Guanidinium Derivatives Act as High Affinity Antagonists of Na⁺ Ions in Occlusion Sites of Na⁺,K⁺-ATPase*

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We have screened various alkyl- and arylguanidinium derivatives as possible competitors of Na⁺ or Rb⁺ for the cation sites on renal Na⁺,K⁺-ATPase. Alkylmonoguanidinium or alkylbisguanidinium (BisG) compounds (chain lengths of C₂ to C₆) competitively inhibit the occlusion of Rb⁺ and Na⁺ with an order of affinities C₆ > C₅ > C₄ > C₃ > C₂. BisG compounds are approximately twice as effective as the equivalent alkylmonoguanidinium compounds. In media of high ionic strength, affinities of tens of micromolar are observed, e.g., 26 μM for BisG 8-m-(mXBG)- and p-xylenebisguanidinium were synthesized and were found to compete with Rb⁺ or Na⁺ with intrinsic affinities of 7.7 and 8.2 μM, respectively. The hydrophobicity rather than the degree of proximity of the guanidinium groups in all BisG compounds appears to determine the binding affinity. A systematic search has been made of conditions in occlusion assays for which the inhibitor affinities are highest. When the pH is raised from 7.0 to 8.5, a 5-fold increase in affinity is observed, suggesting that the guanidinium derivatives compete with protons at sites of pKₐ ≈ 7.5. Replacing Tris-HCl with choline chloride-containing media raised apparent affinities ~2-fold.

All guanidinium derivatives stabilize the E₁ conformation of fluorescein-labeled Na⁺,K⁺-ATPase, acting as competitive Na⁺ analogues. In media containing only 1 mM Tris-HCl, pH 8.55, very high affinities were observed for binding to the fluorescein-labeled enzyme (e.g., 0.08 μM for mXBG). In very low ionic strength medium, the inhibition was still competitive with Rb⁺ ions. However, there was also evidence for nonspecific adsorption to the membranes.

The following findings show that mXBG, a typical guanidinium derivative, behaves as a Na⁺-like antagonist. (a) It inhibits Na⁺,K⁺-ATPase activity, competing strongly with Na⁺ but only weakly with K⁺ ions. (b) It inhibits phosphorylation from ATP, competing with Na⁺ ions. (c) Like Na⁺ ions, it blocks phosphorylation from inorganic phosphate. Based on these results, we propose that the guanidinium group binds to a relatively wide vestibule at the cytoplasmic surface; but, unlike Na⁺ or K⁺ ions, it cannot pass into a narrower region of the cation transport path within the membrane. Therefore, it blocks the occlusion and active transport of cations.

In the future, high affinity guanidinium derivatives may serve the purpose of locating cation-binding domains of the pump protein after being converted to reactive affinity or photoaffinity covalent labels.

The Na⁺,K⁺-ATPase has been purified, cloned, and sequenced (Ovchinnikov, 1987; Jørgensen and Andersen, 1988). Much is known about different transport modes, cation binding and “occlusion,” and accompanying conformational transitions (Glynn, 1985; Jørgensen and Andersen, 1988; Glynn and Karlish, 1990). By contrast, much less is known about the structure and location of the cation-binding sites. Chemical modification by carboxyl reagents or reactive cation analogues combined with selective proteolytic digestion, site-directed mutagenesis, observations on charge transfer properties or voltage sensitivity of pumps, and also the use of cation analogues as probes in fluorescence or NMR studies are providing some insight for the different cation pumps (see Glynn and Karlish (1990) for a recent review).

Recent work from this laboratory describes the use of tryptic digestion and labeling with the carboxyl reagent di-cyclohexylicarbodiimide to investigate the nature of cation sites in renal Na⁺,K⁺-ATPase (Shani-Sekler et al., 1988; Karlish et al., 1990, 1991). This paper represents a first step in the development of the complementary approach, namely to search for a suitable organic cation analogue. If found, such a molecule could serve as a precursor for synthesis of probes for photochemical or chemical labeling in order to pinpoint the cation-binding site or its close surroundings. We have proposed that within cation occlusion sites, the ligating groups include 2 carboxyl residues that are associated with the binding of two K⁺ or Na⁺ ions, whereas an additional “neutral” site binds the third Na⁺ (Goldshleger et al., 1987; Shani et al., 1987; Shani-Sekler et al., 1988). The notion that the cation sites contain negatively charged residues has led to the screening of positively charged organic compounds as possible high affinity competitors of Na⁺ and/or K⁺.

