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THE EFFECT OF CATECHOLAMINES ON TRANSPORT (Na,K) ADENOSINE TRIPHOSPHATASE*

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Abstract—The effects of norepinephrine and dopamine were investigated on NaK-ATPase (ATP phosphohydrolase EC 3.6.1.3) activity from a beef brain microsomal preparation. These catecholamines can produce a 1.5-fold activation in the NaK-ATPase activity. The catecholamines exert their effect by reversal of divalent metal inhibition of NaK-ATPase activity. The physiologically important divalent metals, Cu^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{2+} are well-known inhibitors of NaK-ATPase. Norepinephrine and dopamine could reverse only the inhibitory effects of Fe^{2+}. A model system was devised wherein the effects of several catecholamines and related compounds were tested for their ability to reverse the Fe^{2+} inhibition of NaK-ATPase activity. Effective agents must have an ethylamine side chain and two hydroxyl groups on the phenyl ring to completely reverse the inhibitory effects of Fe^{2+} on NaK-ATPase activity. The order of efficacy is isoproterenol > epinephrine = norepinephrine > phenylephrine. Propranolol can block the effect of isoproterenol. No effect of cyclic AMP either in the presence or absence of theophylline was observed.

Since 1957, when Skou [1] first demonstrated the presence of an adenosine triphosphatase in crab nerve that could be stimulated by Na and K [ATP phosphohydrolase, EC 3.6.1.3. (NaK-ATPase)], a great deal of information has accumulated on the mechanism of action of this enzyme and on conditions that modify its activity (for recent reviews, see Refs 2–4). All available data support the contention that this enzyme is part of the sodium pump which regulates the Na and K content of animal cells. Recently, several workers have shown that certain biogenic amines can modify NaK-ATPase activity *in vitro* [5–13].

In 1970, Herd *et al.* [5] reported that the NaK-ATPase from brown adipose tissue could be maximally stimulated with 6 mM norepinephrine and that this effect could be blocked by low doses of propranolol (4 μM). Subsequently other workers [6–8] using crude preparations of NaK-ATPase from rat and cat brain reported the stimulation of NaK-ATPase activity by catecholamines. Schaefer *et al.* [6] reported the requirement of a soluble, heat stable, dialyzable factor for the stimulation by norepinephrine. Schaefer *et al.* [9] also observed that tetrabenazine could mimic the effects of dopamine, another stimulatory catecholamine, and that chlorpromazine could antagonize the effect of dopamine. Clausen and Formby [10] and Schaefer *et al.* [11] have shown that the K activated phosphatase which is believed to be part of the NaK-ATPase [2–4] is also stimulated by norepinephrine. Tria *et al.* [12] and Glebov and Dmitrieva [13], however, have reported that epinephrine and norepinephrine are inhibitors of NaK-ATPase.

The mechanism by which catecholamines increase NaK-ATPase activity is unclear. However, it is well known that NaK-ATPase activity is inhibited *in vitro* by such biologically important divalent metal ions as Ca^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} [14, 15]. Furthermore it is known that catecholamines can form stable complexes with these metals [16, 17]. Therefore, in consideration of the above reported phenomena, one possible explanation for the activating effect of catecholamines on NaK-ATPase activity is the relief of divalent metal inhibition through the formation of a complex. Indeed Schaefer *et al.* [11] have shown in a reconstituted system that the activating effect of catecholamines on NaK-ATPase activity requires ascorbic acid and a metal, possibly Fe^{2+}.

The purpose of this paper is to investigate the activating effects of catecholamines on NaK-ATPase activity from beef cerebral cortex microsomes, by determining: (a) the requirement for additional factors if any; (b) the structural features of catecholamines required for activation of the NaK-ATPase; (c) the involvement of cyclic 3′,5′-AMP in the activation; and (d) whether any adrenergic blockers can antagonize the effects of the catecholamines.

MATERIALS AND METHODS†

Microsomes containing NaK-ATPase activity were prepared from beef cerebral cortex as described previously [18]. The microsomes were treated with high concentrations of sodium iodide essentially as described by Nakao *et al.* [19]. Magnesium chloride was omitted from the sodium iodide solution without any effect on purification. The NaI treated microsomes (NaI-enzyme) had a sp. act. ranging from 50–70 μmoles P_i/mg protein/hr.

