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BRAIN (Na^+,K^+)-ATPase: BIPHASIC INTERACTION WITH ERYTHROSIN B

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Abstract—Brain (Na^+,K^+)-adenosine triphosphatase (EC 3.6.1.3) has both high and low affinity ouabain binding sites. It has been proposed that the high affinity ouabain binding sites characterize a nerve-specific form of the enzyme. Erythrosin B has been reported to inhibit high affinity ouabain binding selectively. The experiments in this paper were carried out in order to characterize the interactions of erythrosin B with (Na^+,K^+)-ATPase and to examine the specificity of erythrosin B for enzyme with high affinity for ouabain. Inhibition by erythrosin B was biphasic, with a rapid and a slow phase. The rapid phase appeared to be relatively specific for enzyme with high affinity for ouabain, while the slow phase was not. Inhibition by erythrosin B was accelerated by Mg^{2+} and was retarded by ATP, K^+, or Na^+ and ATP. Erythrosin B increased apparent affinity of the enzyme for K^+ and decreased apparent affinity for Na^+ and for ATP. These results indicate that erythrosin B interacts with an ATP site and has effects on cation affinities opposite to those of ATP. Erythrosin B inhibition is proportional to high affinity ouabain binding if brief incubation times and moderate concentrations are used.

(Na^+,K^+)-ATPase, the enzymatic basis of coupled Na^+ and K^+ transport [1, 2], has important but partially distinct roles in excitable and non-excitable cells [2, 3]. This fact suggests the possibility that there is a nerve-specific form of enzyme. The existence of a nerve-specific form is suggested by the presence of two affinities for ouabain binding in brain tissue [4, 5] and the isolation of catalytic subunits having two different molecular weights from brine shrimp [6] and from several mammalian brain and nerve preparations [5]. The putative nerve-specific enzyme has higher molecular weight and higher affinity for cardiac glycosides than does the other form [5]. The cardiac glycoside site may be a receptor for an endogenous inhibitor of (Na^+,K^+)-ATPase [7–9], in which case the inhibitor may interact differentially with nerve and non-nerve enzyme.

A biochemical probe for the two forms of enzyme would be valuable for: (1) characterizing selective pharmacologic or physiologic effects, and (2) comparing nerve and non-nerve enzyme with respect to regulation by cations, ATP, and other ligands. Silbergeld had recently presented evidence that erythrosin B, which inhibits catecholamine uptake and has been implicated in hyperactive behavior [10], can selectively inhibit the high affinity binding of ouabain to (Na^+,K^+)-ATPase [11]. Erythrosin B could thus be a probe for the nerve-specific enzyme. The mechanism of inhibition by erythrosin B is unclear, since displacement of ouabain binding can occur either through direct interactions with the ouabain binding site or through inhibition of the ouabain-binding conformation of enzyme [12]. For this and the above reasons, we have examined the effects of erythrosin B on (Na^+,K^+)-ATPase regulation with respect to interactions with its ligands, enzyme conformation, and conditions for selectivity for enzyme with high or low affinity for ouabain.

MATERIALS AND METHODS

Enzyme source. We used crude microsomes from rat brain, prepared as in our earlier work [13], as an enzyme source. Brain microsomes have a mixture of high and low affinity sites for ouabain, and the difference in affinity is particularly large in rat brain [5].

K^+-p-*Nitrophenylphosphatase assays.* Regulation of K^+-*p*-nitrophenylphosphatase activity by cations, substrate, and ATP demonstrates the major phases of (Na^+,K^+)-ATPase regulation [1, 2, 13–16]. *p*-Nitrophenylphosphatase activity associated with (Na^+,K^+)-ATPase was assayed by the K^+-dependent production of *p*-nitrophenol. Reaction mixtures contained, in a total volume of 60 μl, about 10 μg protein, 5 mM $MgCl_2$, 10 mM *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO), and other ligands as described below. Incubation was started by addition of *p*-nitrophenylphosphate and was terminated 10 min later with 0.6 ml of 0.1 N NaOH; *p*-nitrophenol was determined from its absorption at 410 nm. Activity due to (Na^+,K^+)-ATPase was taken to be the difference between activity with and without added KCl.

