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Acute and Chronic Catecholamine-Ethanol Interactions on Rat Brain ($Na^+ + K^+$)-ATPase

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RANGARAJ, N. AND H. KALANT. *Acute and chronic catecholamine-ethanol interactions on rat brain ($Na^+ + K^+$)-ATPase*. PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 183–189, 1980.—Noradrenaline (N) sensitizes rat brain ($Na^+ + K^+$)-ATPase to inhibition by low concentrations of ethanol (E). Only l-N and not d-N was effective. The sensitization is also produced by other α-adrenergic agonists (adrenaline, phenylephrine), but not by isoproterenol, and is prevented by phentolamine but not by propranolol. The sensitization is greater with partially purified enzyme than with crude homogenates. N + E, like much higher concentrations of E alone, produced competitive inhibition with respect to K^+ but uncompetitive or mixed inhibition with respect to Na^+, Mg^{++} and ATP, and a reduced "physiological efficiency" of ATP utilization. All these changes were abolished by increasing K^+ to 20 mM. After 3-week E treatment, with or without withdrawal, the N + E interaction was markedly reduced, though basal ATPase activity was increased only after withdrawal. Temperature-dependence studies (Arrhenius plots) indicated that sensitization occurs by alteration of activation energy only above the transition temperature. These findings suggest that α-agonists fluidize membrane lipids and thus facilitate conformational change of the enzyme by E, resulting in inhibition.

Rat brain (Na⁺ + K⁺)-ATPase Adrenergic agonists Aα-Receptor blockers Kinetics Ethanol Acute Chronic Arrhenius plot

THE enzyme ($Na^+ + K^+$)-ATPase of the cell membrane is involved in the active transport of monovalent cations across the membrane and maintenance of normal resting membrane polarization [2, 5, 27, 28]. The enzyme activity is inhibited by ethanol (E) *in vitro* [1, 7, 10–12, 16, 32, 33] by a mechanism involving competition with K^+. The lowest E concentration required to produce significant inhibition *in vitro* was 0.054 M, provided the K^+ concentration was 1 mM or less [11]. Since the K^+ concentration in mammalian blood and extracellular fluid is well above 1 mM, higher E concentration is needed for enzyme inhibition *in vivo*. Therefore it seemed unlikely that inhibition of the enzyme *in vivo* would occur at E concentrations typical of mild to moderate intoxication.

However, we have previously demonstrated that catecholamines (CAs) sensitize the ($Na^+ + K^+$)-ATPase to inhibition by low concentrations of E at a physiological concentration of K^+ [20,21]. Only l-noradrenaline (N) was effective and not d-N [21]. This interaction was prevented by an α-blocker, phentolamine, but not by a β-blocker, propranolol [21]. The present work is aimed at exploring the mechanism of this CA-E interaction and its possible significance for E actions and E tolerance *in vivo*.

The inhibition of rat brain ($Na^+ + K^+$)-ATPase by E appears to be produced by an allosteric effect on the affinity of the K^+ binding site [12]. The enzyme activity is dependent on the relative concentrations of Na^+, K^+, Mg^{++}, and ATP [2, 22–31]. Therefore, it was of interest to determine whether the sensitization of the enzyme to E by N was exerted via an enhancement of the allosteric effect of E on the K^+ site or by changes in binding of other ligands. We have studied the effects of varying K^+, Na^+, Mg^{++} and ATP concentrations on rat brain ($Na^+ + K^+$)-ATPase activity in the presence and absence of N and E separately and in combination.

($Na^+ + K^+$)-ATPase is a membrane-bound enzyme [2, 4, 27], the activity of which is markedly influenced by its lipid microenvironment in the membrane [6,13]. Levental and Tabakoff [15] have described temperature-dependent effects of E on the enzyme that apparently result from alteration of the lipid microenvironment. We report here the effect of temperature on the acute N-E interaction. Chin and Goldstein [3] and Littleton [17] have reported that neuronal membranes from E-tolerant animals show altered lipid composition and reduced susceptibility to fluidization by E *in vitro*. These changes might be expected to affect the N-E interaction that we have described. We have therefore examined the N-E interaction in preparations from animals rendered tolerant to E *in vivo*.