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† Abbreviations used are: NE, norepinephrine; DOPA, dihydroxyphenylalanine; and DA, dopamine.

NaK-ATPase activity was determined by measuring the amount of P_i produced in 5 min at 37° under the appropriate assay conditions. Each reaction tube contained in a final vol. of 1.0 ml: 30 mM imidazole-HCl, pH 7.0; 130 mM NaCl; 20 mM KCl; 8 mM $MgCl_2$, 4 mM Na_2ATP and 30–40 μg of NaI-enzyme protein. The medium for measuring Mg-ATPase activity was the same except for the addition of ouabain to a final concentration of 0.1 mM. After all additions except enzyme were made, the reaction tubes were incubated at 37° for 3 min in a shaking water bath. The reaction was initiated with enzyme and terminated after 5 min by the addition of 1 ml of 5% trichloroacetic acid in chloroform–methanol (1:1). After vigorous mixing, the tubes were centrifuged for 10 min at 1500 rev/min. The P_i in the upper phase was measured using a modified Fiske-SubbaRow method [20]. NaK-ATPase activity (ouabain-sensitive) was determined by subtracting the amount of P_i produced in the presence of ouabain from the amount of P_i produced in the absence of ouabain. All determinations were made in duplicate. Protein was measured according to the method of Lowry *et al.* [21] using bovine serum albumin, Cohn Fraction V, as the standard.

All chemicals were of the highest grade commercially available and were used without further purification. $FeCl_2$, $CuCl_2$, and $Zn(C_2H_3O_2)_2$ solutions (see Ref 18) were freshly prepared. The drugs used in this study were *d*-norepinephrine *d*-bitartrate and *d*-isoproterenol HCl (Sterling-Winthrop); *d,l*-propranolol HCl (Ayerst); dopamine HCl and *l*-norepinephrine *d*-bitartrate (Schwarz-Mann); phentolamine mesylate (Ciba); and *l*-epinephrine *d*-bitartrate, *l*-DOPA, *l*-phenylephrine HCl, tyramine HCl, *l*-tyrosine HCl, catechol, β-phenylethylamine, *d,l*-3-methoxy,4-hydroxymandelic acid, *d,l*-normetanephrine, dihydroxyphenylacetic acid, and *d,l*-3,4-dihydroxymandelic acid (Sigma). To prevent auto-oxidation each drug was freshly prepared as a 5 mM solution in 5×10^{-5} NaEDTA, pH 7.0, that was 1×10^{-5} M in 2-mercaptoethanol. The EDTA-mercaptoethanol solution, which was diluted at least 10-fold by the assay medium, had no effect on the inhibition of NaK-ATPase activity by divalent metal. Water was glass triple distilled and all glassware was cleaned in chromic acid.

RESULTS

Activation of NaK-ATPase by norepinephrine and dopamine. The addition of norepinephrine or dopamine to the assay mixture for NaK-ATPase from beef cerebral cortex causes a dose-dependent increase in enzyme activity (Figs. 1 and 2). The nature of the increase in activity was established by testing the effects of these catecholamines on NaK-ATPase from six different brain preparations. The means and standard errors for NaK-ATPase activity from the six brain preparations were calculated for the effects of each of the drugs and plotted as shown. Slightly greater enhancement of activity occurred at lower concentrations of norepinephrine but the character of the curves is essentially the same. Norepinephrine and dopamine were equally potent at 5×10^{-4} M. Preliminary experiments showed the activation to be linear with time.

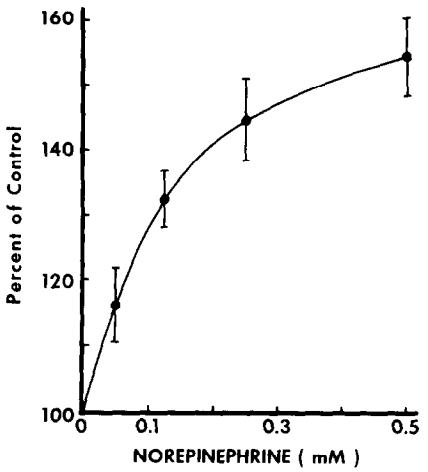

Fig. 1. The effect of norepinephrine on NaK-ATPase activity from beef cerebral cortex. The assays were performed in duplicate as described in Methods. *l*-Norepinephrine was added as the bitartrate salt. The means and standard errors were calculated from sp. act. obtained from the assay of six individual beef brain preparations of NaK-ATPase. Fold stimulation was calculated by dividing the sp. act. obtained with norepinephrine by the sp. act. obtained in the absence of norepinephrine.

