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STUDIES ON THE REACTION CATALYZED BY TRANSPORT (Na, K) ADENOSINE TRIPHOSPHATASE—II.

IN VITRO AND *IN VIVO* EFFECTS OF PHENOXYBENZAMINE*

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Abstract—Phenoxybenzamine is a potent inhibitor of NaK-ATPase both *in vitro* ($I_{50} = 3.5 \times 10^{-5}$ M) and *in vivo* (3 mg/kg inhibits NaK-ATPase in 100 per cent of the animals tested). The inhibition of the enzyme, *in vitro*, was time- and temperature-dependent, being maximal at 37° for 30 min. The inhibition was irreversible and was not specifically influenced by incubation in the presence of Na, K, Mg, ATP or varying pH. Phenoxybenzamine did not alter the concentration of Na or K required for half-maximal activation of the enzyme. Incubation of the enzyme with phenoxybenzamine under conditions where the phosphorylated form of the enzyme exists gave an I_{50} identical to that observed when no phospho-enzyme is formed. K-NPPase activity was inhibited by phenoxybenzamine ($I_{50} = 5.1 \times 10^{-5}$ M) over the same concentration range as required for inhibition of the NaK-ATPase reaction. Neither norepinephrine nor phentolamine could prevent the inhibition by phenoxybenzamine *in vitro*. Ouabain did not alter the sensitivity of the enzyme for phenoxybenzamine.

Catecholamines have been shown to influence the sodium–potassium stimulated adenosine triphosphatase [ATP phosphoxydrolase, EC 3.6.1.3. (NaK-ATPase)] activity from a variety of sources [cf. Ref. 1]. While investigating the catecholamine activation of NaK-ATPase in this laboratory, it was decided to test the antagonistic effects of adrenergic blocking agents on the action of catecholamines. One of the agents, phenoxybenzamine (POB), was observed to be a potent inhibitor of NaK-ATPase from beef cerebral cortex. Roufogalis and Belleau [2] made a similar observation in a study designed to determine the nature of the alpha adrenergic receptor. The studies reported here describe the inhibition of NaK-ATPase by POB and the determination of whether POB, due to its ability to alkylate proteins [3], could inhibit NaK-ATPase *in vivo*.

MATERIALS AND METHODS

Enzyme preparation. NaK-ATPase (NaI-enzyme) was prepared from beef cerebral cortex as described previously [1].

Inhibition studies. A membrane preparation of NaK-ATPase containing 30–50 μg protein was preincubated for 30 min with POB in the presence of 0.03 M imidazole HCl, pH 7.0, at 37°. Additional reagents were present in some of the experiments (see Fig. legends). At the end of the preincubation period, the tubes were placed on ice and the remaining reactants for the NaK-ATPase assay added. The tubes were then warmed to 37° and the reaction was started by the addition of ATP, or, if ATP was present during the 30-min preincubation, Na and K. Unless stated otherwise, the final concentrations of reactants in the NaK-ATPase assay are: 0.130 M NaCl, 0.02 M KCl, 0.008 M $MgCl_2$, 0.004 M Na_2ATP, 0.03 M imidazole HCl, pH 7.0, and 1×10^{-4} M ouabain. All determinations were made in duplicate. The reaction was stopped after 5 min by the addition of 5% trichloroacetic acid in chloroform–methanol (1:1). After vigorous mixing, the tubes were centrifuged at 1500 r.p.m. The P_i in the upper phase was measured using a modified Fiske–SubbaRow method [4]. 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. Protein was measured according to the method of Lowry *et al.* [5] using bovine serum albumin, Cohn fraction V, as the standard.

Incubation of potassium activated *p*-nitrophenylphosphatase [ortho-phosphoric monoester phosphohydrolase EC 3.1.3.1 (K-NPPase)] with POB was performed in duplicate under the conditions described above for the incubation of NaK-ATPase with POB. After the 30-min preincubation in 0.03 M imidazole HCl, pH 7.0, the tubes were placed on ice, and KCl and $MgCl_2$ were added to final concentrations of 0.015 and 0.003 M respectively. The reaction was started by the addition of 0.003 M Tris *p*-nitrophenylphosphate, pH 7.0. The reaction was stopped at 5 min by the addition of 10% trichloroacetic acid. All tubes were centrifuged at 1500 r.p.m. for 10 min. Aliquots were taken and alkalinized with 1 N NaOH. The extinction of *p*-nitrophenol was read at 410 nm.