METHOD

Male Wistar rats weighing 200–300 g were obtained from Biobreeding Laboratories (Ottawa, Canada) and Canadian Breeding Laboratories (Montreal, Canada). Na_2ATP, l-arterenol (N), adrenaline bitartrate (A), phenylephrine HCl (P), and isoproterenol HCl (I) were purchased from Sigma Chemical Co. Phentolamine HCl was a gift from Ciba-Geigy (Montreal, Canada) and propranolol was a gift from Ayerst Laboratories (Montreal, Canada). All other chemicals used were of ACS reagent grade.

The rats used in acute experiments were maintained on Purina rat chow and water ad lib. For chronic experiments, animals were housed singly, and E-treated and sucrose con-

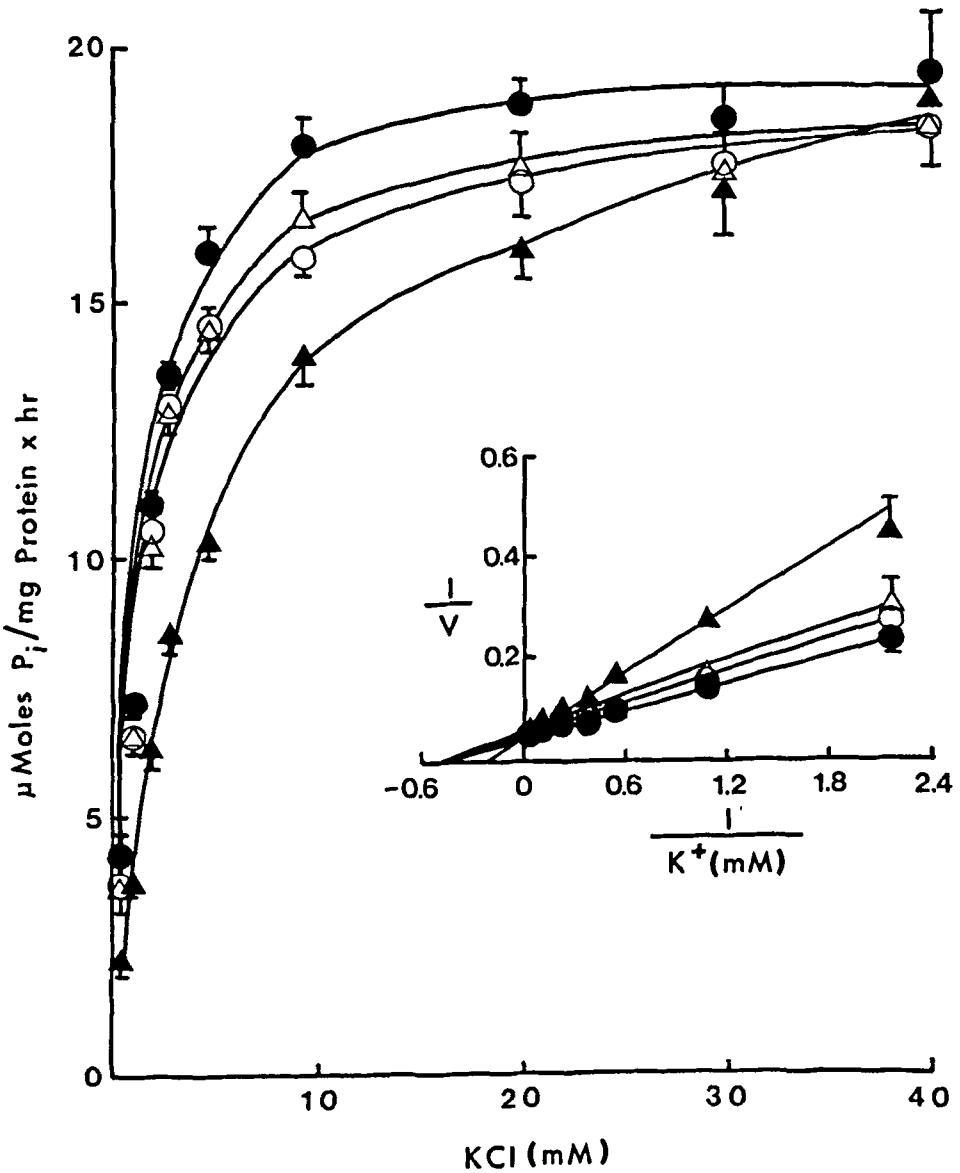

FIG. 1. Effect of 0.1 mM N and 0.05 M E added separately and in combination on (Na^+ + K^+)-ATPase at various K^+ concentrations. Inset: Lineweaver-Burk plot of the same data. ○ control, △ 0.05 M E, ● 0.01 mM N, ▲ 0.1 mM N + 0.05 M E. Each point represents mean of four experiments with duplicate determinations in each. Regression lines were calculated by the method of least squares. Vertical bars indicate SEM for each point.

