Novel Aromatic Isothiouronium Derivatives Which Act as High Affinity Competitive Antagonists of Alkali Metal Cations on Na/K-ATPase*

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This paper describes properties of a novel family of aromatic isothiouronium derivatives, which act as Na⁺-like competitive antagonists on renal Na/K-ATPase. The derivatives are reversible competitors of Rb⁺ and Na⁺ occlusion. $K_0$ values of the most potent compounds, 1-bromo-2,4,6-tris(methylisothiouronium)benzene (Br₂-TITU) and 1,3-dibromo-2,4,6-tris(methylisothiouronium) benzene (Br₂-TITU), 0.65 and 0.32 $\mu M$, respectively, are 15-30-fold lower than $K_0$ values of the bis-guanidinium derivatives described previously (David, P., Cohen, H., and Karlish, S. J. D. (1992) J. Biol. Chem. 267, 1141-1149), and represent the lowest reported values for cation antagonists. Using fluorescein-labeled Na/K-ATPase, all derivatives have been shown to stabilize the $E_2$ conformation when bound at high affinity sites (i.e., they are sodium-like). In addition, in one condition (10 mM Tris-Cl, pH 8.1), high concentrations of Br₂-TITU ($K_0 \approx 10 \mu M$) appear to stabilize an $E_2$ conformation. We propose that an approach which allows for simultaneous binding of the antagonists to high affinity cytoplasmic sites and low affinity sites, which may be at the extracellular surface. Blockage of cation occlusion by the isothiouronium derivatives at the cytoplasmic surface probably occurs at the entrance to the occlusion sites, which is recognized both by Na⁺ antagonists and by Na⁺ or K⁺ ions. Unlike the alkali metal cations, the Na⁺ antagonists are not occluded or transported (see also Or, E., David, P., Shainskaya, A., Tal, D. M., and Karlish, S. J. D. (1993) J. Biol. Chem. 268, 16929-16937). The isothiouronium derivatives appear to be promising candidates for further development as affinity labels of cation binding domains, for kinetic analysis of isoforms or mutated Na/K pumps, or as probes of other cation transport proteins.

Knowledge of the structure and organization of cation occlusion sites is central to an understanding of the mechanism of active transport by the Na/K pump (Glynn and Karlish, 1990). In the absence of detailed molecular structure, a variety of techniques is being applied to obtain information on the sites, including chemical modification with carboxyl-specific labels (Goldshleger et al., 1992; Argüello and Kaplan, 1994) and site-directed mutagenesis (Van Huysse et al., 1993; J ewell-Motz and Lingrel, 1993; Vilsen, 1993). Another approach is the use of extensive proteolysis in order to define the minimal peptide components capable of cation occlusion. In a specifically trypsinized preparation of renal Na/K-ATPase, so-called “19-kDa membranes,” trans-membrane segments and extracellular loops remain, while cytoplasmic loops are removed. Cation occlusion capacity and ouabain binding are intact but ATP binding is destroyed (Karlish et al., 1990; Capasso et al., 1992; Shainskaya and Karlish, 1994; Schwappach et al., 1994). The concept that emerges from these approaches is that a cation occlusion “cage,” is located within the membrane domain, and consists of carboxyl and neutral ligating groups contributed by several trans-membrane segments. Nevertheless each technique is subject to inherent limitations and there is no firm evidence as to which residues or segments are directly involved in cation binding.

Affinity labeling of cation sites is, potentially, an important approach. The objective would be to use the reactive derivative to label and map the region of polypeptide chain in the vicinity of the cation sites. In addition an affinity label of cation sites with a known sidedness of action could help to distinguish between different topological models. An affinity or photoaffinity label could be based on an organic analogue of the alkali metal cations and should bind with a $K_0$ about equal to the concentration of the sites, in practice, 0.1-1 $\mu M$.