Effects of erythrosin B on ligand affinities were examined using 60 min of preincubation with erythrosin B (Sigma Chemical Co.). Affinity for the moderate affinity K^+ site was determined by measuring activity as a function of KCl concentration (0.1 to 50 mM). Affinity for the high affinity K^+ site was measured using activity as a function of KCl concentration (0.01 to 5 mM) in the presence of 25 mM NaCl and 0.1 mM Tris ATP [13, 15] (Sigma "vanadium-free" grade ATP). Affinity for the Na^+ regulatory site that produces K^+ sensitive enzyme was measured using NaCl concentrations from 0.02 to 50 mM in the presence of 0.1 mM Tris ATP and

0.5 mM KCl [13, 16]. ATP affinity was measured by inhibition of activity by 15 μM to 2 mM Tris ATP in the presence of 2 mM KCl [13]. Effects of ligands on erythrosin B affinity were measured after 60 min of preincubation with erythrosin B at 10^{-9} to 10^{-5} M in the presence of other substances as described in Results. Apparent affinities were estimated using least squares curve-fitting to a form of the Hill equation [13].

Ouabain inhibition curves. Biphasic inhibition of K^+-*p*-nitrophenylphosphatase as a function of ouabain concentration was measured using modifications of our previous method [17]. Tissue was preincubated at 37° for 60 min with 2 mM $MgCl_2$, 2 mM inorganic phosphate (adjusted to pH 7.5 with Tris), 50 mM imidazole HCl, pH 7.5, and ouabain at a series of concentrations from 10^{-9} to 10^{-3} M. Tris *p*-nitrophenylphosphate, $MgCl_2$, and imidazole HCl, pH 7.5, were then added to give final concentrations of 5 mM $MgCl_2$, 10 mM Tris *p*-nitrophenylphosphate, 50 mM imidazole HCl, 0.67 mM Tris P_i, and 0 or 25 mM KCl. The resulting inhibition curves were fitted to an equation describing low and high affinity inhibition by the same ligand, as described previously; in rat brain tissue, this method produces a clear delineation between high and low affinity inhibition by ouabain [17].

Erythrosin B inhibition. Rate of erythrosin B inhibition was estimated by preincubation for various periods at 25° under the following conditions: (1) Tris–HCl buffer only, (2) Tris + 3 mM $MgCl_2$, (3) ligands in (2) + 3 mM inorganic phosphate neutralized with Tris, (4) ligands in (2) + 50 μM ATP, (5) ligands in (2) + 20 mM KCl, and (6) ligands in (4) + 25 mM NaCl. This made possible the examination of effects of ATP, phosphorylation, and enzyme conformation (ATP form vs K^+ form) on erythrosin B inhibition [13–16, 18]. The erythrosin B concentration was 4×10^{-7} M; after preincubation, K^+-*p*-nitrophenylphosphatase activity was measured as described under ouabain inhibition. In other experiments, erythrosin B was added after the addition of *p*-nitrophenylphosphate.

Ouabain binding. Ouabain binding was measured using 10 μg protein in a total volume of 150 μl containing 50 mM imidazole HCl, pH 7.5, 100 mM NaCl, and 10^{-7} M [^{3}H]ouabain (New England Nuclear Corp., Boston, MA; sp. act. 10 Ci/mmole). MgATP was present at concentrations ranging from 0 to 10 mM. Tissue was preincubated with buffer or erythrosin B (4×10^{-7} M) for 60 min before addition of ouabain and MgATP. After 60 min, 2 ml of cold imidazole, 50 mM at pH 7.5, was added, the samples were filtered on Millipore type HA filters (pore size 0.45 μm), and the filters were washed three times with buffer and their ^{3}H was determined by liquid scintillation counting.

