Failure of vanadate contamination in ATP to account for noradrenaline stimulation of Na^+, K^+-ATPase in rat brain homogenates

J. W. PHILLIS*, P. H. WU, *Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0*

It has recently been shown that vanadium may be present in muscle tissue at concentrations sufficient to inhibit the sodium potassium stimulated adenosine triphosphatase (Na^+,K^+)-ATPase (Josephson & Cantley 1977). There are a number of reports in the literature of catecholamine activation of (Na^+,K^+)-ATPase in vitro (Schaefer et al 1972; Yoshimura 1973; Iwangoff et al 1974; Logan & O'Donovan 1976; Lee & Phillis 1977; Wu & Phillis 1978) and it has been proposed that these compounds simply reverse the vanadate inhibition which is the result of vanadium contamination of substrate ATP (Josephson & Cantley 1977). Reversal of vanadate inhibition of the enzyme by noradrenaline (NA) and other catecholamines is reportedly acomplished through complexation and reduction of vanadate (Cantley et al 1978a,b).

It is difficult to reconcile this explanation for the catecholamine-induced stimulation of (Na^+,K^+)-ATPase with reports that noradrenaline stimulation of the enzyme can be blocked by adrenolytic agents (Iwangoff et al 1974; Gilbert et al 1975; Wu & Phillis 1978). Phillis et al (1978) have shown that both phentolamine, an α-adrenoceptor antagonist, and propranolol, a β-adrenoceptor antagonist, can abolish the enhancement of (Na^+,K^+)-ATPase activity by noradrenaline and they suggested that this could be a receptor-mediated phenomenon.

To show that noradrenaline stimulation of (Na^+,K^+)-ATPase is not merely caused by reversal of inhibition produced by the contaminating vanadium in substrate ATP, we have studied the interactions of vanadate, noradrenaline and (Na^+,K^+)-ATPase in rat brain homogenates and compared the effects of three batches of substrate adenosine 5'-triphosphate (ATP) (Sigma regular equine muscle ATP, Sigma vanadium-free equine muscle ATP and Sigma synthetic ATP) on noradrenaline-stimulation of brain (Na^+,K^+)-ATPase.

The cortex from male Sprague-Dawley rats (150–300 g) was removed and homogenized in 50 volumes of distilled H_2O (pH 7·5). 50 μl of this homogenate was used for the incubation. The (Na^+,K^+)-ATPase activity was determined by subtracting Mg^{2+}-ATPase activity (ouabain insensitive) from total ATPase activity. The medium used for the estimation of total ATPase activity consisted of (mM) in final concentration: Tris, 115; $MgCl_2$, 5·0; KCl, 6·25; NaCl 72·5. Mg^{2+}-ATPase activity was measured in the K^+-free medium which was composed of (mM) in final concentration; Tris, 172·5; $MgCl_2$, 5·0; NaCl, 14 and ouabain 1·0. In all experiments, the homogenate was preincubated for 10 min at 37 °C in the presence of agonist and/or antagonist. The reaction was terminated 6 min after the addition of disodium adenosine-5'-triphosphate (ATP) (2mM of synthetic ATP, vanadium-free ATP, or equine muscle ATP) by adding 500 μl ice cold 12% trichloroacetic acid solution in an ice bath. The content of inorganic phosphate in the supernatant was measured by the method of Fiske & Subbarow (1925). Noradrenaline and ATP solutions were freshly made for each experiment.

Chemicals: adenosine 5'-triphosphate (Grade 1) synthesized by phosphorylation of adenosine (Sigma, A-2383, Lot 96C–7140); adenosine 5'-triphosphate from equine muscle, vanadate-free (Sigma, A5394, Lot 78C–7110); adenosine 5'-triphosphate from equine muscle (Sigma A6144, Lot 96C–7340); (–)-noradrenaline bitartrate (Sigma A9512). Other chemicals were of analytical grade.

