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INHIBITORY CHARACTERISTICS OF THE MYCOTOXIN PENICILLIC ACID ON (Na^+–K^+)–ACTIVATED ADENOSINE TRIPHOSPHATASE*

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Abstract— Penicillic acid, a cardioactive mycotoxin produced by various *Penicillium* molds, is a potent and selective inhibitor of membrane (Na^+–K^+)-adenosine triphosphatase (ATPase). A broad range of inhibition of activity by the toxin was demonstrated with a half-maximal concentration equal to 1.8×10^{-8}M. Inhibition was time and pH dependent and complete after 20–30 min preincubation within a narrow range of physiological pH. Kinetic evaluation of cationic substrate activation of (Na^+–K^+)-ATPase indicated competitive inhibition with regard to Na^+ concentration and noncompetitive inhibition with regard to K^+ concentration. Also K^+-dependent *p*-nitrophenyl phosphatase activity was not significantly altered by penicillic acid, and uncompetitive inhibition with regard to ATP activation of the enzyme was demonstrated. Preliminary binding studies indicated that inhibition of ATPase activity could be partially restored by repeated washing and by incubation with dithiothreitol and cysteine. Penicillic acid (high concentrations) impaired [^{3}H]ouabain binding to membrane preparations but this effect was noncompetitive, indicating different sites of action for the two inhibitors. A significant linear correlation between reactive enzyme sulfhydryl content [SH] and ATPase activity in the presence of varying concentrations of toxin also was noted. It is postulated that penicillic acid inhibition of (Na^+–K^+)-ATPase occurs via critically accessible membrane thiol receptors regulating Na^+-dependent phosphorylation of the transport enzyme.

Penicillic acid, a tautomeric mycotoxin ($\Delta\alpha\beta$-γ-hydroxylactone or open ring substituted γ-keto hexenoic acid), was first isolated from the mold *Penicillium puberulum* by Alsberg and Black in 1913 [1]. It possesses antimicrobial and antitumor properties (lethal dose, 50 per cent) values in the range of 80–100 mg/kg. It also is carcinogenic in rats, with doses as low as 0.1 mg. initiating tumor development [4]. Specific biological effects of penicillic acid have not been studied, although Murnaghan [5] indicated that penicillic acid has "digitalis-like" action on frog heart, rabbit auricle, perfused cat heart and canine heart–lung preparation. Penicillic acid also had a dilator action on coronary and pulmonary vessels and produced a significant rise in blood pressure when injected into the whole animal. These findings were of interest since we recently showed that penicillic acid inhibited membrane (Na^+–K^+)-ATPase *in vitro* and *in vivo* [6]. The toxin was selective in that mitochondrial Mg^{2+} ATPase (oligomycin-sensitive and insensitive) activity was not affected at concentrations which significantly inhibited (Na^+–K^+)-ATPase. As is the case with many cardioactive steroids which also are potent inhibitors of (Na^+–K^+)-ATPase, the *in vivo* effect of penicillic acid on this enzyme system may be involved in observed toxicity. It also is possible that, since this mycotoxin is a selective inhibitor of (Na^+–K^+)-ATPase, it will be useful as a probe in understanding the mechanism of the (Na^+–K^+)-ATPase transport enzyme. Thus, the present study was initiated to characterize and to elucidate the inhibitory action of penicillic acid on this enzyme complex.

EXPERIMENTAL PROCEDURES

Penicillic acid was obtained from Makor Chemicals, Jerusalem, Israel. Toxin purity (99.8 per cent) was established by melting point, thin-layer chromatography, and infrared and mass spectra. The mycotoxin was stored in the dark at room temperature. Fresh solutions of the toxin were prepared for each experiment using double-distilled deionized water. [^{3}H]Ouabain (sp. act. 14.4 Ci/mmole) was purchased from New England Nuclear, Boston, MA. Fluoralloy TLA, BioSolv BBS-3 and scintillation grade toluene were from Beckman Instruments, Inc, Irving, CA. All other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.

Enzyme. Highly specific (Na^+–K^+)-activated ATPase from swine brain cerebral cortex microsomes (ATP phosphohydrolase, EC 3.1.6.3) (Sigma Chemical Co., St. Louis, MO), prepared according to the method of Nakao *et al.* [7], was used as the enzyme source. Enzyme preparations (pH 7.5) containing 0.32 M sucrose, 10 mM imidazole and 1.0 mM EDTA were quick frozen in liquid nitrogen, were stored in the dark at −80° until used for ATPase analysis, and were discarded after 25 per cent of the original activity was lost. These preparations were demonstrated consistently to be highly specific as to

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(Na^+–K^+)-ATPase activity (approximately 99.5 to 100 per cent of the total activity was ouabain sensitive). The average specific activity of the (Na^+–K^+)-activated component of enzyme preparations was 35.5 μmole/mg of protein/hr (ΔP_i), and, although somewhat lower than that reported by Nakao *et al.* [7], was consistently reproducible. The remaining activity (when present) was the basal Mg^{2+} component.