Studies in vivo. Male Sprague–Dawley rats (200 g) were used in all studies. Experimental animals

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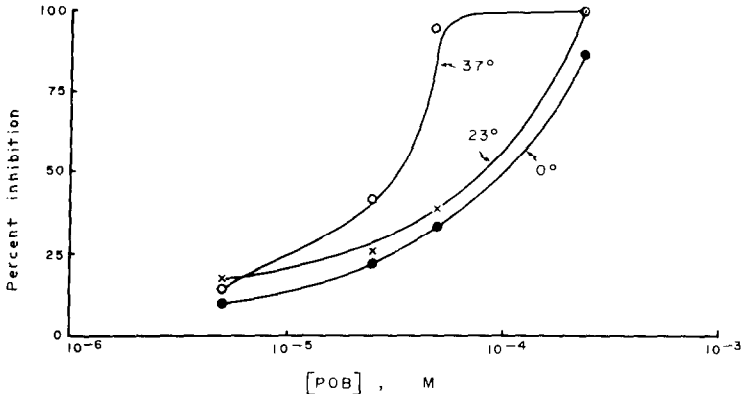

Fig. 1. Temperature dependence of NaK-ATPase inhibition by POB. Increasing concentrations of POB were preincubated with NaK-ATPase (30–50 μg protein) in 0.03 M imidazole HCl, pH 7.0, for 30 min at the temperatures indicated. At the end of this period, tubes were chilled on ice, the remaining reactants were added, the tube contents were warmed to 37° and the reaction was started by the addition of ATP to a final concentration of 0.004 M. After 5 min, the reaction was stopped and the specific activity determined as described in Methods. The specific activity of Nak-ATPase not treated with POB was 50 μmoles/mg/hr. Per cent inhibition was calculated by dividing the sp. act. of POB-treated enzyme by the sp. act. of enzyme not treated with POB but incubated for the same time period.

were injected in the tail vein with 0.6 ml of 1 mg/ml of POB in 0.9% saline solution, i.e., 3 mg/kg, while under light ether anesthesia. Each injection was given over a 5-min period. Control rats were injected similarly with 0.6 ml of 0.9% saline solution. After 4 hr, the rats were sacrificed by decapitation. The brains were removed and made into a 10% (w/v) homogenate using 0.32 M sucrose containing 0.001 M EDTA (Na salt), pH 7.0. Homogenization was performed using a Potter–Elvehjem homogenizer (seven passes) driven by a Tri-R Stir-R, model K41, at a speed setting of 5. Synaptosomes were prepared according to the method of Gray and Whittaker [6] except that the discontinuous sucrose gradients were centrifuged at 22,000 r.p.m. in the SW 41 rotor of the Spinco model L3-50 centrifuge. NaK-ATPase activity was determined as described previously [7].

Chemicals. All chemicals were of the highest grade commercially available and were used without further purification. POB was freshly prepared at the start of each experiment since stored solutions produced varying degrees of inhibition. Smith, Kline & French Labs, Philadelphia, PA, generously provided the POB.

RESULTS

Effect of time and temperature on preincubation. Preliminary experiments showed that preincubation of NaK-ATPase with 2.5×10^{-5} M POB at 37° results in a time-dependent increase in enzyme inhibition. The increase becomes maximal and constant at 30 min. The activity of NaK-ATPase not treated with POB was constant throughout the time course. Preincubation of increasing concentrations of POB with NaK-ATPase at 0, 23 and 37° (Fig. 1) for 30 min results in a dose-dependent increase in inhibition of the enzyme at each temperature tested. Increasing amounts of inhibition are seen as the temperature of preincubation is raised. The I_{50} values at 0, 23 and 37° are 1.0×10^{-4}, 8.1×10^{-5} and 3.3×10^{-5} M respectively. The activity of POB-treated enzyme is compared against enzyme not treated with drug but preincubated under the same conditions. The enzyme was completely stable for 30 min at each temperature tested.

Effect of pH on preincubation. NaK-ATPase was preincubated with 5×10^{-5} M POB at several pH values ranging from 6.0 to 8.0 (Fig. 2). Preincubation was at 0° for 30 min due to the reduced stability of the enzyme at the pH extremes when preincubations were carried out at 37°. The results show the same degree of inhibition at all pH values tested with a broad pH optimum between 7.0–7.5.