trol rats were pair-fed with respect to the chow ration. E was administered by intragastric intubation at 3 g/kg daily and increased by 0.5 g/kg every 3 days to a final dosage of 6 g/kg; total duration of treatment was 3 weeks. Control rats were given sucrose in a dose equicaloric with that of E. Tolerance to E was tested by the hypothermic response produced 1 hr after IP administration of 3 g/kg [14].

Preparation of Microsomes and Synaptosomes

Rats were decapitated and the whole brain was removed as quickly as possible, wiped clean of blood and weighed. It was immediately homogenized at 0–4°C in 9 volumes of 0.25 M sucrose containing 1 mM Na_2 EDTA and 20 mM Tris HCl buffer at pH 7.4. For the temperature-dependence studies the homogenate was diluted 10-fold with cold distilled water and 0.05 ml aliquots were assayed. For other experiments sodium deoxycholate solution was added to 0.1% final concentration and microsomes were prepared as described by Nakao *et al.* [19]. They were washed once in buffer and recentrifuged at 24,000×g, then resuspended in the buffer (3 mls) and diluted 10-fold with cold distilled water. A 0.05 ml aliquot was assayed for (Na^+ + K^+)-ATPase activity. After 3 weeks of chronic E treatment, some animals were sacrificed 24 hrs after the last E administration and others were given 3 g/kg E by gavage prior to sacrifice. After decapitation, blood was collected for E determination, and the brain was removed and homogenized in 9 volumes of 0.32 M sucrose with 5 mM Tris HCl, pH 7.4. Synaptosomes were prepared according to the method of Cotman and Matthews [4].

