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Effects of Inhibitors of (Na^+ + K^+)-Dependent Adenosine Triphosphatase on the Uptake of Norepinephrine by Synaptosomes

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SUMMARY

The effects of various (Na^+ + K^+)-dependent adenosine triphosphatase inhibitors on synaptosomal adenosine triphosphatase activity and norepinephrine uptake were examined under similar conditions. Ouabain (1 mM) and oligomycin (10 μg/ml) inhibited both synaptosomal adenosine triphosphatase activity and norepinephrine uptake. However, strophanthidin (1 mM) and lower concentrations of ouabain (0.1 and 0.01 mM) inhibited adenosine triphosphatase activity to the same extent as 1 mM ouabain but did not depress the uptake of norepinephrine by synaptosomes. Thus, in these circumstances, the capacity of synaptosomes to accumulate norepinephrine is not correlated with their (Na^+ + K^+)-dependent adenosine triphosphatase activity. It also appears that ouabain can inhibit norepinephrine uptake by a mechanism which does not involve (Na^+ + K^+)-dependent adenosine triphosphatase.

INTRODUCTION

The uptake of norepinephrine by isolated nerve endings (synaptosomes) requires physiological concentrations of Na^+ and K^+ in the incubation medium (1), is inhibited by metabolic inhibitors (2), and is depressed by ouabain (3). These requirements for norepinephrine uptake are very similar to those for (Na^+ + K^+)-ATPase activity, and Bogdanski *et al.* (4) have postulated that the energy for transport of norepinephrine into synaptosomes is derived from the inward-directed Na^+ concentration gradient across the synaptosomal membrane, which is maintained by (Na^+ + K^+)-ATPase.

Work in this laboratory, however, has recently demonstrated that an inward-directed Na^+ concentration gradient and/or an outward-directed K^+ concentration gradient cannot alone supply the energy for the uptake of norepinephrine by synaptosomes (2). The question therefore arises whether (Na^+ + K^+)-ATPase might be involved in some other way in the transport of norepinephrine across the synaptosomal membrane.

In the present study, sets of parallel experiments were performed to examine the effects of various (Na^+ + K^+)-ATPase inhibitors on the ATPase activity of synaptosomes and on their ability to accumulate [^{3}H]norepinephrine, in order to determine whether there is a correlation between ATPase activity and uptake capacity.

METHODS

General methods. Ouabain (octahydrate), oligomycin (15% oligomycin A and 85% oligomycin B), strophanthidin, and ATP were purchased from the Sigma Chemical

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Company, Ltd., London. DL-[7-^{3}H]Norepinephrine (5 Ci/mmole) was obtained from the Radiochemical Centre, Amersham. It was periodically assayed for purity by chromatography as described by Roberts (5). Protein was determined by the method of Lowry *et al.* (6).

Preparation of synaptosomes. Synaptosomes were prepared from the brains of four male Wistar rats, Porton strain, 200–250 g, as described previously (2). Each of the synaptosomal fractions from four Ficoll density gradients was diluted with 10 ml of an incubation medium which contained Na^+, 120 mM; K^+, 5 mM; Mg^{2+}, 3 mM; Ca^{2+}, 2.5 mM; Cl^-, 136 mM; and Tris-HCl (pH 7.4), 20 mM. The material was centrifuged at 13,000 × *g* for 20 min; each pellet was gently resuspended with 10 ml of the incubation medium, and the suspension was centrifuged at 13,000 × *g* for 10 min. The washed synaptosomal pellets were finally resuspended with a total of 2 ml of cold incubation medium.

Determination of [^{3}H]norepinephrine uptake by synaptosomes. Fifty microliters of synaptosomal suspension were added to a centrifuge tube containing 0.7 ml of ice-cold incubation medium, together with inhibitors where appropriate, and incubated for 3 min at 37° in a Dubnoff shaking incubator, following which 0.01 ml of [^{3}H]norepinephrine (150 μCi/ml) was added. The incubation was stopped by removing the tubes to ice and immediately centrifuging them at 8500 × *g* for 3 min. Each pellet was allowed to drain for 30 min, and the insides of the tubes were carefully wiped dry.

[^{3}H]Norepinephrine was extracted from the pellet with 0.5 ml of 0.4 N $HClO_4$ for 90 min, the tube was centrifuged, and 0.2 ml of the supernatant fluid was assayed for radioactivity by liquid scintillation spectrometry. The counts per minute per milligram of protein were calculated, and the zero time values were subtracted to give the amount of [^{3}H]norepinephrine taken up during the 15-min incubation period. Previous experiments have shown that total radioactivity in the pellet is a valid measure of [^{3}H]norepinephrine uptake (2).

