On the Inhibition of [Na^+,K^+]-ATPases by the Components of *Naja mossambica mossambica* Venom: Evidence for Two Distinct Rat Brain [Na^+,K^+]-ATPase Activities†

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ABSTRACT: We have compared the effects of highly purified preparations of cardiotoxins and phospholipases A_2 from *Naja mossambica mossambica* venom on rat brain [Na^+,K^+]-ATPase activity. The results were the following: (i) micromolar concentrations of cardiotoxin preparations were required to inhibit [Na^+,K^+]-ATPase activity to the extent achieved by picomolar concentrations of phospholipases A_2; i.e., the inhibitory effect of cardiotoxins appeared to be related to the contamination of the preparations by trace amounts of phospholipase A_2; (ii) comparing phospholipases A_2 from varied origins, a correlation was observed between [Na^+,K^+]-ATPase inhibition, isoelectric point, and toxicity for mice; (iii) when rat brain membranes were used, incubation for extended times with the most basic *N. mossambica mossambica* phospholipase A_2 resulted in a biphasic [Na^+,K^+]-ATPase inhibition, suggesting that two distinct [Na^+,K^+]-ATPases were affected differentially. In contrast, incubation of rat brain membranes with either porcine pancreatic phospholipase A_2, notexin, or β-bungarotoxin and also incubation of erythrocyte membranes with the most basic *N. mossambica mossambica* phospholipase A_2 produced monophasic [Na^+,K^+]-ATPase inhibitions. We discuss a possible specific action of toxic, basic phospholipase A_2 on one of the [Na^+,K^+]-ATPase isoforms of excitable membranes.

The Na^+- and K^+-dependent Mg^{2+}-activated adenosinetriphosphatase ([Na^+,K^+]-ATPase)[1] is a protein present in almost all higher eucaryotic cells [Skou, 1951; reviewed by Jörgensen (1982)]. This enzyme directly couples the hydrolysis of ATP to the vectorial transport across the plasma membrane of Na^+ and K^+, resulting in electrochemical gradients involving these ions. A large variety of cellular processes depend upon these ionic gradients including nerve and muscle excitability. The [Na^+,K^+]-ATPase complex is made of two noncovalently bound subunits, a catalytic one (α, $M_r \approx 100\,000$) bearing the binding sites for ATP and for the potent inhibitor ouabain and a glycosylated one (β, $M_r \approx 50\,000$) whose function remains unknown. Primary structures of both subunits have been recently deduced from cDNA sequences (Shull et al., 1985, 1986a; Kawakami et al., 1985; Schneider et al., 1985). Evidence exists in mammals for the presence of at least two isozymes of [Na^+,K^+]-ATPase that differ in their affinity toward ouabain (Tobin et al., 1972), in the structure of the α-subunit (Sweadner, 1979; Sweadner & Gilkeson, 1985), and in their enzymatic properties (Sweadner, 1985).

The elapid snake venoms are known to inhibit [Na^+,K^+]-ATPase activity. Among their polypeptidic components, both phospholipases A_2 (Schatzmann, 1962; Taniguchi & Tonomura, 1971; Roelofsen & Van Deenen, 1973; Lin-Shiau & Chen, 1982) and cardiotoxins (Lankisch et al., 1972; Zaheer et al., 1975; Vincent et al., 1976) have been so far implicated in the inhibitory process. Indeed, a high specificity in lipid requirement seems to exist for the [Na^+,K^+]-ATPase system [reviewed by Roelofsen (1981)], and it is known that phospholipases A_2, obviously, but also cardiotoxins (Dufourcq & Faucon, 1978; Vincent et al., 1978; Bougis et al., 1981; Batenburg et al., 1985) have a strong affinity toward membranous phospholipids (leading to hydrolysis or phase changes, respectively). These lipid changes are thought to result in the inhibition of [Na^+,K^+]-ATPase activity. The effective inhibitory concentrations are very different, i.e., in the nanomolar range for phospholipases A_2 and in the micromolar range for cardiotoxins. One has also to consider that cross contaminations of phospholipase A_2 and cardiotoxin preparations are well documented (Delori & Tessier, 1980).

We have conducted a set of new experiments in order to identify in the elapid *Naja mossambica mossambica* venom the component(s) responsible for the inhibition of rat brain [Na^+,K^+]-ATPase activity. The results obtained suggest that the apparent inhibitory activity of cardiotoxin preparations was due to their contamination by trace amounts of phospholipase(s) A_2. Furthermore, we have showed that at least one of the toxic phospholipases A_2 present in this venom was able to differentiate between two rat brain [Na^+,K^+]-ATPase

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [K^+]-PNPase, K^+-dependant *p*-nitrophenyl phosphatase; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide, reduced; [Na^+,K^+]-ATPase, Na^+- and K^+-dependent Mg^{2+}-activated adenosinetriphosphatase (EC 3.6.1.3); NTX Nmm, CTX Nmm, and PH Nmm, α-neurotoxin, cardiotoxin, and phospholipase A_2 from *Naja mossambica mossambica* venom, respectively; PEP, phosphoenolpyruvate; PK, pyruvate kinase; Tris, tris(hydroxymethyl)aminomethane; TEA, triethanolamine.

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activities, whereas porcine pancreatic phospholipase A_2 was not.