In preliminary experiments, the time course of erythrosin B inhibition of ouabain binding was measured as above using 2 mM MgATP and added erythrosin B at a series of times before and after addition of ouabain.

Assay of (Na^+,K^+)-ATPase. Activity of (Na^+,K^+)-ATPase as a function of MgATP was measured using about 8 μg protein in 120 μl containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM KCl, and a series of MgATP concentrations ranging from 0 to 10 mM. At each concentration, the difference between matched triplicates containing NaCl and KCl or 1 mM ouabain was taken as (Na^+,K^+)-ATPase activity. After 10 min, reactions were stopped with 400 μl of 8.3% trichloroacetic acid. The tubes were centrifuged, and inorganic phosphate in the supernatant fraction was determined, using the method of Bonting [19].

RESULTS

Rate of inhibition by erythrosin B. Under a wide range of conditions, there appeared to be an initial rapid phase of inhibition followed by a slower phase. The data summarized in Table 1 describe the inhibition of K^+-*p*-nitrophenylphosphatase activity by erythrosin B after 0 or 60 min of preincubation. Less than half of the enzyme was inhibited by erythrosin B added immediately before the addition of substrate, but all of the enzyme was inhibited after 60 min of preincubation. Both the amount of initial inhibition and the rate of inhibition also varied according to incubation conditions. Inhibition was accelerated by Mg^{2+} and retarded by ATP, K^+, or ATP + Na^+, as summarized in Table 2. Incubation with Tris–HCl only or with ATP resulted in relatively large initial inhibition relative to the rate of subsequent inhibition.

The rate of inhibition by erythrosin B added during the course of the *p*-nitrophenylphosphate reaction was determined next. There was essentially no inhibition by erythrosin B added after *p*-nitrophenylphosphate. This may have been a consequence of the presence of K^+ in the reaction medium.

Further experiments examined the reversibility of inhibition by erythrosin B. Microsomes were preincubated for 60 min with 4×10^{-7} M erythrosin B, centrifuged, and resuspended in reaction medium without erythrosin B. As shown in Table 3, inhibition was only slowly reversible.

Apparent affinity for erythrosin B. After 60 min of preincubation, inhibition of K^+-*p*-nitrophenylphosphatase activity by erythrosin B was nearly complete under most conditions. As summarized in Table 4, the apparent $[EB]_{0.5}$ ranged from about 10^{-6} M to 10^{-7} M depending on the incubating conditions. ATP, ATP + Na^+, and K^+ reduced the apparent affinity without affecting the maximum extent of inhibition.

Inhibition of enzyme activity by erythrosin B added immediately before the reaction started was

Table 1. Inhibition of K^+-*p*-nitrophenylphosphatase activity by erythrosin B

Preincubation*	Maximum inhibition (% total activity)	$[EB]_{0.5}$†
0	43	1.28×10^{-6} M
60	100	1.99×10^{-7} M

* Duration of preincubation with erythrosin B at 25° before addition of *p*-nitrophenylphosphate and KCl.

† Concentration of erythrosin B required for half-maximum inhibition.

Table 2. Biphasic inhibition of K^+-*p*-nitrophenylphosphatase by erythrosin B

Preincubation additions*	Initial inhibition, 4×10^{-7} M erythrosin B†	Rate constant, slow inhibition‡
Tris–HCl only	0.110	0.049 ± 0.005§
Mg^{2+}, 3 mM	0.130	0.138 ± 0.018‖
Mg^{2+} + P_i, 3 mM	0.125	0.103 ± 0.012‖
Mg^{2+} + ATP, 50 μM	0.088	0.036 ± 0.006§
Mg^{2+} + K^+, 20 mM	0.025	0.024 ± 0.003§‖
Mg^{2+} + ATP + Na^+, 25 mM	0.100	0.024 ± 0.003§‖

* Crude microsomes were incubated with the additions shown in 50 mM Tris–HCl, pH 7.5, for 60 min at 25°.