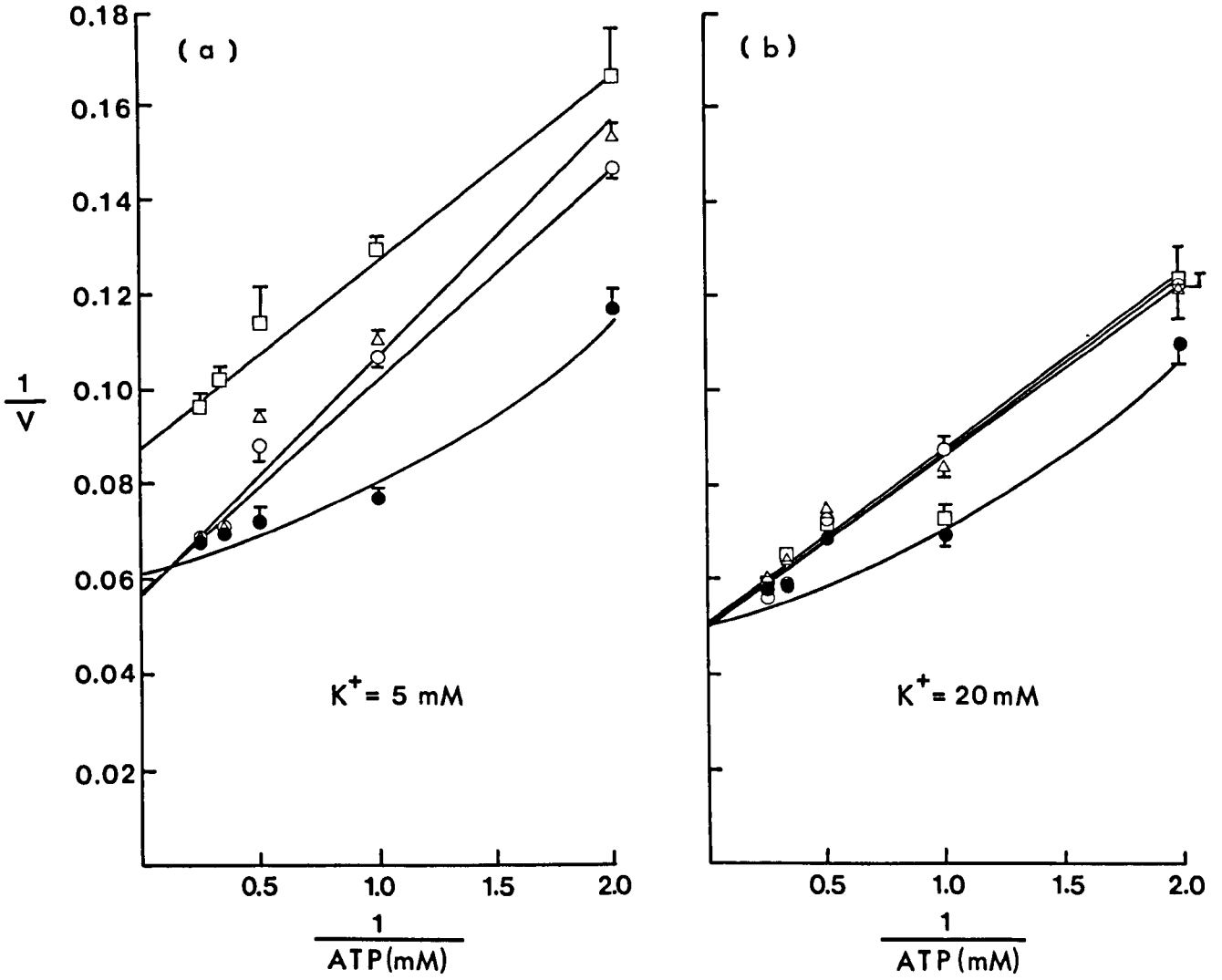

FIG. 2. Lineweaver-Burk plot of the effect of E (0.05 M) and N (0.1 mM), separately and together, on (Na⁺ + K⁺)-ATPase activity of rat brain homogenates at various ATP concentrations. (a) 5 mM K⁺, (b) 20 mM K⁺, ○ control, △ 0.05 M E, ● 0.1 mM N, □ 0.1 mM N + 0.05 M E.

Homogenate was centrifuged at 1000×g for 10 min and the precipitate was discarded after washing twice in the buffer. The supernatant and washings were centrifuged at 17,500×g for 20 min and the precipitate (P_2 fraction) was washed twice and then resuspended in the buffer. This was mixed with 3.3 volumes of 19% Ficoll in 0.32 M sucrose with 5 mM Tris HCl at pH 7.4, and on top of this mixture was layered 7% Ficoll in 0.32 M sucrose in 5mM Tris at pH 7.4; the tubes were then centrifuged at 105,000×g for 90 min. The myelin layer (top of the gradient) and the precipitated mitochondrial pellet at the bottom were discarded. Synaptosomes at the interface of the 7 and 13% Ficoll layers were collected, diluted with 3 volumes of 0.32 M sucrose in 5 mM Tris HCl, pH 7.4, and centrifuged at 35,000×g for 30 min. The synaptosomal pellet was washed once again in 0.32 M sucrose in 5 mM Tris HCl at pH 7.4 and suspended in the buffer. This was diluted 10 fold with cold distilled water and assayed for (Na⁺ + K⁺)-ATPase. Protein concentrations were determined by the method of Lowrey *et al.* [18].

(Na⁺ + K⁺)-ATPase Assay

The usual assay mixture contained 30 mM imidazole, 30 mM glycylglycine, 3 mM Na_2 ATP, 120 mM NaCl and 5 mM KCl plus 0.05 ml enzyme in a final volume of 1.3 ml. In another tube, 1 mM ouabain was added and NaCl and KCl were omitted. The components were preincubated for 3 min at 37°C or at other selected temperatures, and the reaction was started by addition of the enzyme. Duration of incubation varied with the temperature: 30 min. at 13°, 20 min at 15–37°, and 10 min at 40° or 42°C. The reaction was stopped in each case by addition of 0.5 ml of 1.2 M $HClO_4$. Inorganic phosphate (P_i) was determined by the method using ammonium molybdate and 1-amino-2-naphthol-4-sulfonic acid [12,20]. After 5 min at room temperature, absorbance was read at 825 nm.