Previous studies have examined competitive effects of different alkyl- or arylamines on active K⁺ transport in red cells (Kropp and Sachs, 1977; Ellory et al., 1979), Na⁺-dependent phosphorylation of Na⁺,K⁺-ATPase (Schuurmans Stekhoven et al., 1988, 1989; Takada et al., 1990; Fukushima, 1987), or the rate of release of occluded ⁸⁸Sr (Forbush, 1988). Where tested systematically, the order of potency of inhibition has been found to be quaternary > tertiary > secondary > primary amines. Within each group, the more hydrophobic the compounds, the more effective competitors they are up to a limited size (Ellory et al., 1979; Forbush, 1988). Recently, it has been shown that primary amines are superior to monoamines in blocking Na⁺-dependent phosphorylation and for dianimes with chain lengths of C₂ to C₆, effectiveness was best at C₆ (ethylenediamine), which was 180-fold better than C₆ (1,6-

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Guanidinium Is a Na⁺-like Antagonist on Na⁺,K⁺-ATPase

Na⁺,K⁺-ATPase was prepared from fresh pig kidney red outer medulla as described by Jørgensen (1974a). Before use, the enzyme was dialyzed at 4°C for 16 h against 1000 volumes of a solution containing 25 mM histidine, pH 7.0. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

EXPERIMENTAL PROCEDURES

Na⁺,K⁺-ATPase Activity—This was determined as described by Jørgensen (1974b). Where NaCl and KCl concentrations in the medium were changed, e.g. Fig. 7, the total salt concentration was maintained at 150 mM with Tris-HCl. The specific activity of ATP hydrolysis was measured as described by Jørgensen (1974a). The enzyme was incubated for 3 min at room temperature in 50 µl of a medium containing choline chloride and various NaCl concentrations up to a total of 100 µM, 25 mM Tris-HCl, pH 8.35, 1 mM MgCl₂ in the presence and absence of 1 mM mXBG. From ATP, 75 µg of enzyme was incubated for 5 s at room temperature in 125 µl of a medium containing choline chloride and various NaCl concentrations up to a total of 100 µM, 25 mM Tris-HCl, pH 8.35, 1 mM MgCl₂, and 10 µM ATP + 17 nM BAPTA without or with 1 mM mXBG.

RESULTS

The structures of the guanidinium derivatives used in this study are shown in Fig. 1. Two groups were used, consisting of an alkyl chain of variable length with a single terminal guanidinium group in a monovalent cation-binding site with a much higher affinity. 

1 The abbreviations used are: mXBG, m-xylylenesiguanidinium; FITC, fluorescein 5’-isothiocyanate; MonoG, n-alkyl-1-guanidinium; BisG, n-alkyl-α,ω-bisguanidinium.

2 C. Miller, personal communication.
groups (BisG) (Fig. 1B), or aromatic \textit{m}- and \textit{p}-xylenebis-guanidinium derivatives (Fig. 1, C and D, respectively). In the text the numbers following MonoG or BisG refer to the number of carbon atoms in the hydrocarbon chain.

Are Guanidinium Derivatives Competitors of K$^+$ or Na$^+$ in Occlusion Assays?

Fig. 2A shows the result of a Rb$^+$ occlusion assay with varying Rb$^+$ concentrations (0.125–2 mm in 100 mM Tris-HCl, pH 7.0) in the absence and presence of 0.5 mM BisG 8. The data were well fitted to a single-site binding model showing a significant decrease in apparent Rb$^+$ affinity ($K_{a,s}$ = 0.234 ± 0.02 and 0.684 ± 0.12 mM Rb$^+$, respectively), but no significant change in maximal binding ($B_{max}$ = 2.21 ± 0.06 and 2.45 ± 0.18 nmol of Rb$^+$/mg of protein, respectively). The same trend was observed with all alkylguanidinium derivatives up to BisG 10. Similar behavior was also seen in Na$^+$ occlusion assays (for example, that shown in Fig. 2B, where the reaction mixture contained 0.25–16 mm Na$^+$ balanced with Tris-HCl, pH 7.0, to a total salt concentration of 100 mM. Oligomycin was added at 125 pg/ml. The continuous line is that using the best fit to the Hill equation.