† Initial inhibition is the fraction of activity inhibited by erythrosin B, 4×10^{-7} M, added at the same time that the reaction was started.

‡ The rate constant for the slow phase of inhibition was obtained by a plot of

$$(a - b)^{-1} \ln \frac{b(a - x)}{a(b - x)},$$

where a = erythrosin B = 4×10^{-7} M, b = enzyme (calculated by ouabain receptor binding) = 2×10^{-8} M, and x = enzyme inhibited (assuming 1:1 stoichiometry with erythrosin B), as a function of time. The initial inhibition was subtracted before calculating the rate constants for slow inhibition. The rate constant is given in units of $10^4\ M^{-1} \cdot sec^{-1}$, ± the standard error of the estimate (six degrees of freedom).

§ Different from Mg^{2+}, $P < 0.01$.

‖ Different from Tris–HCl only, $P < 0.05$.

Table 3. Reversibility of inhibition by erythrosin B*

Number of washes	0	3	6	10
Activity remaining (control = 1.000)	0.015	0.014	0.129	0.599

* Microsomes were preincubated with erythrosin B or Tris–HCl only (control) for 60 min, diluted with buffer, centrifuged and resuspended in buffer; K^+-*p*-nitrophenylphosphatase activity was measured after 20 min of preincubation with buffer not containing erythrosin B.

also concentration dependent, but maximum inhibition was only about 43% of total enzyme activity. Half-maximum inhibition was obtained in the presence of 1.28×10^{-6} M erythrosin B (Table 1).

Effect of erythrosin B on ligand affinities of K^+-p-*nitrophenylphosphatase.* We have previously described moderate and high affinity sites for K^+ [13, 15]. This high affinity site is only accessible when the enzyme is in the conformation that has phosphatase activity [13, 15, 18, 20]. ATP and K^+ have antagonistic effects on the transition to this form when it is mediated by K^+ at its moderate affinity site [13, 16, 18]. Figure 1 shows that erythrosin B at 1×10^{-7} M increased apparent K^+ affinity 2-fold at the moderate affinity site. Figure 2 shows that the same concentration of erythrosin B decreased apparent affinity for ATP. Apparent affinity for Na^+ in the presence of ATP was decreased (apparent K_m = 2.1 mM without and 3.4 with erythrosin B) while affinity of the high affinity K^+ site was not affected.

Table 4. Apparent affinities for inhibition of K^+-*p*-nitrophenylphosphatase by erythrosin B*

Preincubation	$[EB]_{0.5}$
Tris–HCl only	$2.19 \pm 0.13 \times 10^{-7}$ M
Mg^{2+}, 3 mM	1.23 ± 0.15†
Mg^{2+} + P_i, 3 mM	1.54 ± 0.17†
Mg^{2+} + ATP, 50 μM	3.89 ± 0.21‡
Mg^{2+} + K^+, 20 mM	5.32 ± 0.44‡
Mg^{2+} + ATP + Na^+, 25 mM	9.45 ± 0.54§

* Microsomes were incubated for 60 min with the ligands shown and a series of erythrosin B concentrations. $[EB]_{0.5}$ is given ± the standard error of the estimate (seven degrees of freedom). In all cases, there was complete inhibition at infinite erythrosin B.

† Different from Tris–HCl only, $P < 0.05$.

‡ Different from Tris–HCl only, $P < 0.01$.

§ Different from Tris–HCl only, $P < 0.001$.

The effects of erythrosin B on MgATP dependence of ouabain binding, (Na^+,K^+)-ATPase activity, and inhibition of K^+-*p*-nitrophenylphosphatase activity are summarized in Table 5. Each of these measurements has certain advantages as probes for ATP interactions: K^+-*p*-nitrophenylphosphatase is sensitive, initial rates can readily be measured, and inhibition by ATP is largely a function of the high affinity ATP site if the K^+ concentration is relatively low [13]. Further, ATP is not hydrolyzed. Ouabain binding has the advantages of being selective for the high affinity ATP site [12] and of being measurable in the absence of K^+. ATPase activation represents a mixture of high and low affinity site effects and, thus, has lower apparent affinity for ATP. As shown in Table 5, apparent affinity for ATP was consistently reduced by erythrosin B.

