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INHIBITION OF DOG BRAIN SYNAPTOSOMAL Na^+–K^+ ATPase AND K^+-STIMULATED PHOSPHATASE ACTIVITIES BY LONG CHAIN *n*-ALKYL-AMINE AND -PIPERIDINE, AND *N*″-ALKYLNICOTINAMIDE DERIVATIVES*

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Abstract—The effects of piperidine, primary amine, and nicotinamide aliphatic derivatives on dog brain snyaptosomal Na^+–K^+ ATPase were investigated. These derivatives inhibited the enzyme activity in a manner dependent on alkyl chain length. Kinetic studies revealed that inhibition of Na^+–K^+ ATPase activity by long chain alkyl derivatives (C_{12}–C_{18}) was biphasic and non-competitive with respect to the inhibitor and substrate (ATP) concentrations respectively. These long alkyl derivatives caused changes in the Hill coefficient that suggest the occurrence of a possible conformational change in the enzyme molecule. Dual-inhibitor experiments showed that both saturated and unsaturated pentadecylpiperidine (C_{15}-pip) derivatives inhibited Na^+–K^+ ATPase by the same mechanism. Oleylamine ($C_{18:1}$–NH_2), and *N*-dodecylnicotinamide (C_{12}–NA^+Cl^-) derivatives apparently inhibited the enzyme activity by a mechanism different from that of piperidine derivatives. Low concentrations of $C_{15:1}$-pip, $C_{18:1}$–NH_2, and C_{12}–NA^+Cl^- inhibited dog brain Na^+–K^+ ATPase activity only, but at higher inhibitor concentrations K^+-stimulated phosphatase activity was inhibited as much as Na^+–K^+ ATPase. It is concluded that long chain *n*-alkyl derivatives of piperidines, amines, and nicotinamide have a cationic detergent-like action on Na^+–K^+ ATPase, possibly by the disruption of protein–phospholipid interactions. The mechanism by which these compounds inhibit the overall Na^+–K^+ ATPase may involve two different inhibitory sites: one, a high affinity inhibitory site, binding to which inhibits the Na^+-stimulated phosphorylation reaction, and the other, a low affinity inhibitory site, binding to which inhibits the K^+-stimulated dephosphorylation reaction. These possible mechanisms may provide an explanation for the observed biphasic inhibition kinetics.

Na^+–K^+ adenosine triphosphatase (ATPase, EC 3.6.1.3) is a membrane-bound allosteric enzyme, which regulates the Na^+ and K^+ gradients across plasma membranes [1–3]. It is well documented that Na^+–K^+ ATPase is concentrated in the plasma membrane of the nerve-ending particles from the central nervous system [4]. Na^+–K^+ ATPase in the cortical synaptic membrane has been reported to have a direct role in the uptake of biogenic amines [5]. Piperidine (a biogenic amine) is known to cause a potent nicotine-like action on the peripheral [6, 7] and central nervous system [8, 9]. A subcellular distribution study of [^{3}H]piperidine in rat brain revealed that this compound was mainly localized in the nerve ending vesicles ("synaptosomes") [10].

Venom from the imported fire ant, *Solenopsis invicta*, contains, to a large extent, *n*-alkyl piperidine derivatives [11, 12]. Some of these derivatives were found to inhibit Na^+–K^+ ATPase activity isolated from fire ant head, and rat brain [13, 14].

The present studies were undertaken to examine the mechanism(s) of inhibition of dog brain Na^+–K^+ ATPase by long chain *n*-alkyl-piperidine derivatives in comparison with a homologous series of *n*-alkylamines and *N*′-alkylnicotinamides. Particular emphasis was put on inhibition kinetics and the role of the alkyl chain length in the inhibition of Na^+–K^+ ATPase and K^+-stimulated *p*-nitrophenyl phosphatase activities in a dog brain synaptosomal preparation.