The data were well fitted to a single-site binding model showing a significant decrease in apparent Na$^+$ affinity ($K_{a,s}$ = 2.63 ± 0.37 and 4.59 ± 0.26 mM Na$^+$, respectively) and the Hill number ($n_H$) = 1.57 ± 0.21 and 2.12 ± 0.08, respectively (see “Discussion”).

To estimate the intrinsic affinity of the guanidinium derivatives ($K_a$), Rb$^+$ occlusion was measured at different Rb$^+$ concentrations with various fixed concentrations of MonoG or BisG (range of 0.25–4 mM), and the apparent affinities for Rb$^+$ ($K_{app}$) were plotted against the concentration of inhibitor. A linear correlation was found as expected for simple competitors, where $K_{app}$ = $K_a(1 + [I]/[S])$, from the slope of which the $K_a$ can be found. Table I lists the $K_a$ for each of the compounds tested. Evidently, the longer the alkyl chain, for either MonoG or BisG derivatives, the better competitors they are. The affinities of BisG compounds are approximately 2-fold higher than those of the corresponding MonoG compounds, except for the C$_7$ derivative. This small difference between MonoG and BisG derivatives suggests that the effectiveness of the BisG compound depends only on the concentration of guanidinium groups, i.e., there is no significant cooperativity between the pairs of guanidinium groups.

The apparent affinities of the alkylguanidinium derivatives for the monovalent cation-binding sites were relatively low. Therefore, attempts to improve the affinity of the guanidinium compounds were made.

Effect of Medium on Affinity of Guanidinium Derivatives

Changing the buffer from 100 mM Tris-HCl to 100 mM choline chloride plus 10 mM Tris-HCl (both at pH 7.0) increased the apparent affinity of Rb$^+$ by ~2-fold (0.234 ± 0.02 and 0.113 ± 0.15 mM Rb$^+$, respectively) (data not shown). BisG 8 had a similar inhibitory potency in either buffer (for example, with 0.136 mM Rb$^+$, 0.5 mM BisG 8 inhibited 48% and 42%, respectively). Therefore, the intrinsic affinity of BisG 8 was ~2-fold higher in the choline chloride medium.

Effect of pH on Affinity of Guanidinium Derivatives

The inhibitory potency of a fixed concentration of BisG 8 (0.2 mM) was tested on the occlusion at a subsaturating concentration of Rb$^+$ (0.27 mM) in the choline chloride medium over a pH range of 7.0–9.15 (Fig. 3). The inhibitory effect of BisG 8 rose from <20% to >90%. At each pH, the apparent affinity for Rb$^+$ was determined. In the pH range of 7.0–8.2, there is a slight decrease in the Rb$^+$ affinity from 67 ± 0.02 to 91 ± 0.15 μM Rb$^+$. The intrinsic affinity for the inhibitor is estimated to rise ~5-fold in this range. Elevation of the pH above 8.2 significantly reduces the Rb$^+$ affinity, and the intrinsic affinity of BisG 8 is not elevated further (or may even be slightly reduced compared to that at pH 8.2).

Table II shows the affinity of MonoG 8 and BisG 8 in 100 mM choline chloride plus 10 mM Tris-HCl, pH 8.5, determined

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_a$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisG</td>
<td>725</td>
</tr>
<tr>
<td>3</td>
<td>650</td>
</tr>
<tr>
<td>4</td>
<td>865</td>
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<tr>
<td>8</td>
<td>243</td>
</tr>
<tr>
<td>10</td>
<td>73</td>
</tr>
<tr>
<td>MonoG</td>
<td>7620</td>
</tr>
<tr>
<td>3</td>
<td>2200</td>
</tr>
<tr>
<td>4</td>
<td>1530</td>
</tr>
<tr>
<td>8</td>
<td>510</td>
</tr>
<tr>
<td>10</td>
<td>128</td>
</tr>
</tbody>
</table>
Guanidinium Is a Na⁺-like Antagonist on Na⁺,K⁺-ATPase

**FIG. 3. Effect of pH on inhibitory potency of BisG 8.** Enzyme (0.8 mg/ml) was incubated in 50 μl of reaction mixture containing 100 mM choline chloride plus 10 mM Tris-HCl, pH 7.0-9.15, with 270 μM RbCl + 32P-Rb⁺ in the presence or absence of 200 μM BisG 8.