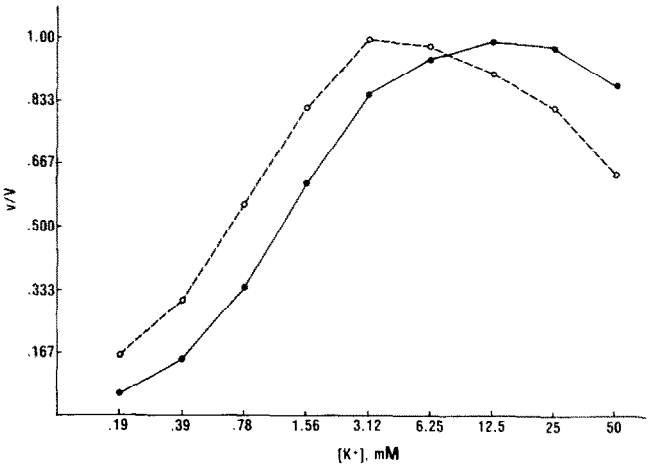

Fig. 1. Effect of erythrosin B on activation of K^+-*p*-nitrophenylphosphatase by K^+. The x-axis shows the concentration of KCl in mM; the y-axis, K^+-*p*-nitrophenylphosphatase activity as a fraction of maximum activity. Solid line: control; dashed line: 60 min of preincubation with 4×10^{-7} M erythrosin B. Erythrosin B decreased $[K^+]_{0.5}$ from 1.3 mM to 0.7 mM.

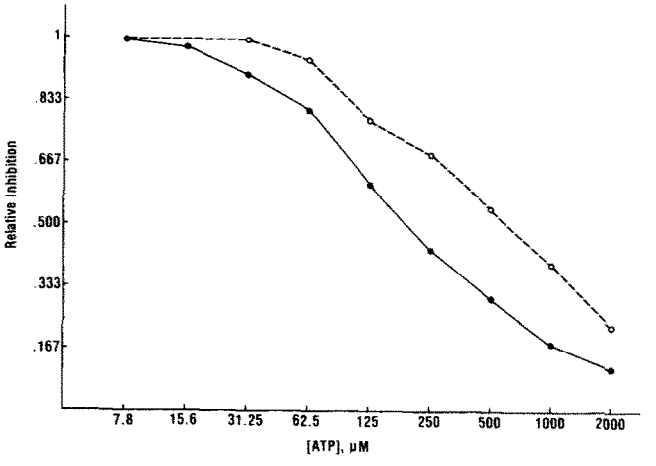

Fig. 2. Effect of erythrosin B on inhibition of K^+-*p*-nitrophenylphosphatase by ATP. The x-axis shows ATP concentration in μM; the y-axis, K^+-*p*-nitrophenylphosphatase activity as a fraction of that in the absence of ATP; KCl = 2 mM. Solid line = control; dahsed line = 60 min of preincubation with 4×10^{-7} M erythrosin B. Erythrosin B increased $[ATP]_{0.5}$ from 180 μM to 520 μM (uncorrected for *p*-nitrophenylphosphate).