Reversibility of the inhibition by POB. The ability of 2-halo-alkylamines such as POB to react with various chemical groups of protein molecules and irreversibly bind probably via an ethylenimonium ion intermediate is well known [3, 9]. These facts suggest that the inhibition of NaK-ATPase by POB should be irreversible. Microsomes were treated

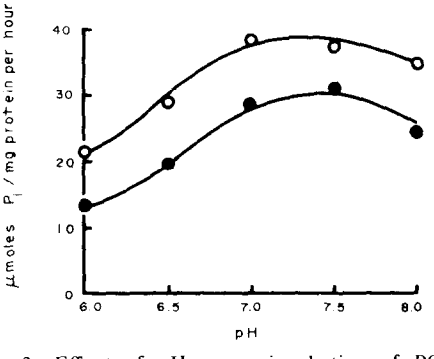

Fig. 2. Effect of pH on preincubation of POB with NaK-ATPase. NaK-ATPase was preincubated with 5×10^{-5} M POB for 30 min at 0° in 0.03 M imidazole HCl, at the pH indicated. At the end of this period, the remaining reactants were added, the tube contents were warmed to 37°, and the reaction was started by the addition of ATP to a final concentration of 0.004 M. After 5 min, the reaction was stopped and the sp. act. determined as described in Methods. Key: ○–○, without POB; ●–●, with POB.

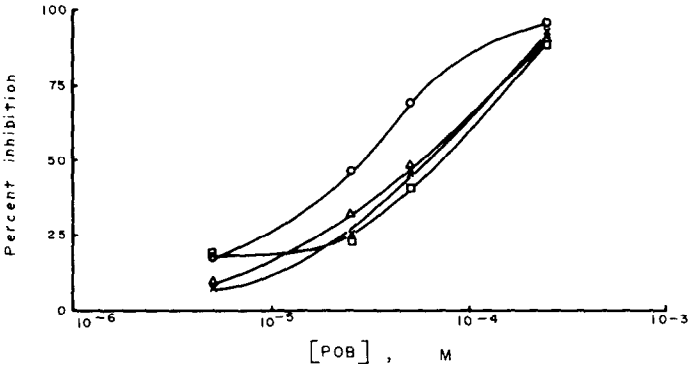

Fig. 3. Effect of ATP and ions on POB inhibition of NaK-ATPase. NaK-ATPase was preincubated (37° for 30 min) with increasing concentrations of POB in the presence of: 0.03 M imidazole HCl, pH 7.0 (○–○); 0.004 M Na ATP, pH 7.0 (×–×); 0.02 M KCl (□–□); and 0.008 M $MgCl_2$ (△–△). At the end of this time period, the tubes were chilled on ice, the remaining reactants added, and the tube contents warmed to 37°. The reactions (1 ml volume) were started by the addition of 0.1 ml of 0.04 M Na_2ATP, pH 7.0, except when ATP was present in the preincubation mixture. In the latter case, the reaction was started by the addition of 0.25 ml of 0.52 M NaCl, 0.8 M KCl and 0.032 M $MgCl_2$. After 5 min, the reaction was stopped and the sp. act. determined as described in Methods. The sp. act. of NaK-ATPase not treated with POB was 45 μmoles/mg/hr. Per cent inhibition was calculated as described in Fig. 1.

with 0, 7.5×10^{-5} and 3.0×10^{-4} M POB for 30 min at 37°, centrifuged at 100,000 *g* for 30 min and washed twice. The per cent of inhibition of NaK-ATPase before and after centrifugation and washing was 42 per cent at 7.5×10^{-5} M and 88 per cent at 3.0×10^{-4} M. This demonstrates the irreversible nature of NaK-ATPase inhibition by POB.

Effect of ions and substrate on POB inhibition. The effect of K, Mg or Na and ATP on NaK-ATPase inhibition by POB was examined (Fig. 3). The data show a shift of the dose–response curve to the right when K, Mg or Na and ATP are added compared to the curve obtained with imidazole alone. There is at least a 2-fold increase in the I_{50} with the addition of ions or ATP.

It was then decided to test whether formation of the phospho-enzyme would have any effect on POB inhibition. NaK-ATPase was preincubated with 0.13 M NaCl, 0.008 M $MgCl_2$, 0.03 M imidazole HCl, pH 7.0, with and without 0.004 M Na_2ATP for 3 min at 37°. Then increasing concentrations of POB were added and the preincubation was continued for an additional 30 min at 37°. ATP was added to the control tubes and the reaction started by the addition of KCl to a final concentration of 20 mM. The results showed no difference in the inhibition of NaK-ATPase in either the phosphorylated or non-phosphorylated form at any concentration of POB tested. The extent of POB inhibition was similar to that seen when K, Mg or Na and ATP are present (Fig. 3).