Materials and Methods

Reagents. The water used to prepare solvents and buffers was obtained with a Milli-Ro/Milli-Q (Millipore) system. Most of the reagents were analytical grade from Merck. *p*-Bromophenacyl bromide was from Fluka. Pyrene lipids, 1-palmitoyl-2-(6-pyrenylhexanoyl)-*sn*-glycero-3-phosphocholine (FC.3024) and 6-pyrenylhexanoic acid (FC.4006), were from KSV Chemicals (Finland). NADH, PEP, and PK/LDH were from Boehringer Mannheim.

Venom and Toxins. N. mossambica mossambica venom was supplied by a professional catcher (D. Muller, Johannesburg, South Africa). From this venom 11 toxins were purified in our laboratory: α-neurotoxins (NTX Nmm I–III) according to Rochat et al. (1974), cardiotoxins (CTX Nmm I–V) according to Bougis et al. (1983), and phospholipases A_2 (PH Nmm I–III) according to Joubert (1977). Phospholipase A_2 contamination was subsequently removed from CTX Nmm II by either anti-phospholipase A_2 immunoaffinity chromatography (Delori & Tessier, 1980) or high-performance liquid chromatography (Bougis et al., 1986). In the same way, PH Nmm III was also ultimately purified by means of an anti-cardiotoxin immunoaffinity chromatography. *Cerastes cerastes* phospholipase A_2 has been purified in our laboratory by Dr. F. Laraba, and porcine pancreatic phospholipase A_2 was given by Dr. R. Verger (CBBM, CNRS–Marseille, France), *Apis mellifera* phospholipase A_2 by Dr. J. Dufourcq (CRPP, CNRS–Talence, France), and notexin by Dr. A. Menez (CEA–Saclay, France). β-Bungarotoxin was from Boehringer Mannheim.

Phospholipase A_2 catalytic inactivation was carried out by covalent modification of His_{48} by *p*-bromophenacyl bromide as originally described by Volwerk et al. (1974). The remaining phospholipase A_2 activity was spectrofluorometrically estimated (see below) and the reaction stopped by acidification when less than 0.1% of the initial phospholipase A_2 activity was still detectable.

The isoelectric point (pH_i) of phospholipases A_2 was determined on an ampholyte gradient according to the manufacturer instructions (Pharmacia).

Phospholipase A_2 activity was spectrofluorometrically assayed with pyrene lipid as substrate (Bougis et al., 1986).

Preparation of Membrane Fragments. The crude synaptosomal fraction (P_2) from cerebral cortex of Wistar rats (Evic-Ceba, 33 Blanquefort, France) was prepared according to Gray and Whittaker (1962) as modified by Blaustein and Ector (1976). Synaptosomal membrane fragments were subsequently prepared according to Morel et al. (1977), using a multilayer saccharose gradient (0.4, 0.6, 0.8, 1.0, and 1.2 M). The 0.6/0.8 interfacial layer was recovered, buffered in 25 mM Tris–HEPES and 10 mM KCl, pH 7.4, to a final protein concentration of 1.2–1.6 mg/mL. Human erythrocyte membrane fragments were prepared according to Hanahan and Ekholm (1974). Heart membrane fragments from 5–8-day-old rats were prepared by homogenization of whole heart for 3 min with an Ultra Turax (Ika-Werk) apparatus at maximum speed in 10% w/v of buffer (5 mM NaH_2PO_4/Na_2HPO_4, 0.32 M saccharose, pH 7.4). The homogenate was centrifuged at 1000*g* for 10 min, and the resulting supernatant was centrifuged at 10000*g* for 30 min. The pellet was suspended in buffer (25 mM Tris–HEPES, 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, pH 7.2). Membrane fragments from *Torpedo marmorata* electric organ were prepared as previously described (Bougis et al., 1986). All membrane fragment preparations were kept in liquid nitrogen until use.

[K^+]-PNPase and [Na^+,K^+]-ATPase Assays. Phosphatase activity, [K^+]-PNPase, was often measured instead of [Na^+,K^+]-ATPase activity. However, in some cases, both activities were determined. The data shown on the figures are the mean of more than two experiments (±standard error).

[K^+]-PNPase activity was spectrophotometrically measured (410 nm), at 37 °C, by following versus time the release of *p*-nitrophenol from *p*-nitrophenyl phosphate. The assay medium contained 50 mM TEA-HCl, 1 mM DTT, 20 mM $MgCl_2$, 20 mM KCl, 1 mM EDTA, and 25 mM *p*-nitrophenyl phosphate disodium salt, pH 7.4. [K^+]-PNPase activity was the difference between *p*-nitrophenol produced in the absence and in the presence of 1 mM ouabain.

[Na^+,K^+]-ATPase activity was measured, at 37 °C, by the method of Scharschmidt et al. (1979). They assay medium had the following composition: 50 mM TEA-HCl, 100 mM NaCl, 0.3 mM NADH, 2 mM PEP, 13 μL (53 μg) of PK/LDH, and 3.8 mM Mg-ATP, pH 7.5. [Na^+,K^+]-ATPase activity was the difference between ATP hydrolysis measured in the absence and the presence of 1 mM ouabain.

[Na^+,K^+]-ATPase and [K^+]-PNPase specific activities of synaptosomal membranes were 0.04 and 0.18 μmol min^{-1} (mg of protein)$^{-1}$, and in the presence of ouabain, residual activities accounted for 52% and 33% of total activities, respectively. [Na^+,K^+]-ATPase and [K^+]-PNPase half-inhibitions by Ca^{2+} ion were obtained at 0.4 and 1.6 mM, respectively. These Ca^{2+} concentrations were used in assays made in the presence of either venom or phospholipase A_2. [K^+]-PNPase activity of synaptosomal membranes was stable for at least 12 h.