Determination of synaptosomal ATPase activity. Synaptosomes were incubated as described above, except that the incubation medium contained 3 mM ATP (disodium salt, adjusted to pH 7.4 with Tris base) and the [^{3}H]norepinephrine was replaced by an equivalent amount of nonradioactive DL-norepinephrine. The incubation was terminated by the addition of 0.20 ml of 12.5% trichloracetic acid, and the contents of the tube were mixed and centrifuged at 8500 × *g* for 3 min. An aliquot (0.5 ml) of the supernatant was added to 3.8 ml of distilled water, and the inorganic phosphate present was determined by the method of Fiske and SubbaRow (7). The quantities (micromoles) of P_i released per milligram of synaptosomal protein were calculated, and the zero time values were subtracted to give micromoles of P_i released from ATP per milligram of protein during the 15-min incubation.

Incubation media containing (Na^+ + K^+)-ATPase inhibitors. Incubation media containing cardioactive steroids and oligomycin were prepared by dissolving a suitable amount of each substance in 0.20 ml of 95% ethanol and then diluting this to 10 ml with incubation medium. A control medium containing ethanol without inhibitor was also prepared. This amount of ethanol was shown to have no effect on either synaptosomal ATPase activity or [^{3}H]norepinephrine uptake.

RESULTS

Effect of (Na^+ + K^+)-ATPase inhibitors on [^{3}H]norepinephrine uptake by synaptosomes. Table 1 shows the effect of various inhibitors of (Na^+ + K^+)-ATPase on [^{3}H]norepinephrine accumulation by synaptosomes. Ouabain (1 mM) and oligomycin (10 μg/ml) depressed uptake; strophanthidin (1 mM), on the other hand, had no effect.

Effect of (Na^+ + K^+)-ATPase inhibitors on synaptosomal ATPase activity. Figure 1 shows that only a small amount of P_i was released from ATP during the 3-min incubation before the addition of norepinephrine. Most of the ATP hydrolysis occurred during the 15-min incubation, so that this ATPase activity can be directly compared with the uptake of [^{3}H]norepinephrine by synaptosomes shown in Table 1.

In the presence of 1 mM ouabain, 1 mM

TABLE 1

Effect of ($Na^+ + K^+$)-ATPase inhibitors on [^3H]norepinephrine uptake by synaptosomes

The synaptosomes were prepared and incubated as described in METHODS. Each value is the mean of six determinations (three experiments).

Incubation medium	[^{3}H]Norepinephrine accumulated during 15-min incubation	Per cent of control	Significance of difference from control by *F*-test
	cpm/mg protein (± *SE*)	%	
Control	21,810 ± 1,440	100	
Oligomycin (10 μg/ml)	16,960 ± 922	77.8	$p < 0.02$
Ouabain (1 mM)	15,820 ± 2,130	72.6	$p < 0.05$
Strophanthidin (1 mM)	23,010 ± 2,000	105.5	N.S.

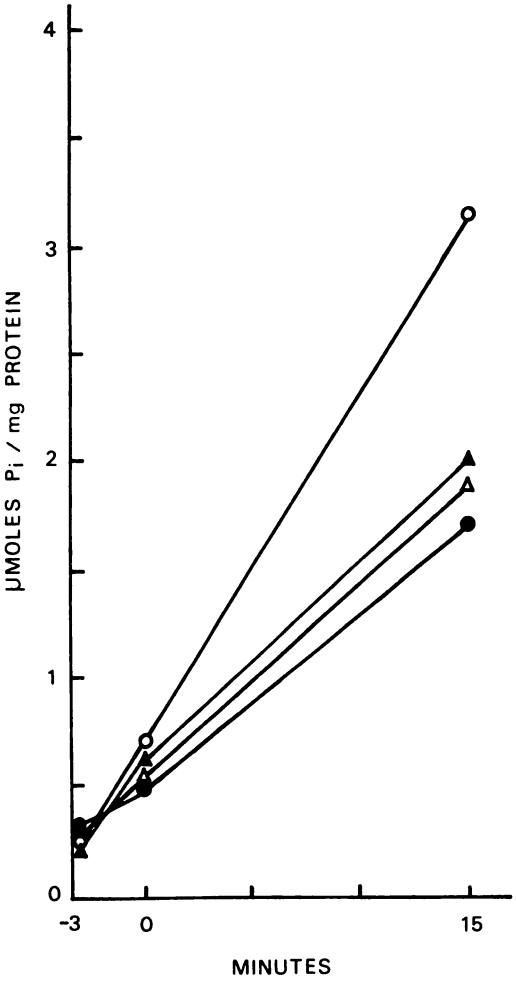

FIG. 1. *Effect of ($Na^+ + K^+$)-ATPase inhibitors on synaptosomal ATPase activity*

The synaptosomes were prepared and incubated as described in METHODS. ○, control medium; ●, 10 μg/ml of oligomycin; △, 1 mM ouabain; ▲, 1 mM strophanthidin. Each point is the mean of five determinations (three experiments).

strophanthidin, or 10 μg/ml of oligomycin, synaptosomal ATPase activity was reduced (Table 2). Strophanthidin (1 mM) inhibited ATPase activity to the same extent as did 1 mM ouabain, even though these drugs had different effects on [^{3}H]norepinephrine uptake by synaptosomes (Table 1).