ATPase determination. Adenosine triphosphatase activity was determined using endpoint phosphate analyses and methods described previously [8,9]. A one-ml reaction mixture contained in final concentration: 5.0 mM ATP (vanadium free), 5.0 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 135 mM imidazole/HCl buffer (pH 7.5) and 5–40 μg of enzyme protein. Total cationic ligand-stimulated ATPase activity of enzyme aliquots was measured with Na^+, K^+ and Mg^{2+} present in reaction mixtures. The basal Mg^{2+} component was measured by omitting both Na^+ and K^+. Delineation of the (Na^+–K^+)-activated component was obtained from the difference between total ATPase ($Na^+ + K^+ + Mg^{2+}$) and basal Mg^{2+} activity (Mg^{2+} only). When present in reaction mixtures, penicillic acid was added in water (5 μl) to tubes containing either Na^+, K^+, and Mg^{2+} or Mg^{2+} only. The (Na^+–K^+)-ATPase activity was determined by the difference between the two. Water (5 μl) only was added to control reaction mixtures. Treated and control preparations, reagent and enzyme blanks were incubated simultaneously at 37° for various intervals prior to initiation of the hydrolysis reaction with ATP. Incubation was stopped after 10–20 min with the addition of trichloroacetic acid (5%, w/v) to the reaction mixture. Then samples were assayed for inorganic phosphate using the modified method of Lowry and Lopez [10]. Protein was determined by the method of Lowry *et al.* [11], using bovine serum albumin as the standard. Maintenance of ionic strength and osmolarity in reaction mixtures was achieved by adding inert choline chloride when either Na^+, K^+ or Mg^{2+} concentrations were varied from optimal experimental concentrations.

Analysis of p-nitrophenyl phosphatase. The K^+-stimulated phosphatase activity of enzyme preparations was measured utilizing hydrolysis of the substrate *p*-nitrophenyl phosphate (5.0 mM) in the presence of 5.0 mM Mg^{2+}, 10 mM K^+, 100 mM Tris–HCl buffer (pH 7.4) and 40 μg of microsomal protein at 37° in a final volume of 1.0 ml [12,13]. After incubation, aliquots of reaction supernatant fluids were diluted with 1M Tris (pH 10.4), and the optical density was determined at 410 nm against a suitable blank. K^+-stimulated phosphatase activity was expressed as nmoles P_i/mg of protein/min in the presence of $Mg^{2+} + K^+$ minus activity in the presence of Mg^{2+} only. Penicillic acid was added to reaction mixtures as described earlier.

Kinetic evaluation. Methods of analysis, with minor variations in substrate ligand concentrations, incubation times and protein content, were as described previously by Ahmed *et al.* [14], Phillips and Hayes [8] and Phillips *et al.* [9]. Two inhibitor (toxin) concentrations, in most cases the concentrations which produced 50 per cent inhibition (I_{50}) and 25 per cent inhibition (I_{25}), were plotted against the control. Variations in toxin concentration, as well as mean apparent K_m and V_{max} values for independent studies, are listed under Results or in the figure legends.

Reactive membrane sulfhydryl [SH] group determination. Sulfhydryl reactivity for toxin-membrane protein was determined utilizing a lead mercaptide reaction and analysis of free thiol groups as described by Pasto and Johnson [15]. Total sulfhydryls per mg of protein also were determined by the method of Ellman [16] utilizing the reaction of enzyme preparation with DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)]. Absorbance of the liberated reaction product (5-thio-2-nitrobenzoic acid) was monitored against a suitable blank to correct for protein and imidazole using a Beckman Acta III recording spectrophotometer. The incubation medium consisted of 50–100 μg of enzyme protein/ml in 135 mM imidazole buffer (pH 7.5). Penicillic acid was added to reaction mixtures as described previously but prior to addition to DTNB. Fully reactive membrane thiol was calculated using cysteine-HCl as a standard and expressed in terms of nanoequivalents [SH]/mg of enzyme protein (n-equiv.). The free sulfhydryl concentration also was estimated from the molar extinction coefficient of the *p*-nitrothiophenol anion ($13{,}600\ M^{-1}cm^{-1}$).

Assay of [^{3}H]ouabain-ATPase binding. The binding of [^{3}H]ouabain to microsomal (Na^+–K^+)-ATPase was carried out under experimental conditions favourable for rapidly saturable "Complex I" formation, as described by Van Winkle *et al.* [17]. Binding medium consisted of 1.25 mM Na^+–ATP, 1.25 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 7.4) and various concentrations of [^{3}H]ouabain (0–700 nM), in a volume of 2–10 ml/tube. Binding experiments were initiated with enzyme (50–100 μg/ml of binding medium) in tubes prewarmed to 37° in a water bath and were terminated using the rapid millipore filtration technique described by Van Winkle *et al.* [17] and Wallick *et al.* [18]. Aliquots (1.0 ml) taken at specific intervals were suctioned rapidly through Millipore filters (0.45 μpore size) followed by three rapid washes of 5 ml of deionized water. Filters were removed carefully and placed in scintillation vials containing 10 ml of 10% BBS-3 Fluoralloy TLA/toluene scintillation medium and counted in a Beckman LS-250 liquid scintillation spectrometer using appropriate blanks and standards. External channel ratio (calibrated with internal standards) was used to monitor counting efficiency. Ouabain–receptor binding was expressed in terms of pmoles [^{3}H]ouabain bound/mg of enzyme protein and was determined from saturable, specific binding in all experiments. Nonspecific, nonsaturable binding (<2.0 per cent of total binding) was determined by preincubation of the enzyme with 10^{-3} M unlabeled ouabain for 10 min prior to initiation of binding or by using a heat inactivated enzyme preparation.

