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THE ACTION OF EGTA ON THE CATECHOLAMINES STIMULATION OF RAT BRAIN Na-K-ATPase

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It has recently been reported that catecholamines increase the activity of the ouabain-sensitive Na-K-ATPase in rat brain homogenates (1). This increase has been interpreted as being the result of either a direct action of catecholamines on the enzyme or as a consequence of adenylate cyclase activation (2). In those experiments the incubation medium did not contain a Ca chelating agent which would have complexed the tissue calcium present in the solution. Since Ca inhibits Na-K-ATPase (3), we have designed experiments so as to determine whether the action of catecholamines on Na-K-ATPase is indirect and due to activation of a cellular Ca-sequestering mechanism sufficient to reduce the medium pCa. Therefore, the action of catecholamines has been studied in the presence of EGTA, a specific Ca complexing agent (4, 5, 6). The present experiments indicate that the action of catecholamines on Na-K-ATPase activity in rat brain homogenates is indirect and might be due to an activation of a Ca-pump.

METHODS

Wistar rats (± 200 g) were decapitated and the whole brain was rapidly removed. It was homogenized in 9 volumes ice-cold deionized water, the homogenate was diluted ten times before use.
The assay mixture was pre-incubated at 37°C in the absence of ATP-Tris for 10 min and the reaction was started by adding ATP-Tris solution. The final volume of the enzyme mixture was 1 ml and contained (unless otherwise stated) mM : NaCl 100, KCl 20, $MgCl_2$ 6, ATP-Tris 4, Tris-maleate 10, pH 7.4, brain diluted homogenate 200 µl (= 2 mg wet weight, 232 µg proteins).

The reaction was stopped after 10 minutes by trichloroacetic acid being added to make the final concentration 5%.
The enzymatic activity was estimated by determination of inorganic phosphates in the protein free supernatant by the method of Fiske and Subbarow (8). The protein concentration was determined by the biuret method.
The calcium content was measured by atomic absorption spectrometry; after mineralization in an oven at 500°C for 18 hr, the residue was dissolved in HCl 0.01 N before assay.
In some experiments the calcium concentration was buffered with EGTA (ethyleneglycol-bis(2aminoethylether)tetraacetic acid) and Ca-EGTA. The apparent stability constant K' for Ca-EGTA is not only dependent on pH and temperature (4,5) but also on the nature of the buffer (6). Under the present experimental conditions, K' was found to be $10^{5.9}$. In order to calculate ionic Ca^{2+} concentration, the Ca affinity for ATP and the presence of Mg were taken into account (7). Whenever possible, values are presented as means $\pm$ S.E. of mean. Significance of differences between means was checked by Student's t test.

RESULTS

Ca and catecholamines action

ATP hydrolysis by rat brain homogenates has been studied under various conditions. A typical experiment performed with one enzyme preparation is reported in table 1. Na-K-ATPase activity was estimated as the difference in P_i liberated in the absence and in the presence of ouabain 10^{-3}M which totally inhibits microsomial Na-K-ATPase (9). In the presence of noradrenaline (5×10^{-4}M) and of dopamine (10^{-3}M), there was an enhanced ATP hydrolysis ($P<0.01$) due to a large stimulation of Na-K-ATPase and a slight stimulation of ATP hydrolysis insensitive to ouabain ($P<0.01$). When EGTA 1mM was added to the incubation medium, ATP hydrolysis was enhanced ($P<0.01$). The increased hydrolysis was due to a stimulation of Na-K-ATPase and it was associated with a decrease of ouabain insensitive ATP hydrolysis ($P<0.01$). In the presence of EGTA 10^{-3}M, both noradrenaline and dopamine failed to stimulate further ATP hydrolysis.

TABLE I : Influence of Noradrenaline, Dopamine and EGTA on ATPase activities in rat brain homogenates. Enzymatic activities are expressed in μmoles P_i liberated mg protein^{-1} hr^{-1}. Na-K-ATPase activity was estimated as the difference between Total ATPase and ATPase in the presence of ouabain 10^{-3}M. The results shown are averages ± S.E. The number of determinations is given between brackets.

Treatment	Total ATPase	ATPase activity in the presence of ouabain	Na-K-ATPase
Controls	15.3 ± 0.6 (8)	10.7 ± 0.1 (8)	4.6
Noradrenaline 5×10^{-4} M	20.6 ± 0.1 (4)	12.0 ± 0.1 (4)	8.6
Dopamine 10^{-3} M	19.1 ± 0.4 (4)	11.2 ± 0.2 (4)	7.9
EGTA 10^{-3} M	17.9 ± 0.3 (8)	9.2 ± 0.1 (8)	8.7
EGTA 10^{-3} M + Noradrenaline 5×10^{-4} M	18.7 ± 0.3 (4)	9.3 ± 0.2 (4)	9.4
EGTA 10^{-3}M + Dopamine 10^{-3} M	17.4 ± 0.4 (4)	9.3 ± 0.2 (4)	8.1