Effect of ATP on activation of NaK-ATPase by catecholamines. During the course of experimentation it became necessary to utilize ATP of a different lot number. A comparison of the effects of dopamine on NaK-ATPase activity using ATP from two different lot numbers is shown in Table 1. The activating effect of dopamine (or norepinephrine—not shown) on NaK-ATPase activity varies with the ATP supply and dopamine could no longer increase NaK-ATPase activity when ATP from lot No. 2 was used (Table 1). These data suggest the presence of an inhibitor in the

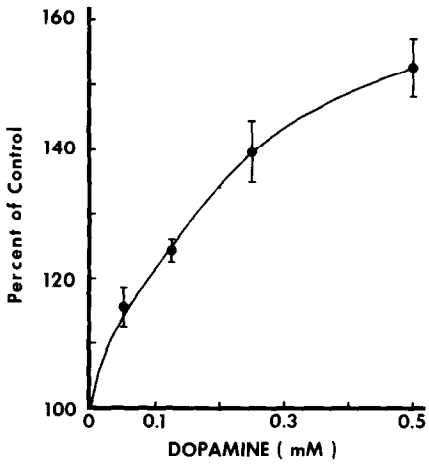

Fig. 2. The effect of dopamine on NaK-ATPase activity from beef cerebral cortex. The assays were performed in duplicate as described in Methods. Dopamine was added as the hydrochloride salt. The means and standard errors were calculated from sp. act. obtained from the assay of six individual beef brain preparations of NaK-ATPase. Fold stimulation was calculated as explained in Fig. 1.

Table 1. Variability of dopamine stimulation of NaK-ATPase activity depending on supply of ATP*

Dopamine mM	ATP lot No. 1	ATP lot No. 2
	μmoles P_i/mg protein/hr	
0	54.1	80.2
0.125	65.9	82.0
0.5	83.1	85.2

* Dopamine was added to the final concentrations indicated. NaK-ATPase activity was assayed as described in Methods. ATP lot No. 1 and ATP lot No. 2 indicate Na_2 ATP of different batches from the chemical supplier.

ATP, which varied with the supply of ATP, and whose effects could be reversed by catecholamines. Attempts to identify the inhibitory species were not successful (cf Specht and Robinson [22]). Barium, which is frequently a contaminant of ATP preparations, is not an inhibitor of NaK-ATPase activity up to 1×10^{-4} M (T. D. Hexum, unpublished observation).

Effect of EDTA on activation of NaK-ATPase by catecholamines. EDTA can mimic the effects of the catecholamines (Table 2). The effect of EDTA was dose-dependent and maximal at 2×10^{-3} M. Norepinephrine in the presence of a sub-maximal amount of EDTA can no longer activate NaK-ATPase activity to any significant degree (Table 3, lot No. 1 ATP contains the inhibitory substance). A submaximal amount of EDTA was used in the presence of norepinephrine since higher concentrations of EDTA may alter the concentration of the substrate, MgATP. The increase in activity produced by norepinephrine at an EDTA concentration of 0.5 mM is only 10 percent (Table 3) whereas in the absence of EDTA the activation is 40 percent (Fig. 1). Since (a) EDTA is a chelator of divalent metals, (b) ATP complexes to divalent metals [23], (c) norepinephrine and dopamine form stable complexes with divalent metals [24–26], and (d) divalent metals are inhibitors of NaK-ATPase activity [14, 15, 18], it appears that the inhibitor is a divalent metal or metals. The NaK-ATPase was not the source of inhibitory metal in the experiments reported here because EDTA was included in each step of the enzyme preparation. Therefore by proper choice of the source of ATP the activating effects of norepinephrine and dopamine could be reduced to an insignificant level.