(Na⁺ + K⁺)-ATPase activity was obtained by subtracting the ouabain-insensitive activity from the total activity in the presence of Na⁺, K⁺, and Mg⁺⁺ and the absence of ouabain. The activity was expressed as micromoles of Pi produced per mg protein per hour.

The kinetic studies of Na⁺, Mg⁺⁺ and ATP were carried out at K⁺ concentrations of both 5 and 20 mM. The effect of 0.1 mM N and 0.05 M E separately and together were tested in all kinetic studies. Stock solutions (2.6 mM) of adrenergic

TABLE 1

KINETIC CONSTANTS FOR Na^+ AND Mg^{2+} WITH (Na^+ + K^+)-ATPase. EFFECT OF 0.1 mM N AND 0.05 M E

	Na^+		Mg^{2+}	
	Control	N + E	Control	N + E
A) At 5 mM K^+				
K_m (mM)	4.06 ± 0.43 (7)	1.75 ± 0.36 (7)	0.37 ± 0.03 (5)	0.19 ± 0.03 (4)
V_{max}	19.78 ± 0.99	13.63 ± 0.88	16.10 ± 0.27	10.57 ± 0.56
B) At 20 mM K^+				
K_m	4.93 ± 0.42 (5)	5.89 ± 0.30 (5)	0.57 ± 0.05 (3)	0.74 ± 0.16 (3)
V_{max}	23.30 ± 1.54	21.89 ± 1.41	17.74 ± 0.37	16.81 ± 0.72

V_{max} expressed in μmoles of $Pi \cdot hr^{-1} \cdot mg\ protein^{-1}$. Values reported as mean ± SEM. Numbers of experiments in parentheses; each experiment performed in duplicate.

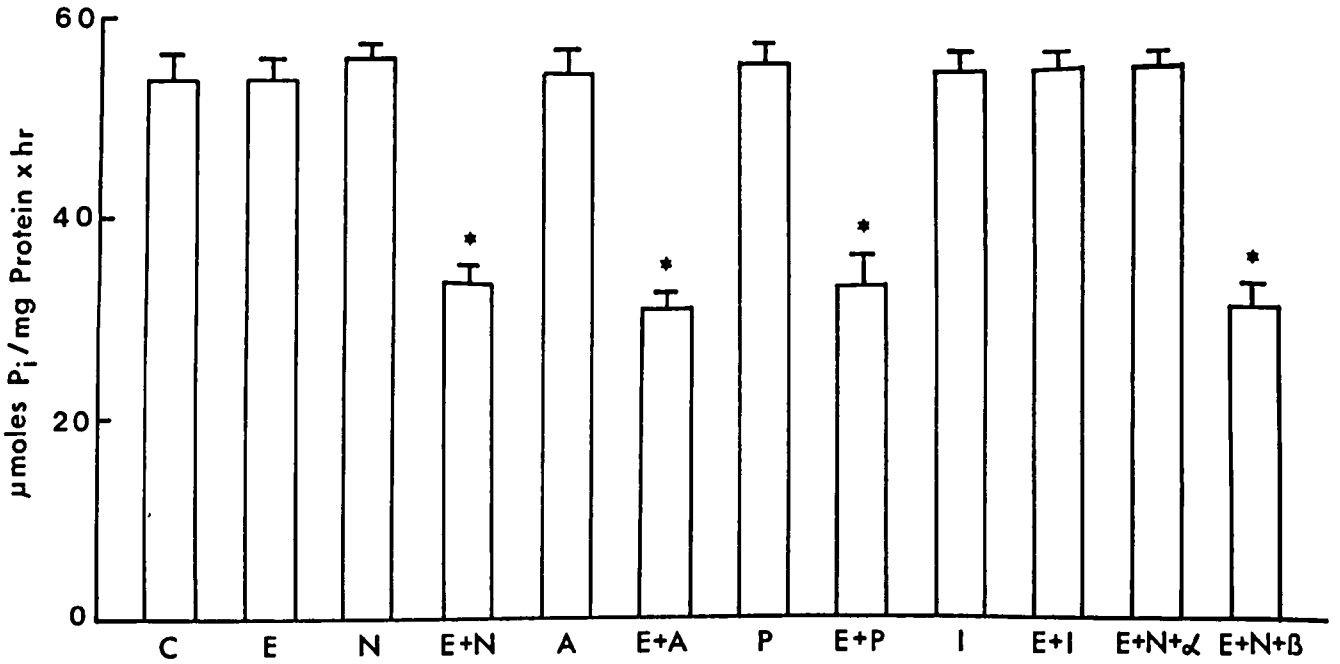

FIG. 3. Effects on microsomal (Na^+ + K^+)-ATPase of various adrenergic agonists alone and together with 50 mM E, and modification of E-N interaction by adrenergic blockers: N—noradrenaline, A—adrenaline, P—phenylephrine, I—isoproterenol, α—phentolamine (0.1 μM) and β—propranolol (100 μM). All agonists 100 μM.