**TABLE II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisG 8</td>
<td>25.6</td>
</tr>
<tr>
<td>MonoG 8</td>
<td>45.0</td>
</tr>
<tr>
<td>mXBG</td>
<td>7.7</td>
</tr>
<tr>
<td>pXBG</td>
<td>8.2</td>
</tr>
</tbody>
</table>

**Determination of Affinity of Xylylenebisguanidinium Derivatives**

To examine the effects of replacing alkyl chains with an aromatic nucleus and also possible proximity effects of the guanidinium groups, two xylylenebisguanidinium derivatives were synthesized with the guanidinium groups in either meta- or para-positions (Fig. 1, C and D, respectively). Synthesis of the ortho-derivative was also attempted. This synthesis started with phthalazine, which was first reduced in two stages to produce o-xylylenediamine (Elsager et al., 1968). However, bisguanidination of the diamine by the method used for the other isomers failed to give the expected o-xylylenebisguanidinium, probably because of steric hindrance. The m- and p-compounds were found to be competitive inhibitors of Rb⁺ in the occlusion assay (data not shown). In the choline chloride medium at pH 8.5, the intrinsic affinities of the meta- and para-derivatives were similar (7.7 and 8.2 μM, respectively) (Table II) and ~3-fold higher as compared with BisG 8, the alkyl derivative with the same number of carbon atoms (Table II). Thus, the aromatic nucleus provided a significantly stronger binding interaction with the protein; and no effect of position of the guanidinium groups was observed, consistent with the lack of cooperativity of guanidinium groups in the BisG derivatives (Table I).

**Effects of mXBG on Conformational Changes of Fluorescein-labeled Enzyme**

In principle, a competitor of either Rb⁺ or Na⁺ in the occlusion assay could act like Rb⁺ in stabilizing the E₂ state or like Na⁺ in stabilizing the E₁ state. These possibilities were distinguished by looking at the conformational state of the protein using FITC-labeled Na⁺,K⁺-ATPase (see Rephaeli et al. (1986) and Karlish (1980)). Addition of Rb⁺ ions to the labeled protein in a high ionic strength medium induces the transition from the initial E₁ conformation to the E₂(2Rb⁺) conformation and is accompanied by up to 35% fluorescence...
Guadininium Is a Na⁺-like Antagonist on Na⁺,K⁺-ATPase

Enhancement of Affinity of mXBG for FITC-labeled Na⁺,K⁺-ATPase at Very Low Ionic Strength

When fluorescence changes were examined in media of very low ionic strength (1 mM Tris base, pH 8.55), the affinity of mXBG was greatly enhanced compared to the standard medium. In a medium consisting of 1 mM Tris base, pH 8.55, the fluorescence quench with Rb⁺ was very small; saturation of the Rb⁺ quench was achieved with 37.5 μM Rb⁺; and subsequent addition of 0.75 μM mXBG reversed most of the quench (Fig. 4C). Addition of 0.75 μM mXBG in the absence of Rb⁺ resulted in a strong fluorescence enhancement, reversed by 0.5 mM Rb⁺ (Fig. 4D), and Na⁺ ions were able to reverse the fluorescence change (data not shown). These observations show that the protein is largely in the E₂ state in the low ionic strength medium (see Skou and Esmann (1980)). The apparent affinity of mXBG for the fluorescence enhancement in the absence of Rb⁺ was very high (Kᵋ ≈ 0.12 μM). Fig. 5A demonstrates titrations of the mXBG response without Rb⁺ or with 37.5 μM Rb⁺ and clearly suggests competition between mXBG and Rb⁺. A linear relation between the Kᵋ for mXBG and Rb⁺ concentration (Fig. 5B) confirms competition between mXBG and Rb⁺ under these conditions.

Affinities of Guadininium Compounds in Low Ionic Strength Media

The Kᵋ values for all the different guadininium compounds for inducing conformational transition at low ionic strength are summarized in Table III. Evidently, all the BisG derivatives show a very high affinity and that of the MonoG derivatives is also significantly enhanced compared to the standard medium of occlusion assays. The trend that the longer alkyl chain endows a better affinity is maintained for both groups. However, the BisG derivatives appear much more than twice as effective as the comparable MonoG derivatives, unlike the situation in the high ionic strength medium of Table I.