MATERIALS AND METHODS

Adenosine triphosphate (ATP, Na_2), phosphoenolpyruvate (PEP), reduced nicotinamide adenine dinucleotide (NADH), bovine serum albumin (BSA), pyruvate kinase (PK, EC 2.7.1.40), lactate dehydrogenase (LDH, EC 1.1.1.2), ouabain, ethylenediaminetetraacetic acid (EDTA), imidazole, Tris (hydroxymethyl aminoethane), choline chloride, *p*-nitrophenyl phosphate (*p*-NPP) and *p*-nitrophenol (*p*-NP) were purchased from the Sigma Chemical Co., St. Louis, MO. $MgCl_2$, KCl, and NaCl were obtained from the Fisher Chemical Co., Memphis, TN. Aliphatic hydrocarbons and *n*-alkylamine derivatives were purchased from the Aldrich Chemical Co., Milwaukee, WI. *N*′-Alkylnicotinamide derivatives were provided by Dr. Bruce Anderson, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA. Derivatives of *n*-alkylpi-

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peridine were provided by Dr. M. S. Blum, University of Georgia, Athens, GA.

Tris-ATP was prepared from disodium ATP using a Dowex AG-50B column pre-equilibrated with Tris as described by Schwartz *et al.* [15]. Similarly, Tris-PEP and Tris-*p*-NPP were prepared from sodium and dicyclohexyl ammonium salts respectively.

Preparation and purification of dog brain Na^+–K^+ *ATPase.* Dog brain was removed immediately after exsanguination of an animal that had been injected with succinylcholine. The head was dissected, and the brain tissue was removed and placed in ice-cold buffered sucrose solution containing: 0.32 M sucrose, 200 mM imidazole–HCl (pH 7.5) and 1 mM EDTA. The gray matter of the brain cortex was separated from the white matter. Small portions of the brain cortex were homogenized in buffered-sucrose solution (10 vol.) using an ice-cold Thomas-Ten Broeck ground glass homogenizer, and the homogenate was centrifuged at 900 *g* for 10 min. The supernatant fraction was recentrifuged for 30 min at 13,000 *g*. The sediment, which contained mainly synaptosomes and mitochondria, was resuspended in sucrose solution using a teflon piston homogenizer, frozen in liquid nitrogen and stored at −20°.

Na^+–K^+ ATPase was partially purified by treatment with a high ionic strength solution as described by Hegyvary and Post [16], suspended at a concentration of 4 mg protein/ml, quick frozen in liquid nitrogen and stored at −20°. Protein was determined as described by Lowry *et al.* [17] using bovine serum albumin as a standard.

Na^+–K^+ *ATPase assay method.* Na^+–K^+ ATPase was assayed in a multi-enzyme coupled reaction system, where ATP is regenerated and its concentration maintained constant as described by Pullman *et al.* [18] and reported by Koch and Gilliland [19]. Ouabain-sensitive Na^+–K^+ ATPase activity was estimated by subtracting the enzyme activity in the presence of 1.0 mM ouabain from the total ATPase activity.

K^+*-stimulated* p-*NPPase assay method.* K^+-stimulated, *p*-NPPase activity was determined using *p*-NPP as a substrate. The reaction mixture (1.0 ml) contained 20 mM imidazole–Cl buffer (pH 7.5), 10 mM KCl, 2 mM $MgCl_2$, and 3 mM Tris-*p*-NPP freshly prepared. The reaction mixture was preincubated at 37° for 1 min before adding the enzyme preparation. The enzymatic reaction was initiated by the addition of 11–12 μg of enzyme preparation protein. The change in absorbance of the released *p*-NP was measured at 420 nm as a function of time using a Gilford 2400 recording spectrophotometer. The reaction was linear for at least 30 min. Ouabain-sensitive K^+-stimulated *p*-NPPase activity was determined by subtracting the enzyme activity in the presence of 1 mM ouabain from the total *p*-NPPase activity in the absence of ouabain.

Inhibitor stock solutions were prepared by dissolving the appropriate quantity of each inhibitor in 95% ethanol or water. One microliter of each inhibitor solution was added with a Hamilton microsyringe to a rapidly stirred reaction mixture. None of the inhibitors used in this study had an effect on PK or LDH activities at the concentrations used. The inhibition of Na^+–K^+ ATPase activity was confirmed by the discontinuous assay method where the released inorganic phosphate was measured colorimetrically [20].