agonists and blockers were prepared fresh daily in distilled water. Appropriate dilutions were prepared, such that 0.05 ml would yield the desired final concentrations in the assay.

Determination of Kinetic Constants for N-E Interaction

Rat brain was homogenized in 19 volumes of 0.32 M sucrose. The homogenate was diluted 5-fold with cold distilled water and 0.05 ml was assayed for (Na^+ + K^+)-ATPase activity. The concentration of each ligand (Na^+, K^+, Mg^{++}, ATP) in turn was systematically varied while the others were held constant. At each concentration of a given ligand, K_m and V_{max} were estimated in control preparations and in the presence of 0.1 mM N and 0.05 M E separately and in combination.

RESULTS

Kinetic Constants for N-E Interaction

Effect of varying K^+. With vanadium-free ATP as substrate, the effect of varying K^+ (0.5–40 mM) on the enzyme activity in the presence of 0.05 M E and 0.1 mM N individually and in combination is shown in Fig. 1. A Lineweaver-Burk plot (Fig. 1 insert) of the same data indicated a competitive type of inhibition in presence of N + E. In control preparations, the K_m for K^+ was 1.96 mM; this was not affected by N or E alone but was increased to 5.48 mM by N + E. V_{max} was not affected. Similar results were obtained with vanadium-containing ATP.

Effect of varying ATP (Fig. 2). In controls at 5 mM K^+, K_m for ATP was 0.88 ± 0.05 mM. This was not affected by E