Effect of Protein Concentration on mXBG Affinity

The large difference in effectiveness between MonoG and BisG derivatives in the low ionic strength medium suggested that there may be nonspecific interactions with the lipid that differentially affect the free concentrations of the compounds. This possibility was tested by measuring the apparent affinity at different protein concentrations. Indeed, Fig. 6 demonstrates a linear relation between the protein concentration of FITC-labeled enzyme (3, 6, 15, and 30 μg) and the apparent

![Graph A](image)

**Fig. 5.** Competition between Rb⁺ and mXBG on FITC-labeled Na⁺,K⁺-ATPase at low ionic strength and equilibrium titrations of fluorescein fluorescence signals. For the titrations in A, fluorescein-labeled enzyme (6 μg) was suspended in 1.5 ml of 1 mM Tris base, pH 8.55; and then aliquots of mXBG were added in the absence (A) or presence (B) of 37 μM Rb⁺. The fluorescence enhancement changes were recorded. The continuous line is that using the best fit of binding to a single site after normalization of the total fluorescence changes. B, same as A, but in the presence of different Rb⁺ concentrations. The Kᵋ = Kᵋ for mXBG values are shown as a function of Rb⁺ concentration, and the line is the best fit to a linear regression.

**Table III**

Affinities of guadininium derivatives for FITC-labeled Na⁺,K⁺-ATPase at low ionic strength

Fluorescein-labeled enzyme (6 μg) was suspended in 1.5 ml of 1 mM Tris base, pH 8.55, and then aliquots of each of the compounds present below were added until saturation of the fluorescence signal was reached. The fluorescence enhancement changes were recorded, and a fit to binding to a single-site model was done. The Kᵋ values found for each of the compounds are presented.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵋ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisG</td>
<td>0.263</td>
</tr>
<tr>
<td>3</td>
<td>0.512</td>
</tr>
<tr>
<td>6</td>
<td>0.474</td>
</tr>
<tr>
<td>8</td>
<td>0.184</td>
</tr>
<tr>
<td>10</td>
<td>0.126</td>
</tr>
<tr>
<td>MonoG</td>
<td>262.3</td>
</tr>
<tr>
<td>3</td>
<td>61.8</td>
</tr>
<tr>
<td>6</td>
<td>8.8</td>
</tr>
<tr>
<td>8</td>
<td>1.7</td>
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<tr>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>mXBG</td>
<td>0.125</td>
</tr>
<tr>
<td>pXBG</td>
<td>0.219</td>
</tr>
</tbody>
</table>
Guanidinium Is a Nu+-like Antagonist on Na+, K+-ATPase

**Fig. 6.** Effect of protein concentration on mXBG affinity. Fluorescein-labeled enzyme (3, 6, 15, and 30 μg of protein) was suspended in 1.5 ml of 1 mM Tris base, pH 8.55. Aliquots of mXBG were added, and the fluorescence changes were recorded. The best fit of binding to a single site was done, and the \( K_{\text{app}} \) values were calculated. The \( K_{\text{app}} \) values as a function of protein concentration are presented, and the line is the best fit to a linear regression.

**Fig. 7.** Inhibition of ATP hydrolysis by mXBG. Enzyme (20 μg) was suspended in 1 ml of reaction mixture containing 3 mM MgCl2, 3 mM Tris/ATP, and 1, 10, and 100 mM NaCl in the presence of 0.5 or 13.4 mM KCl plus Tris-HCl, pH 7.0, to a 150 mM final salt concentration. The enzyme was incubated at 37 °C in the presence or absence of 1 mM mXBG. The percent inhibition as a function of the reaction mixture composition is given.

affinity of mXBG. The correlation found was: \( K_{\text{app}} = 0.0315 \times \text{[protein]} \ (\mu\text{g/ml}) + 0.079 \ \mu\text{M} \) mXBG. It is quite clear there is nonspecific binding (probably to the lipid), which reduces the free concentration of the derivative and hence the apparent affinity. Extrapolation to zero protein reveals a \( K_i \) of 79 nM for mXBG.