In Vivo Assay. Toxicity was assayed in male NMRI white mice (Evic-Ceba, 33 Blanquefort, France) weighing 20 ± 3 g. Samples were dissolved in 0.9% NaCl containing 0.1% BSA, and 0.2 mL was injected intravenously in the tail vein. Intracerebroventricular injection was performed as described by Haley and McCormick (1957). The LD_{50} value was determined according to Behrens and Karber (1935) after a 48-h period of observation.

Results

N. mossambica mossambica venom completely deactivated the rat brain synaptosomal membrane ATPase activity, the half-inhibition (IC_{50}) being obtained for a venom quantity of 3.5 ng/mL, after 5 min of incubation at 37 °C in the presence of 1.6 mM Ca^{2+}. Characterization of the venom component(s) responsible for this inhibitory activity was subsequently performed. The availability in our laboratory of all the purified venom toxic polypeptidic components thus far identified, i.e., three α-neurotoxins, five cardiotoxins, and three phospholipases A_2 (Bougis et al., 1986), was a prerequisite for this systematic search.

Effect of α-Neurotoxin on Rat Brain [K^+]-PNPase Activity. NTX Nmm I, at 70 μM, after 1 h of incubation at 37 °C, was found to have no effect on [K^+]-PNPase activity.

Effect of Cardiotoxin on Rat Brain [K^+]-PNPase and [Na^+,K^+]-ATPase Activities. CTX Nmm I–IV were tested for their effect on [K^+]-PNPase: only CTX Nmm III was found to be noninhibitory in the conditions tested (Figure 1). For the others the kinetics of inhibition were very different, CTX Nmm II showing the greatest efficiency. This preparation of CTX Nmm II was further treated to eliminate a possible contamination by phospholipase(s) A_2. Two different kinds of additional purification steps were set up: anti-phospholipase A_2 immunoaffinity chromatography and high-performance liquid chromatography (see Materials and

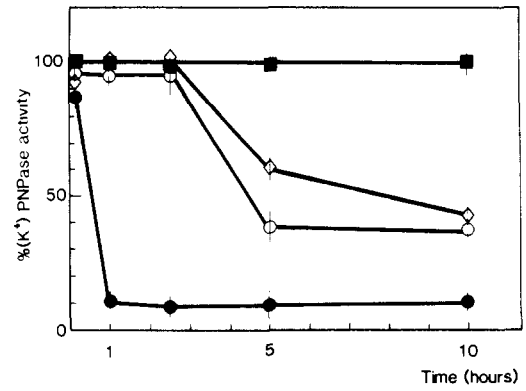

FIGURE 1: Kinetics of [K+]-PNPase inhibition by several cardiotoxins. The incubation of rat brain synaptosomal membranes was performed, at 37 °C, in the absence of Ca^{2+} and in the presence of 5×10^{-5} M of either (○) CTX Nmm I, (●) CTX Nmm II, (■) CTX Nmm III, or (◊) CTX Nmm IV. The [K+]-PNPase activity was measured as described under Materials and Methods.

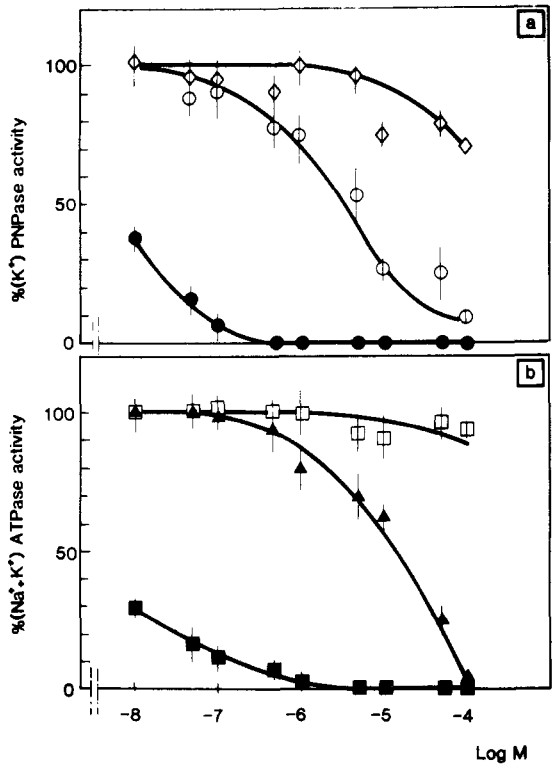

FIGURE 2: Effects of two phospholipase A_2 effectors on either (a) [K+]-PNPase or (b) [Na+,K+]-ATPase inhibition by either native or *p*-bromophenacyl bromide treated CTX Nmm II. Rat brain [K+]-PNPase and [Na+,K+]-ATPase inhibitions were determined after 1 h of incubation, at 37 °C, of synaptosomal membranes. (a) CTX Nmm II (○) in the absence of Ca^{2+} and (●) in the presence of 1.6 mM of Ca^{2+}; (◊) CTX Nmm II fraction beforehand treated with *p*-bromophenacyl bromide and in the absence of Ca^{2+}. (b) CTX Nmm II (▲) in the absence of Ca^{2+} and (■) in the presence of 0.4 mM of Ca^{2+}; (□) CTX Nmm II fraction beforehand treated with *p*-bromophenacyl bromide and in the absence of Ca^{2+}. The [K+]-PNPase and [Na+,K+]-ATPase activities were measured as described under Materials and Methods.