Initial velocity studies where Mg–ATP (2:1) were varied during preincubation with NaK-ATPase and three concentrations of POB resulted in data exhibiting "non-competitive" inhibition when a Lineweaver–Burk plot was constructed (data not shown). This is to be expected for an irreversible inhibitor since the concentration of active enzyme is reduced similar to the case for true-non-competitive inhibition.

Initial velocity studies were also carried out where Na was varied at several concentrations of K and where K was varied at several concentrations of Na with NaK-ATPase in the presence and absence of 2.5×10^{-5} M POB. Hyperbolic plots were obtained both in the presence and absence of POB (data not shown). Determination of the concentration of monovalent cations required for half-maximum activation of the NaK-ATPase gave the results shown in Table 1. POB inhibition did not alter the half-maximum concentrations of either Na or K when either subsaturating or saturating amounts of K or Na were present respectively.

Table 1. Concentrations of Na^+ and K^+ required for half-maximum velocity in the presence of absence of POB*

	K^+ (M)	
Na^+ (M)	With POB	Without POB
0.01	0.0007	0.0006
0.1	0.0008	0.001
	Na^+ (M)	
K^+ (M)	With POB	Without POB
0.001	0.0025	0.0025
0.02	0.0125	0.012

* NaK-ATPase was preincubated (37° for 30 min) with increasing concentrations of KCl at constant NaCl (0.01 or 0.1 M) and imidazole HCl (0.03 M, pH 7.0) concentrations in the presence and absence of POB (upper portion of the table), or with increasing concentrations of NaCl at constant KCl (0.001 or 0.02 M) and imidazole HCl (0.03 M, pH 7.0) concentrations in the presence and absence of POB (lower portion of Table). After 30 min, the tubes were placed on ice, the remaining reactants added, and the tube contents warmed to 37°. The reactions were started by the addition of ATP to a final concentration of 0.004 M. The final concentration of POB was 2.5×10^{-5} M. After 5 min, the reaction was stopped and the sp. act. determined as described in Methods.

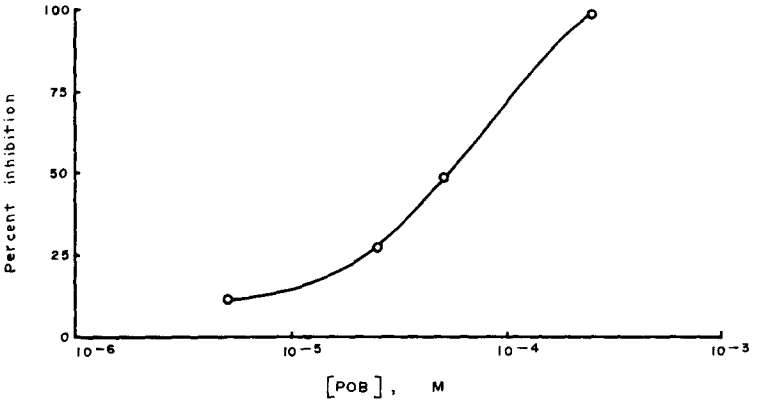

Fig. 4. Effect of POB on K-NPPase. Potassium-activated *p*-nitrophenyl phosphatase (K-NPPase) associated with the NaK-ATPase was preincubated with 0.03 M imidazole HCl, pH 7.0, at 37° for 30 min with increasing concentrations of POB. At the end of this period, the tubes were chilled on ice, the remaining reactants added, the tube contents were warmed to 37° and the reaction was started by the addition of NPP. After 5 min, the reaction was stopped and the sp. act. determined as described in Methods. The sp. act. of K-NPPase not treated with POB was 17 μmoles/mg/hr. Per cent inhibition was calculated as described in Fig. 1.

Effect of POB on K-*NPPase.* The effects of POB were tested on the neutral phosphatase reaction since the bulk of reported data show that this reaction mimics the dephosphorylation step in the NaK-ATPase reaction [8]. Preincubation of K-NPPase (37° for 30 min) with increasing concentrations of POB gave a dose-dependent increase in inhibition of the enzyme (Fig. 4). The I_{50} is 5.1×10^{-5} M, which is similar to that obtained for the NaK-ATPase.