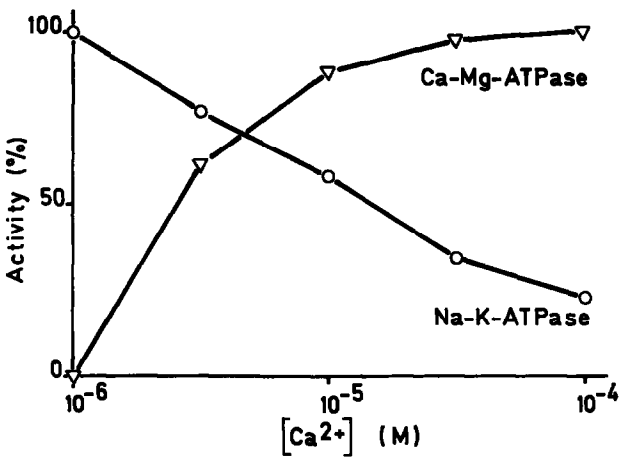

FIG. 1 : Na-K-ATPase inhibition and Ca-Mg ATPase activation in rat brain homogenate as a function of calcium concentration. ATPase activity was assayed as described under Methods. Values are mean of 4 determinations. Na-K-ATPase is defined as the ouabain sensitive activity under all conditions. Per cent inhibition of Na-K-ATPase is expressed relative to ouabain-sensitive ATPase in the absence of calcium. Per cent activation of Ca-Mg-ATPase is expressed relative to maximal Ca-Mg-ATPase activation. The limits of S.E. dit not exceed the diameter of the symbol.

Since these experiments indicated that the presence of calcium ions is necessary for catecholamine activation of Na-K-ATPase, it is tempting to suggest that catecholamines stimulated a cell process controlling the pCa in the homogenate. In order to determine whether the calcium contamination was sufficient to inhibit Na-K-ATPase to the level observed in control conditions, ATP hydrolysis sensitive to ouabain has been measured in the presence of Ca-EGTA buffers. As shown in fig.1, Na-K-ATPase activity was progressively reduced by increasing ionic Ca^{2+} concentration. In the preparation of the experiment illustrated in fig.1, Na-K-ATPase activity in the absence of EGTA was equal to 3 µmole P_i $mg^{-1}hr^{-1}$ and it was equal to 4.5 in the presence of EGTA 1mM. There was a 33% inhibition of the enzyme in the absence of EGTA. The calcium concentration in this mixture was equal to 5.2×10^{-6}M. As shown in fig. 1, a controlled ionic Ca^{2+} concentration equal to 5×10^{-6} M evoked a 30 percent inhibition of Na-K-ATPase, an observation suggesting that contaminant calcium was responsible for the inhibition in the ab-

sence of EGTA.

ATP hydrolysis insensitive to ouabain was increased by increasing the calcium concentration of the medium, the activation was maximum for a concentration equal to 10^{-4} M (fig.1).

Noradrenaline dose-effect curve

As shown in fig. 2, the noradrenaline activation of Na-K-ATPase was dose-dependent. The threshold concentration was near 10^{-7}M and the maximum effect was obtained with noradrenaline 10^{-4}M; noradrenaline 10^{-3}M evoked a lower stimulation than noradrenaline 10^{-4}M. Noradrenaline ED_{50} for activation was equal to 6 x 10^{-6}M. The noradrenaline activation of the Ca-sensitive, ouabain insensitive ATP hydrolysis, was also dose-dependent. The threshold concentration of noradrenaline was 10^{-5}M, noradrenaline 10^{-3}M evoked a higher activation than noradrenaline 10^{-4}M.

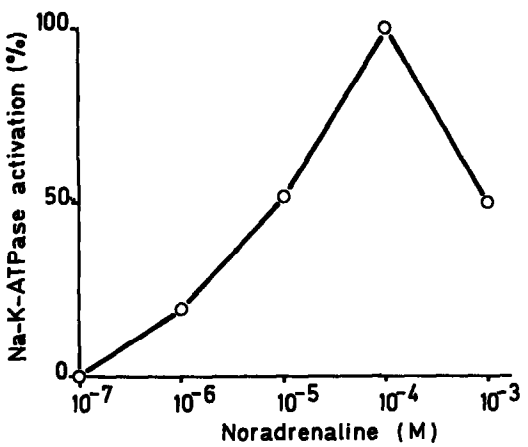

FIG. 2 : Na-K-ATPase activation in rat brain homogenate as a function of noradrenaline concentration. Na-K-ATPase is defined as the ouabain-sensitive activity assayed as described under Methods. Per cent activation is expressed relative to maximal stimulation in the presence of noradrenaline 10^{-4} M. Each point is the mean of 3 determinations. The limits of S.E. did not exceed the diameter of the symbol.

DISCUSSION

The present experiments confirm that catecholamines stimulate Na-K-ATPase in rat brain homogenates. This stimulation did not take place in the absence of free Ca^{2+} and the maximal stimulation evoked by catecholamines was of the same magnitude as the stimulation observed in the presence of EGTA 1mM. This indicates that the action of catecholamines was indirect and that the stimulation of Na-K-ATPase was likely due to the suppression of the inhibition exerted by calcium on this enzyme.

It was also observed that catecholamines activated the ouabain insensitive ATPase activity; this stimulation did not occur in the presence of EGTA. Ouabain insensitive ATPase activity was reduced in the presence of EGTA and was increased by increasing pCa, an indication for the existence of a Ca-Mg-ATPase already found in brain microsomes (10). This Ca-Mg-ATPase was stimulated by catecholamines but apparently at higher dosages than needed for the stimulation of Na-K-ATPase.

It has been proposed that catecholamines may act by chelating divalent cations (11). In the present experiments, such an action might be excluded since Ca-Mg-ATPase was stimulated by catecholamines and inhibited by EGTA. The receptors responsible for catecholamines activation of Na-K-ATPase need to be identified. It is unlikely that beta adrenergic receptors could be involved : the addition of cyclic AMP does not evoke a significant stimulation of Na-K-ATPase (1) and adenylate cyclase is reduced in the presence of EGTA (12).

In conclusion, the present experiments indicate that the catecholamines stimulation of Na-K-ATPase in rat brain homogenate is an indirect process probably mediated by the stimulation of a cellular calcium sequestering mechanism.

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