RESULTS

Synaptosomal Na^+–K^+ ATPase was partially purified from dog brain cortex. About 95 percent of the total ATPase activity in the purified preparation was ouabain-sensitive. The specific activity was about 40 μmoles ATP hydrolyzed · (mg protein)$^{-1}$ · hr^{-1}. The enzyme requirements for Na^+ and K^+ in the presence of Mg^{2+} for activity is well documented and reported elsewhere [3]. The K^+ requirement for maximal activity of ouabain-sensitive K^+-stimulated *p*-NPPase was 2.0 mM in the presence of 2.0 mM Mg^{2+}. The K_m value for K^+ was 0.4 mM indicating a high affinity for K^+. Ouabain-sensitive *p*-NPPase activity was about 10–20 percent that of ouabain-sensitive Na^+–K^+ ATPase in the partially purified dog brain synaptosomal preparation.

Dodecane (22 mM), a long chain aliphatic hydrocarbon, had no inhibitory effect on dog brain Na^+–K^+ ATPase. Ammonium chloride (20–100 mM) showed no inhibitory effect on Na^+–K^+ ATPase and *p*-NPPase activities. Long chain *n*-alkyldiamine derivatives, i.e. 1,6-hexanediamine and 1,12-dodecanediamine, showed only slight inhibition of Na^+–K^+ ATPase. Only slight inhibition and/or stimulation of Na^+–K^+ ATPase and K^+-stimulated *p*-NPPase activities were observed at low concentrations of 4-phenylpiperidine, 4-dipiperidine-di-ol and 4-dipiperidine.

Kinetic studies of the effects of long chain alkylamines, piperidines and nicotinamides on dog brain N^+–K^+ ATPase revealed that all inhibit the enzyme activity in a non-competitive manner with respect to substrate concentration. A Lineweaver–Burk plot (Fig. 1) shows the kinetic response of dog brain Na^+–K^+ ATPase activity to ATP or to ATP plus *cis*-2-methyl-6-pentadecanyl piperidine ($C_{15:1}$-pip); the inhibition was non-competitive with the substrate. A Dixon plot of inhibition of Na^+–K^+ ATPase by oleylamine (Fig. 2), shows one example of the biphasic nature of inhibition by the *n*-alkyl-*N* derivatives. At low concentrations (0–6 μM) a much smaller increase of inhibition was noted (to about 60 percent inhibition), whereas at slightly higher concentrations (> 6–12 μM) a sharp increase in inhibition occurred. Because of the latter observation, the apparent inhibition constant (K_i) values were determined using the lower concentrations of these inhibitors.

Figure 3 shows the relationship between pK_i values and the number of carbon atoms in the alkyl group of *n*-alkylamine and *N'*-alkylnicotinamide derivatives. The pK_i values of the *N'*-alkylnicotinamide derivatives (C_8–C_{12}) were linearly related to the number of carbons in the alkyl chain. The pK_i values of C_{15}-pip and $C_{15:1}$-pip were located on the extrapolated line of the *N'*-alkylnicotinamide derivatives (Fig. 3). The pK_i values of *n*-alkylamine derivatives also were linearly related to the number of carbon atoms between C_6 and C_{12}. Deviation from this linearity