Expression of results. Kinetic data were transformed to double reciprocals and plots were constructed according to the method of Lineweaver and Burk [19]. Data were subjected to regression analysis and also analyzed by Student's *t*-test. Differences

from controls were considered significant at $P \leq 0.05$. Other methods or modifications of a described procedure are detailed in the appropriate figure legend or Results.

RESULTS

Inhibition of (Na^+–K^+)-*ATPase.* Penicillic acid significantly inhibited microsomal (Na^+–K^+)-ATPase activity in a concentration-dependent manner with an I_{50} of 1.8×10^{-8} M (Fig. 1). Impairment of enzyme activity also was observed over a broad toxin concentration range with inhibition still present at 5×10^{-12} M penicillic acid. Inhibition of enzyme activity was specific for (Na^+–K^+)-ATPase; the basal Mg^{2+}-activated ATPase activity of preparations was not affected by penicillic acid at the concentrations tested.

Inhibition during preincubation of penicillic acid with (Na^+–K^+)-ATPase was time and concentration dependent, approaching steady state after 10–30 min reaction time (Fig. 2). No further inhibition of activity occurred.

Time course. Inhibition of (Na^+–K^+)-ATPase by penicillic acid was independent of incubation time and enzyme concentration (after 30 min preincubation prior to initiation) (Fig. 3). Linear rates of ATP hydrolysis were observed with 10 and 40 μg of enzyme protein through 40 and 20 min of incubation time, respectively, with and without penicillic acid. Linearity at the highest protein concentration was maintained until 0.6 to 0.7 μmole P_i was formed in the reaction mixture, which suggests product inhibition or negative feedback of the ATPase system by ADP at these levels [8,9,14].

Effect of pH on inhibition of (Na^+–K^+)-*ATPase by penicillic acid.* The pH of separate incubation mixtures was varied from 6.0 to 9.0 by using a mixture of Tris/imidazole/HCl buffers (30 mM) [8,9,14]. Under the experimental conditions employed, optimal (Na^+–K^+)-ATPase activity was observed at pH 7.5 (Fig. 4). Inhibition by penicillic acid varied with pH. Maximal inhibition of (Na^+–K^+)-ATPase activity occurred between pH 7.0 and 8.0, with significant impairment of inhibitory potency at both

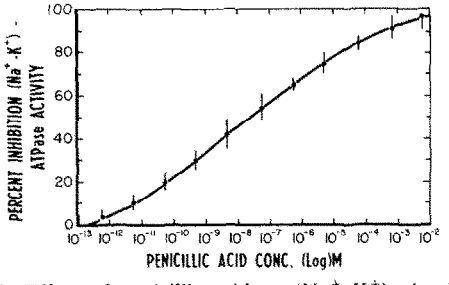

Fig. 1. Effect of penicillic acid on (Na^+–K^+)-stimulated ATPase activity. Each point represents the $\bar{X} \pm$ S.E. percent inhibition of activity (μmoles P_i/mg of protein/hr) of three independent replicate experiments each assayed in triplicate. Toxin was preincubated for 30 min at 37° with enzyme (40 μg) in the incubation mixture before initiation of the reaction.

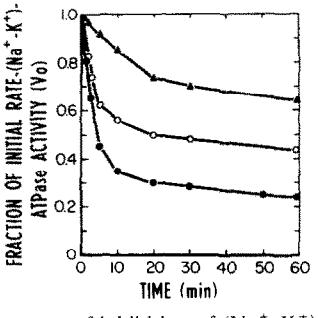

Fig. 2. Time course of inhibition of (Na^+–K^+)-ATPase by penicillic acid. Toxin was preincubated for different time intervals at 37° with enzyme (40 μg) prior to ATPase assay. Data, expressed as a fraction of the initial control rate of ATP hydrolysis, represent $\bar{X}$ activity from three independent replicates, each assayed in triplicate. (Na^+–K^+)-ATPase was expressed as activity in the presence of $Na^+ + K^+ + Mg^{2+}$ minus activity in the presence of Mg^{2+} Key: (▲) 5×10^{-9} M toxin; (○) 1.8×10^{-8} M toxin; (●) 2.0×10^{-6} M toxin.

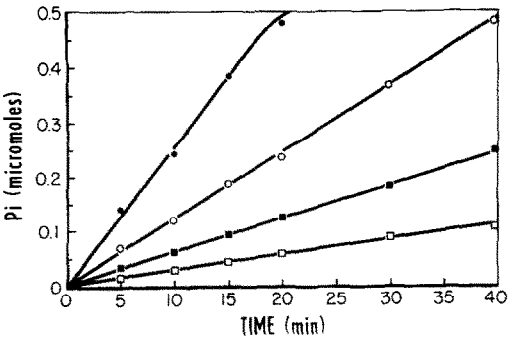

Fig. 3. Time course of incubation reaction with and without penicillic acid in reaction mixtures. P_i represents activity (μmoles) in the presence of optimal concentrations of Na^+, K^+ and Mg^{2+} minus that in the presence of Mg^{2+} alone. Forty μg of enzyme protein (●, control; and ○, 1.8×10^{-8} M penicillic acid). Ten μg of enzyme protein (■, control; □, 1.8×10^{-8} M penicillic acid). Each point represents the $\bar{X}$ activity from duplicate experiments assayed in triplicate.