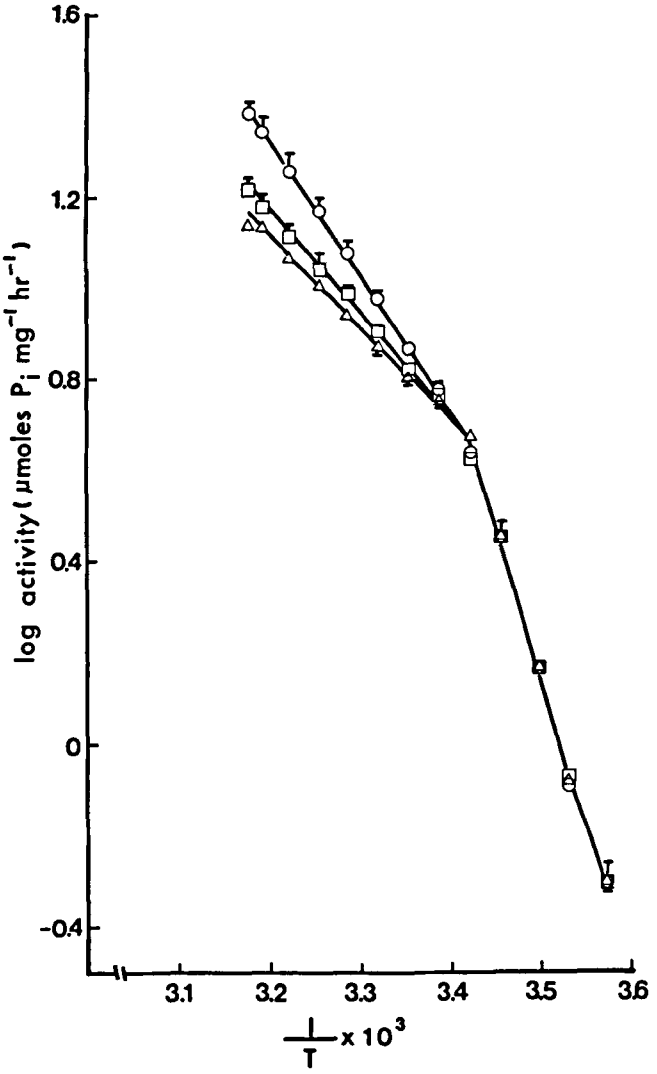

FIG. 4. Effects of 1 μM N + 0.05 M E (□), and of 0.44 M E alone (△), on Arrhenius plot of rat brain (Na^+ + K^+)-ATPase. Enzyme activity was measured from 7–42°C at 2-3 degree intervals. Each point is the average of four separate experiments and each experiment included duplicate determinations. Vertical bars indicate SEM. Where no bar is shown SEM falls within the diameter of the symbol. Results with N 1–100 μM or 0.05 M E added separately were the same as those of controls (○).

alone, but was decreased to 0.47 ± 0.04 by N alone, and was not further altered by N + E. V_{max} was 17.54 ± 0.41 in controls; this was unaffected by E or N alone, but was reduced to 11.18 ± 0.75 by N + E. At 20 mM K^+, V_{max} was increased about 18% in the controls, but the effects of E, N and N + E were completely prevented.

Effect of varying Na^+ and Mg^{++} (Table 1). Neither E nor N alone had any effect on either K_m or V_{max} for Na^+ or Mg^{++}. However, at 5 mM K^+, N + E reduced the K_m by more than 50% for each of these ligands, while reducing V_{max} by about 30%. The effects on K_m were reversed by increasing K^+ to 20 mM, and the effects on V_{max} were almost abolished.

Interaction of Adrenergic Agonists and Blockers with E and (Na^+ + K^+)-ATPase

The interaction of adrenergic agonists + E was studied in

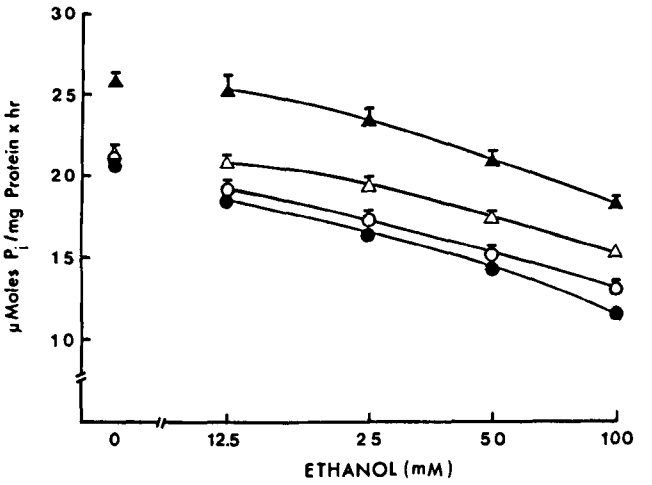

FIG. 5. Effect of various E concentrations (12.5 mM–100 mM) on synaptosomal (Na^+ + K^+)-ATPase sensitized by 1 μM N, from control rats, chronically E treated rats and after 24 hr withdrawal. ▲ chronically E treated for 3 weeks, last dose administered 24 hrs prior to experiment; ● control rats received equicaloric sucrose; △ chronically E treated for 3 weeks; 3 g/kg E given 1 hr prior to sacrifice; ○ control rats received equicaloric sucrose for 3 weeks and 1 hr prior to sacrifice.

partially purified microsomes. Addition of 0.05 M E alone or of 0.1 mM N, A, P or I separately had no effect on the enzyme activity (Fig. 3). However, the α-agonists, A or P, together with E, produced about 45% inhibition of enzyme activity, similar to the effect of N + E. I + E did not produce inhibition. N + E inhibition was prevented by an α-blocker (phentolamine 0.1–10 μM) but not by a β-blocker (propranolol, 100 μM).

Temperature Dependence of N-E Interaction on ATPase

The enzyme activity in rat brain homogenates increased with temperature (Fig. 4). An Arrhenius plot of the data shows a transition temperature (T_d) at 19.10 ± 0.11°C and an energy of activation (E_a) of 30.00 ± 1.02 Kcal/mol below T_d and 13.8 ± 0.38 Kcal/mol above T_d. Addition of E (0.05 or 0.22 M) or N (1 μM or 100 μM) separately had no effect on T_d or E_a. In the presence of 1 μM N + 0.05 M E, the enzyme activity was inhibited progressively more with increasing temperature above T_d, but no inhibition was observed below T_d. The interaction of N + E had no effect on E_a below T_d but decreased it significantly to 10.5 ± 0.37 Kcal/mol above T_d. A similar lowering of E_a (9.2 Kcal/mol) was observed with 0.44 M E above T_d, suggesting that N sensitized the enzyme to E inhibition by about 8-fold.