Table 2. Stimulation of NaK-ATPase activity by EDTA*

EDTA mM	Specific activity μmoles P_i/mg protein/hr
0	39.9
0.1	40.7
0.5	49.4
1.0	65.0
2.0	72.8

* EDTA (sodium salt) was added to the final concentrations indicated. NaK-ATPase activity was assayed as described in Methods. The ATP used in the NaK-ATPase assay contained an inhibitory substance.

Table 3. Stimulation of NaK-ATPase activity by norepinephrine in the presence of EDTA*

Norepinephrine mM	Specific activity μmoles P_i/mg protein/hr
0	67.5
0.05	64.6
0.125	69.1
0.25	74.4
0.5	74.0

* Norepinephrine was added to the final concentrations indicated. NaK-ATPase activity was assayed as described in Methods. Each tube contained 0.5 mM EDTA (sodium salt). The ATP used in the NaK-ATPase assay contained an inhibitory substance.

Effect of dopamine and norepinephrine on inhibition of NaK-ATPase by Ca^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+}. Divalent metals which are present in nervous tissue and particularly at the synapse are Cu^{2+}, Zn^{2+}, Fe^{2+}, [16, 17] and Ca^{2+}. These metals are also inhibitors of NaK-ATPase [14, 15, 18]. Therefore in light of the fact that catecholamines can increase NaK-ATPase activity by relieving divalent metal inhibition, it seemed plausible to investigate whether any of these metals may be involved in the activation of NaK-ATPase by catecholamines.

The effects of norepinephrine in reversing the inhibition produced by low concentrations of Cu^{2+}, Fe^{2+}, Zn^{2+} and Ca^{2+} are shown in Table 4. Norepinephrine is completely effective in reversing the inhibitory effects of Fe^{2+} but ineffective in reversing the inhibitory effects of Cu^{2+}, Zn^{2+} and Ca^{2+}. Similar results were obtained for dopamine (not shown).

Effects of catecholamines and related compounds on Fe^{2+} inhibition of NaK-ATPase activity. As a result of the findings that catecholamine activation of NaK-ATPase can be mediated specifically by Fe^{2+}, it was decided to determine the structural features for catecholamines to be active in this system. The effect of increasing concentrations of various compounds on Fe^{2+} inhibition of NaK-ATPase activity are presented in Fig. 3. Dopamine, norepinephrine, epinephrine, and isoproterenol at 5×10^{-4} M can all com-

Table 4. The effect of norepinephrine in reversing the inhibition of NaK-ATPase activity by divalent metals*

Norepinephrine mM	Cu^{2+}	Fe^{2+}	Zn^{2+}	Ca^{2+}
	μmoles P_i/mg protein/hr			
0	(66.7)†	(63.7)	(66.7)	(62.9)
0	12.7	31.9	39.9	47.2
0.005	11.9	32.7	39.1	44.2
0.025	11.9	42.1	36.1	46.3
0.25	9.3	62.0	37.8	47.2
0.5	8.5	64.6	40.8	50.1

* Norepinephrine was added to the final concentrations as indicated. The final concentrations of inhibitory divalent metals are Cu^{2+}, 0.025 mM; Fe^{2+}, 0.05; Zn^{2+}, 0.05 mM; and Ca^{2+}, 0.2 mM. NaK-ATPase activity was assayed as described in Methods.

† The specific activities given in parentheses are values obtained in the absence of added norepinephrine or divalent metal and are control values.