Is mXBG Agonist or Antagonist of Na⁺ Ions?

To answer this question, the following tests were carried out.

**ATPase Activity**—Fig. 7 shows the inhibition by 1 mM mXBG of Na⁺, K⁺-ATPase activity at various Na⁺ concentrations (1, 10, and 100 mM NaCl) in the presence of 0.5 mM KCl or at near-saturating concentrations of the ions (100 mM NaCl and 13.5 mM KCl). ATPase activity is inhibited strongly at the low K⁺ concentration, and an increase in Na⁺ concentration from 1 to 10 to 100 mM is moderately effective in antagonizing the inhibition (95, 86, and 70%, respectively). In contrast, elevation of [KCl] from 0.5 to 13.4 mM in the presence of 100 mM NaCl reduced the inhibitory potency to only 6.2%. Thus, both Na⁺ and K⁺ ions appear to competitively antagonize the effects of mXBG, but K⁺ ions are much more effective (see "Discussion").

**Phosphorylation from ATP**—Fig. 8 demonstrates the inhibition of phosphorylation from ATP by 1 mM mXBG at different Na⁺ concentrations (nominally 0, 0.5, 1, and 20 mM NaCl). The higher the Na⁺ concentration, the lower is the inhibition (58.5, 51.2, 23.4, and 3.1%, respectively), as expected for an antagonist of Na⁺.

**Phosphorylation from Inorganic Phosphate**—The inhibitory potency of 1 mM mXBG on the phosphorylation of the enzyme from inorganic phosphate after 1 h was examined. The presence of mXBG was effective and inhibited the phosphorylation, as did a high concentration of Na⁺ (Fig. 9).

**DISCUSSION**

Characterization of Guanidinium Derivatives as Na⁺ and K⁺ Competitors

All the alkylmono- and bisguanidinium derivatives and m- and p-xylylenbisguanidinium tested in this series of experiments compete with Rb⁺ or Na⁺ in an occlusion assay (Fig. 10).
Guanidinium is a Na⁺-like Antagonist on Na⁺,K⁺-ATPase

**Fig. 8.** Inhibition of phosphorylation from ATP by mXBG. Enzyme (75 µg) was incubated for 5 s at room temperature in 125 µl of a medium containing 100 mM choline chloride, 25 mM Tris-HCl, pH 8.35, 1 mM MgCl₂, 10 µM ATP + [γ-³²P]ATP, and various NaCl concentrations as indicated. The experiments were done in the absence (dark shaded bars) or presence (light shaded bars) of 1 mM mXBG. The percent inhibition is given above the light shaded bars.

**Fig. 9.** Inhibition of phosphorylation from inorganic phosphate by mXBG. Enzyme (75 µg) was incubated for 1 h in 50 mM Tris-HCl, pH 8.35, 2 mM P/Tris + 4-6 × 10⁶ cpm P/sample, and 1 mM MgCl₂ in the presence or absence of 1 mM ouabain, or 1 mM mXBG, or 20 mM NaCl.

24 and Table I). The compounds decreased the apparent affinities of both Rb⁺ or Na⁺ ions without reducing the maximal level of their occlusion. It is of interest that the cooperativity index (nH) for Na⁺ is raised in addition to the Kᵥ₅ value (see Fig. 2B). The cooperativity index is one measure of the relative affinities of the three Na⁺ sites (see Karlish and Stein (1985)), and this finding probably implies that the guanidinium derivatives do not compete equally well with all three Na⁺ sites.

The guanidinium derivatives, like Na⁺ ions, stabilize the E₁ conformational state of the FITC-labeled enzyme (Fig. 4, A and B). In high ionic strength medium, the different derivatives fail to quench the fluorescence, but they successfully reverse fluorescence quenching induced by Rb⁺. Thus, the guanidinium derivatives are "Na⁺-like." This behavior is evident also in low ionic strength media where the FITC-labeled enzyme is 90% stabilized in the E₁ conformational state. Here, the guanidinium derivatives, like Na⁺, enhance the fluorescence upon binding and stabilize the E₁ conformation. The fluorescence enhancement is reversed by Rb⁺ (Fig. 4, C and D). mXBG and other derivatives, like Na⁺ ions, compete with Rb⁺ in the conformational transition in both high and low ionic strength media (see "Results" and Fig. 5, A and B).