Effect of various concentrations of ouabain and strophanthidin on synaptosomal ATPase activity and [^3H]norepinephrine uptake. If ouabain inhibits norepinephrine transport into synaptosomes by inhibiting ($Na^+ + K^+$)-ATPase, one would expect there to be a correlation between ATPase activity and norepinephrine uptake at various ouabain concentrations. Therefore the synaptosomal ATPase activity and [^{3}H]norepinephrine uptake were determined at several concentrations of ouabain. The results of these experiments are shown in Tables 3 and 4.

As little as 0.01 mM ouabain and 0.1 mM strophanthidin inhibited ATPase activity to the same extent as did 1 mM ouabain and 1 mM strophanthidin (Table 3). However, 0.1 and 0.01 mM ouabain had no effect on [^{3}H]norepinephrine uptake by synaptosomes (Table 4). Still lower concentrations of ouabain (1 and 0.1 μM) appeared to increase the uptake of [^{3}H]norepinephrine, but this increase was not statistically significant (Table 4).

DISCUSSION

The uptake of norepinephrine by synaptosomes is depressed in the presence of metabolic inhibitors (2), suggesting that ATP is required for the uptake process. Moreover, uptake is virtually abolished in the absence of K^+ (1, 2) and is reduced in

TABLE 2

Effect of ($Na^+ + K^+$)-ATPase inhibitors on synaptosomal ATPase activity

The synaptosomes were prepared and incubated as described in METHODS. Each value is the mean of five determinations (three experiments).

Incubation medium	ATPase activity	Per cent of control	Significance of difference from control by *F*-test
	μmoles P_i released/mg protein/15 min (± *SE*)	%	
Control	2.46 ± 0.183	100	
Oligomycin (10 μg/ml)	1.21 ± 0.049	49.3	$p < 0.001$
Ouabain (1 mM)	1.34 ± 0.083	54.7	$p < 0.001$
Strophanthidin (1 mM)	1.38 ± 0.199	56.1	$p < 0.01$

TABLE 3

Effect of various concentrations of ($Na^+ + K^+$)-ATPase inhibitors on synaptosomal ATPase activity

The synaptosomes were prepared and incubated as described in METHODS. Each value is the mean of four determinations (two experiments).

Incubation medium	ATPase activity	Per cent of control	Significance of difference from control by *F*-test
	μmoles P_i released/mg protein/15 min (± *SE*)	%	
Control	1.83 ± 0.116	100	
Ouabain (1 mM)	1.18 ± 0.073	64.4	$p < 0.01$
Ouabain (0.1 mM)	1.22 ± 0.089	66.8	$p < 0.01$
Ouabain (0.01 mM)	1.28 ± 0.080	70.1	$p < 0.01$
Strophanthidin (1 mM)	1.16 ± 0.056	63.4	$p < 0.01$
Strophanthidin (0.1 mM)	1.12 ± 0.172	61.1	$p < 0.02$

TABLE 4

Effect of various concentrations of ouabain on [^3H]norepinephrine uptake by synaptosomes

The synaptosomes were prepared and incubated as described in METHODS. Each value is the mean of six determinations (three experiments).

Incubation medium	[^{3}H]Norepinephrine accumulated during 15-min incubation	Per cent of control	Significance of difference from control by *F*-test
	cpm/mg protein (± *SE*)	%	
Control	13,800 ± 611	100	
Ouabain (1 mM)	10,900 ± 544	79.4	$p < 0.02$
Ouabain (100 μM)	13,800 ± 511	100	N.S.
Ouabain (10 μM)	13,800 ± 490	100	N.S.
Ouabain (1 μM)	15,400 ± 763	112	N.S.
Ouabain (0.1 μM)	15,300 ± 856	111	N.S.

the presence of ouabain (3), implying that ($Na^+ + K^+$)-ATPase may be involved in the transport process. Bogdanski *et al.* (4) have suggested that the energy for the transport of norepinephrine into synaptosomes is derived from an inward-directed Na^+ concentration gradient across the synaptosomal membrane, which is maintained by ($Na^+ + K^+$)-ATPase.