Effect of norepinephrine, phentolamine and ouabain on POB inhibition. POB interacts with the alpha adrenergic receptor and blocks the action of alpha receptor agonists. Therefore, it seemed reasonable to test whether other agents known to interact with the alpha receptor could prevent the inhibition of NaK-ATPase by POB. NaK-ATPase was preincubated with either 5×10^{-4} M norepinephrine bitartrate or 1×10^{-4} M phentolamine mesylate and 2.5×10^{-5} M POB for 30 min at 37°. Neither norepinephrine nor phentolamine had any effect on the inhibition by POB.

NaK-ATPase was also preincubated with ouabain (1×10^{-8} M) and then increasing concentrations of POB were added (Fig. 5). This concentration of ouabain inhibited the enzyme about 35 per cent. The per cent inhibition of the NaK-ATPase by POB was the same in the presence and absence of ouabain.

Effect of POB on NaK-*ATPase* in situ. Since POB is an irreversible inhibitor of NaK-ATPase *in vitro*, it seemed reasonable to test whether this drug had any effect on NaK-ATPase *in vivo*. Male rats were injected in the tail vein with POB in saline (3 mg/kg) or saline (control) as described in Methods. The

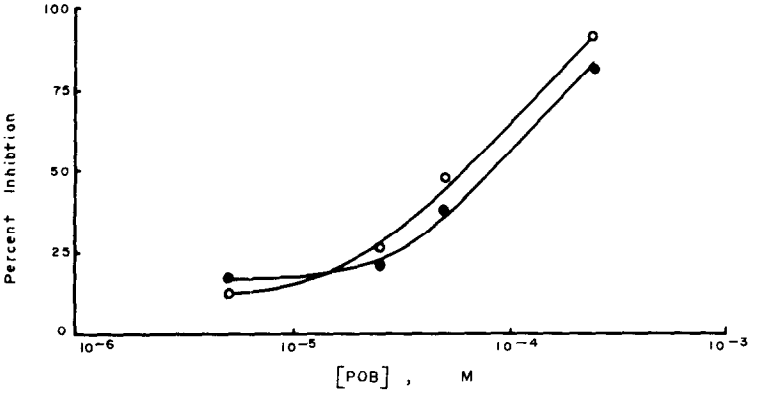

Fig. 5. Effect of ouabain on POB inhibition of NaK-ATPase. NaK-ATPase was preincubated with 0.1 M NaCl, 0.005 M $MgCl_2$, 0.004 M Na_2ATP, pH 7.0, and 0.03 M imidazole HCl, pH 7.0, for 3 min at 37°, with and without 1×10^{-8} M ouabain. Then increasing concentrations of POB were added and the preincubation was continued for an additional 30 min at 37°. At the end of this period, the tubes were chilled on ice, the remaining reactants added, and the tube contents warmed to 37°. The reaction was started by the addition of KCl to a final concentration of 0.02 M. After 5 min, the reaction was stopped and the sp. act. determined as described in Methods. The sp. act. of NaK-ATPase not treated with POB or ouabain was 30 μmoles/mg/hr. After the addition of 1×10^{-8} M ouabain, the sp. act. was 20 μmoles/mg/hr. Per cent inhibition was calculated by dividing the sp. act. of POB-treated enzyme by the sp. act. of untreated NaK-ATPase (○——○), or by dividing the sp. act. of ouabain- and POB-treated enzyme by the sp. act. of ouabain-treated enzyme (●——●).

Table 2. Reduced activity of NaK-ATPase in brains of rats injected with POB*

Treatment	N	Sp. act. (μmoles P_i/mg protein/hr)	P
Control	9	17.4 ± 1.79	
+ POB	9	13.7 ± 1.92	< 0.005

* Rats were injected with either POB (3 mg/kg) in 0.9% saline solution or the injecting vehicle alone. After 4 hr, the rats were decapitated, their brains removed, synaptosomes prepared and NaK-ATPase activity was measured (see Methods). The level of significance was determined using Student's *t* test.

effective dose for POB administered to rats parenterally is 0.25 to 20 mg/kg. Isolation of synaptosomes and assay of NaK-ATPase activity showed that animals receiving POB had NaK-ATPase activity that was significantly lower than animals receiving only saline (Table 2). Animals receiving POB were sedated compared to control animals. Blood pressures were not measured.

DISCUSSION

In an earlier report, Roufogalis and Belleau [2] investigated the effects of a series of adrenergic blocking (alkylating) agents on beef brain NaK-ATPase. They showed that POB was one of the most potent inhibitors ($I_{50} = 1 \times 10^{-4}$ M) of the compounds tested. The results of the present study confirm this observation and further characterize the inhibition of NaK-ATPase by POB. In addition, the effectiveness of POB as an inhibitor of NaK-ATPase *in vivo* is also demonstrated.