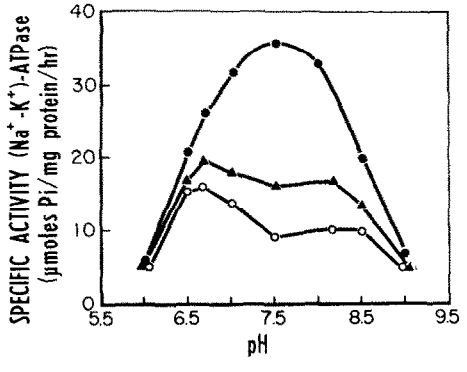

Fig. 4. Effect of pH on penicillic acid inhibiton of (Na^+–K^+)-ATPase. Various pHs were obtained using mixtures of imidazole/Tris–HCl buffers. Protein (40 μg) was present in reaction mixtures, and (Na^+–K^+)-ATPase was expressed as activity in the presence of $Na^+ + K^+ + Mg^{2+}$ minus activity in the presence of Mg^{2+} alone. Key: (●) control; (▲) $1.8 \times 10^{+8}$ M penicillic acid; and (○) 5.0×10^{-4} M penicillic acid. Each point represents $\bar{X}$ activity of duplicate experiments assayed in triplicate.

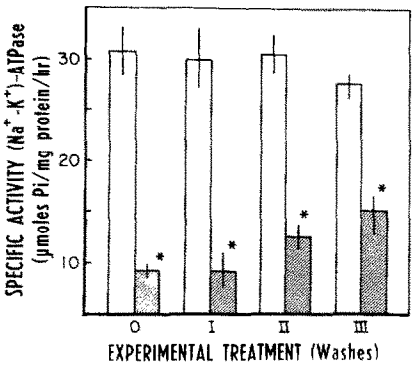

Fig. 5. Reversibility of penicillic acid inhibition of (Na^+–K^+)-ATPase with washing. Three enzyme preparations were appropriately diluted in sucrose solution (0.32 M sucrose, 10 mM imidazole and 1.0 mM EDTA, pH 7.5); after removing an aliquot (containing 35 μg protein/50 μl) for control assay the remaining protein was treated with 5×10^{-4} M penicillic acid and aliquots were removed for treated analysis. Control and treated protein suspensions were diluted 10-fold with ice-cold sucrose solution and centrifuged at 70,000 *g* for 1 hr at 4°. The pellets were resuspended in suitable volumes, and control and treated aliquots were removed and assayed for (Na^+–K^+)-ATPase activity. This procedure was repeated twice. Data represent $\bar{X} \pm$ S.E. activity of triplicate assays from three different enzyme preparations. Key: (□) control; (■) treated; and (*) statistically significant from the control, $P<0.05$.

acid and alkaline pH ranges. For example, at pH 7.5, 1.8×10^{-8} and 5×10^{-5} M toxin inhibited activity by 51 per cent and 72 per cent respectively, while at the pH extremes of 6.0 and 9.0 inhibition was insignificant even at the higher toxin concentration.

Reversibility of inhibition by washing. A slow recovery of activity of penicillic acid-treated (Na^+–K^+)-ATPase was demonstrated by repeated washing of enzyme preparations with 0.32 M ice-cold sucrose solution (containing 10 mM imidazole and 1.0 mM EDTA, pH 7.5). No significant recovery of control activity was achieved through three washes, but after the third dilution, centrifugation, and resuspension of treated and control enzyme aliquots, approximately 33 per cent of the control activity was restored (Fig. 5), suggesting tight binding to preparations.

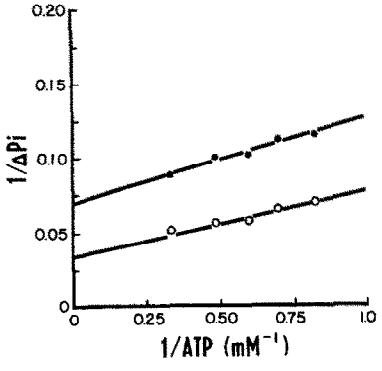

Fig. 6. Effect of penicillic acid on the kinetics of activation of (Na^+–K^+)-ATPase by ATP. The concentration of ATP was varied from 1.2 to 3.3 mM while maintaining optimal concentrations of Na^+, K^+ and Mg^{2+}. ΔP_i represents specific activity (μmoles P_i/mg of protein/hr). The incubation reaction was carried out for 10 min. Key: (○) control; and (●) 1.8×10^{-8} M penicillic acid. Data represent $\bar{X}$ activity from three independent replicate experiments assayed in triplicate.

Kinetic analysis. The effects of penicillic acid on various cationic and substrate activation parameters of (Na^+–K^+)-ATPase were examined to determine the site of inhibitory action.