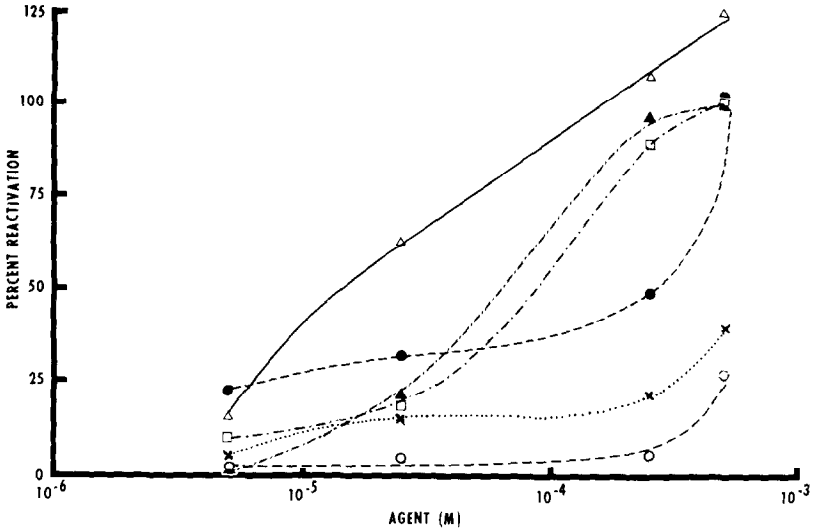

Fig. 3. The effect of various agents on Fe^{2+} inhibition of NaK-ATPase. The assays were performed in duplicate as described in Methods. Percent reactivation was calculated by dividing the differences obtained when the activity in the presence of Fe^{2+} is subtracted from the activity obtained in the presence of Fe^{2+} plus agent, by the difference obtained when the activity in the presence of Fe^{2+} is subtracted from the activity obtained in the absence of both Fe^{2+} and agent. The various agents tested are △, *l*-isoproterenol; ▲, *l*-norepinephrine; □, *l*-epinephrine; ●, dopamine; ○, *l*-phenylephrine; and ×, tyramine.

pletely reverse the inhibition of NaK-ATPase by Fe^{2+}. The non-catecholamines are only weakly effective in reversing the inhibition.

Table 5 gives a comparison of the effects of these agents with the effects of several other catecholamines and related compounds. Of all the other compounds tested only *l*-DOPA and *d,l*-3,4-dihydroxymandelic acid can give any appreciable reactivation of the Fe^{2+} inhibited NaK-ATPase activity. In addition to containing an intact catechol moiety these compounds

Table 5. Comparison of the effects of various agents on Fe^{2+} inhibition of NaK-ATPase activity*

Agent	Control†	$+Fe^{2+}$	$+Fe^{2+}$ + agent	Percent Reactivation
	μmoles P_i/mg protein/hr			
	a	b	c	d
l-isoproterenol	50.1	27.1	55.7	124.3
l-epinephrine	51.2	25.1	51.4	100.8
l-norepinephrine	53.7	27.0	53.7	100.0
d-norepinephrine	61.9	36.3	62.9	103.9
dopamine	51.2	35.3	51.5	101.9
l-DOPA	68.5	38.5	65.4	89.7
l-phenylephrine	59.8	31.4	39.2	27.5
tyramine	54.9	34.1	42.3	39.4
l-tyrosine	64.6	41.8	40.9	−3.0
catechol	59.5	28.1	35.1	22.3
hydroquinone	58.0	30.5	11.2	−70.2
β-phenylethylamine	84.8	59.9	57.5	−9.6
d,l-3-methoxy, 4-hydroxy mandelic acid	66.6	37.4	39.5	7.2
d,l-normetanephrine	36.0	10.2	16.3	23.6
dihydroxyphenylacetic acid	56.2	29.0	39.8	39.7
d,l-3,4-dihydroxy mandelic acid	66.0	37.1	58.1	72.7

* The NaK-ATPase activity was measured as described in Methods. All agents were added to a final concentration of 5×10^{-4} M except tyrosine which was added to 1×10^{-4} M due to its lower solubility. All agents were freshly prepared in 5×10^{-5} M Na_2EDTA, pH 7.0 containing 1×10^{-5} M 2-mercaptoethanol and diluted at least 10-fold in the assay medium. The concentration of Fe^{2+} was 0.05 mM during the assay. Each value is the average of three separate experiments done in duplicate (see Methods). The percent reactivation is calculated by dividing the difference obtained when the value in column b is subtracted from the value in column c by the difference obtained when column b is subtracted from the value in column a; i.e. (c − b)/(a − b) = percent reactivation.

† Control values were obtained in the absence of both Fe^{2+} and agent.