The inhibition of NaK-ATPase from beef cerebral cortex is time dependent and reaches a maximal level at 30 min. The slow rate of reaction between POB and NAK-ATPase is probably a consequence of the time required for formation of the ethylenimonium ion [9]. The rate of formation of the ethylenimonium ion intermediate is temperature dependent as well [9], resulting in less inhibition of the enzyme (larger values for the I_{50}) at temperatures of 0 and 23° (Fig. 1).

Variation of the pH of the preincubation medium from 6.0 to 8.0 had no effect on NaK-ATPase inhibition by POB. The specific activity of the enzyme was reduced at the pH extremes but the degree of inhibition did not vary. Roufogalis and Belleau [2] showed that the rate of inhibition by an analogue of POB was slower at pH 6.0 but produced the same degree of inhibition within 30 min of preincubation.

The addition of various ligands to the NaK-ATPase has been reported to produce different conformations of the enzyme (for review, see Ref. 8). Therefore, it might be expected that the addition of selected ligands would result in changes in POB inhibition since reactive groups may be sequestered. The inclusion of K, Mg or Na and ATP during the preincubation of NaK-ATPase with POB results in a shift of the dose–response curve to the right and a 2-fold increase in the I_{50} (Fig. 3). Since only a non-selective increase in the I_{50} occurred, it appears that the site of POB interaction with the NaK-ATPase is not affected specifically by enzyme conformational changes produced by these ligands. The increase in ionic strength of the preincubation medium might explain the changes in I_{50} seen here. The degree of interaction between NaK-ATPase and inhibitor may be dependent on the accessibility of reactive groups on the enzyme which is controlled by ionic strength. Alternatively, an increase in ionic strength could slow the formation of the ethylenimonium ion intermediate. In addition, formation of the phosphorylated form of the enzyme, the so-called E_1-P intermediate (cf. Ref. 8), does not produce any change in the enzyme that renders it any more, or less, susceptible to inhibition by POB.

NaK-ATPase activity has been shown to be dependent on the presence of intact sulfhydryl groups [10]. Furthermore, Na and ATP can protect these groups from attack by compounds such as *N*-ethylmaleimide. Since POB reacts predominantly with sulfhydryl groups *in vitro* [3], it might be expected that these groups are the site of action for POB. However, the interaction is not the same as that seen with *N*-ethylmaleimide because Na and ATP provide only weak protection against the effects of POB, unlike their more pronounced protective effects afforded against *N*-ethylmaleimide inhibition [10,11]. In addition, the site of action of POB does not appear to involve either the Na or K activation site since POB does not alter the concentration of either Na or K required for half-maximal activation of the enzyme. POB does react with the E_2 form of the enzyme since the neutral phosphatase reaction (K-NPPase) was inhibited by POB ($I_{50} = 5.1 \times 10^{-5}$ M). Thus, it appears that POB reacts non-specifically with groups on the enzyme and reveals little new information about the reaction mechanism.

It is unlikely that the NaK-ATPase comprises any portion of the alpha adrenergic receptor [2] although catecholamines can influence the NaK-ATPase (cf. Refs. 1 and 12). To test whether there was any similarity between the NaK-ATPase described here and the alpha adrenergic receptor, norepinephrine or phentolamine was added to the NaK-ATPase preincubation to determine if either could prevent POB from inhibiting the NaK-ATPase. Inhibition of NaK-ATPase by POB was the same in the presence and absence of norepinephrine or phentolamine. These data are consistent with the non-specific nature of the POB inhibition. Similarly, ouabain had no effect on the inhibition by POB (Fig. 5), although the curve was shifted to the right due to the presence of Na, Mg and ATP.

The irreversible nature of the inhibition provided by this pharmacologically important compound provided a unique opportunity to test the effects of POB on NaK-ATPase *in vivo*. The injection of pharmacologic concentrations (3 mg/kg) into the tail vein of rats and the subsequent isolation of brain synaptosomal NaK-ATPase revealed that a significant reduction (Table 2) of NaK-ATPase had occurred. Although the NaK-ATPase is probably not involved in alpha adrenergic receptor mechanisms, this reduction in enzyme activity may provide insight into the previously unexplained means by which

POB produces sedation and other effects on the central nervous system. The central effects of POB are probably not related to its ability to block alpha adrenergic receptors [13].

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