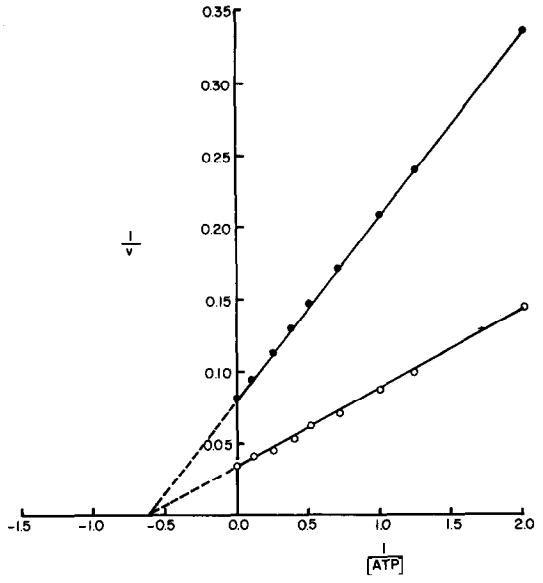

Fig. 1. Lineweaver–Burk plot for dog brain Na^+–K^+ ATPase. The enzyme activity was measured in the absence (○) and the presence (●) of 7×10^{-6} M $C_{15:1}$-piperidine with various ATP concentrations. Each point represents an average of three different observations; S.E.M. was <10 percent.

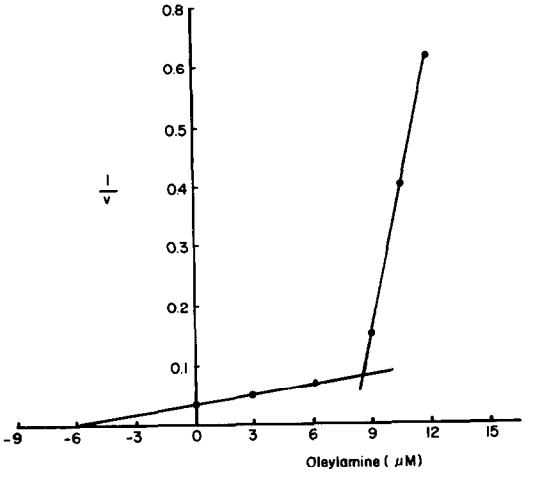

Fig. 2. Dixon plot showing the biphasic inhibition kinetics of Na^+–K^+ ATPase with increasing concentrations of oleylamine.

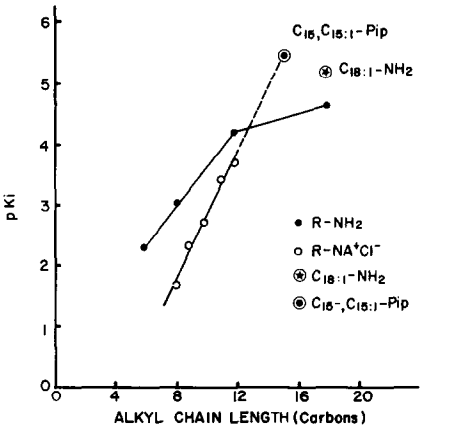

Fig. 3. Relationship between pK_i and the number of carbons in the alkyl chain of *n*-alkyl derivatives of amines (●), and piperidine ◉ and *N'*-alkylnicotinamide (○). Oleylamine $C_{18:1}$-NH_2 is represented by the symbol ⊛.

was observed with the longest alkyl carbon chains tested, C_{18}-NH_2 and $C_{18:1}$-NH_2 (Fig. 3).

A two inhibitor combination kinetic study was conducted to determine the interaction of C_{12}-NH_2, C_{12}-NA^+Cl^-, or C_{15}-pip with $C_{15:1}$-pip, when inhibiting dog brain Na^+–K^+ ATPase activity. Figure 4 shows that C_{15}-pip (3.6×10^{-6} M) (B) did not change the slope of the line of $C_{15:1}$-pip, but it reduced the maximum velocity of the catalytic reaction. The interaction coefficient (α) in this case equaled unity. C_{12}-NH_2 (1.25×10^{-6} M) (C) increased the slope of the $C_{15:1}$-pip line, and the apparent K_i value of $C_{15:1}$-pip was changed by a factor of 0.3. C_{12}-NA^+Cl^- (1×10^{-4} M) (D) caused an even greater change in the slope of the $C_{15:1}$-pip line, with an interaction coefficient of 0.14.

Dog brain Na^+–K^+ ATPase was found to have a high affinity for Na^+ in a cooperative manner. The K_m value for Na^+ was 2.5 mM in the presence of 20 mM K^+ and 5 mM Mg^{2+}. The ionic strength was maintained constant with choline chloride. A Hill plot (Fig. 5) shows cooperative activation of dog brain Na^+–K^+ ATPase by Na^+ in the absence or presence of $C_{15:1}$-pip, C_{15}-pip, $C_{18:1}$-NH_2, and C_{12}-NA^+Cl^-. Cooperativity coefficient values (n) are given in Table 1.