Our previous studies (Wu & Phillis 1978) had shown that the optimal reaction conditions for rat cortical (Na^+,K^+)-ATPase activity required 5mM Mg^{2+} and 2mM ATP. The reaction is linear for 20 min of incubation, and in the present experiments a 6 min incubation was used.

The effects of noradrenaline at different concentrations on rat brain (Na^+,K^+)-ATPase using the three substrate ATPs are shown in Table 1. There were no significant differences between the basal levels of activity of the enzyme, regardless of the type of ATP used as a substrate. Noradrenaline in concentrations ascending from 10^{-6} to 10^{-3}M elicited increasing levels

Table 1. (Na^+,K^+)-ATPase Activity (μmol Pi mg^{-1} tissue h^{-1})*.

NA (M)	Equine Vanadate free ATP†	Synthetic ATP†	Equine Muscle ATP†
Control (0)	0·760 (0·038) (100%)	0·734 (0·107) 100%	0·726 (0·065) 100%
NA (10^{-6})	0·958 (0·060)‡ 126% (3%)	0·928 (0·096)* 120% (11%)	0·842 (0·145) 116% (17%)
NA (10^{-5})	1·126 (0·100)‡ 147% (12%)	1·100 (0·130)‡ 140% (15%)	1·144 (0·123)‡ 158% (15%)
NA (10^{-4})	1·514 (0·176)‡ 199% (20%)	1·460 (0·158)† 185% (19%)	1·594 (0·154)‡ 219% (25%)
NA (10^{-3})	1·595 (0·174)‡ 207% (22%)	1·555 (0·200)† 196% (22%)	1·565 (0·190)‡ 219% (20%)

† Each value is the mean (with s.d.) of the five experiments.
* 1 mg of rat brain cortical tissue was incubated with the media described in the text. The inorganic phosphate released during 6 min. of reaction at 37°C was measured by method of Fiske & Subbarow (1925).

1. The *t*-test was applied to compare the levels of enzyme activity using different sources of ATP and in all cases the results were not significant.
2. The *t*-test was applied to compare the levels of enzyme activity in the presence of various concentrations of noradrenaline bitartrate to enzyme activity in the absence of noradrenaline(control) for each type of ATP. * $0{\cdot}05 \geqslant P \geqslant 0{\cdot}01$, † $0{\cdot}01 \geqslant P \geqslant 0{\cdot}001$ ‡ $0{\cdot}001 \geqslant P$.

* Correspondence.

of (Na^+,K^+)-ATPase activity. Comparisons of the levels of activity at any given NA concentration, clearly fail to show any difference in the amount of stimulation of the enzyme, regardless of the type of ATP used as the substrate.

Rat brain (Na^+,K^+)-ATPase activity was measured in the presence of 5 mM Mg^{2+} and various concentrations (10^{-10}–10^{-4}M) of vanadate (Fig. 1). Fifty percent inhibition of the enzyme occurred with a vanadate concentration of 10^{-5}M.

Our comparison of the basal levels of (Na^+,K^+)-ATPase activity in rat brain homogenates using the three different substrate ATPs has failed to reveal any significant differences. Nor was the amount of stimulation of the enzyme by noradrenaline affected. Two of the ATPs we used (Sigma vanadate-free equine ATP and Sigma Grade 1 synthetic ATP) are essentially vanadate-free, in that they contain less than 0·5 ppm of vanadium—the detection limit for the vanadium assay used by Sigma (Sigma, personal communication). Regular Sigma equine muscle ATP preparations contain between 5 to 40 ppm of vanadium (Sigma bulletin, February 1978). Using these values, it is possible to calculate a vanadium concentration in an incubation medium containing 2 mM of the non-vanadium-free ATP (the level used in these experiments) as being in the range of 10^{-7}–8×10^{-7}M. A similar calculation based on the data provided by Cantley et al (1977), predicts that there would be a vanadium concentration of 6×10^{-7} M in our incubation medium. According to the data presented in Fig. 1, at a vanadium concentration of 10^{-7} M, the level of inhibition of the enzyme would be of the order of 10%, and even at 10^{-6} M there is only a 25% loss of enzyme activity.