mXBG is Na⁺-like in stabilizing the E₁ state, but it is an antagonist of Na⁺ ions. It inhibits hydrolysis of ATP (Fig. 7) and phosphorylation of the enzyme from ATP (Fig. 8) and from inorganic phosphate (Fig. 9). The inhibition of Na⁺,K⁺-ATPase is antagonized weakly by Na⁺, but strongly by K⁺ ions (Fig. 7). This is exactly the behavior one might predict of a cation analogue that binds tightly to the cytoplasmic surface, stabilizes the E₁ state, and competes strongly with Na⁺ ions, but binds poorly to the extracellular surface in the E₃P state and so competes weakly with K⁺ ions (Fig. 4, A and B).

The inhibitory potency of mXBG in the ATPase activity assay and the different phosphorylation assays was relatively poor compared to that in occlusion assays. This is expected in situations when the form of the protein that binds the inhibitor most tightly (E₁) is not the major steady-state
intermediate, i.e., this is an independent if indirect confirmation of the different affinities of the $E_i$ and $E_P$ forms with cation sites at the cytoplasmic and extracellular surfaces, respectively. Preliminary experiments with reconstituted proteoliposomes confirm that mXBG competes with Na$^+$ ions at the cytoplasmic surface and inhibits active Na$^+$/K$^+$ exchange.

**Affinity of Guanidinium Compounds**

Much effort was invested in: 1) establishing experimental conditions and 2) finding the structure of the guanidinium derivatives with the highest affinity for the protein. This was important since the success of subsequent attempts to synthesize and make use of covalent labels based on these pre-derived positions depended critically on affinity. Any attempt to label the protein with low affinity analogues would probably lead only to unselective labeling. In this process of optimization, interesting facts were learned about the cation-binding site and interactions between the guanidinium derivatives and the protein.

Two experimental conditions were found to affect the apparent affinity of the guanidinium compounds: medium composition and pH.

**Medium Composition**—Tris-HCl has a Na$^+$-like effect (Skou and Esnmann, 1980). Reduction of the Tris-HCl content in the medium from 100 mM at pH 7.0 to 10 mM Tris-HCl plus 100 mM choline at pH 7.0 increased the Rb$^+$ affinity and the inhibitory potency of the guanidinium derivatives by 2-fold. Apparently, Tris-HCl is a better competitor of the guanidinium derivatives than choline. Under these conditions, there was no evidence for any nonspecific binding of the compounds to the membranes.

Removing the salt altogether produced a very large increase in the apparent affinity of all the bisguanidinium derivatives of $\sim$100-fold (compare Tables II and III). Table II shows that of all the bisguanidinium derivatives with the highest affinity for the protein. This was found that an increased alkyl chain length (aliphaticity) of mono- and bisguanidinium derivatives increased their affinity. It would not be surprising if the approach to or micro-environment of the cation sites contained hydrophobic residues. In high ionic strength medium, the 2–3-fold higher affinity of BisG compared to MonoG derivatives reflects only the 2-fold higher concentration of guanidinium groups in the molecule. As mentioned above, this appears to rule out the notion that two guanidinium groups interact simultaneously with the site. Changing the structure of the molecule from aliphatic to the aromatic xylylene derivative (with an eight-carbon atom chain) (Table II). Presumably, the aromatic ring interacts more strongly with the protein in the surroundings of the cation-binding site. Changing the position of the guanidinium groups from the meta- to para-position had no effect (Table II), supporting the idea that only a single guanidinium group interacts with the site at one time.

In summary, one may assume that the positively charged guanidinium group interacts with the cation-binding region in a manner competitive with Rb$^+$ and Na$^+$; and, in addition, the side chain or ring also interacts with the protein so as to raise the binding affinity, under some conditions, even above that of Na$^+$ or Rb$^+$.

Our findings with the BisG derivatives differ from those of Schuurmans Stekhoven et al. (1988) with diamines, for which the longer the chain length from C$_2$ to C$_6$, the poorer was the compound as an inhibitor. These authors inferred that binding of both amine groups in ethylenediamine to two proximal sites gives rise to a cooperative effect on the affinity. Conceivably, two guanidinium groups, each of radius $\sim$2.88 Å, may be sterically hindered from entering a space that accommodates two protonated amines (radius $\approx$ 1.4 Å). A less welcome explanation for the progressive reduction in apparent affinity of diamines from C$_2$ to C$_6$ could be that this reflects only a greater degree of nonspecific adsorption to the lipid, particularly of the nonprotonated species, and so reduces the concentration of free protonated diamine in the medium available to bind to the site.