Table 6. Relative potencies of catecholamines in reversing Fe^{2+} inhibition of NaK-ATPase activity*

Agent	n	Percent reactivation	P
Epinephrine	3	100.7 ± 3.2	
Norepinephrine	3	99.7 ± 5.3	N.S.
Isoproterenol	3	124.3 ± 3.3	<0.005
Phenylephrine	3	28.0 ± 8.5	<0.001

* The experimental conditions and calculation of percent reactivation are as given in Table 5. The ability of each agent to reactivate Fe^{2+} inhibited NaK-ATPase was compared to that of epinephrine. The level of significance was determined using student's t-test (N.S.—not significant).

also have an ethyl side chain attached to a strongly electronegative group (carboxyl). All other compounds lack either the catechol groups or an ethyl side chain. It appears that the catechol group specifically is required since hydroquinone which has hydroxyl groups in the *para* position is inhibitory in character. Monohydroxylated compounds such as tyramine and phenylephrine are only weakly effective; tyrosine is completely ineffective. The stereospecificity of the β-carbon does not have to be maintained since both the *d*-, and *l*-forms of norepinephrine are equally effective. The increase in lipid solubility brought about by the addition of the isopropyl group may be responsible for the increased effectiveness of *l*-isoproterenol (Table 6). Further inspection of Table 6 shows that the effectiveness of catecholamines follows the beta adrenergic "pattern" of isoproterenol > epinephrine = norepinephrine > phenylephrine.

Role of cyclic AMP in the reversal of Fe^{2+} inhibition of NaK-ATPase. Since catecholamines exert their effect in some tissues by increasing levels of cyclic AMP it was decided to test whether cyclic AMP had any effect on the inhibition by Fe^{2+} or could potentiate the reversal of Fe^{2+} inhibition of NaK-ATPase by norepinephrine. Cyclic AMP was found to be completely without effect in either situation at concentrations ranging from 5×10^{-7} M to 5×10^{-4} M. The possibility was then considered that the NaK-ATPase preparation may contain an active phosphodiesterase. The effect of theophylline, from $1 \times 10^{-6} - 1 \times 10^{-5}$ M, was tested on the reversal of the Fe^{2+} inhibition by catecholamines. Neither theophylline alone nor theophylline with cyclic AMP (5×10^{-7} M to 5×10^{-4} M) had any effect on the reversal of Fe^{2+} inhibition.

Effects of various blocking agents on Fe^{2+} inhibition of NaK-ATPase. Propranolol, a beta adrenergic blocker, and the alpha blocker, phentolamine were tested for their ability to block the reversal of Fe^{2+} inhibition of NaK-ATPase activity by catecholamines (Table 7). Propranolol at a concentration equal-molar (5×10^{-4} M) with isoproterenol prevents the reactivation of Fe^{2+} inhibited NaK-ATPase activity ($P < 0.025$). Phentolamine, however, cannot block the effects of norepinephrine at equal-molar concentrations (5×10^{-4} M). Both blockers were slightly inhibitory when added to the NaK-ATPase assay system in the absence of Fe^{2+}. Hence each of the controls given in Table 7 contained the appropriate blocking agent. Both norepinephrine and isoproterenol produced dose-dependent responses in the presence of the blocking agent (not shown).

DISCUSSION

The results of this study confirm the earlier findings of others [6–8], that norepinephrine can increase the NaK-ATPase activity from brain. Evidence provided here indicates that the mechanism by which norepinephrine and dopamine increase the NaK-ATPase activity is by reversal of divalent metal inhibition. This evidence includes the following facts: (a) norepinephrine and dopamine form stable complexes with divalent metals [24–26]; (b) divalent metals are inhibitors of NaK-ATPase activity [14, 15, 18]; (c) EDTA can mimic the effects of catecholamines; (d) EDTA can block the effects of catecholamines on NaK-ATPase activity; and (e) the likelihood that the ATP used in the assay contained a divalent metal contaminant. Consistent with this evidence are the findings of Schaefer *et al.* [6] who reported their catecholamine effect to be dependent on a heat stable, dialyzable factor. Reversal of divalent metal inhibition by norepinephrine may also explain the results of Yoshimura [7] and Iwangoff [8]. These authors did not take steps to bind divalent metals that might be present in their assay media.