Methods). The contaminating phospholipase A_2 activity was detected with a highly sensitive fluorometric assay and [K+]-PNPase inhibitory activity again determined (Table I). Data were in agreement with a direct relation between the presence of phospholipase A_2 activity and the ATPase inhibition. To support the conclusion that inhibition of the ATPase was due to phospholipase A_2, and not to cardiotoxin, two sets of additional experiments were performed. On the one hand, the inhibitory activity of CTX Nmm II on [K+]-PNPase and

Table I: Comparison between the Inhibition of [K+]-PNPase Activity by Different CTX Nmm II Preparations and Their Respective Contents in Phospholipase A_2

preparations	phospholipase A_2 content[a] (% w/w)	[K+]-PNPase inhibition[b] (%)
CTX Nmm II originally used	1.200	100
CTX Nmm purified by immunochromatography	0.020	39
CTX Nmm II purified by HPLC	0.007	26

[a] The % w/w ratio was determined by expressing the phospholipase A_2 activity in terms of PH Nmm III specific activity: 0.36 μmol of fatty acid min^{-1} mg^{-1}. [b] The rat brain [K+]-PNPase inhibition was determined after 1 h of incubation of synaptosomal membranes at 37 °C in the presence of 5×10^{-5} M CTX Nmm II preparations and in the absence of Ca^{2+}.

Table II: Comparison between the [K+]-PNPase Inhibition Activity, the pH_i, and the Lethality of Different Phospholipases A_2

phospholipase A_2	[K+]-PNPase IC_{50}[a] (M)	pH_i	LD_{50} in mice (μg per mouse, iv)
PH Nmm III	5×10^{-12}	9.5	4.5
A. mellifera	6×10^{-10}	9.0	80
PH Nmm II	1×10^{-9}	8.5–8.8	6.6
PH Nmm I	4×10^{-8}	6.1	19
C. cerastes	4×10^{-8}	5.5	>300
p-bromophenacyl-PH Nmm III	$>1 \times 10^{-7}$	ND	>120
porcine pancreatic	$>1 \times 10^{-7}$	5.9	>120

[a] The rat brain [K+]-PNPase inhibition (IC_{50}) was determined after a 5-min incubation of synaptosomal membranes at 37 °C in the presence of 1.6 mM Ca^{2+} and of increasing concentrations of phospholipase A_2. ND, not determined; iv, intravenous injection.

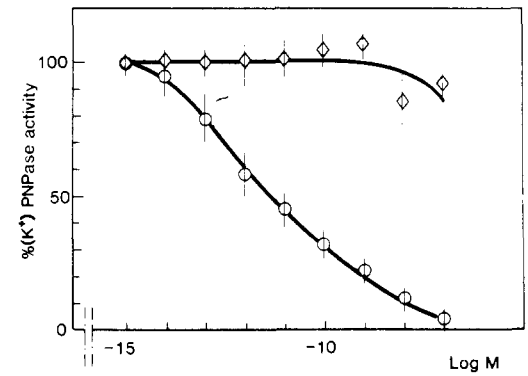

FIGURE 3: Dose-dependent [K+]-PNPase inhibition by PH Nmm III. The phospholipase A_2 was (○) native or (◊) chemically inactivated by *p*-bromophenacyl bromide. Rat brain [K+]-PNPase inhibition was determined after 5 min of incubation of synaptosomal membranes, at 37 °C, in the presence of 1.6 mM Ca^{2+}. The [K+]-PNPase activity was measured as described under Materials and Methods.

[Na+,K+]-ATPase was measured in the presence of Ca^{2+}, a potent activator of phospholipase A_2, resulting in a strong increase in [K+]-PNPase and [Na+,K+]-ATPase inhibitions (Figure 2). On the other hand, the same CTX Nmm II preparation was initially allowed to react with *p*-bromophenacyl bromide, a covalent inhibitor of phospholipase A_2, resulting in a strong decrease in [K+]-PNPase and [Na+,K+]-ATPase inhibitions (Figure 2).

Effect of Phospholipases A_2 on [K+]-PNPase and [Na+,K+]-ATPase Activities. PH Nmm I–III as well as several other phospholipases A_2 from varied origins were assayed, in the presence of Ca^{2+}, in order to estimate their ability to inhibit rat brain [K+]-PNPase activity and then compared in terms of their basicity and their toxicity (Figure 3, Table II). IC_{50}'s obtained after 5 min of incubation of synaptosomal

Table III: Comparison of the Inhibition Effect of PH Nmm III and Porcine Pancreatic Phospholipase A_2 on [K^+]-PNPase Activity of Membranes of Varied Origins

membrane origin	PH Nmm III IC_{50}[a] (M)	porcine pancreatic phospholipase A_2 IC_{50}[a] (M)
rat brain	5×10^{-12}	$>1 \times 10^{-7}$
rat heart	5×10^{-12}	$>1 \times 10^{-7}$
T. marmorata electric organ	5×10^{-12}	$>1 \times 10^{-7}$
human erythrocyte	3×10^{-8}	8×10^{-9}

[a] The IC_{50} of [K^+]-PNPase inhibition was determined after a 5-min incubation of membranes at 37 °C in the presence of 1.6 mM Ca^{2+} and of increasing concentrations of the phospholipase A_2 considered.