Effects of penicillic acid on ATP and Mg^{2+} activation kinetics. Activation of (Na^+–K^+)-ATPase by ATP (low affinity) was demonstrated by varying ATP concentrations from 1.2 to 3.3 mM while maintaining otherwise optimal reaction conditions. Double-reciprocal plots of ATP-stimulated (Na^+–K^+)-ATPase activity demonstrated a reduction in the apparent V_{max} from 29.4 to 14.7 μmoles P_i/mg of protein/hr in the presence of 1.8×10^{-8} M penicillic acid (Fig. 6). A change in the K_m also was observed, resulting in parallel lines suggesting uncompetitive or coupled inhibition with ATP. The Mg^{2+} concentration in reaction mixtures also was varied from 0.5 to 7.0 mM while maintaining all other conditions optimal. These conditions did not significantly affect the inhibition of (Na^+–K^+)-ATPase by 1.8×10^{-8} or 5.0×10^{-10} M penicillic acid, suggesting that the action of the toxin on (Na^+–K^+)-ATPase was independent of Mg^{2+} (data not shown).

Effects of variation in Na^+ and K^+ concentrations. Initial studies of the toxin effects on cationic activation of (Na^+–K^+)-ATPase involved changing the concentrations of either Na^+ or K^+ in reaction mixtures while maintaining all other conditions optimal and constant. At optimal Na^+ (100 mM), the K^+ concentration was varied from 1 to 20 mM, while at optimal K^+ (20 mM), the Na^+ concentration was varied from 5 to 100 mM. A significant and concentration-dependent difference in percent inhibition of ATPase (relative to specific activity) was observed at lower Na^+/K^+ ratios (72 per cent at Na^+/K^+ = 0.25 vs 48.7 per cent at $Na^+/K^+ = 5.0$). No difference in inhibition was produced by changing the K^+ concentration (Table 1). These results indicated possible inhibitor interaction at Na^+ sites. To determine the kinetic nature of this interaction, the effects of penicillic acid on Na^+ and K^+ activation kinetics of (Na^+–K^+)-ATPase (in the presence of low, noninterfering concentrations of the cation held constant) were studied. Results from these experiments follow.

Na^+ activation of (Na^+K^+)-ATPase. Due to a strong cooperative interaction between Na^+ sites, linear plots of Na^+ activation data at limiting K^+ concentration (1.0 mM) were demonstrated by taking the square root of the reciprocal of ΔP_i and replotting these data against the reciprocal of the Na^+ concentration in the reaction mixture, thus accounting for two available Na^+ activation sites [20,21]. Activation of (Na^+–K^+)-ATPase was observed by varying the Na^+ concentration between 0.5 and 2.0 mM (Fig. 7 inset). Double reciprocal plots of the data demonstrate that penicillic acid competitively inhibited the (Na^+–K^+)-ATPase, resulting in a slope change of the inhibited plot without change in the intercept or apparent V_{max} (Fig. 7). Kinetic parameters were significantly affected in the presence of two concentrations of

Table 1. Inhibition of $(Na^+–K^+)$-ATPase by penicillic acid at various cationic activity states

NaCl* (mM)	KCl* (mM)	$(Na^+–K^+)$-adenosine triphosphatase activity† (% inhibition)
100	1	51.9 ± 3.6‡
100	2	52.3 ± 2.2
100	4	48.9 ± 1.9
100	6	50.0 ± 2.5
100	8	52.1 ± 2.0
100	10	54.2 ± 1.7
100	20	50.5 ± 1.5
5	20	72.0 ± 2.3
10	20	68.8 ± 2.6
20	20	66.3 ± 1.7
30	20	63.9 ± 1.3
50	20	54.3 ± 2.2
100	20	48.7 ± 1.8

* Cationic concentrations of Na^+ and K^+ were varied in reaction mixtures while maintaining all other conditions constant. Enzyme protein (40 μg) was incubated in the presence and absence of penicillic acid (1.8×10^{-8} M) at the various concentrations of Na^+ and K^+.

† Specific activity (umoles P_i/mg of protein/hr) refers to activity in the presence of $(Na^+ + K^+ + Mg^{2+})$ minus activity in the presence of (Mg^{2+}) only. Control and treated activities were determined at each activity state tested. Optimal ionic strength was maintained using choline chloride in reaction mixtures.

‡ Data represent the $\bar{X}$ ± S.E. percent inhibition of duplicate independent studies assayed in triplicate.

penicillic acid. A change in the apparent K_m from 0.53 to 0.91 and 1.7 mM in the presence of 1.8×10^{-10} M toxin, respectively, was observed. These results indicate that the inhibitory effect of penicillic acid was dependent on Na^+.

K^+ Activation of $(Na^+–K^+)$-ATPase. K^+ activation of $(Na^+–K^+)$-ATPase was demonstrated by varying the K^+ concentration at 10 mM Na^+ to avoid interaction at K^+ sites (Fig. 8, inset). Double reciprocal plots of the data demonstrate noncompetitive inhibition kinetics as evidenced by no change in the apparent K_m (2.0 mM) with decreasing apparent V_{max} values in the presence of 1.8×10^{-8} and 5.0×10^{-10} M penicillic acid (Fig. 8). These results suggest that the inhibitory action of penicillic acid was independent of K^+.