Cation analogues which act on the Na/K-ATPase with dissociation constants of 0.1-1 $\mu M$ have not been described. By contrast, the pyridineimidazole class of potassium-competitive inhibitors of gastric H/K-ATPase and gastric acid secretion represents an example of high affinity cation antagonists (Wallmark et al., 1987), derivatives of which have been used to successfully chemically modify the related H/K-ATPase (Munson et al., 1991). Competition with alkali metal cations of different alkyl or aryl amines and diamines on the Na/K-ATPase have been described (Kropp and Sachs, 1977; Schuurmans-Stekhoven et al., 1988; Forbush, 1988), but the dissociation constant of ethylenediamine, the compound with the highest affinity, is only about 25 $\mu M$ (Forbush, 1988). A photoaffinity probe based on a precursor with such a low affinity would probably be unselective, due to rapid dissociation of the active species and labeling of regions of the protein other than the cation sites. Recently, we have described synthesis and characterized functional effects of alkyl and aryl bis-guanidinium derivatives (m- or p-xylylene bis-guanidinium, mXBG or pXBG) (David et al., 1992). The guanidinium derivatives

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1 The abbreviations used are: mXBG, m-xylylene bis-guanidinium; Br₂-TITU, 1-bromo-2,4,6-tris(methylisothiouronium) benzene; Br₂-TITU, 1,3-dibromo-2,4,6-tris(methylisothiouronium) benzene; DCCD, N,N'-dicyclohexylcarbodiimide; FITC, fluorescein 5'-isothiocyanate; pXBG, p-xylylene bis-guanidinium; Tricine, N,N'-hydroxy-1,1-bis(hydroxymethyl) ethanolglycine; TPCK, 1-1-tosylamide-2-phenylethyl chloromethyl ketone.

2 These abbreviations are used: Br₂-TITU, 1-bromo-2,4,6-tris(methylisothiouronium) benzene; DCCD, N,N'-dicyclohexylcarbodiimide; FITC, fluorescein 5'-isothiocyanate; pXBG, p-xylylene bis-guanidinium; Tricine, N,N'-hydroxy-1,1-bis(hydroxymethyl) ethanolglycine; TPCK, 1-1-tosylamide-2-phenylethyl chloromethyl ketone.
Inhibition of Na/ K-ATPase by Isothiouronium Derivatives

Competitively inhibit Na⁺ or Rb⁺ occlusion, stabilize the E₁ conformation of the enzyme, and block Na/K-ATPase activity. Thus they are competitive Na⁺ antagonists. pXBG or mXBG compete with a Kₐ ≈ 8 µM.

Systematic structure-activity studies on the guanidinium derivatives (David et al., 1992) and amiloride analogues (David et al., 1993) led to a number of provisional conclusions concerning expected properties of a putative high affinity Na⁺ antagonist. These conclusions form the basis for a search and design of a new set of compounds of higher affinity. In order to simplify syntheses, the positively charged guanidinium has been replaced by the isosterically positively charged isothiourea moiety. This paper describes the interactions of the new high affinity antagonists with renal Na/K-ATPase. Detailed syntheses of the compounds are described elsewhere (Tal and Karlisch, 1995).

EXPERIMENTAL PROCEDURES

Enzyme Preparations—NaK-ATPase was prepared from fresh kidney red outer medulla by the simpler of the procedures developed by Jørgensen (1974). Before use, the enzyme was dialyzed overnight against 1,000 volumes of a solution containing 25 mM histidine, 1 mM EDTA, pH 7.0, at 4°C. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

ATPase Activity—This was determined as described by Swarts et al. (1990). The specific activity of ATP hydrolysis for the Na-K-ATPase was in the range of 12–20 µmol of P₄/mg of protein at 37°C.

Rb⁺ and Na⁺ Occlusion Assays—The Rb⁺ and Na⁺ occlusion assays were performed as described by Shani et al. (1987) and Shani-Sekler et al. (1988). The assay is based on elution of the Rb⁺- or Na⁺-enzyme complex on Dowex 50W (Tris) columns at 0°C. In the standard Rb⁺ occlusion assay 20–50 µg of enzyme or 19-kDa membranes were incubated for 15 min at room temperature in a medium, containing 10 mM Tris-HCl, pH 8.0, 100 mM choline chloride, 2–5 mM RbCl plus 2–8 × 10⁵ cpm of ²²NaCl. After incubation, 2 µl of sodium cyanide (1 mg/ml) was added and incubated for another 10 min.