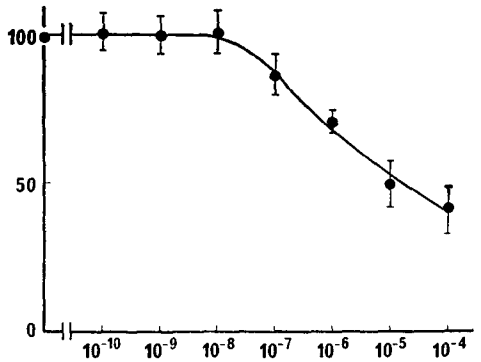

FIG. 1. Effect of vanadate on rat brain (Na^+-K^+) ATPase. Rat brain cortical homogenate (equivalent to 1 mg brain weight) was preincubated for 10 min at 37 °C in the presence of various concentrations of vanadate. (Na^+-K^+)-ATPase activity was measured according to the procedures described in the text. Each point represents the mean (s.d.) of three experiments. Abscissa: vanadate concentration (M). Ordinate: relative (Na^+-K^+)-ATPase activity (%).

Our failure to observe a significant decrease in the level of enzyme activity when non-vanadium-free ATP was used as a substrate is not, therefore, unexpected, especially if this particular lot of ATP had a low vanadate content.

It is equally apparent that vanadium contamination of the substrate ATP will not account for a large part of the stimulation of (Na^+,K^+)-ATPase activity by noradrenaline. Our observation of a 100% increase in the level of enzyme activity with the higher levels of noradrenaline, cannot be explained on the basis of vanadium contamination of the three ATP samples used as substrate, since at the low levels present in these samples, vanadium could not have caused more than a minimal amount of inhibition of the (Na^+,K^+)-ATPase. The alternative explanation, namely that NA is opposing the actions of endogenous vanadium in the rat brains, is more difficult to exclude on the basis of the data presented here. It seems highly unlikely that vanadium would be present in concentrations in excess of 10^{-5} M (the I50) in the brain, but the possibility that (Na^+,K^+)-ATPase is able to concentrate this element cannot be overlooked. Also mitigating against a putative role of vanadium as the responsible factor in NA-stimulation of brain (Na^+,K^+)-ATPase is our observation that the NA effect can be antagonized by α- and β-adrenolytic agents (Wu & Phillis 1978; Phillis et al 1978).

In conclusion therefore, the observations presented in this communication clearly exclude vanadium-contamination of the substrate ATP as a major factor in the NA-evoked stimulation of brain (Na^+,K^+)-ATPase. The existence of a receptor-mediated step between catecholamine and enzyme appears to be a more likely possibility.

January 24, 1979

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Dopamine receptor changes after long-term haloperidol treatment in rats

RICHARD P. EBSTEIN*, DALIA PICKHOLZ, ROBERT H. BELMAKER, *Jerusalem Mental Health Center-Ezrath Nashim, POB 140, Jerusalem, Israel*

Tardive dyskinesia is a neurological syndrome associated with prolonged neuroleptic treatment of schizophrenic patients (Crane 1968; Faurbye 1970; Faurbye et al 1964). It has been suggested that tardive dyskinesia results from chemical denervation of central dopamine neurons and subsequent development of supersensitivity of the postsynaptic receptor (Rubovits & Klawans 1972). Experiments based on animal models of tardive dyskinesia support this hypothesis. Chronic neuroleptic treatment increases postsynaptic dopamine receptor sensitivity when measured either behaviourally or biochemically (Christensen et al 1976; Klawans & Rubovits 1972; Moore & Thornburg 1975; Sayers et al 1975; Tarsy & Baldessarini 1974; Von Voigtlander et al 1975). The molecular basis for this behavioural change has been reported to be an increase in the number of receptor sites with no change in their affinity as measured by ^{3}H-neuroleptic binding to striatal membrane homogenates (Burt et al 1977; Klawans et al 1977; Muller & Seeman 1977). Previous biochemical reports of the development of model tardive dyskinesia after neuroleptic treatment of animals have generally used treatment periods of 3 weeks or less (Burt et al 1977; Klawans et al 1977; Muller & Seeman 1977). Only one report treated animals for longer periods (Clow et al 1978). In the present experiments we studied the kinetics of [^{3}H]spiroperidol binding to rat caudate nucleus homogenates after 3 and 10 weeks of haloperidol treatment.