Effect of penicillic acid on p-nitrophenyl phosphatase activity. K^+-stimulated *p*-nitrophenyl phosphatase represents a model for the phosphatase step in the overall $(Na^+–K^+)$-ATPase reaction [12,22,23]. The mean specific activity of enzyme *p*-nitrophenyl phosphatase (at optimal conditions) was 70.9 nmoles P_i/mg of protein/min and was only slightly inhibited (16.4 per cent) by penicillic acid at 5×10^{-4} M, suggesting an indirect action on this component of the enzyme–complex (data not shown). The same concentration inhibited total $(Na^+–K^+)$-ATPase activity by more than 90 per cent.

Alteration of penicillic acid inhibition by sulfhydryls. Protection from the inhibitory effect of penicillic acid on $(Na^+–K^+)$-ATPase was demonstrated in the presence of the sulfhydryl reagents cysteine and dithiotrietol. These compounds were added separately to incubation mixtures prior to the addition of penicillic acid, and then tubes were preincubated at 37° for 30 min prior to initiation of the reaction with ATP. Cysteine and dithiothreitol reduced inhibition of ATPase in a dose-dependent manner.

Preincubation of equimolar concentrations of cysteine or dithiothreitol with penicillic acid reduced the amount of lead mercaptide formed. Loss of free thiol was time and pH dependent (extremely slow reaction at acidic pH), suggesting a correlation between inhibition of the enzyme by penicillic acid and involvement with sulfhydryls (data not shown).

Effect of penicillic acid on reactive membrane sulfhydryl [SH]: correlation with ATPase inhibition. The average free reactive sulfhydryl in control enzyme preparations from three separate experi-

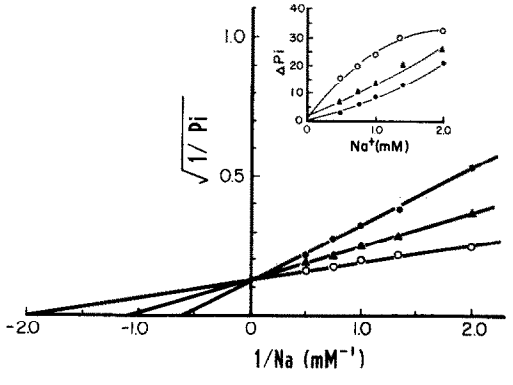

Fig. 7. Effect of penicillic acid on Na^+ activation of $(Na^+–K^+)$-ATPase. Membrane protein (40 μg) was incubated for a duration of 20 min upon initiation with ATP, and the concentration of Na^+ was varied from 0.5 to 2.0 mM while maintaining the K^+ concentration at 1.0 mM (inset). ΔP_i represents activity in the presence of Na^+, K^+ and Mg^{2+} minus that in the presence of Mg^{2+} only. Key: (○) control; (▲) 5×10^{-10} M penicillic acid; and (●) 1.8×10^{-8} M penicillic acid. Data represent $\bar{X}$ activity from three independent replicates assayed in triplicate.

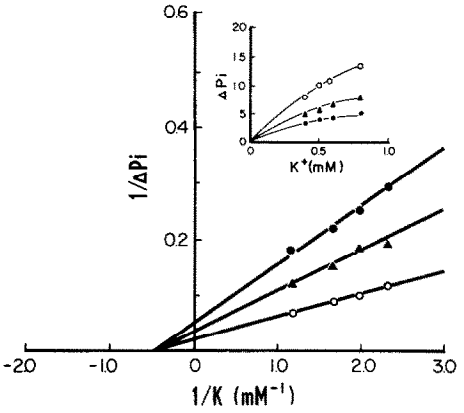

Fig. 8. Effect of penicillic acid on K^+ activation of $(Na^+–K^+)$-ATPase. The concentration of K^+ was varied from 0.40 to 0.80 mM while keeping the Na^+ concentration constant at 10 mM (inset). Membrane protein (40 μg) was incubated for 15 min upon initiation with ATP. ΔP_i represents specific activity of $(Na^+–K^+)$-ATPase. Key: (○) control; (▲) 5×10^{-10} M penicillic acid; and (●) 1.8×10^{-8} M penicillic acid. Data represent $\bar{X}$ activity from three independent replicates, assayed in triplicate.

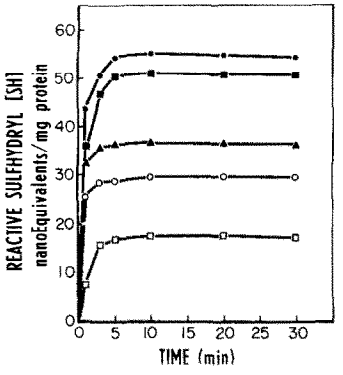

Fig. 9. Effect of penicillic acid on free reactive sulfhydryl content of membrane protein enzyme. Total free sulfhydryl [SH] is expressed as n-equiv./mg of enzyme protein. Key: (●) control; (■) 5×10^{-10} M penicillic acid; (▲) 1.8×10^{-8} M penicillic acid; (○) 5×10^{-5} M penicillic acid; and (□) 2×10^{-4} M penicillic acid. Data represent $\bar{X}$ [SH] from three studies each assayed in triplicate.