In both cases, 0.5 ml of ice-cold 200 mM sucrose was added to dilute and cool the mixture, which was immediately transferred to ice-cold Dowex columns. The enzyme with occluded Rb⁺ or Na⁺ was eluted with 1.0 ml of the ice-cold 200 mM sucrose. ²²Rb⁺ was measured as its Cerenkov radiation. For ²²Na⁺, 3 ml of scintillation liquid (Lumac: xylene: 1:3) was added and radioactivity was measured in a scintillation counter.

Fluorescence Measurements on FITC-labeled Na/K-ATPase—FITC labeling of the Na-K-ATPase was done exactly as described by Rephaeli et al. (1986) and Karlisch (1988). Equilibrium fluorescence measurements were performed at room temperature using a Perkin Elmer MFP 4A spectrophotofluorimeter. Excitation and emission wavelengths were 495 and 520 nm, respectively. Silt widths were 10 nm and the time constant was 0.3 s. In the standard procedure 10 µg of labeled enzyme was suspended in 1.5 ml of either 10 mM Tris·HCl, pH 8.1, 100 mM choline chloride (high ionic strength medium), 10 mM Tris·HCl, pH 8.1 (moderate ionic strength medium), or 1 mM Tris·HCl, pH 8.5 (very low ionic strength medium), under constant magnetic stirring. For equilibrium titrations, increasing amounts of Rb⁺, Na⁺, or Na/K-ATPase derivatives were added, until the signal became constant. The amplitude of the change of fluorescence was measured and corrected for the dilution due to the added volume.

Inactivation of Na/K-ATPase by DCCD—Inactivation by DCCD was done as described by Shani-Sekler et al. (1988) with the modifications described by Or et al. (1993).

Digestion of Na/K-ATPase—Extensive digestion of renal Na/K-ATPase with TPCK-trypsin and resolution of fragments using the Tricine SDS-PAGE system was done as described by Capasso et al. (1992).

Synthesis of Isothiouronium Derivatives—Syntheses and structural characterization of the derivatives are described in a separate publication (Tal and Karlisch, 1995). The concentration of synthesized isothiouronium derivatives was verified (a) by quantitative hydrolysis at pH ≈ 11 to the sulfhydryl derivative (1 h in 1 M Tris base), and (b) determination of the sulphydryl concentration with Ellman’s reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman, 1959; Riddles et al., 1983).

Calculations—Best fit parameters of theoretical equations for the experimental data were calculated by nonlinear regression analysis, using the program Enzfitter (Elsevier- Biosoft) for the IBM-PC.

Materials—²²RbCl and ²²NaCl were obtained from Du Pont. Dowex 50W-X8 (50–100 mesh) was from Fluka. Dowex was converted in the Tris form before use. Ouabain, oligomycin (mixture of A-C), trypsin inhibitor (type I-S from soybean), bovine serum albumin (fraction V), FITC (isomer I), and ATP (disodium salt, converted to the Tris form by passage over Dowex 50 columns) were from Sigma. TPCK-trypsin (240 units/mg) was from Worthington. DCCD was from Merck.

RESULTS

The structures, IUPAC names, and abbreviations, of the new isothiouronium derivatives are shown in Fig. 1. The benzyl, xylol, or mesityl derivatives contain either one, two, or three isothiouronium groups, respectively, without or with methyl substituents, and none, one, or two bromine atoms in the aromatic ring.