Rat food containing 0·01% haloperidol was prepared by grinding regular rat pellets to a fine powder and thoroughly mixing with drug. Control rats received the same powdered food without haloperidol. Male Sabra strain were used in all experiments and weight gain on this diet was normal. The approximate daily oral dose was 3 mg of haloperidol per rat.

Rats were killed 4 days after cessation of haloperidol feeding and the striatum was dissected and stored at —70 °C until assayed. The binding of [^{3}H]spiroperidol to striatal homogenate was determined as described by Burt et al (1976). Striatum was homogenized using a glass-teflon homogenizer in 100 volumes of 50 mM Tris buffer pH 7·7 containing the following components: (mM) NaCl 120, KCl 5, $CaCl_2$ 2, $MgCl_2$, 0·1% ascorbic acid and 10 μM pargyline. The membranes were collected by centrifugation (50 000 g for 10 min) and resuspended in 285 volumes (original wet weight) of buffer. The reaction mixture contained 800 μl membrane suspension, 100 μl [^{3}H]spiroperidol (NEN, 23 Ci mM^{-1}) from 0·1 to 1·0 nM (5 different concentrations) and either 100 μl 0·1% ascorbic acid or 10 μM dopamine (blank) in 0·1% ascorbic acid. After 10 min incubation at 37 °C the reaction was stopped by rapidly filtering through Whatman GF/B glass fibre filters and washing with ice-cold buffer (2 × 10 ml). The filters were counted in 10 cc Instagel after shaking vigorously for 2 h. Specific binding of [^{3}H]spiroperidol was calculated as the number of counts in excess over the dopamine blank. The number of receptor sites and the K_D was determined by Scatchard plot (Scatchard 1949) for each individual rat striatum. Plots were fitted with a standard computer program.

Table 1 shows the change in number of [^{3}H]spiroperidol binding sites after 3 and 10 weeks of neuroleptic treatment. After 10 weeks there is a mean 128% increase in the number of binding sites compared with a mean 59% increase after 3 weeks, suggesting a trend towards increased number of receptors with increased exposure to haloperidol. Analysis of the data of Table 1 reveals a significantly increased variance ($F = 9{\cdot}31$, $P < 0{\cdot}05$) in the number of receptor binding sites after

Table 1. The effect of chronic haloperidol on the number of [^{3}H]spiroperidol binding sites in striatum

	No of receptors (pmol g^{-1} tissue)		
	Control	Haloperidol	% change
3 weeks	32·27 ± 2·30 (±s.e.m., n = 5)	51·30 ± 3·95 (±s.e.m., n = 5)	+58·9*
10 weeks	26·51 ± 2·35 (±s.e.m., n = 6)	60·56 ± 7·87 (±s.e.m., n = 5)	+128**

* $t = 2{\cdot}81$ Student's t, $P < 0{\cdot}05$.
** $t = 3{\cdot}04$, $P < 0{\cdot}02$.
Numbers in parentheses are the number of individual rat striatum assayed.
Behavioural supersensitivity as measured by amphetamine (5 mg kg^{-1})-induced stereotypy developed in a parallel group of haloperidol-fed animals ($t = 4{\cdot}94$ Student's t, $P < 0{\cdot}01$).

* Correspondence.