Table 5. Effects of erythrosin B on interactions of (Na^+,K^+)-ATPase with ATP*

Activity	Preparation	Maximum activity	$[ATP]_{0.5}$, mM
Ouabain binding	Control	42 pmoles/mg	0.052
	Erythrosin B	32	0.133
(Na^+, K^+)-ATPase (activation)	Control	0.200 μmole/(mg protein · min)	0.490
	Erythrosin B	0.213	0.855
K^+-*p*-Nitrophenylphosphatase (inhibition)	Control		0.180 (0.050)
	Erythrosin B		0.520 (0.146)

* Ouabain binding, (Na^+,K^+)-ATPase activity, and K^+-*p*-nitrophenylphosphatase activity were determined as functions of MgATP concentration as described in Materials and Methods. Microsomes were preincubated for 60 min with 50 mM Tris–HCl, pH 7.5, with or without 4×10^{-7} M erythrosin B. The values in parentheses for inhibition of K^+-*p*-nitrophenylphosphatase were corrected for the presence of 10 mM *p*-nitrophenylphosphate [13].

Table 6. Relative effects of erythrosin B on high and low affinity inhibition of K^+-*p*-nitrophenylphosphatase by ouabain*

Erythrosin B concn (M)	Preincubation (min)	Percent inhibited by erythrosin B	
		High affinity	Low affinity
4×10^{-7}	0	30	0
8×10^{-7}	0	40	0
2×10^{-6}	0	60	5
8×10^{-6}	0	90	10
8×10^{-8}	60	80	18
4×10^{-7}	60	95	50

* Brain microsomes were preincubated with oubain and inorganic phosphate, as described in the text, for 1 hr before *p*-nitrophenylphosphatase activity was measured. Control experiments were compared to experiments with erythrosin B added at the concentrations and time before initiation of the reaction shown in the table. For each set of conditions, ouabain inhibition curves with and without erythrosin B were compared. Amounts of enzyme with high and low affinity were calculated as described in Ref. 17, and the reduction in each by erythrosin B is shown in the table. Erythrosin B did not affect apparent affinity for ouabain.

Selectivity of erythrosin B for enzyme with high affinity for ouabain. Ouabain inhibition curves for K^+-*p*-nitrophenylphosphatase have high and low affinity components [17]. These components may represent the populations of enzyme molecules having high and low affinity for cardiac glycosides [4, 5, 17]. To determine the selectivity of erythrosin B for enzyme with high affinity, the effect of preincubation with erythrosin B on ouabain inhibition curves was examined. For example, selective inhibition of the enzyme with high affinity for ouabain would result in a curve with only low affinity inhibition and with total activity (in the absence of ouabain) equal to the low affinity component of a curve obtained without erythrosin B. The results are summarized in Table 6. The concentrations of ouabain causing half-maximum inhibition were not affected by erythrosin B. The partial inhibition occurring immediately after addition of erythrosin B was limited almost entirely to high affinity enzyme. After preincubation, progressively more low affinity enzyme was also inhibited.

DISCUSSION

The purposes of the experiments in this paper were: (1) to examine the selectivity of inhibition by erythrosin B with respect to ouabain affinity in mixtures of high and low affinity enzyme, and (2) to examine the mechanism of inhibition by erythrosin B. The results show: (1) that erythrosin B inhibited both forms of enzyme, but with different time courses, and (2) that inhibition appeared to involve interaction with an ATP site (either directly or through enzyme conformation) and prevention of ATP effects.

(Na^+,K^+)-ATPase appears to exist in two forms with different moleculear weights [5, 6] and cardiac glycoside affinities [4, 5]; one form may be nerve-specific [5]. The two forms can only be resolved in mixtures by gel electrophoresis [5, 6], which inactivates the enzyme. Either form can be purified [5], but this does not afford quantitative measurement of their activities in mixtures such as brain tissue preparations. Further, the only known preparation of pure high affinity enzyme is very time consuming and results in a very small yield that does not reflect the amount of high affinity enzyme originally present [5].

One method for resolving the two forms of enzyme quantitatively in active mixtures is through analysis of the biphasic inhibition of brain enzyme by cardiac glycosides [4, 5, 17]. This method has the disadvantages of being relatively cumbersome and feasible only in tissue from species having a large difference in ouabain affinity between the two forms.