Competitive Inhibition of Cation Occlusion

Fig. 2A shows typical dose-response curves for the two most potent derivatives, Br-TITU and Br₂-TITU. In the presence of 50 µM BrCl, IC₅₀ values of 1.4 µM and 0.75 µM were found for these compounds, respectively. Fig. 2B shows results of an experiment in which Rb⁺ occlusion was measured at different Rb⁺ concentrations (0.05–1.2 mM), in the absence and presence of 3.13 ± Br-TITU at pH 8.0. The curves were fitted to hyperbolic functions, with a large difference in Kᵣ for Rb⁺ (Kᵣ = 44 ± 2 and 310 ± 66 µM without and with inhibitor, respectively) but no significant change in maximal binding (Vₘₐₓ = 3.4 ± 0.3 and 3.5 ± 0.3 nmol of Rb/mg of protein, respectively). Thus Br-TITU is a competitive inhibitor. Competitive inhibition was observed also for other derivatives, similar to previous findings with the guanidinium derivatives (David et al., 1992), but not all compounds were tested for competition. Table I presents Kᵣ values of the different derivatives, estimated from curves such as those in Fig. 2, A or B (see "Discussion"). Note that the Kᵣ for Br-TITU is 0.65 µM while that for Br₂-TITU is 0.32 µM. The third line from the bottom records the result of an experiment with 19-kDa membranes (Karlisch et al., 1990; Capasso et al., 1992). Evidently the Br-TITU interacts competitively but much less strongly with these digested membranes than with the native enzyme. A similar phenomenon was found previously for the bis-guanidinium derivatives (Or et al., 1993). Competitive behavior was also observed in Na⁺ occlusion experiments, and in addition the maximal inhibitory potency was observed at pH 8.0 as found previously for the bis-guanidinium derivatives (David et al., 1992) (data not shown).

Conformational Changes

A competitor of either Rb⁺ or Na⁺ in the occlusion assay might act either like Rb⁺ in stabilizing the E₂ state or like Na⁺ in stabilizing the E₁ state. One can distinguish between these alternatives by looking at the conformational states, with the use of FITC-labeled NaK-ATPase (Karlisch, 1988; David et al., 1992).

Fig. 3 shows the effect of Br-TITU on FITC-labeled NaK-ATPase. In the high ionic strength medium at pH 8.1, the enzyme is in the E₁ conformation. If Rb⁺ (1 mM) is added first to induce the quench, subsequent addition of Br-TITU (25 µM) largely reverses the signal (Fig. 3A). (The lack of complete reversal of the fluorescence signal by Br-TITU (25 µM) is due to a small contribution of nonspecific fluorescent quenching, observed when high concentrations of Br-TITU are added after RbCl.) At lower concentration of RbCl (300 µM) and Br-TITU (8 µM), reversal of fluorescence quenching was complete (not
shown). Thus, Br-TITU behaves essentially like Na$^+$ ions in stabilizing the E$_1$ form. Accordingly, if Br-TITU is added first, it does not affect the fluorescence and subsequent addition of Rb$^+$ has no effect (Fig. 3B). Stabilization of the E$_1$ conformation was observed for all the isothiouronium derivatives, except Me$_2$-Br-TITU, due apparently to precipitation of the FITC-labeled enzyme. At very low ionic strength the enzyme is mainly in the E$_2$ state (Skou and Esmann, 1980). Addition of Br-TITU in the absence of Rb$^+$ resulted in a strong enhancement, which can be reversed by Rb$^+$ (Fig. 3C). When Rb$^+$ is added first, almost no change was observed, showing that the enzyme is indeed mainly in the E$_2$ conformation. Subsequent addition of Br-TITU did not reverse the signal at the low concentration used, due to competition with the Rb$^+$ (Fig. 3D).

Experiments with other derivatives confirm that all are competitive Na$^+$ analogues which stabilize the E$_1$ state. In fluorescence experiments, observed $K_i$ values are significantly lower than in occlusion experiments. This result is attributable to the higher enzyme concentration in occlusion experiments, for the derivatives are bound to the membranes, reducing the free concentration in the medium (see David et al., 1992).