The effect of temperature on dog brain Na^+–K^+ ATPase activity and its response to C_{15}-pip and $C_{15:1}$-pip was investigated. The enzyme activity was enhanced as a function of temperature up to 45°. A sharp decline in the enzyme activity was observed at 50°. A small break was observed between 25° and 30°. The inhibitory effect of C_{15}-pip (1.8 μM) or $C_{15:1}$-pip (1.5 μM) was not temperature dependent.

Inhibition of dog brain Na^+–K^+ ATPase and K^+-stimulated *p*-NPPase activities by $C_{15:1}$-pip, $C_{18:1}$-NH_2 and C_{12}-NA^+Cl^- is shown in Figs. 6 and

Table 1. Effect of C_{15}- and $C_{15:1}$-piperidine, $C_{18:1}$-NH_2, and C_{12}-NA^+Cl^- on the cooperativity coefficients on Na^+ activation of Na^+–K^+ ATPase activity

Compound	(M)	Hill plot cooperativity coefficient* (n)
Control	0.0	1.19 ± 0.11 (5)
C_{15}-Piperidine	3.6×10^{-6}	1.5 ± 0.07 (4)
$C_{15:1}$-Piperidine	3.0×10^{-6}	1.8 ± 0.17 (4)
$C_{18:1}$-NH_2	6.0×10^{-6}	1.9 ± 0.13 (3)
C_{12}-NA^+Cl^-	1.5×10^{-4}	2.2 ± 0.18 (3)

* *n*-Values were determined from the slopes of the Hill plots presented in Fig. 6. *n*-Values are averages of the number of runs indicated in parentheses ± S.D.

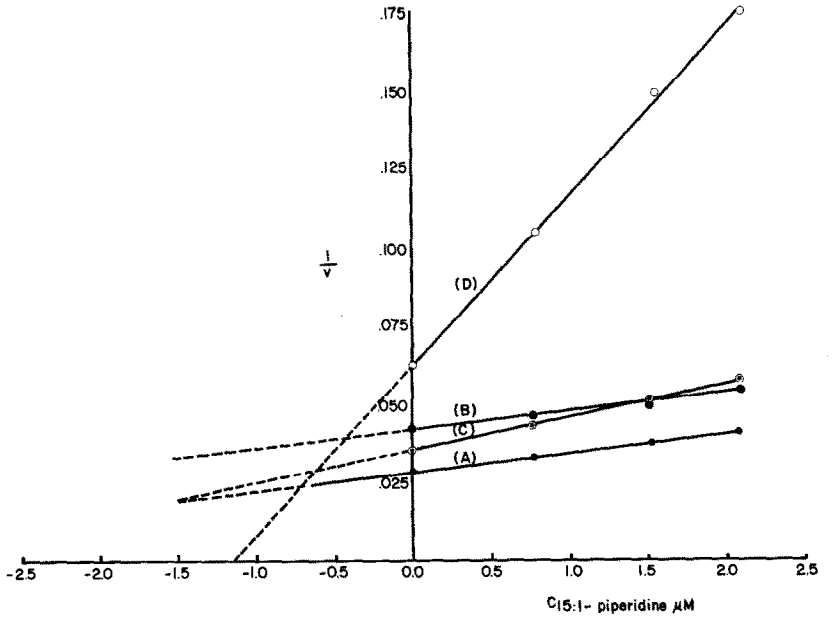

Fig. 4. Dual-inhibitor analysis. Inhibition of dog brain Na^+–K^+ ATPase activity by $C_{15:1}$-piperidine in the absence (●) and the presence of 3.6×10^{-6} M C_{15}-piperidine ◉, 1.2×10^{-6} M C_{12}-NH_2 ⊛, and 1.0×10^{-4} M C_{12}-NA^+Cl^- (○). The enzyme activity was measured as described in the text. Each point represents an average of two observations; S.D. was < 10 percent.