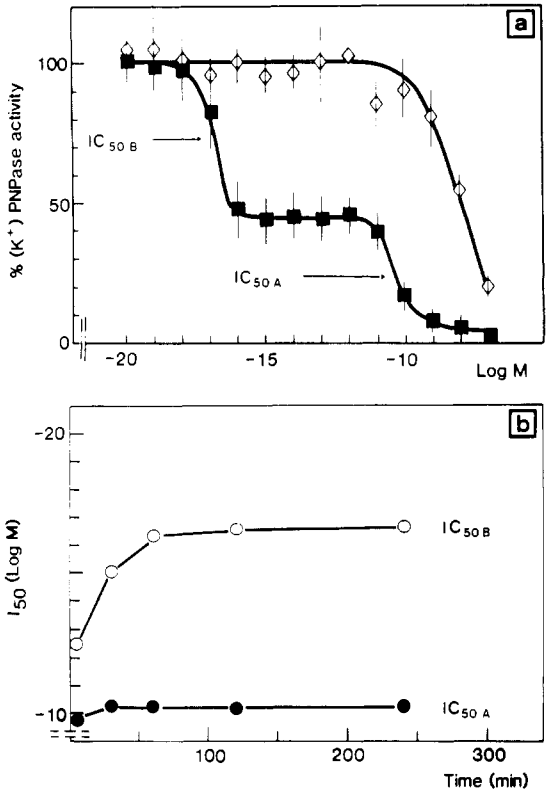

FIGURE 4: Dose- and time-dependent [K^+]-PNPase inhibition by PH Nmm III, native or chemically inactivated by *p*-bromophenacyl bromide. Incubation at 37 °C of rat brain membranes was in the presence of phospholipase A_2 and 1.6 mM Ca^{2+}. (a) Data obtained after 1 h of incubation of membranes in the presence of (■) native or (◊) *p*-bromophenacyl-PH Nmm III. (b) Variation computed versus time of (●) IC_{50A} and (○) IC_{50B} values as determined in (a). The [K^+]-PNPase activity was measured as described under Materials and Methods.

membranes in the presence of 1.6 mM Ca^{2+} covered a wide range of concentrations. At one extreme, PH Nmm III was found to be not only the most inhibitory but also the most basic and the most toxic. However, after reaction with *p*-bromophenacyl bromide, it was found to have lost almost all of its inhibitory (Figure 3) and toxic effects (Table II). On the other extreme, porcine pancreatic phospholipase A_2, which is acidic and nontoxic, was found to be poorly inhibitory (Table II). PH Nmm III and porcine pancreatic phospholipase A_2 were further studied with respect to their inhibitory activities on [K^+]-PNPase from varied membranes (Table III). [K^+]-PNPase activity of excitable membranes was found to be much more susceptible to inhibition by PH Nmm III than that of erythrocytes. On the contrary, porcine pancreatic phospholipase A_2 was more active than PH Nmm III toward human erythrocyte [K^+]-PNPase.

The major aspect of the dose-dependent inhibition of rat

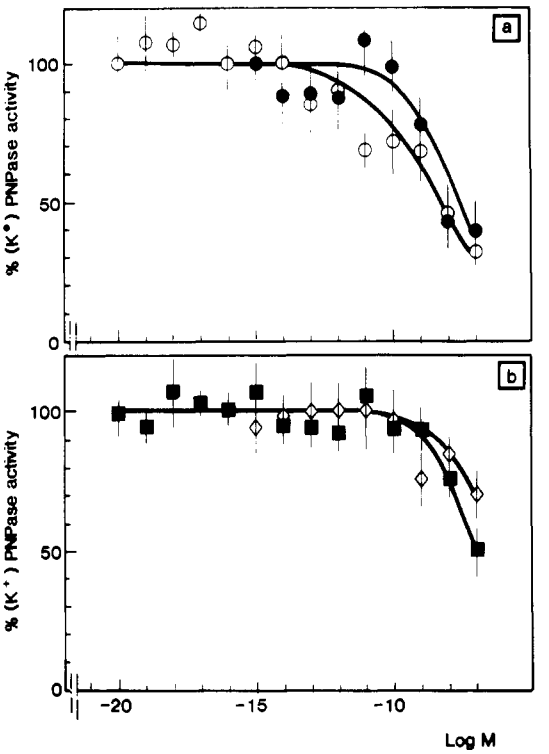

FIGURE 5: Comparative study on dose-dependent inhibition of [K^+]-PNPase activity of (a) erythrocyte membranes incubated with PH Nmm III or (b) rat brain synaptosomal membranes incubated with porcine pancreatic phospholipase A_2. Respective to both assays, incubations were carried out during (● and ◊) 5 min or (○ and ■) 1 h, at 37 °C, in the presence of 1.6 mM Ca^{2+}. The [K^+]-PNPase activity was measured as described under Materials and Methods.

brain [K^+]-PNPase activity determined after 5 min of incubation of membranes with PH Nmm III was the large range of the dose effect, which was much greater than 2 orders of magnitude with a IC_{10}/IC_{90} of about 1×10^6 (Figure 3). Thus, the effect of the incubation time was investigated: after 1 h, a biphasic dose–effect curve appeared, suggesting that two [K^+]-PNPase activities (referred to as activities A and B) were differentially affected with regard to the PH Nmm III concentration (Figure 4a). The IC_{50A} and IC_{50B} values were computed, and their variation in terms of incubation time was plotted in Figure 4b. While IC_{50A} decreased only slightly down to 5×10^{-11} M, IC_{50B} decreased dramatically to reach its minimum value after 1 h of incubation: 2×10^{-17} M. In these conditions, the two inhibition processes occurred each within 2 orders of magnitude of the PH Nmm III concentration. In the case of *p*-bromophenacyl-PH Nmm III a monophasic inhibition curve for rat brain [K^+]-PNPase activity ($IC_{50} = 1 \times 10^{-8}$ M) was observed after 1 h of incubation (Figure 4a). Identical results were obtained when addressing [Na^+,K^+]-ATPase activity.