These findings suggest the possibility that physiologically important catecholamines such as norepinephrine and dopamine might regulate NaK-ATPase activity by relieving *in situ* inhibition by divalent metals. The plausibility of the hypothesis is supported by the following observations: (a) the inhibitory metals Cu^{2+}, Zn^{2+}, Fe^{2+}, and Ca^{2+} are present in nervous tissue [16, 17]; (b) Cu^{2+}, Zn^{2+} and Fe^{2+} are potent inhibitors of NaK-ATPase activity with I_{50}s of 1 μM, 1 μM and 3 μM, respectively [18]; (c) Cu^{2+}, Zn^{2+} and Fe^{2+} are present in whole brain tissue at concentrations sufficient to inhibit NaK-ATPase activity: 1.7×10^{-4} M, 3×10^{-4} M and 11×10^{-4} M

Table 7. Effect of adrenergic blockers on catecholamine reversal of Fe^{2+} inhibition of NaK-ATPase activity*

Addition	n	Specific activity	P
		μmoles P_i/mg protein/hour	
PTA	3	44.8 ± 3.2	
Fe^{2+} + PTA	3	22.9 ± 5.2	<0.001
NE + Fe^{2+} + PTA	3	43.5 ± 3.6	N.S.
PROP	3	45.3 ± 0.8	
Fe^{2+} + PROP	3	31.4 ± 1.2	<0.001
i-PROT + Fe^{2+} + PROP	3	42.1 ± 1.0	<0.025

* The assays were performed as described in Methods. The values are reported as the average of three separate experiments done in duplicate. The table shows for each adrenergic blocker, phentolamine (PTA) or propranolol (PROP), the sp. act. in the presence of blocking agent, the inhibition by Fe^{2+} and the reactivation after addition of catecholamine, norepinephrine (NE) or isoproterenol (i-PROT). Concentration of added agents are PTA, 5.3×10^{-4} M; Fe^{2+}, 5×10^{-5} M; NE, 5×10^{-4} M; PROP, 5×10^{-4} M; and i-PROT, 5×10^{-4} M. The level of significance was determined using student's t-test (N.S.—not significant).

for Cu^{2+}, Zn^{2+} and Fe^{2+}, respectively [27] and (d) the relief of inhibition by catecholamines occurs in a rapid fashion. A mechanism for regulating NaK-ATPase activity by relieving divalent metal inhibition has been suggested by Bader *et al.* [28] although catecholamines were not specifically implicated in their hypothesis.

A model system was constructed of the effects of various catecholamines and related compounds on Fe^{2+} inhibition of NaK-ATPase activity. Fe^{2+} was chosen as the hypothetical inhibitor although it has not been shown to be a regulator of the enzyme *in situ* by direct measurement. Support for the use of Fe^{2+} in the model system comes from the observation that norepinephrine and dopamine were effective in reversing only the inhibition of the NaK-ATPase cause by Fe^{2+} and not the inhibition caused by Cu^{2+}, Zn^{2+}, and Ca^{2+} although norepinephrine and dopamine can form complexes with all four of these divalent metals [16]. The results of the study to determine the effects of a series of catecholamines and related compounds on Fe^{2+} inhibited NaK-ATPase activity gave an efficacy pattern strikingly similar to that of the beta adrenergic "pattern" (Table 6), i.e., isoproterenol > epinephrine = norepinephrine > phenylephrine.

As can be seen from the structure activity relationships (Table 5) ring hydroxyl groups *ortho* to each other are required for maximal activity. Removal of one of the hydroxyl groups, *para* placement of the hydroxyl groups, or methylation of one of the hydroxyl groups all result in reduced ability of a compound to reverse divalent metal inhibition of NaK-ATPase activity. The importance of the ring hydroxyl groups in Fe^{2+} chelation is well known (cf Graziano *et al.* [29]). In addition to the hydroxyl groups on the ring, the ethylamine side chain is also important. For example, the percent reactivation with dopamine is 101.9 per cent whereas for catechol it is only 22.3 per cent (Table 5). The ethylamine side chain by itself contributes little to the reactivating effect since β-phenylethylamine is devoid of activity. Hence, neither the ring hydroxyl groups nor the ethylamine side chain by themselves are sufficient for maximal activity. Only when both are present on the molecule does the resultant structure produce optimal activity.

Other sites on the molecule which may increase the efficacy of the compounds include the amino nitrogen and the β-carbon. Alkyl groups on the amino nitrogen may increase the lipid solubility of the catecholamines and thus account for the increased potency of isoproterenol. The stereospecificity of the β-hydroxyl group is not important for the reversing action of the catecholamines (Table 5), since the (d)-isomer of norepinephrine is as potent as the (*l*)-isomer.