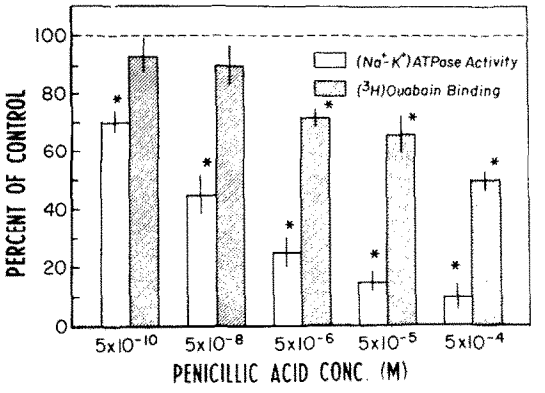

Fig. 11. Effect of penicillic acid on (Na^+–K^+)-ATPase activity (open bars) and [^{3}H]ouabain binding to the enzyme (hatched bars). Data represent $\bar{X} \pm$ S.E. percent of control activity. Values statistically significant from control levels ($P < 0.05$) are marked by an asterisk. For details see Experimental Procedures.

ments was 55 ± 2.8 n-equiv./mg of membrane protein (Fig. 9). Formation of the reaction product, 5-thio-2-nitrobenzoic acid, stabilized after 5–10 min and was reduced (in a concentration-dependent manner) in the presence of 5×10^{-10}, 1.8×10^{-8}, 5×10^{-5} and 2×10^{-4} M penicillic acid. A 70 per cent reduction in total reactive [SH] was observed at the higest toxin concentration.

The relationship between reduction in reactive enzyme sulfhydryl and inhibition of (Na^+–K^+)-ATPase at different toxin concentrations if illustrated in Fig. 10. A significant linear correlation of the data ($r = 0.973$, $b = 14.5$) indicates that inhibition of (Na^+–K^+)-ATPase by penicillic acid and reaction with accessible membrane sulfhydryls are related. Extrapolation of the regression line suggests that 14.5 n-equiv. [SH]/mg of protein (or 26.4 per cent of the total [SH]) were not affected by penicillic acid at concentrations which completely inhibit (Na^+–K^+)-ATPase activity.

Penicillic acid interaction with ouabain binding. Conditions favourable for "Complex I" formation [17], as described in Experimental Procedures, were used for binding studies. Incubation of enzyme with penicillic acid produced a decrease in the amount of [^{3}H]ouabain (3.5 nM) bound after 60 min reaction time. Control binding, 15.86 pmoles [^{3}H]ouabain/mg of protein, was reduced to 7.80 pmoles [^{3}H]ouabain bound/mg of protein (50.8 per cent inhibition) by 5.0×10^{-4} M penicillic acid. Nonsaturable binding was only 1.95 per cent of the total. Ouabain binding was not impaired except in the presence of high concentrations of penicillic acid (Fig. 11). Also, at 5×10^{-4} M toxin more than 90 per cent (Na^+–K^+)-ATPase activity was inhibited whereas only a 50 per cent reduction in ouabain binding occurred, suggesting that the two inhibitors of ATPase act at independent sites. Saturable ouabain binding to

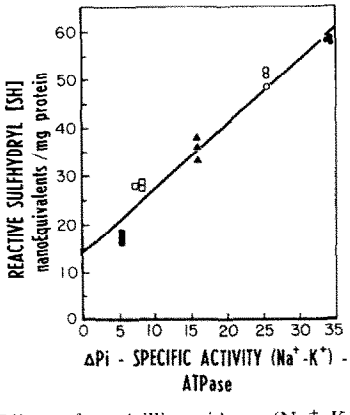

Fig. 10. Effect of penicillic acid on (Na^+–K^+)-ATPase activity and free membrane sulfhydryls. Each data point represents the $\bar{X}$ of triplicate assays from a separate experiment. Key: (●) control; (○) 5×10^{-10} M toxin; (▲) 1.8×10^{-8} M toxin; (□) 5×10^{-5} M toxin; and (■) 2×10^{-4} M toxin. Linear regression analysis indicated $r = 0.973$, $a = 1.3$, $b = 14.5$.

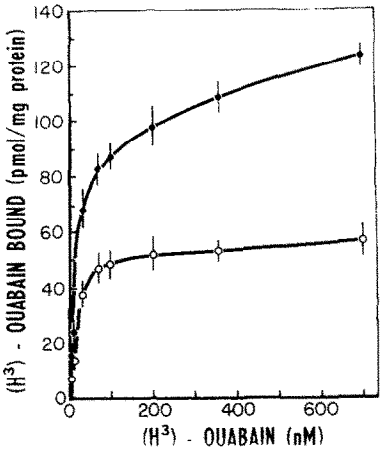

Fig. 12. Effect of penicillic acid on [^{3}H]ouabain binding to microsomal (Na^+–K^+)-ATPase preparation. Key: (●) control; (○) 5×10^{-4} M penicillic acid. Data represent $\bar{X} \pm$ S.E. specific [^{3}H]ouabain binding from three independent replicates. Nonspecific, nonsaturable binding was substracted from total binding for each point. For details see Experimental Procedures.

enzyme over a concentration range of 7.0 to 700 nM labeled ouabain was demonstrated both with and without toxin (1×10^{-4} M) (Fig. 12). Regression analysis of double-reciprocal plots of the data indicates noncompetitive interaction of the toxin with ouabain binding (data not shown). These results further support the suggestion that the two inhibitors do not act at the same site.