Results expressed in Figure 5 demonstrate that only monophasic inhibition curves for [K^+]-PNPase activity were obtained ($IC_{50} > 1 \times 10^{-7}$ M) when human erythrocyte membranes were incubated (5 min or 1 h) with PH Nmm III (Figure 5a) or rat brain synaptosomal membranes were incubated (5 min or 1 h) with porcine pancreatic phospholipase A_2 (Figure 5b).

Finally, two additional phospholipases A_2 described as presynaptically acting neurotoxins, i.e., β-bungarotoxin and notexin, were also tested on rat brain synaptosomal membranes. For both of them monophasic inhibition curves for [K^+]-PNPase activity ($IC_{50} > 1 \times 10^{-7}$ M) were observed after 1 h of incubation (Figure 6).

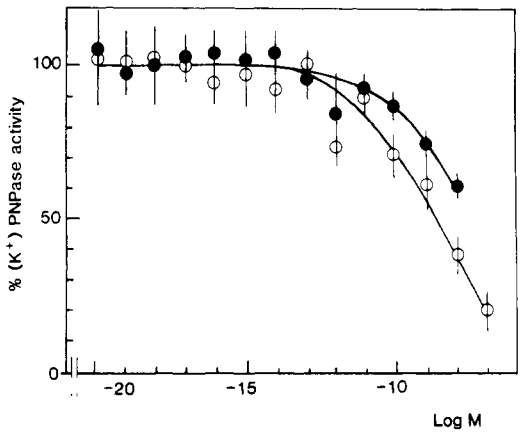

FIGURE 6: Dose-dependent [K+]-PNPase inhibition by (O) notexin or (●) β-bungarotoxin. Rat brain [K+]-PNPase inhibition was determined after 1 h of incubation of synaptosoal membranes at 37 °C in the presence of 1.6 mM Ca^{2+}. The [K+]-PNPase activity was measured as described under Materials and Methods.

Discussion

The elapid venoms, genus *Naja*, can be separated into several active components. Among them α-neurotoxins, phospholipases A_2, and cardiotoxins are recognized to be mainly responsible for the lethality of these venoms. To address the question of the nature of *N. mossambica mossambica* venom components that inhibit rat brain [Na+,K+]-ATPase, we have planned new experiments, keeping in mind the possible contamination of the available preparations of each component by the other components of this venom. At the venom quantity allowing half-inhibition of the enzyme activity, i.e., 3.5 ng/mL, the respective concentrations of NTX Nmms, PH Nmms, and CTX Nmms can be calculated when considering, on the one hand, the average molecular weights of 7000 for both α-neurotoxins and cardiotoxins and 14 000 for phospholipases A_2 and, on the other hand, the amounts of these three types of components in the venom (Bougis et al., 1983, 1986). The calculated concentrations are 6.0×10^{-12} M for NTX Nmms, 6.6×10^{-11} M for PH Nmms, and 2.6×10^{-10} M for CTX Nmms. It appears that NTX Nmms, described as specific competitive antagonists for the nicotinic acetylcholine receptor (Lee & Chang, 1966; Changeux et al., 1984), cannot be involved in the inhibitory effect on [Na+,K+]-ATPase since NTX Nmm I at concentrations up to 70 μM was found ineffective. As concerns cardiotoxins, they are not present at a concentration high enough to be relevant for the inhibition of [Na+,K+]-ATPase. Indeed, CTX Nmm preparations, when active, demonstrated this activity in the micromolar range (Figures 1 and 2). The molecular mechanism of action of cardiotoxins on axonal membrane has been previously proposed to be related to the inhibition of [Na+,K+]-ATPase in its membranous environment. The hypothesis was that the toxin binding to a lipid-type receptor structure would trigger a structural rearrangement in the membrane domain resulting in the inactivation of the enzyme (Vincent et al., 1976). How can the discrepancy between this hypothesis and our results be explained? We are convinced that the preparation of cardiotoxin from *N. mossambica mossambica* used by these authors, a preparation that is very similar to the ones we used, was contaminated by phospholipase(s) A_2, even though at an extremely low percentage. Our data are in accordance with this statement because of the following: (i) very different kinetics of [K+]-PNPase inhibition characterize the various CTX Nmm preparations (Figure 1), in spite of LD_{50} values quite similar for all these toxins (Bougis et al., 1983); (ii) it is noteworthy that the apparent inhibitory activity of CTX Nmm II is related to its mode of purification, mainly as a function of phospholipase A_2 contamination (Table I); (iii) known effectors of phospholipase A_2 activity such as Ca^{2+}, on the one hand, and the selective covalent modification by *p*-bromophenacyl bromide of the His_{48} of the enzyme active site, on the other hand, modulate the apparent inhibitory activity of CTX Nmm II in the expected ways (Figure 2). Accordingly, this fact implies that phospholipases A_2 should be very potent inhibitors of [Na+,K+]-ATPase: after incubation of synaptosomal membranes for only 5 min with PH Nmm III, the IC_{50} value of [K+]-PNPase inhibition is 5×10^{-12} M, a value that agrees well with the phospholipase A_2 concentration estimated to be present in the venom quantity necessary to obtain half-inhibition of [K+]-PNPase (see above). Finally, the various activities reported in the literature for cardiotoxins are obtained in the micromolar range rather than the picomolar range (Lee, 1972; Harvey, 1985; Bougis et al., 1983). Consequently, the cardiotoxin molecular mechanism of action linked to a deactivation of [Na+,K+]-ATPase previously proposed by Vincent et al. (1976) seems, to us, to be invalid. Only phospholipases A_2 must be considered as active components in the venom responsible for the inhibition of [Na+,K+]-ATPase.

The inactivation of [Na+,K+]-ATPase by phospholipases A_2 appears to be a complex phenomenon that depends on the source of both enzymes. Among phospholipases A_2 from *N. mossambica mossambica* venom, PH Nmm III is the most active as well as the most basic and toxic. A close correlation seems to exist between [Na+,K+]-ATPase inhibitory activity, basicity, and toxicity for phospholipases A_2 (Table II). Moreover, as suggested by data obtained with *p*-bromophenacyl-PH Nmm III (Figure 3, Table II), it is obvious that [Na+,K+]-ATPase inhibition, as well as toxicity, depend on the enzymatic activity of phospholipase A_2.

The influence of the incubation time upon the IC_{50} value of [Na+,K+]-ATPase inhibition by PH Nmm III has been assayed. As an unexpected result, an increase of the incubation time reveals a biphasic phenomenon (Figure 4a). This result might be explained by the existence, in rat brain membranes, of two [Na+,K+]-ATPase activities (referred to as A and B) that are differentially inhibited by PH Nmm III. Activity B is more sensitive to inhibition at low PH Nmm III concentrations than activity A. Moreover, activity B appears to be distinctive of excitable-type membranes. This statement is fully supported by the data in Table III and Figure 5. Indeed, PH Nmm III acting on erythrocyte membranes does not promote a biphasic inhibition, even when membrane incubation is extended up to 1 h. Likewise, porcine pancreatic phospholipase A_2 is able to inhibit [Na+,K+]-ATPase of synaptosomal membranes only when used at high concentrations and only within a monophasic shape, as it is for [Na+,K+]-ATPase of erythrocyte membranes. It is most likely that the two activities in question originate from two enzymes displaying the same type of activity, but differing in some way. The existence of multiple isozymes of [Na+,K+]-ATPase in mammals has emerged (Sweadner, 1979). The catalytic subunit of the α form found in kidney differs from the catalytic subunit of the α(+) form found in brain axolemma. Rat brain has been proven, on a genetic basis, to possess both forms (Shull et al., 1986b), and rat heart seems also to possess both (Sweadner & Farshi, 1987). May these structural differences among [Na+,K+]-ATPases explain our results? Alternatively, may the lipid environment of these two isozymes be distinctive and lead to a preferential hydrolysis depending on the phospholipase A_2? Indeed, even though it is well recognized that

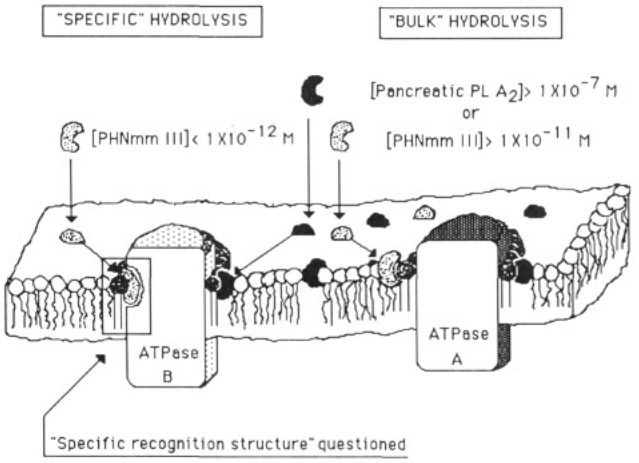

FIGURE 7: Model showing the two putative forms of $[Na^+,K^+]$-ATPase, A and B, and the different ways of their inhibition by porcine pancreatic phospholipase A_2 and PH Nmm III. The phospholipase A_2 concentration ranges that are effective on form A and form B of $[Na^+,K^+]$-ATPase are indicated. Boxed is the "specific recognition structure" in question. Explanations for "specific" and "bulk" hydrolysis are found under Discussion. Note that, after their initial electrostatic binding to the lipid–water interface, the molecules of phospholipase A_2 are assumed to enter the membrane through their lipid-binding domain and to be able to move around in the membrane.

$[Na^+,K^+]$-ATPase activity depends on its lipid surrounding, the exact nature of the lipids involved is still a matter of controversy (Roelofsen, 1981). More work is required to answer these questions.

