharmacology, Medico-Biological Faculty, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR S. S. Debov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 83, No. 2, pp. 158-160, February, 1977. Original article submitted June 9, 1976.

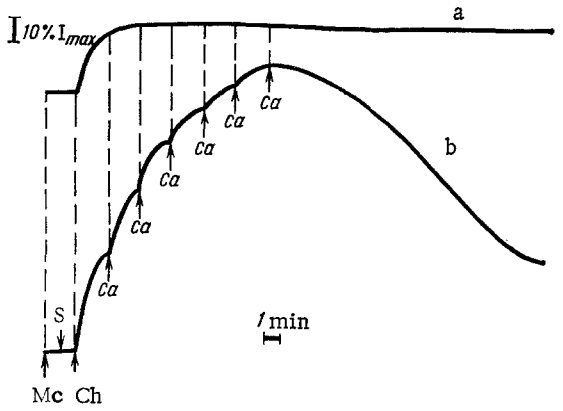

Fig. 1. Effect of succinate on change in fluorescence of Ca-tetracycline complex. Incubation medium: 110 mM KCl, 10 mM Tris-HCl, 10 μM $MgCl_2$, pH 7.2. Substances added: mitochondria (Mc) 2 mg/ml, succinate (S) 10 mM, chlortetracycline (Ch) 50 μg/ml, $CaCl_2$ (Ca) 20 μM. a) Change in fluorescence on incubation of mitochondria in medium without succinate; b) the same, in medium containing succinate.

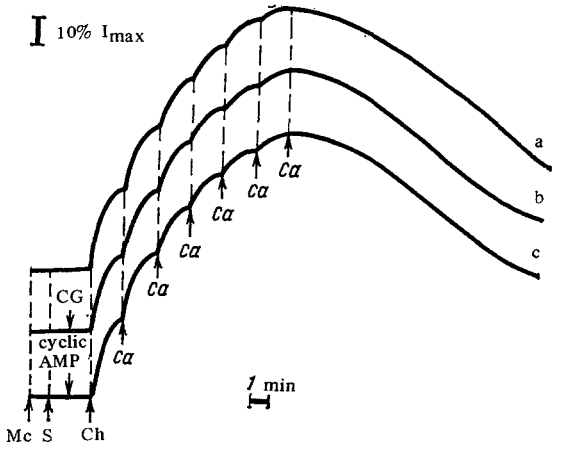

Fig. 2. Effect of ouabain and cyclic AMP on Ca-accumulating capacity of mitochondria: a) change in fluorescence in absence of preparation; b) the same in presence of oubain (CG) in final concentrations 10^{-9} to 10^{-4} M; c) the same in presence of cyclic AMP in final concentrations of between 10^{-9} and 10^{-4} M. Remainder of legend as in Fig. 1.

Ouabain and cyclic AMP did not affect the Ca-accumulating capacity of mitochondria from the rat heart (Fig. 2).

In the next series of experiments the effect of oubain and cyclic AMP on the outflow of Ca^{2+} from mitochondria previously loaded with them (20 nmoles Ca^{2+}/1 mg protein) was investigated. To rule out any possible harmful action of the additive itself, experiments were carried out in which Ca^{2+} was added 10 and 25 min after addition of the first portion. As Fig. 3 shows, the first addition of Ca^{2+} induced fluorescence, the degree of which persisted throughout the period of observation. The second addition of Ca^{2+} at the times specified induced a further increase in the intensity of fluorescence; the number of ions bound in these cases (80-100 nmoles/mg protein) was indistinguishable from that obtained previously. On the addition of ouabain (in concentrations of 10^{-9} to 10^{-4} M) to such a system no difference was found from

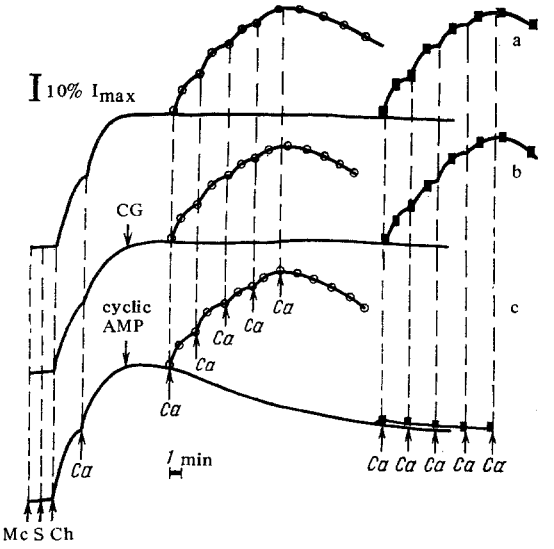

Fig. 3. Effect of ouabain and cyclic AMP on outflow of Ca ions from loaded mitochondria: a) change in fluorescence in absence of preparation; b) the same in presence of ouabain in final concentrations of 10^{-9} to 10^{-4} M; c) the same, in presence of cyclic AMP in final concentrations of 10^{-7} to 10^{-4} M. Continuous line: change in fluorescence in absence of repeated addition of Ca^{2+}; line with circles: the same, with repeated addition of Ca^{2+} 10 min after beginning of experiment; line with squares: the same, with repeated addition of Ca^{2+} 25 min after beginning of experiment. Remainder of legend as in Fig. 2.