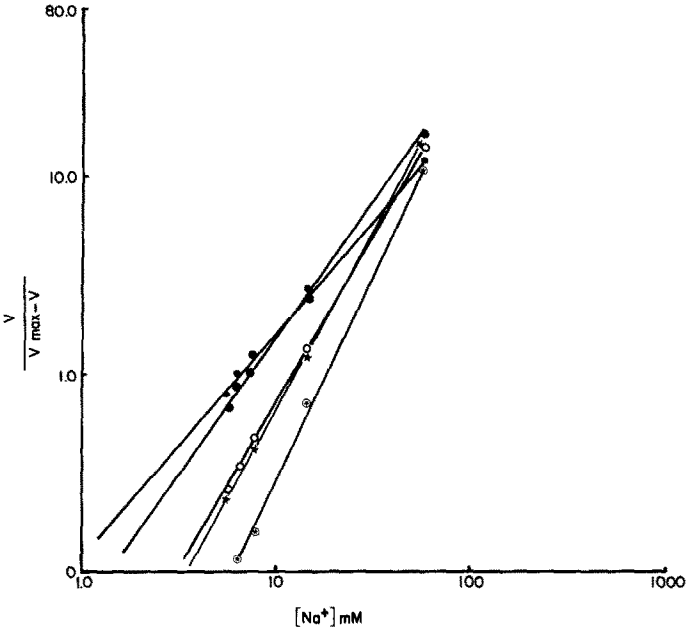

Fig. 5. Hill plots indicating the activation of dog brain synaptosomal Na^+–K^+ ATPase by Na^+ in the absence (●) or in the presence of 3.6×10^{-6} M C_{15}-piperidine ◉, 3×10^{-6} M $C_{15:1}$-piperidine (○), 6×10^{-6} M, $C_{18:1}$-NH_2 (★), or 1.4×10^{-6} M C_{12}-NA^+Cl^- ⊛. Na^+–K^+ ATPase was assayed as described in the text except at various Na^+ concentrations. The ionic strength of the reaction medium was maintained constant with choline chloride. Velocity is μmoles $P_i \cdot (mg\ protein)^{-1} \cdot hr^{-1}$. Each point represents an average of four observations; S.E.M. was < 10 percent.

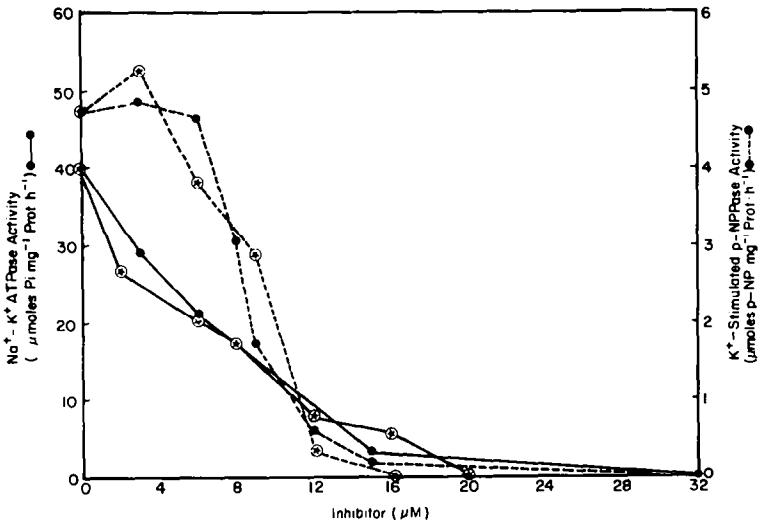

Fig. 6. Comparison between dog brain synaptosomal Na^+–K^+ ATPase (solid lines) and K^+-stimulated *p*-NPPase (dotted lines) sensitivity to $C_{15:1}$-piperidine ⊛ and $C_{18:1}$-NH_2 (●). Each point represents an average of four observations; S.E.M. was < 10 percent.