In the course of systematic testing of effects of conditions on fluorescence responses, an unexpected phenomenon was observed for a medium of intermediate ionic strength (10 mM Tris-HCl, pH 8.1, Fig. 3E). The $K_i$ of Br-TITU for reversing the Rb$^+$-induced quenching was lower than in the other media, ($K_i$ 0.13 $\mu$M in Fig. 3E), and addition of higher concentrations of Br-TITU caused the fluorescence to be quenched again. Subsequent addition of Na$^+$ ions, 20 $\mu$M, largely reversed this quench. The apparent affinity for this E$_2$-stabilizing effect of Br-TITU effect is about 10 $\mu$M, i.e. about 80-fold lower than that of the high affinity site. The same effect was observed for the mono- and dimethyl-Br-TITU derivatives, although they are much less potent inhibitors of occlusion than Br-TITU (Table I). In a medium of high ionic strength (100 mM choline chloride, 10 mM Tris-HCl, pH 8.1) addition of a very high concentration of Br-TITU caused quenching of fluorescence, but this effect appears to be nonspecific since it was not reversed by Na$^+$ (data not shown).

Mode of Interaction with the Protein

Protection against DCCD—The hydrophobic carboxyl reagent DCCD inactivates Rb$^+$ and Na$^+$ occlusion on Na/K-ATPase (Shani-Sekler et al., 1988; Goldshleger et al., 1992). Rb$^+$ ($K_1$), Na$^+$, and Na$^+$ antagonists such as the guanidinium derivatives protect against this inactivation (Or et al., 1993). Table II shows clearly that, in a medium of constant ionic strength, Br-TITU, like Rb$^+$ ions, protects against inactivation by DCCD of both the Rb$^+$ occlusion and the ATPase activity.

Lack of Protection of 19-kDa membranes against Tryptic Digestion—The experiment in Fig. 4 compares the ability of Rb$^+$ ions and Br-TITU to protect the 19-kDa and other fragments of the $\alpha$ subunit, and the 16-kDa fragment of the $\beta$ subunit, from complete digestion by trypsin, and to preserve Rb$^+$ occlusion capacity (Karlish et al., 1990). Evidently the presence of Br-TITU, 200 $\mu$M, during extensive digestion of renal Na/K-ATPase is completely unable to protect these fragments. Accordingly, Rb$^+$ occlusion was destroyed in the sample digested with Br-TITU, unlike that digested in the presence of Rb$^+$ ions (result not shown). In this respect, Br-TITU behaves similarly to the lower affinity Na$^+$-antagonists we have described previously (see Or et al., 1993).

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DISCUSSION

Structure-activity Relations of Isothiouronium Derivatives—The dissociation constants of Br₂-TITU (0.32 \(\text{mM}\)) and Br-TITU (0.65 \(\text{mM}\)) are the lowest reported to date for competitive cation antagonists on Na/K-ATPase, being 15–30-fold lower than those, in comparable conditions, of the aromatic bis-guanidinium derivatives, \(\text{pXBG}\) and \(\text{mXBG}\) (David et al., 1992), and 50–100-fold lower than those reported for competitive inhibitors such as ethylenediamine (\(\sim 25 \text{mM}\)) (Forbush, 1988). The data in Table I on competition between the isothiouronium derivatives and Na\(^+\) or K\(^+\) (Rb\(^+\)) ions allow one to conclude that the highest binding affinity is achieved with compounds having the greatest number of alkyl isothiouronium residues per molecule, three > two > one residue, the greatest number of Br atoms in the ring, two > one > no Br atoms, while \(N\)-substituted methyl derivatives are much less potent. These features are consistent with predictions based on structure-activity relations of the guanidinium derivatives and amiloride analogues (David et al., 1992; David et al., 1993). The increase
Ka-methylisothiourea (pS be above 9, as is found for other alkyl isothioureas such as mono- and bis-guanidinium derivatives (David cooperative effect of this sort was not observed in work with effect of the number of isothiouronium moieties (Table I). A derivatives, is larger than could be predicted for the statistical subunit against tryptic digestion.

Described by Capasso ofinhibitorisnecessaryforinhibition(Fig.2, occlusion assays, suggesting that binding of only one molecule competitive inhibition with respect to the alkali metal cations in derivatives. 1) The isothiouronium derivatives display simple com-

Metal Cations Sites—The following points summarize proper-

2-fold lower affinity and higher degree of co-operativity for occlusion of sodium ions at three sites (Karlish, 1990; Lau ¨ger, 1991). The Na ions (Fig. 1) or thermal inactivation (not shown).