The results of recent experiments, in which the internal and external ion concentrations of synaptosomes were altered

and the synaptosomes then were incubated in the presence and absence of metabolic inhibitors, indicate that ion gradients do not provide the energy for norepinephrine uptake (2). If ion gradients are not important, the role, if any, of $(Na^+ + K^+)$-ATPase in the norepinephrine transport system remains to be determined.

In the present investigations an attempt has been made to determine the correlation between synaptosomal ATPase activity and [^{3}H]norepinephrine uptake in the presence of various $(Na^+ + K^+)$-ATPase inhibitors.

[^{3}H]Norepinephrine uptake occurs only into intact synaptosomes. Hence it is imperative that ATPase activity also be measured in intact synaptosomes, since the effect of inhibitors on uptake might be governed by their ability to penetrate the synaptosomal membrane. It is also important to use the same incubation medium in both types of experiment, since ATPase activity and the action of inhibitors are known to be affected by the concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} in the medium. The necessity of examining ATPase activity and [^{3}H]-norepinephrine uptake under identical conditions gives rise to two problems.

1. ATP added to the incubation medium is outside the synaptosome. Caldwell *et al.* (8) have shown that squid axons can only hydrolyze intracellular ATP and that exogenous ATP does not penetrate the cell membrane. However, results of our own preliminary experiments and those of Abdel-Latif *et al.* (9, 10) and Tissari *et al.* (11) show that synaptosomal ATPase activity is only slightly increased by rupturing the synaptosomes, indicating that synaptosomes can hydrolyze exogenous ATP. Whether the ATP is hydrolyzed on the outside of the membrane or penetrates the membrane before it is hydrolyzed is not known. Perhaps this apparent peculiarity in ATP metabolism represents a special modification of the neuron at its synapse.

2. Synaptosome membranes contain two types of ATPase: an Mg^{2+}-ATPase and an $(Na^+ + K^+)$-ATPase (12). Under the conditions usually employed for ATPase assays, the activity of each of these two enzymes can be measured by removing the appropriate ions from the incubation medium. This cannot be done in an intact synaptosomal preparation, since it is obviously impossible to remove the ions contained within the synaptosomes. Hence, in the work reported here, $(Na^+ + K^+)$-ATPase has been estimated as that part of the total ATPase activity which is inhibited by 1 mM ouabain, as suggested by Albers (13). This concentration of ouabain inhibited ATPase activity by 40–50% (Tables 2 and 3), which is the proportion of total synaptosomal ATPase activity which Abdel-Latif *et al.* (9) found to be due to $(Na^+ + K^+)$-ATPase.

Comparison of Tables 1–4 shows that while 1 mM ouabain and 10 μg/ml of oligomycin inhibited both ATPase activity and norepinephrine uptake, strophanthidin (1 mM) and lower concentrations of ouabain (0.1 and 0.01 mM) inhibited ATPase activity to the same extent as 1 mM ouabain, but did not depress norepinephrine uptake. These findings must cast some doubt on the involvement of $(Na^+ + K^+)$-ATPase in the uptake of norepinephrine by synaptosomes. A similar lack of correlation between the extent to which cardioactive steroids inhibit ATPase activity, on one hand, and the active transport of amino acids, on the other, has been reported in rat diaphragm (14) and the isolated intestine (15).

The finding that ouabain can inhibit synaptosomal norepinephrine uptake by some mechanism other than $(Na^+ + K^+)$-ATPase means that inhibition of a transport process by ouabain cannot be taken as conclusive evidence of $(Na^+ + K^+)$-ATPase involvement. Experiments to test the ion gradient hypothesis for norepinephrine uptake by synaptosomes by more direct methods have failed to confirm the hypothesis (2). Insofar as these studies overlap, they agree with the findings of Tissari *et al.* (11), who have recently shown that the uptake of norepinephrine, unlike that of 5-hydroxytryptamine, cannot be restored when Na^+ is added to synaptosomes which have previously been incubated with ouabain in the absence of Na^+. Under these conditions, an inward-directed Na^+ concentration gradient, independent of $(Na^+ + K^+)$-ATPase activity, should exist, and yet this preparation did not accumulate norepinephrine.

Oligomycin is known to inhibit mito-

chondrial ATP synthesis (16) as well as (Na^+ + K^+)-ATPase, and it may be this reduction of intrasynaptosomal ATP which depresses norepinephrine uptake.

Strophanthidin, which did not inhibit norepinephrine uptake, is an aglycone and may not penetrate membranes as readily as the glycoside, ouabain (17). Perhaps access to the ouabain-sensitive component of norepinephrine transport is unavailable to aglycones such as strophanthidin. A more thorough investigation of the mechanism of cardioactive steroid inhibition of norepinephrine uptake by synaptosomes is now in progress.

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