The mechanism by which the catecholamines exert their effect on the Fe^{2+} inhibition of NaK-ATPase activity is probably by removal of Fe^{2+} through chelation. Rajan *et al.* [24] have established the ability of norepinephrine to form a ternary chelate with Fe^{2+} and ATP by potentiometric equilibrium studies. The alternative hypothesis, that Fe^{2+} is involved in the oxidation of catecholamines, can be ruled out on the basis of the findings that preincubation of Fe^{2+} with catecholamines under assay conditions (minus enzyme) and the subsequent use of the catecholamines to activate Fe^{2+} inhibited NaK-ATPase, does not reduce their ability to reactivate the inhibited enzyme (T. D. Hexum, unpublished observation, see also Ref. 16).

Other workers have suggested that catecholamines exert their effects on NaK-ATPase activity through a similar mechanism. They have, however, suggested different requirements for this effect. Schaefer *et al.* [11] suggested that Fe^{2+} is the inhibitory metal. Unlike the study reported here these workers have postulated a requirement for ascorbic acid in the catecholamine effect on NaK-ATPase activity. As can be seen from the above reported results the effect of catecholamines on Fe^{2+} inhibition of NaK-ATPase can be demonstrated in the absence of ascorbic acid. Furthermore ascorbic acid had no effect on the system described here (T. D. Hexum, unpublished observation). Godfraind *et al.* [30] have suggested that the effect of catecholamines on NaK-ATPase activity is due to relief of Ca^{2+} inhibition of the NaK-ATPase. This seems unlikely since no direct effect of catecholamines on Ca^{2+} inhibition of NaK-ATPase activity could be demonstrated in the experiments reported here or by Schaefer *et al.* [11].

Additional studies which were designed to establish the possible physiological significance of the catecholamine facilitated reversal of inhibition of NaK-ATPase activity included the effects of cyclic AMP and modification by adrenergic receptor blocking drugs. Cyclic AMP has no action of its own on NaK-ATPase activity or on the Fe^{2+} inhibition of NaK-ATPase activity, and could not potentiate the action of norepinephrine in reversing the inhibition of NaK-ATPase activity by Fe^{2+}. Furthermore, the addition of theophylline did not result in an effect by cyclic AMP. Of the two blocking agents tested only propranolol was effective in preventing the action of isoproterenol to completely reactivate the Fe^{2+} inhibited enzyme at a concentration equal-molar to that of isoproterenol. Propranolol may be exerting its effect by binding to the NaK-ATPase at a site near that where Fe^{2+} interacts and thus sterically prevent the action of isoproterenol.

The structure activity relationships reported here together with the blocking action of propranolol suggest that the catecholamine facilitated relief of inhibition of NaK-ATPase activity by Fe^{2+} may be of physiological significance. In addition to the blocking effect of propranolol reported here, Schaefer *et al.* [9] have shown that chlorpromazine can antagonize the action of dopamine on the NaK-ATPase. Clearly more work is needed to further establish the validity of the hypothesis and is in progress. Points needing further clarification include the relatively high concentration of catecholamine (5×10^{-6} M – 5×10^{-4} M) needed to produce the effect, the relatively high concentration of propranolol needed to block the action of isoproterenol and the lack of stereospecificity required of the β-carbon. The disruption of the tissue through homogenization and the subsequent fractionation may be largely responsible for the lack of sensitivity of the enzyme.

The possible significance of these findings may be related to the postulated role of NaK-ATPase in transmitter function. For example, the NaK-ATPase

has been implicated as the driving force in the uptake of some neurotransmitters [31]. In addition, Gilbert and co-workers [32] have suggested that norepinephrine may regulate its own release by stimulation of nerve terminal NaK-ATPase. These workers suggest that the NaK-ATPase is part of the alpha presynaptic receptor believed to be involved in the feedback regulation of norepinephrine release. Finally, Phillis [33] has suggested that catecholamine induced hyperpolarization of central neurones is a result of the stimulation of NaK–ATPase. Alternatively, this particular system may fail to prove physiologically significant but might reflect a mechanism important in adrenergic function.

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