7 respectively. Na^+–K^+ ATPase activity was inhibited in a concentration-dependent manner. The I_{50} values for these inhibitors [5 μM C_{15}- and $C_{15:1}$-pip; 6 μM $C_{18:1}$-NH_2 (Fig. 6); and 1.8 mM C_{12}-NA^+Cl^- (Fig. 7)] were in close agreement with the K_i values (Fig. 3). At these latter inhibitor concentrations (which caused about 50 percent inhibition of Na^+–K^+ ATPase activity), K^+-stimulated-*p*-NPPase was not affected (Figs. 6 and 7). The K^+-stimulated *p*-NPPase activity of dog brain synaptosomol preparation, however, was strongly inhibited by C_{15}-pip, $C_{15:1}$-pip, $C_{18:1}$-NH_2 at concentrations higher than 5 μM and by C_{12}-NA^+Cl^- at concentrations higher than 0.2 mM.

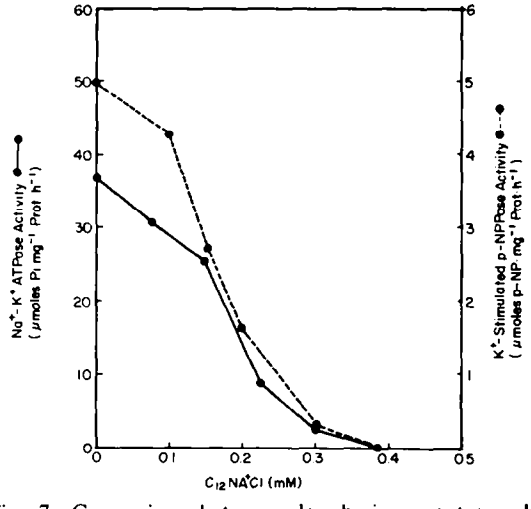

Fig. 7. Comparison between dog brain synaptosomal Na^+–K^+ ATPase (solid line) and K^+-stimulated *p*-NPPase (dotted line) sensitivities to C_{12}–NA^+Cl^-. Each point represents an average of four observations; S.E.M. was < 10 percent.

DISCUSSION

A long chain aliphatic hydrocarbon (dodecane), a simple amine (ammonium chloride), and simple piperidines and dipiperidines did not inhibit dog brain Na^+-K^+ ATPase or K^+-stimulated *p*-NPPase activities even at millimolar concentrations (data not shown). Long chain alkyldiamines, i.e. H_2N-C_{12}-NH_2 and H_2N-C_6-NH_2, had only a slight inhibitory effect at 10^{-5} M concentration (data not shown). These results suggest certain structural requirements for these molecules to be potent inhibitors of Na^+–K^+ ATPase.

In the present paper, kinetic studies of inhibition of dog brain Na^+–K^+ ATPase by a series of *n*-alkylamine (C_6-C_{18}) and *N'*-alkylnicotinamide derivatives revealed that the inhibition was kinetically biphasic, and was non-competitive with the substrate. The biphasic nature of inhibition, as indicated by Dixon plots (Fig. 2), suggests two sites of inhibition, one of high affinity and the other of low affinity. The inhibition of Na^+–K^+ ATPase by these inhibitors was very much dependent on the alkyl chain length (Fig. 3). *n*-Alkylalcohols [21] and free fatty acids [22] inhibit Na^+–K^+ ATPase similarly, in a manner dependent on the alkyl or acyl chain length respectively. *n*-Alkylammonium [23] and *N'*-alkylnicotinamide [24] also inhibited L-α-glycerophosphate dehydrogenase in a manner dependent on the alkyl chain length. A linear relation (Fig. 3) between the pK_i values and the number of carbons in the alkyl chain of *N'*-alkylnicotinamide derivatives indicates an absolute requirement for a long hydrophobic alkyl chain for the molecule to be a potent inhibitor of Na^+–K^+ ATPase.