Most of these features are similar to those of the aromatic bis-guanidinium derivatives, pXBG and mXBG (David et al., 1992; Or et al., 1993). pXBG and mXBG act as competitive Na antagonists, with selectivity for the cytoplasmic surface, but have much lower affinities than the Br-TITU and Br2-TITU. The previous work led to a proposal that cation occlusion from the cytoplasmic surface occurs in two steps (Or et al., 1993). In an initial recognition step, either transported cations or Na antagonists interact with carboxyl groups, while a second step is selective for transported cations, and involves occlusion of either K or Na ions and a conformational change to a compact structure, which is resistant to proteolysis and thermal inactivation. The isothiouronium derivatives, like the lower affinity Na antagonists, are presumed to be sterically hindered from becoming occluded, and so block cation occlusion and Na/K-ATPase activity (see David et al., 1992; Or et al., 1993). In another recent study we have looked at effects of diffusion potentials on inhibition by Na antagonists, using reconstituted proteoliposomes (Or et al., 1994). The data are interpreted on the basis of a model which assumes that two of the three Na transport sites are negatively charged and serve also as the K ion pathway or "ion well" (Goldshleger et al., 1987; Glynn and Karlish, 1990; Lauger, 1991). The Na antagonists do not appear to enter the narrow Na access pathway, or become occluded, but block transport by interacting with the negatively charged sites located nearer the surface. Evidently, these studies provide fairly clear structural implications for affinity labels based on the competitive Na antagonists. One can expect that such affinity labels would modify the protein at the entrance to the cytoplasmic cation sites.

One feature of tris-isothiouonium derivatives, not found previously for the bis-guanidinium derivatives, was observed in experiments with FITC-labeled Na/K-ATPase (Fig. 3). At high ionic strength, Br-TITU and other isothiouronium derivatives, like the pXBG and mXBG, behave like Na ions (Fig. 3, A-D). However, at a lower ionic strength (10 mM Tris-HCl, pH 8.1), whereas the "Na-like" effect was observed at a low concentration (K ≈ 0.13 μM), higher concentrations of Br-TITU (K ≈ 10 μM), and its methylated derivatives, appear to be "potassium-like" (Fig. 3E). The model in Fig. 5 can explain the findings in Fig. 3E. There are two sites for Br-TITU, high affinity, cyto-

plasmic (K1 << K2) and low affinity, (K4 << K2), respectively. In this model KCL2 >> 1 (KCL = [E1L][E2L]/[E1][E2]), while KCL1 << 1, (KCL2 = [E1L]/[E1][E2]). At low concentrations, Br-TITU occupies a high affinity cytoplasmic site and stabilizes the E1 con-

in affinity in the series mono-, bis-, and tris-isothiouonium derivatives, is larger than could be predicted for the statistical effect of the number of isothiouronium moieties (Table I). A cooperative effect of this sort was not observed in work with mono- and bis-guanidinium derivatives (David et al., 1992).

Interaction between Isothiouronium Derivatives and Alkali Metal Cations Sites—The following points summarize properties of the reversible interactions of the isothiouronium derivatives. 1) The isothiouronium derivatives display simple competitive inhibition with respect to the alkali metal cations in occlusion assays, suggesting that binding of only one molecule of inhibitor is necessary for inhibition (Fig. 2, A and B, Table I). 2) At low concentrations, the isothiouronium derivatives act as Na+-like antagonists on fluorescein-labeled enzyme, stabilizing the E1 form and competing with either Na+ or K+ ions (Fig. 3, A-D). 3) The potency of Br-TITU as a reversible competitive inhibitor rises nearly 3-fold upon raising the pH from 7.0 to 8.5 (not shown). A similar finding was described in detail for the guanidinium derivative, pXBG, and the possibility of direct competition between protons and the positively charged guanidinium moiety (pK ≈ 12) was raised (David et al., 1992). The pK, of the isothiouronium moieties in Br-TITU is expected to be above 9, as is found for other alkyl isothiouracil such as S-methylisothiourea (pK ