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INHIBITION OF DOG KIDNEY Na^+, K^+-ATPase ACTIVITY BY PROCAINE, TETRACAINE AND DIBUCAINE*

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Abstract—The potency of local anesthetics as inhibitors of Na^+, K^+-ATPase and K^+-NPPase activities correlated with lipid solubility. The order of potencies was: dibucaine > tetracaine ≫ procaine. Na^+-ATPase activity was remarkably more sensitive to inhibition by tetracaine and procaine, and inhibitory potency did not correlate with lipid solubility. The order of potencies for inhibition of Na^+-ATPase activity was: tetracaine > dibucaine > procaine. We examined interactions between the local anesthetics and monovalent cations in an attempt to explain this observation. Inhibition of Na^+-K^+-ATPase by tetracaine and dibucaine was competitive with respect to Na^+, and inhibition of Na^+-ATPase activity by all three agents was competitive with respect to Na^+. Inhibition of Na^+, K^+-ATPase activity by procaine and tetracaine was competitive with respect to K^+, and inhibition of K^+-NPPase activity by all three agents was competitive with respect to K^+. Dibucaine, the most lipid soluble agent, was equipotent as an inhibitor of all three activities and was generally less effective as a competitor with respect to activation by monovalent cations. These results suggest that dibucaine may interact nonspecifically with membrane lipids to inhibit enzyme activity whereas less lipid soluble agents, such as tetracaine and procaine, may interact more selectively with cation binding sites. It appears that the presence of K^+ in the assay medium specifically decreases the inhibitory potency of tetracaine and procaine. Direct competition between these agents and K^+ may prevent inhibition or, alternately, the presence of K^+ may convert the enzyme to a conformation less susceptible to inhibition by agents of low to intermediate lipid solubility.

Local anesthetics block excitability in nerve by preventing the passive inward movement of Na^+ which initiates the action potential [1]. Local anesthetics also inhibit cation transport ATPases [2–4], and this effect may contribute to the duration of action and toxicity of this class of agents.

Roufogalis [3] showed that the order of potency of a series of depressant drugs as inhibitors of bovine brain Na^+, K^+-ATPase‡ activity correlated with their lipid solubilities. Chazotte *et al.* [5] also demonstrated a correlation between lipid solubility and potency for inhibition of mitochondrial F_1-ATPase by the local anesthetics procaine, lidocaine, tetracaine and dibucaine.

Roufogalis [3] also examined the influence of the depressant drugs on the activation of Na^+,K^+-ATPase by monovalent cations. He found that highly lipid soluble agents, such as chlorpromazine, were competitive inhibitors of Na^+ activation and noncompetitive inhibitors of K^+ activation. Agents with lower lipid solubility, like tetracaine, had the opposite effect, i.e. they were noncompetitive inhibitors of Na^+ activation and competitive inhibitors of K^+ activation. We previously showed that tetracaine inhibited Na^+,K^+- and Ca^{2+}-ATPase activities in human red cell membranes [2]. However, in the case of Na^+,K^+-ATPase, we found that tetracaine was a competitive inhibitor of both Na^+ and K^+ at their respective activation sites. This discrepancy might be due to inherent differences between the Na^+, K^+-ATPase preparations used in the two studies.

The present study was undertaken to compare the influence of three local anesthetics on dog kidney Na^+, K^+-ATPase and two other activities catalysed by this enzyme: K^+-NPPase and Na^+-ATPase. Our objectives were to determine if local anesthetic potency uniformly correlates with lipid solubility, and to examine the influence of these agents on the kinetics of activation of each activity by monovalent cations.

METHODS

Na^+, K^+-ATPase was prepared as a microsomal fraction from the medulla of dog kidney according to the method of Post and Sen [6]. The specific activity of enzyme preparations was between 50 and 100 μmoles P_i per mg protein per hr.

Na^+, K^+- and Na^+-ATPase activities were measured in terms of P_i production by means of the sensitive assay procedure of Muszbek *et al.* [7]. With this method, the presence of local anesthetic in the assay medium does not interfere with the colorimetric determination of P_i, if the malachite green

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‡ Abbreviations: Na^+, K^+-ATPase, ($Na^+ + K^+ + Mg^{2+}$)-adenosine triphosphatase (EC 3.6.1.3); K^+-NPPase, K^+-dependent *p*-nitrophenylphosphatase (EC 3.6.1.3); Na^+-ATPase, Na^+-dependent adenosine triphosphatase (EC 3.6.1.3); EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; and $K_{0.5}$, concentration of drug required to half-maximally inhibit the enzyme.

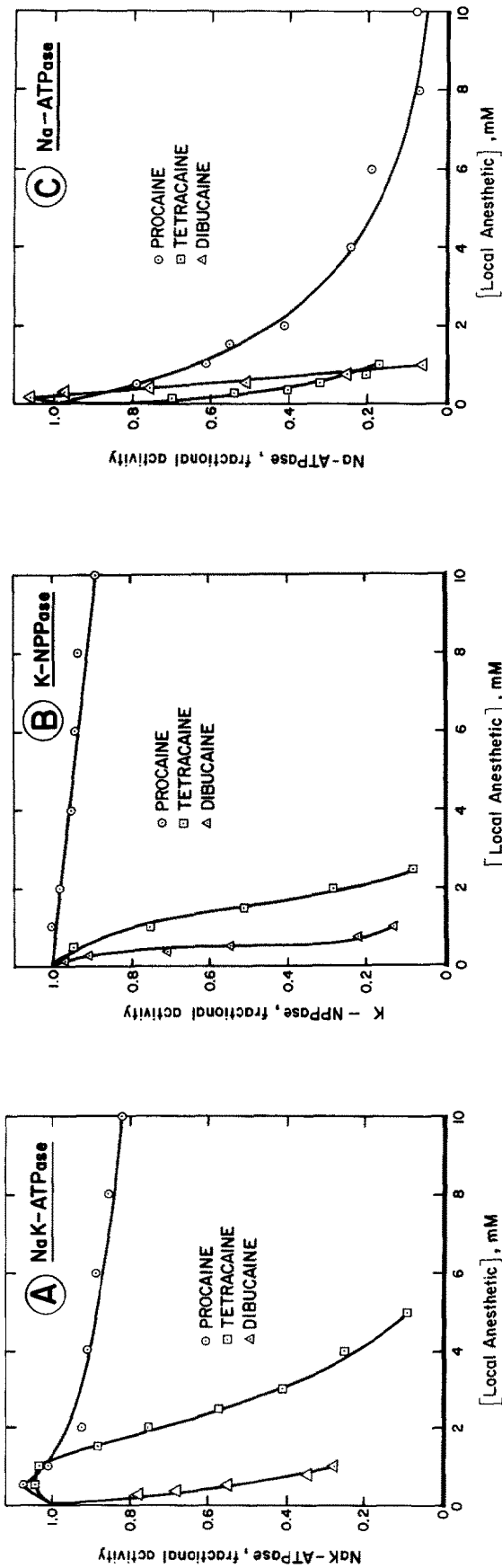

Fig. 1. (A) Na^+, K^+-ATPase activity, (B) K^+-NPPase activity, and (C) Na^+-ATPase activity as a function of local anesthetic concentration relative to control activity in the absence of drug. Key: procaine (—⊙—), tetracaine (—⊡—), and dibucaine (—△—). Monovalent cation concentrations were fixed at 120 mM for Na^+ and 20 mM for K^+. Na^+, K^+-ATPase was determined with both Na^+ and K^+; K^+-NPPase with K^+; and Na^+-ATPase with Na^+. Each point represents the average of three to seven experiments.

reagent is added before the acid molybdate. All Na^+, K^+- and Na^+-ATPase assays were carried out with low ATP concentrations in order to minimize the P_i blanks resulting from the non-enzymatic hydrolysis of ATP. In all cases we verified that activity was linear with time during the assay period.

K^+-NPPase activity was measured at 410 mm in terms of *p*-nitrophenol production. In each case, the activity of interest was calculated by subtracting activity in the presence of 0.25 mM ouabain from total activity. Conditions common to all experiments were: 63 mM Tris–HCl (pH 7.4 at 38°) and 0.25 mM EGTA. For Na^+, K^+-ATPase, ATP was 0.3 mM and Mg^{2+} 1 mM; for Na^+-ATPase, ATP was 0.15 mM and Mg^{2+} 1 mM; and for K^+-NPPase, *p*-nitrophenylphosphate was 6 mM and Mg^{2+} 5 mM. Other conditions are described in the figure legends. Figures represent the average of three or more experiments carried out in duplicate. In most cases, kinetic constants were obtained with the aid of a microcomputer using averaged data fitted by the Michaelis–Menten or Hill equations. The program for analyzing enzyme kinetic data was developed by Knack and Rohm [8].

RESULTS

Kinetics of inhibition of enzyme activities by local anesthetics. Relative enzyme activity plotted as a function of local anesthetic concentration is shown in Fig. 1, A–C. Procaine was not an effective inhibitor of Na^+, K^+-ATPase activity (Fig. 1A) or K^+-NPPase activity (Fig. 1B), but Na^+-ATPase activity was sensitive to inhibition by procaine (Fig. 1C). Tetracaine inhibited all three activities, and the order to potencies for this local anesthetic was Na^+-ATPase > K^+-NPPase > Na^+, K^+-ATPase. Dibucaine was nearly equipotent as an inhibitor of all three activities.

The concentrations of local anesthetic required to half-maximally inhibit enzyme activity ($K_{0.5}$ values) were obtained from Hill plots of these data. $K_{0.5}$ values, Hill coefficients, and partition coefficients for the local anesthetics are summarized in Table 1.

The order of potencies of local anesthetics as inhibitors of Na^+, K^+-ATPase and K^+-NPPase activities correlated with their lipid solubilities. However, in the case of Na^+-ATPase (Fig. 1C), tetracaine was the most effective inhibitor.

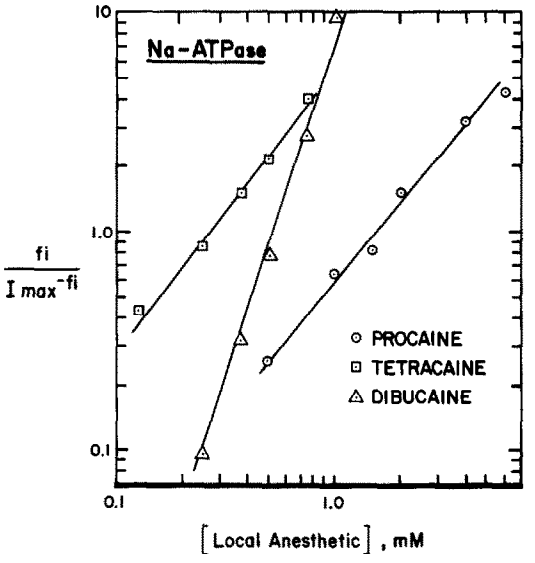

Fig. 2. Hill plot of inhibition of Na^+-ATPase activity by the local anesthetics procaine (—⊙—), tetracaine (—⊡—) and dibucaine (—△—). The Na^+ concentration was 120 mM. Each point represents the average of five to seven experiments. Fractional inhibition = fi.

A Hill plot of inhibition of Na^+-ATPase activity by the local anesthetics is shown in Fig. 2. In this case, the Hill coefficients for inhibition by procaine and tetracaine were remarkably different than for dibucaine.

Interactions between local anesthetics and monovalent cations. In these experiments, Na^+, K^+-ATPase and Na^+-ATPases activities were studied as a function of Na^+ concentration in the absence or presence of fixed concentrations of the local anesthetics. The concentrations of dibucaine and tetracaine used were approximately the $K_{0.5}$ values (Table 1); however, in the case of procaine, a concentration of 5 mM was arbitrarily selected for study. Figure 3 shows that both tetracaine and dibucaine increased the apparent K_m of Na^+, K^+-ATPase for Na^+, but that procaine was without effect.

Activation of Na^+-ATPase by Na^+ is biphasic [10] and, therefore, could not be conveniently analyzed kinetically. Instead, we measured Na^+-ATPase activity at two fixed concentrations of Na^+, 60 and

Table 1. $K_{0.5}$ values and Hill coefficients (n) for procaine, tetracaine and dibucaine as inhibitors of Na^+, K^+-ATPase, K^+-NPPase and Na^+-ATPase activities*

Activity		Procaine	Tetracaine	Dibucaine
Na^+, K^+-ATPase	$K_{0.5}$ (mM)		2.81 ± 0.16	0.54 ± 0.15
	n		3.2 ± 0.37	1.8 ± 0.47
K^+-NPPase	$K_{0.5}$ (mM)		1.68 ± 0.15	0.51 ± 0.04
	n		2.7 ± 0.36	1.7 ± 0.42
Na^+-ATPase	$K_{0.5}$ (mM)	1.57 ± 0.31	0.30 ± 0.06	0.61 ± 0.05
	n	1.1 ± 0.15	1.1 ± 0.14	3.0 ± 0.43
Partition coefficient†		80	5,000	25,000

* Monvalent cation concentrations were fixed at 120 mM for Na^+ and 20 mM for K^+. Na^+, K^--ATPase activity was determined in the presence of both Na^+ and K^+; K^+-NPPase, with K^+; and Na^+-ATPase, with Na^+. Values are mean ± S.D.

† Octanol/water [9].

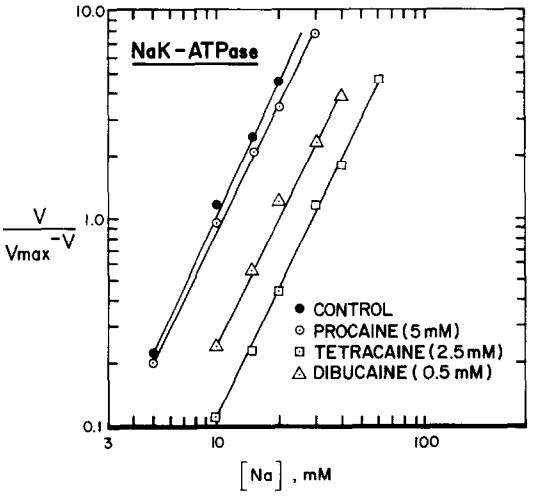

Fig. 3. Hill plot of Na activation of Na^+, K^+-ATPase activity in the absence of local anesthetic (—●—) and in the presence of 5 mM procaine (—⊙—), 2.5 mM tetracaine (—⊡—) or 0.5 mM dibucaine (—△—). The K^+ concentration was 20 mM. Each point represents the average of four experiments.

180 mM, as a function of local anesthetic concentration. $K_{0.5}$ values for the local anesthetics obtained in this series of experiments are presented in Table 2. There was a significant difference between $K_{0.5}$ values obtained at 60 and 180 mM Na^+ in each case ($P < 0.01$). These results suggest a competitive interaction between all three local anesthetics and Na^+ in the case of Na^+-ATPase activity.

The next series of experiments examined the influence of local anesthetics on activation of Na^+, K^+-ATPase and K^+-NPPase by K^+. Figure 4A shows a Hill plot of the activation of Na^+, K^+-ATPase by K^+ in the presence or absence of fixed concentrations of the local anesthetics. Dibucaine did not influence K^+ activation, whereas both procaine and tetracaine markedly increased the apparent K_m for K^+. This value increased from 0.85 mM in control curves to 2.3 mM in the presence of procaine or tetracaine. It is of interest that this concentration of procaine (5 mM) did not inhibit significantly Na^+, K^+-ATPase activity (Fig. 1A) where the K^+ concentration was 20 mM, well above the apparent K_m for K^+ found in the experiment shown in Fig. 4A.

Apparently, when local anesthetic concentration was varied at three fixed concentrations of K^+, curves for Na^+, K^+-ATPase activity were shifted to the left as K^+ concentration decreased (Fig. 4B). These experiments clearly demonstrate a competitive interaction between K^+ and the local anesthetics procaine and tetracaine; however, the potency of dibucaine was not influenced by variation of the K^+ concentration.

Figure 5A shows a Hill plot of experiments that examined the influence of local anesthetics on the activation of K^+-NPPase by K^+. In this case, all three local anesthetics appeared to increase the apparent K_m for K^+ at its activation site. The apparent K_m for K^+ was 2.2 mM in control curves, and this value was increased to 3.2 mM by procaine, to 4.6 mM by dibucaine, and to 5.6 mM by tetracaine. The competitive interaction between local anesthetics and K^+ can also be seen in Fig. 5B where an increase in K^+ concentration shifted curves for inhibition of K^+-NPPase activity by local anesthetics to the right. These results clearly demonstrate that all three agents compete with K^+ at the site where it activates K^+-NPPase.

DISCUSSION

Correlation between lipid solubility and potency of the local anesthetics as inhibitors of Na^+, K^+-ATPase *activity.* Results of the present study confirm the observation that a correlation exists between the potencies of local anesthetics as inhibitors of Na^+, K^+-ATPase activity and their lipid solubilities [3]. The present study also demonstrates that this correlation extends to inhibition of K^+-NPPase activity by these agents. The order of potencies for inhibition of Na^+, K^+-ATPase and K^+-NPPase activities was: dibucaine > tetracaine ≫ procaine.

With regard to the influence of local anesthetics on monovalent cation interactions with Na^+, K^+-ATPase, present results confirm our previous observation that tetracaine is a competitive inhibitor of both Na^+ and K^+ at their respective activation sites [4]. Roufogalis [3] reported that tetracaine was a noncompetitive inhibitor of Na^+ activation of brain Na^+, K^+-ATPase. Although we cannot account for this discrepancy, it is possible that the quality and quantity of lipid associated with brain Na^+, K^+-ATPase differ from that present in human red cell membranes or kidney microsomes.

In general, however, we do confirm the generalization by Roufogalis [3] with respect to Na^+, K^+-ATPase that highly lipid soluble agents (e.g. dibucaine) are competitive inhibitors of Na^+ activation and noncompetitive inhibitors of K^+ activation while

Table 2. $K_{0.5}$ values for procaine, tetracaine and dibucaine as inhibitors of Na^+, K^+-ATPase activity at a 60 or 180 mM concentration of Na^+*

Condition		Procaine	Tetracaine	Dibucaine
60 mM Na^+	$K_{0.5}$ (mM)	1.15 ± 0.09	0.35 ± 0.02	0.53 ± 0.02
	n			3.1 ± 0.17
180 mM Na^+	$K_{0.5}$ (mM)	2.22 ± 0.13	0.81 ± 0.04	0.61 ± 0.01
	n			3.0 ± 0.89

* $K_{0.5}$ values for procaine and tetracaine were determined with the Michaelis–Menten equation. $K_{0.5}$ values and Hill coefficients (n) for dibucaine were determined with the Hill equation. Values are mean ± S.D.

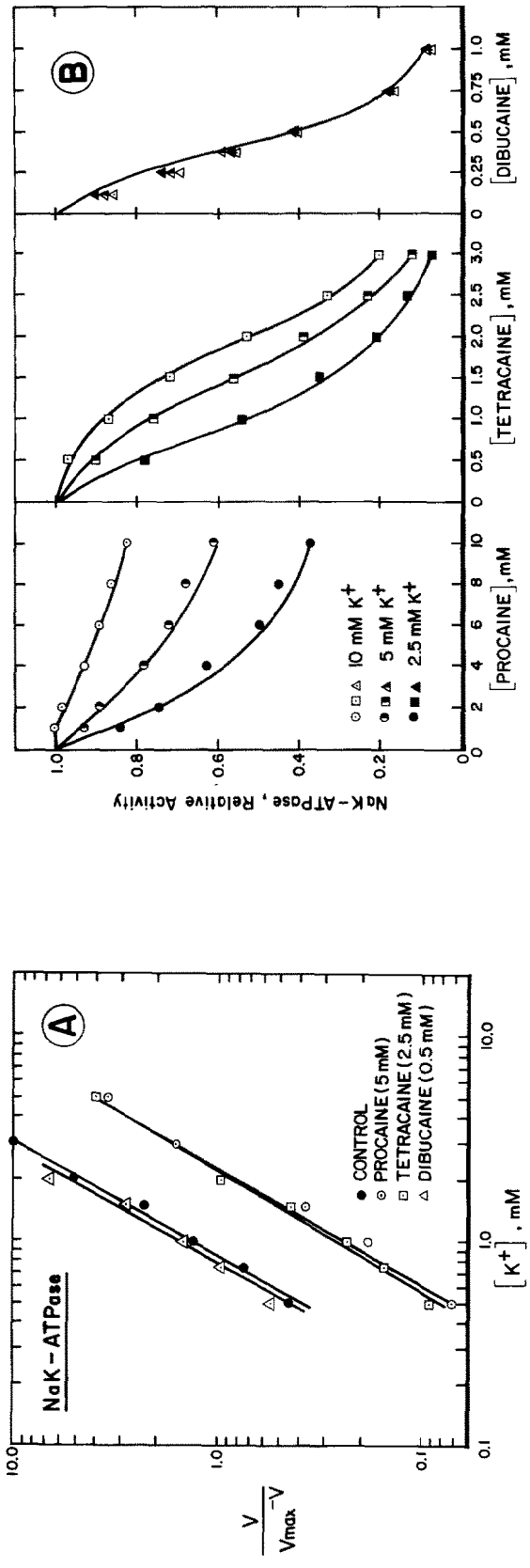

Fig. 4. (A) Hill plot of K^+ activation of Na^+, K^+-ATPase activity in the absence of local anesthetic (—●—) and in the presence of 5 mM procaine (—⊙—), 2.5 mM tetracaine (—□—) or 0.5 mM dibucaine (—△—). The Na^+ concentration was 120 mM. Each point represents the average of three experiments. (B) Na^+, K^+-ATPase activity as a function of local anesthetic concentration relative to control activity in the absence of drug. Open symbols, 10 mM K^+; half-open symbols, 5 mM K^+; and closed symbols, 2.5 mM K^+. Each point represents the average of three experiments.

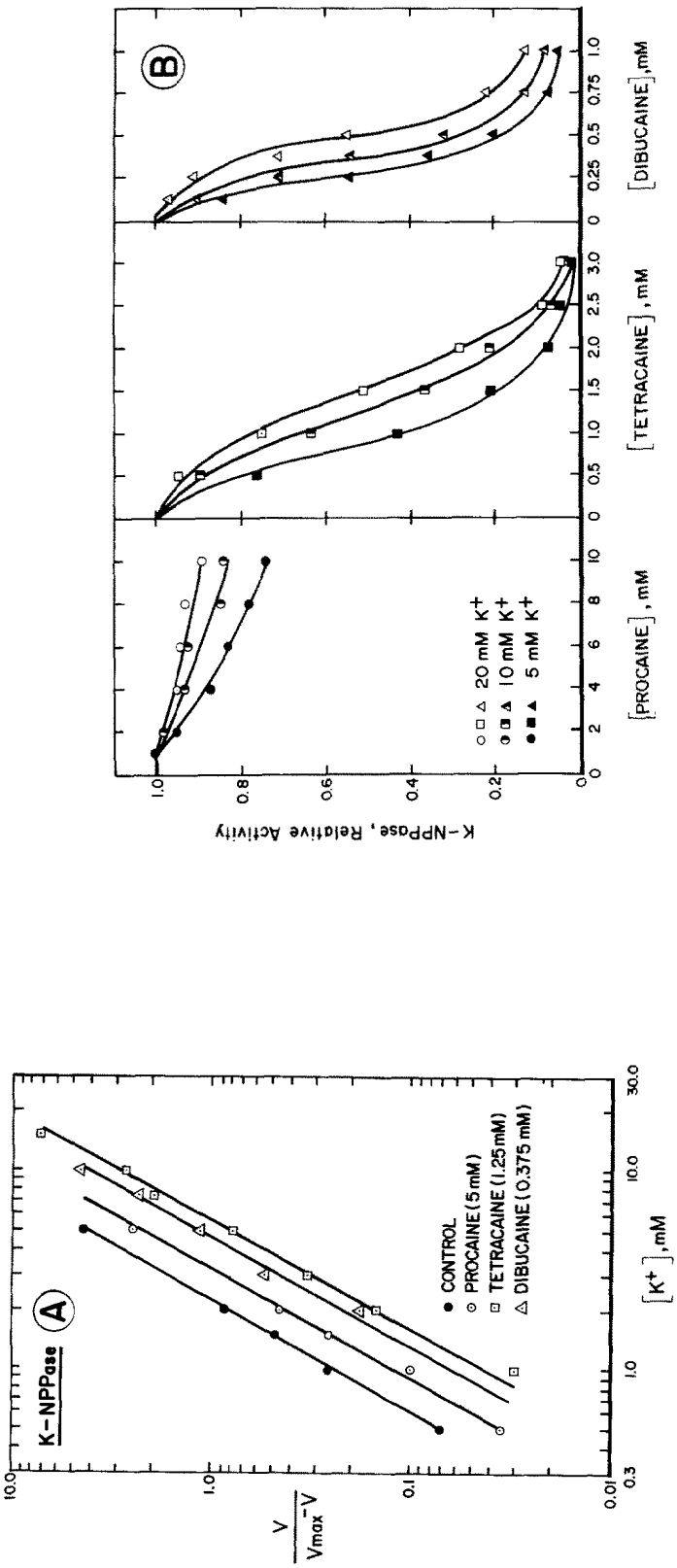

Fig. 5. (A) Hill plot of K^+ activation of K^+-NPPase activity in the absence of local (—●—) and in the presence of 5 mM procaine (—○—), 1.25 mM tetracaine (—□—) or 0.375 mM dibucaine (—△—). Each point represents the average of three to six experiments. (B) K^+-NPPase activity as a function of local anesthetic concentration relative to control activity in the absence of drug. Open symbols, 20 mM K^+; half-open symbols, 10 mM K^+; and closed symbols, 5 mM K^+. Each point represents the average of three to six experiments.

agents with lower lipid solubility (e.g. procaine) are noncompetitive inhibitors of Na^+ activation and competitive inhibitors of K^+ activation (Figs. 3 and 4). Tetracaine, an agent with intermediate lipid solubility, appears to be a competitive inhibitor with respect to both Na^+ and K^+ activation of Na^+, K^+-ATPase.

Influence of local anesthetics on K^+*-NPPase activity*. Tetracaine was more effective as an inhibitor of K^+-NPPase than Na^+, K^+-ATPase activity (Table 1). When fixed concentrations of local anesthetic were present during Na^+ activation of Na^+, K^+-ATPase, tetracaine was a more effective Na^+ competitor than dibucaine (Fig. 2). Thus, tetracaine might be expected to be a more effective inhibitor of K^+-NPPase activity as compared to Na^+, K^+-ATPase activity since the former activity is assayed in the absence of Na^+.

All three local anesthetics, including dibucaine, were competitive inhibitors of K^+-NPPase activity with respect to activation by K^+ (Fig. 5, A and B). The apparent K_m for K^+ at its activation site on K^+-NPPase was 2.2 mM. This value is greater than the apparent K_m for K^+ at its activation site on Na^+, K^+-ATPase (0.85 mM). This difference is consistent with the observation that K^+ activates K^+-NPPase at a moderate-affinity site [11, 12]. Dibucaine, which did not compete with K^+ at its high-affinity activation site on Na^+, K^+-ATPase (Fig. 4, A and B), does compete with K^+ at its moderate-affinity site on K^+-NPPase.

Hill plots of inhibition of K^+-NPPase activity as a function of the concentration of dibucaine and tetracaine yielded essentially parallel curves with Hill coefficients greater than 1 (Table 1). Thus, it appears that more than one molecule of dibucaine or tetracaine interacts with K^+-NPPase to produce inhibition.

Influence of local anesthetics on Na^+*-ATPase activity*. Tetracaine was significantly more potent than dibucaine as an inhibitor of Na^+-ATPase activity (Table 1, Fig. 2). This result was the only exception we found to the generalization that potency of local anesthetics as inhibitors of enzyme was correlated with lipid solubility. It is also surprising that Hill plots of Na^+-ATPase activity as a function of local anesthetic concentration showed that the Hill coefficient for dibucaine was different from that obtained for tetracaine and procaine. These values were 3.0 for dibucaine and 1.1 for both tetracaine and procaine. These results suggest that the interaction between dibucaine and the enzyme is significantly different than the interaction with the more hydrophilic agents, procaine and tetracaine.

Na^+-ATPase activity was far more sensitive to inhibition by procaine than were either Na^+, K^+-ATPase or K^+-NPPase activities. It appears likely that monovalent cation interactions, especially with K^+, may modify the sensitivity of Na^+-ATPase activity to inhibition by both procaine and tetracaine. These agents were particularly effective as competitive inhibitors of K^+ activation of Na^+, K^+-ATPase; thus, the absence of K^+ in these experiments may explain the enhanced sensitivity of Na^+-ATPase activity to inhibition.

Increasing the Na^+concentration increased $K_{0.5}$ values for local anesthetics, suggesting a competitive interaction between Na^+ and these agents for inhibition of Na^+-ATPase activity (Table 2).

Na^+ activates Na^+-ATPase at two distinct sites, one with high affinity and one with low affinity [10, 13]. At 20 mM Na^+, the high affinity sites are saturated, and at higher Na^+ concentrations the occupancy of low affinity sites causes additional activation. In Table 2, the increase in $K_{0.5}$ values for local anesthetics at 180 mM Na^+ as compared to 60 mM Na^+ is due to an effect of Na^+ at low affinity sites. At 60 mM Na^+, activity was approximately 80% of maximum. It has been suggested that Na^+ might have a "K^+-like" effect at the low affinity site to facilitate enzyme turnover [10, 14, 15]. Since all three local anesthetics were also competitive inhibitors of K^+ activation of K^+-NPPase at the moderate affinity site (Fig. 5A), this similarity suggests that the moderate affinity K^+ site may be the low affinity Na^+ site on Na^+-ATPase.

Dibucaine, the most lipid soluble agent included in this study, was nearly equipotent as an inhibitor of all three activities (Table 1). This observation suggests that dibucaine may interact nonspecifically with membrane lipids to alter enzyme activity. Present results are consistent with those of Giraud *et al.* [16]. These workers reported that, in erythrocytes depleted of cholesterol or exposed to an amphiphile (i.e. chlorpromazine, imipramine or benzyl alcohol), membrane fluidity increased and the apparent affinity of the Na^+, K^+-transport system for Na^+ remained unchanged by these manipulations.

On the other hand, less lipid soluble agents, like tetracaine and procaine, may interact more specifically with the enzyme, i.e. with monovalent cation binding sites or with one of the two conformations (E_1 or E_2) which the enzyme can assume [17]. The three agents studied are weak bases with pK_a values in the range of 8.2 to 8.9 [18]; thus, they are predominately ionized at pH 7.4. Local anesthetics less lipid soluble than dibucaine, such as tetracaine and procaine, may remain in the aqueous phase where they interact with negatively charged regions of the enzyme which serve as cation binding sites. Present results are consistent with the possibility that tetracaine and procaine may interact with unoccupied K^+ sites on the enzyme to produce inhibition of activity. Alternatively, the presence of K^+ in the medium may convert the enzyme to the E_2 conformation [17] which may be less susceptible than the E_1 conformation to inhibition by these local anesthetics. It is of interest to note that similar conclusions were reached by Ahmed and Thomas [19] on the basis of studies which examined the influence of long chain fatty acids on the activation of Na^+, K^+-ATPase and K^+-NPPase by monovalent cations.

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