**Mechanism of Inhibition of Guanidinium Derivatives**

How do guanidinium derivatives, which compete with Na$^+$ or Rb$^+$ and behave like Na$^+$ in stabilizing the $E_i$ conformation, block the Na$^+$, K$^+$-ATPase activity and active transport of cations?

Consider the ionic radii of the guanidinium group, Na$^+$ and Na$^+$-like congeners, or K$^+$ and K$^+$-like congeners. Guanidinium is $\sim$2.88 Å. Na$^+$ and Li$^+$ (with hydrated ionic radii of 1.00 and 0.6 Å, respectively) form one group of Na$^+$-like cations. K$^+$ and K$^+$-like congeners Ti$^+$, NH$_4^+$, Rb$^+$, and

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3 E. Or and S. J. D. Karlish, unpublished data.

4 P. David and S. J. D. Karlish, unpublished data.
Ca⁺ (with dehydrated ionic radii of 1.33, 1.40, 1.40, 1.48, and 1.69 Å, respectively) form the second groups of cations (Hille, 1984). Thus, the selectivity properties of the Na⁺- or K⁺-like cations on the Na⁺,K⁺-ATPase appear to be differentiated according to the dehydrated ionic radii of 1.0 Å and smaller or 1.33 Å and larger (see Glynn and Karlish (1990)). Precedents of well-characterized cation-binding proteins suggest that the cation will indeed be largely or wholly dehydrated in the sites. In the case of Gd³⁺ ions, which serve as Ca⁺ congeners in sarcoplasmic reticulum ATPase, there is evidence (Klemens et al., 1986; Klemens and Grisham, 1988) that as the Gd³⁺ ions are first bound and then become occluded, they are progressively and completely stripped of their water molecules.

Assuming that the cation becomes dehydrated when it is occluded, how might guanidinium act as a Na⁺-like antagonist? We remind the reader of Hille’s selectivity filter hypothesis for the voltage-sensitive Na⁺ channel (see Hille (1984)). Based on the finding that guanidinium is the smallest of a series of organic cations that permeate the channel, Hille suggested that Na⁺ ions with a single water of hydration or guanidinium fit the dimensions of the selectivity filter with a cross-section of 3.1 × 5.1 Å. Thus, one may propose by analogy that at the cytoplasmic surface of the Na⁺/K⁺ pump, there is a vestibule or “mouth” of the Na⁺-binding and transport path wide enough to accommodate sodium or potassium ions with a single water molecule or a guanidinium ion, but that a subsequent narrowing of the permeation pathway within the transmembrane segments excludes guanidinium, but permits entry of the dehydrated alkali metal cations. Guanidinium therefore competes with either Na⁺ or K⁺ for access to their occlusion sites and blocks active transport and ATPase activity. The notion of a funnel-like structure at the cytoplasmic surface and subsequent narrowing of the cation path fits in well with proposals for an “Na⁺ well” based on charge transfer properties of the pump (Goldshleger et al., 1987; Stürmer et al., 1991).

Recently, we have shown that lanthanide ions (with ionic radius of 0.86–1.03 Å) (Martin and Richardson, 1979; Martin, 1984), compete with Na⁺ with high affinity and stabilize the E₁, conformation (David and Karlish, 1991). By the present hypothesis, La³⁺ ions behave as Na⁺-like blockers (David and Karlish, 1991) because they fit into the more superficial site, but do not readily lose the final water molecule and enter the narrow transport path due to the very high hydration energy of the triple-charged cation (≈500 kcal/mol).

The hypothesis does not explain why guanidinium behaves more like Na⁺ than K⁺ ions. Conceivably, this is related to the relative hydration energies since Na⁺ (105 kcal/mol) is less likely to lose the final water molecule than is K⁺ (85 kcal/mol), but it does not seem worthwhile to speculate further. Indeed, if one had some information regarding this question, one might obtain insight into the central question of the difference in selectivity between Na⁺ and K⁺ ions.

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