Silbergeld [11] recently reported that erythrosin B inhibited high affinity ouabain binding to, and K^+ uptake by, brain (Na^+,K^+)-ATPase without affecting low affinity binding or red blood cell K^+ uptake. These findings suggested that erythrosin B might selectively inhibit the form of enzyme with high affinity for cardiac glycosides, providing (1) a probe for the relative activities of the two forms *in situ* without the disadvantages of inhibition curves, and (2) a means for comparing properties of the two putative forms of the enzyme.

Biphasic time course of erythrosin B inhibition. The data reported here indicate that inhibition of the K^+-*p*-nitrophenylphosphatase associated with (Na^+,K^+)-ATPase by erythrosin B has two phases. The initial rapid phase of inhibition was limited, in these experiments, to about 43% of total enzyme activity and was selective for enzyme with high affinity for ouabain. The slower phase of inhibition was not selective; by 60 min, enzyme activity was totally inhibited by erythrosin B at concentrations greater than about 10^{-6} M.

Erythrosin B interactions with ligand binding sites. Inhibition by erythrosin B was accelerated by Mg^{2+} and was retarded by ATP, K^+, or ATP and Na^+. It is therefore likely that erythrosin B does not interact directly with the cardiac glycoside site, since that type of interaction would be facilitated by phosphorylating conditions [1, 2, 12, 18]. The reduction in inhibition in the presence of either K^+ or ATP indicates that inhibition by erythrosin B probably requires interaction with a substrate site [10], as does inhibition by structurally similar fluorescein [18]. This hypothesis is supported by the following: (1) the apparent K_i for competition of ATP with erythrosin B, corrected for *p*-nitrophenylphosphate

concentration [10], is about 20 μM, similar to the K_i we have obtained for high affinity ATP inhibition of K^+-*p*-nitrophenylphosphatase [10]; (2) K^+, which antagonizes ATP binding [1, 13, 16, 18, 20], strongly antagonizes inhibition by erythrosin B; and (3) phosphorylating conditions with Na^+ and ATP, which facilitate ouabain binding [12], antagonize erythrosin B inhibition.

Effect of erythrosin B on ligand affinities. The effects of erythrosin B on K^+ and ATP affinities, shown in Figs. 1 and 2 and Table 5, also support interaction with an ATP site, since apparent affinity for ATP was markedly reduced. Erythrosin B increased apparent affinity for K^+ (Fig. 1) and decreased that for Na^+, opposite to the effect of ATP on affinities for these cations [13, 16].

Differential interactions with high affinity ouabain inhibition. One interpretation for the data in Tables 1 and 2 is that there are two distinct forms of (Na^+,K^+)-ATPase and that erythrosin B inhibits them at different rates. An alternative explanation for these data is that high and low ouabain affinity represent different conformations of enzyme; physical differences between the conformations would account for their different rates of migration on polyacrylamide gels [5]. Erythrosin B might bind first to the conformation of enzyme with high affinity for ouabain and convert this to the low affinity form; higher concentrations would saturate the low affinity form as well. This explanation appears unlikely for the following reasons: (1) Sweadner described enzyme sources with only high or low affinity ouabain sites that were not interconvertible [5], (2) the initial erythrosin B binding had lower affinity than the slower binding, (3) under no conditions does erythrosin B appear to increase the absolute amount of enzyme with low affinity for ouabain, and (4) erythrosin B had no effect on apparent affinity for ouabain.

General conclusions. According to the data presented in this paper, erythrosin B can be used, with significant limitations, as a probe for high affinity ouabain inhibition. Low concentrations or short incubation times favor selective inhibition of high affinity ouabain binding but may lead to underestimates of total high affinity binding. Higher concentrations of erythrosin B or longer incubations will overestimate the high affinity binding and eventually lead to complete inhibition of enzyme activity. If the enzyme with high affinity for ouabain represents a distinct nerve-specific form of enzyme, the effects of erythrosin B indicate that the two forms of enzyme may differ in substrate binding properties as well as in cardiac glycoside affinity.

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