DISCUSSION

This study indicates that penicillic acid is a potent inhibitor of brain microsomal (Na^+-K^+)-ATPase, as evidenced by an I_{50} of 1.8×10^{-8} M. At least two classes of critical sulfhydryls are essential for (Na^+–K^+)-ATPase activity [24–27]. Protection of the enzyme from the inhibitory effects of penicillic acid in the presence of the sulfhydryl reagents dithiothreitol and cysteine and direct reduction of lead mercaptide in the presence of added thiol suggest that penicillic acid acts at membrane sulfhydryl sites. Penicillic acid also reacted with (Na^+–K^+)-ATPase and concomitantly reduced the amount of free reactive enzyme sulfhydryl [SH]. A significant correlation at different toxin concentrations suggests a cause–effect relation. Our results indicate that penicillic acid reacted with 73.6 per cent of the total free sulfhydryls. Our data also indicate that 26.4 per cent of these sulfhydryl groups were not reactive to penicillic acid (at concentrations which totally inhibit ATP hydrolysis) and were thus unrelated to ATPase inhibition. Akera and Brody [27] have shown that chlorpromazine free radical, another sulfhydryl-ATPase inhibitor, was unreactive to 26 per cent of the 64 n-equiv. [SH]/mg of protein in their enzyme preparation.

Although a cardioactive agent and a selective inhibitor of (Na^+–K^+)-ATPase, penicillic acid did not inhibit the enzyme at ouabain sites. The binding of [^{3}H]ouabain was disrupted by penicillic acid, but only at high penicillic acid concentrations and the effect was noncompetitive. Chlorpromazine free radical also failed to inhibit ouabain binding significantly [27]. Ouabain has no effect on enzyme sulfhydryls, further suggesting separate receptor sites for the two inhibitors of (Na^+–K^+)-ATPase. The observed reduction of ouabain binding by penicillic acid might be a consequence of conformational changes in the ouabain receptor site resulting from penicillic acid–sulfhydryl interaction at other sites.

Inhibition of enzyme activity by penicillic acid was pH dependent. Maximal inhibition of the toxin was attained at physiological pH, whereas, at pH extremes, inhibition was reduced greatly. Reduction in inhibition at acidic pH might be the result of a slower reaction rate and concurrent reequilibration of the cyclic lactone tautomer (stabilized by neutral to alkaline pH) biased in favour of the open ring γ-keto hexenoic acid. Penicillic acid has been shown to react much slower with sulfhydryls, such as cysteine and glutathione, at acidic pH than at neutral to alkaline ranges [28]. Disappearance of inhibition at pH 9.0 is not as easily explained, although this pH may exert effects on the conformation of critically reative enzyme thiols, either stabilizing or rendering them unreactive to the toxin.

The mechanism of inhibition of ATPase by penicillic acid can be explained by illustrating the partial reactions of the enzyme system:

$$\text{Step 1: } E_1 + ATP \xrightleftharpoons{Na^{++}, Mg^{++}} E_1.P + ADP$$

$$\text{Step 2: } E_1.P \xrightleftharpoons{Mg^{++}} E_2.P$$

$$\text{Step 3: } E_2.P \xrightarrow{K^+} E_1 + P_i$$

In the presence of free dephosphoenzyme (E_1), ATP, Na^+ and Mg^{2+} activate the formation of E_1 phosphoenzyme (transphosphorylation) (Step 1). The $E_1.P$ complex then undergoes a change in conformation to form an E_2 phosphoenzyme complex (Step 2) which is sensitive to hydrolysis in the presence of K^+ [29], and subsequently undergoes dephosphorylation.

Kinetic evaluation showed that penicillic acid competitively inhibited (Na^+–K^+)-ATPase with respect to Na^+ but was uncompetitive with respect to ATP. Inhibition was independent of K^+ concentration. K^+-dependent phosphatase activity was not inhibited significantly by penicillic acid. These results suggest that penicillic acid primarily affected Step 1 in the reaction sequence, possibly at a Na^+-phosphoenzyme regulatory site(s). This conclusion is reinforced by the lack of significant inhibition of the phosphatase step (Step 3). These effects may be due to conformational changes in the enzyme complex affecting the affinity of ATP and Na^+ receptor sites or to specific binding at essential ATPase-thiol receptors by penicillic acid. More definite conclusions await further work with a highly purified enzyme and radiolabeled toxin.

Additional preliminary evidence from our laboratory indicates that the mode of action of penicillic acid and membrane thiol may involve nucleophilic addition across the conjugated α, β unsaturation to the carbonyl or addition across the exocyclic methylene functionality resulting in intermediates which labilize to the respective *S*-alkylated derivatives (unpublished data).

The potency of penicillic acid to the membrane (Na^+–K^+)-ATPase system, both *in vivo* and *in vitro*, suggests a high affinity for ATPase sites. The rather broad range of inhibition demonstrated *in vitro* is somewhat inconsistent with this conclusion, since a range of concentration on the order of 10^5 higher than the I_{50} was required for total inhibition. This observation may indicate a mixed or complex type of binding to the membrane preparation. However, preliminary [^{14}C] penicillic acid–(Na^+–K^+)-ATPase binding data suggest the possibility of intermolecular H-bonding of the cyclic dimer type or self-association of toxin in solution which might adequately explain this effect (unpublished data).

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