The change in the free energy per methylene group was calculated as described by Anderson and Reynolds [23] using the following relationship: $\Delta F = 2.3\,RT\,\Delta pK_i$. The ΔF value for the *N'*-alkylnicotinamide derivatives was about -0.75 kcal·mole^{-1}·CH_2^{-1}. This value is within the range suggested for

interactions through dispersion forces [25] and could indicate that the *N'*-alkyl and *n*-alkyl derivatives exert their actions by disruption of secondary bonding forces of the lipoprotein complex of the membrane bound Na^+–K^+ ATPase.

The results obtained from dual-inhibitor studies indicated that the C_{15}- and $C_{15:1}$-piperidines were acting on the same inhibitory site, or by the same mechanism of inhibition, because the interaction coefficient equalled one. In contrast, C_{12}-NH_2 or C_{12}-NA^+Cl^-, in combination with $C_{15:1}$-pip, reduced the interaction coefficient and apparently acted on inhibitory sites different from that of $C_{15:1}$-piperidine.

Cooperativity studies revealed that a possible conformational change in the Na^+–K^+ ATPase molecule could have occurred as a result of the interaction of C_{15}-pip, $C_{15:1}$-pip, $C_{18:1}$-NH_2, or C_{12}-NA^+Cl^- with the enzyme molecule. This conformational change may have been due to the disruption of the enzyme protein–phospholipid complex. It has been reported that the binding sites for Na^+, K^+ and Mg^{2+}-ATP are exposed to the aqueous phase and that the portion of the enzyme molecule embedded in the hydrophobic matrix contains a high content of nonpolar amino acids [26]. Protein–phospholipid interactions are important in determining the enzyme conformation and, in turn, its activity [27]. Disruption of protein–phospholipid interactions of Na^+–K^+ ATPase by *n*-alkyl derivatives used in this study may have led to conformational changes in the enzyme molecule which could have resulted in enzyme inhibition. We suggest that the mechanism of action of *n*-alkyl piperidines and amines, and *N'*-alkylnicotinamide derivatives, on Na^+–K^+ ATPase is complex and similar to that of cationic detergents, reflecting an interaction with the enzyme protein–phospholipid complex.

Comparative studies on the inhibition of dog brain synaptosomal Na^+–K^+ ATPase and K^+-*p*-NPPase activities at low concentrations of $C_{15:1}$-piperidine ($< 5\,\mu M$), $C_{18:1}$-NH_2 ($< 4\,\mu M$), and C_{12}-NA^+Cl^- (< 0.1 mM), showed that Na^+–K^+ ATPase was strongly inhibited, whereas K^+-stimulated *p*-NPPase was not affected (Figs. 6 and 7). Koch *et al.* [14] reported similar observations for rat brain Na^+–K^+ ATPase inhibition by C_{15}- and $C_{15:1}$-pip at concentrations of $5\,\mu M$. At these concentrations K^+-*p*-NPPase and phosphoenzyme formation were not affected. Their [14] conclusion suggested that these derivatives interfere with the conformation transition of the enzyme from $E_1P \rightarrow E_2P$ state. In the present report, K^+-dependent dephosphorylation was also strongly inhibited by C_{15}-piperidines, $C_{18:1}$-NH_2 and C_{12}-NA^+Cl^-, but at much higher concentrations than those required for inhibition of Na^+–K^+ ATPase activity.

It has been reported that removal of endogenous phospholipid together with 80 percent of the cholesterol from Na^+–K^+ ATPase resulted in a complete loss of Na^+–K^+ ATPase activity, while 50 percent of the original K^+-stimulated activity was maintained [28–30]. It seems possible that the K^+-dependent dephosphorylation reaction of Na^+-K^+ ATPase was less dependent on phospholipids than the Na^+-dependent phosphorylation reaction. This may explain why K^+-*p*-NPPase was more resistant to the effects of long chain hydrocarbon derivatives of amines, piperidines, and nicotinamides. We propose that the mechanism of Na^+-K^+ ATPase inhibition may involve more than one inhibitory site: one a high affinity inhibitory site, binding to which inhibits the Na^+-dependent phosphorylation reaction, and the other a low affinity inhibitory site, binding to which inhibits the K^+-dependent dephosphorylation reaction. This could explain the biphasic behavior of Na^+–K^+ ATPase inhibition kinetics.

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