As concerns the *p*-bromophenacyl-PH Nmm III, we find it surprising that this inactivated phospholipase A_2 still apparently inhibits the $[Na^+,K^+]$-ATPase activity, by displaying a monophasic inhibition curve (Figure 4a). Since no further purification is made after its chemical modification, we expect a few unreacted native molecules of PH Nmm III to be still present. This would explain the apparent inhibition observed when using high concentrations of *p*-bromophenacyl-PH Nmm III. But why is the shape of the inhibition curve monophasic within 2 orders of magnitude instead of biphasic as it is for PH Nmm III? Our assumption, hypothesized in Figure 7, is that two isoforms (A and B) of $[Na^+,K^+]$-ATPase coexist, not necessarily on the same type of cell membrane. On the one hand, low concentrations of *p*-bromophenacyl-PH Nmm III are not able to inhibit either of the two forms of $[Na^+,K^+]$-ATPase. But because of a "specific recognition structure", being part of the intrinsic structure or the surrounding of only form B of $[Na^+,K^+]$-ATPase, competition for form B between *p*-bromophenacyl-PH Nmm III and the unreacted molecules of PH Nmm III could take place, protecting form B from inhibition. If so, this structure should be able to direct specifically the enzymatic activity of PH Nmm III on form B of $[Na^+,K^+]$-ATPase. On the other hand, for high concentrations of *p*-bromophenacyl-PH Nmm III both form A and form B of $[Na^+,K^+]$-ATPase are actually inhibited by the amount of unreacted PH Nmm III still present, as they are for high concentrations of porcine pancreatic phospholipase A_2 (Figure 5b), by a way that may be qualified as "nonspecific" or "bulk" hydrolysis. In this instance, the IC_{50} value relative to *p*-bromophenacyl-PH Nmm III is effectively 1000 times higher than IC_{50A} relative to PH Nmm III, a factor that agrees with the 0.1% estimate for the amount of unreacted PH Nmm III remaining in the *p*-bromophenacyl-PH Nmm III preparation (see Materials and Methods). Furthermore, since erythrocyte membranes should contain only form A of $[Na^+,K^+]$-ATPase, a monophasic inhibition curve is observed whatever the concentrations of PH Nmm III and the length of the incubation (Figure 5a). As mentioned before, the structural support of the so-postulated "specific recognition structure" for PH Nmm III could be of lipid nature as well as of protein nature (Figure 7). The concept of a motionally restricted lipid annulus (Esmann et al., 1985) together with a possible high positive allosteric effect of the annular lipids on the $[Na^+,K^+]$-ATPase activity may be of importance. Finally, our model may support the idea developed by Sargent and Schwyzer (1986) of "membrane catalysis", by which the surface accumulation due to electrostatic attractions would be a way to enhance the target binding of a multiply charged molecule. Thus, after an initial binding through their so-called lipid-binding domain, the final binding within the lipids of the membrane of basic phospholipases A_2, such as PH Nmm III, to a specific target would require only a small energetic contribution. In addition to the enzymatic nature of $[Na^+,K^+]$-ATPase inhibition, such a sequence of events could be an explanation for their ability to inhibit a specific form of $[Na^+,K^+]$-ATPase (form B) in spite of a molar ratio considerably in favor of $[Na^+,K^+]$-ATPase. Afterward, one can suppose that cellular phospholipases A_2 possess also the ability to inhibit the $[Na^+,K^+]$-ATPase activity in a specific way, like we believe PH Nmm III does, thus behaving as endogenous regulators.

In conclusion, the apparent $[Na^+,K^+]$-ATPase inhibitory activity of cardiotoxins which has been previously reported is likely to be due to a contamination of cardiotoxin preparations by phospholipase(s) A_2. Furthermore, the effects of phospholipases A_2 on $[Na^+,K^+]$-ATPases are complex and depend on the origin of both phospholipases A_2 and target membranes. A specificity of action of toxic, basic phospholipases A_2 on a specific form of $[Na^+,K^+]$-ATPase of excitable membranes is a new concept to be better defined.

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Registry No. ATPase, 9000-83-3; phospholipase A_2, 9001-84-7.

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Substrate Variability as a Factor in Enzyme Inhibitor Design: Inhibition of Ovine Brain Glutamine Synthetase by α- and γ-Substituted Phosphinothricins

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ABSTRACT: Ovine brain glutamine synthetase (GS) utilizes various substituted glutamic acids as substrates. We have used this information to design α- and γ-substituted analogues of phosphinothricin [L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid], a naturally occurring inhibitor of GS. These compounds display competitive inhibition of GS, and a correlation between the inhibitor K_i values and the K_m/V_{max} values of the analogously substituted glutamates supports the hypothesis that the phosphinothricins participate in transition-state analogue inhibition of GS. At concentrations greater than K_i these inhibitors caused biphasic time-dependent loss of enzyme activity, with initial pseudo-first-order behavior; k'_{inact} parameters were determined for several compounds and were similar to the 2.1×10^{-2} s^{-1} value measured for PPT. Dilution after GS inactivation caused a non-first-order recovery of activity. Reactivation kinetics were insensitive to inhibitor and ADP concentrations over wide ranges, although very high postdilution concentrations of inhibitor suppressed reactivation. The burst activity level, β, as well as the concentration of inhibitor required to suppress reactivation to this level, μ, expressed as a multiple of the K_i value, was characteristic for each compound in the phosphinothricin series. Increasing substitution of the phosphinothricin parent structure caused an increase in K_i values as well as in the inactivation/reactivation parameters. The kinetic behavior of these inhibitors is consistent with a mechanistic scheme involving initial phosphorylation and rapid partial inhibitor dissociation, followed by slow release of remaining bound inhibitor.

Glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] is a ubiquitous enzyme in both prokaryotes (Stadtman & Ginsburg, 1974; Rhee et al., 1985) and eukaryotes (Meister, 1974, 1985) and catalyzes a central reaction in nitrogen metabolism, the conversion of glutamate to glutamine (eq 1). Prokaryotic GS consists of 12 identical catalytic subunits weighing approximately 45 kDa, while the eukaryotic enzyme is an octomer. The crystal structure of dodecameric GS from *Salmonella typhimurium* has recently been determined to a resolution of 3.5 Å (Almassy et al., 1986;

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