the results of the control series of experiments. Meanwhile, after the addition of cyclic AMP in a concentration of between 10^{-7} and 10^{-4} M to the loaded mitochondria, the intensity of fluorescence was observed to diminish, indicating the outflow of Ca^{2+} ions from the mitochondrial membrane. Under these circumstances the mitochondria remained capable of taking up Ca for 10 min, and the addition of Ca 25 min later did not cause any increase in the intensity of fluorescence. These effects were not seen in the presence of 10^{-8} or 10^{-9} M cyclic AMP: The curves of the change in fluorescence corresponded exactly to the control curves and the Ca-accumulating capacity of the mitochondria was unchanged.

In the modern view, the accumulation of Ca^{2+} by the mitochondria takes place as a result of binding by the inner mitochondrial membrane, or in the presence of penetrating anions, through incorporation into the matrix [10]. The use of the tetracycline probe method and of medium free from penetrating anions enabled the first of these processes to be studied.

The data indicating that rat heart mitochondria can bind 800-100 nmoles Ca^{2+} per milligram protein agree with those published elsewhere [10]. The increase in the intensity of fluorescence on the addition of Ca^{2+} but in the presence of succinate only is evidence that the penetration of ions to the Ca-binding sites of the membrane is an energy-dependent process.

The fact that ouabain had no action on the Ca-binding capacity or on the outflow of Ca^{2+} ions from the loaded mitochondria suggests that the glycoside has no direct effect on these processes. Similar results were obtained in a radioisotope investigation of the effect of ouabain on Ca^{2+} transport by rat mitochondria [9]. However, the absence of an effect of the glycoside in vitro does not rule out the possibility of its indirect action. Cyclic AMP may act as intermediary agent in vivo. Ouabain is known to be capable of activating adenyl cyclase [8]. It has also been shown that the cyclic AMP level rises in systole and falls in diastole [4]. There is much evidence that cardiac glycosides can induce the discharge of catecholamines, which produce most of their effects by increasing the cyclic AMP concentration in the cell, from the tissue depots [2, 7]. In the present experiments cyclic AMP was

found to induce the liberation of Ca^{2+} ions bound by the mitochondrial membrane. Characteristically, on the addition of the nucleotide to loaded mitochondria, their ability to accumulate Ca^{2+} persisted for another 10 min. This suggests the absence of an effect on the binding process. This conclusion is also supported by the absence of any action of cyclic AMP on the Ca-accumulating activity of the mitochondria. It was shown previously that cyclic AMP can liberate Ca^{2+} also from the matrix of unloaded mitochondria without injury to the mechanisms of uptake [3].

Cardiac mitochondria in vitro can thus take up Ca^{2+} into the inner membrane and cyclic AMP can cause the outflow of the cation from the loaded organelles.

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EFFECT OF 3-ACETYLPYRIDINE ON ADRENOCORTICAL FUNCTION

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UDC 615.356:577.164.15.042.2].015.4:612.453

The corticosteroid concentration in the blood of rats was found to be lowered after injection of 3-acetylpyridine. In rats previously given 3-acetylpyridine, ACTH reduced the fall in the corticosteroid level in the blood and adrenal tissue compared with that in intact animals. Glucose-6-phosphate dehydrogenase activity in the adrenals was reduced after administration of 3-acetylpyridine. It is suggested that 3-acetylpyridine exerts its action at the adrenal level by inhibiting NADPH generation in dehydrogenase systems.

KEY WORDS: *3-acetylpyridine*; *corticosteroids*; *adrenals*.

The use of antivitamins in order to reproduce hypo- or avitaminosis is now being increasingly adopted as an experimental procedure. It does away with the need for keeping the animals on a special diet, and so rules out any effect of an unbalanced diet and abolishes the added stressor effect of starvation. The use of antivitamins is particularly promising in species of animals which are capable of endogenous synthesis of the corresponding vitamin. In particular, for the production of hypovitaminosis PP in rats, which can actively synthesize the coenzyme forms of the vitamin from tryptophan [8], more and more often nowadays 3-acetylpyridine (3-AP), an antivitamin PP, is being used [2, 5, 9].

Considering that, when interpreting the changes he has found, the investigator has often to differentiate between the effect of the vitamin deficiency itself and of the accompanying stress, it was interesting to examine how 3-AP affects adrenal function.

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(Presented by Academician of the Academy of Medical Sciences of the USSR N. A. Yudaev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 83, No. 2, pp. 161-162, February, 1977. Original article submitted April 19, 1976.