Effect of Chronic E Treatment on N-E Interaction with (Na^+ + K^+)-ATPase

After 3 weeks of chronic E treatment, the animals had become tolerant to the hypothermic effect of E. A test dose of E produced a temperature fall of 6.20 ± 0.32°F in the controls, vs. 3.32 ± 0.37°F in the E group ($p<0.001$).

After chronic E treatment and withdrawal (blood alcohol concentration=0 mg/dl), there was a 25% increase ($p<0.001$) in the enzyme activity of the synaptosomal fraction, but E administration 1 hr prior to sacrifice abolished this stimulatory effect. The blood alcohol concentration in the latter case was 119.3 ± 15.9 mg/dl at sacrifice. Therefore continued

presence of E prevented the increase associated with withdrawal, as we reported previously [20].

Addition of 0.1 M E or 1 μM N separately had no effect on the enzyme with preparations from either chronic E or withdrawn animals. In presence of 1 μM N, E inhibited the enzyme in a concentration-dependent manner in control rats (Fig. 5). However, after chronic E treatment, E in presence of 1 μM N had to be doubled to produce the same degree of inhibition as in controls. This was true for both non-withdrawn and withdrawn animals, suggesting that the decreased N sensitization for E inhibition is an effect of chronic E administration rather than of withdrawal.

DISCUSSION

Since N sensitized the enzyme to E inhibition, regardless of whether the ATP contained vanadium or not, the N + E interaction is independent of catecholamine stimulation of the enzyme. Inhibition by high concentrations (e.g., 0.22 M) of E alone was competitive with respect to K^+ and appeared to be produced by an allosteric effect on the K^+ binding site [12]. The same features were observed here for the N + E interaction. In contrast, V_{max} and K_m values for Na^+ and Mg^{++} were reduced by N + E at 5 mM K^+, but at 20 mM K^+, V_{max} was the same as that of controls. These findings suggest that N altered the enzyme in such a way that E can convert it to the inwardly facing Na^+-binding form rather than the phosphorylated outward-facing K^+-binding form. N itself lowers the K_m for ATP without affecting V_{max}, thus raising the "physiological efficiency" i.e. the ratio V_{max}/K_m [8]. In contrast, the combination of N + E does not lower K_m further but reduces V_{max}, thus lowering the physiological efficiency of ATP use. Therefore the sensitization by N appears to be a facilitation of the same conformational change in the enzyme that is produced by E alone.

In confirmation of our earlier findings [21], this effect of N appears to be exerted through an α-adrenergic receptor because other α-agonists were also effective while a β-agonist was not, and an α-blocker prevented the sensitization while a 1000-fold higher concentration of a β-blocker did not.

The absence of any N-E interaction below T_d for the ATPase, and the progressively greater interaction with increasing temperature above T_d, suggest that facilitation of conformational change by N is effected through an enhanced fluidization of membrane lipids. This is comparable to the membrane fluidization produced by higher concentrations of E alone, and is reminiscent of the model for a β-adrenergic action on adenyl cyclase proposed by Hirata *et al.* [9].

In membranes from non-withdrawn E-tolerant rats, the N-E interaction was markedly reduced in comparison with controls. Therefore, the change probably represents an effect of chronic E on the structure of the cell membrane, rather than a consequence of withdrawal, even though it was still present 24 hr after withdrawal. The reduction in N-E interaction appears to support the findings of decreased fluidization by E in membranes from tolerant animals [3]. The only apparent effect of withdrawal was an increase in basal activity of the enzyme, as observed previously [20].

CONCLUSIONS

(1) Rat brain (Na^+ + K^+)-ATPase is sensitized to E inhibition by N acting through an α-receptor. (2) The sensitization apparently consists of membrane fluidization which facilitates conformational change of the enzyme by E. (3) The conformational change, like that produced by E alone, is reversible by increase in K^+. (4) Tolerance to E involves an alteration in the cell membrane, which diminishes the sensitizing effect of N.

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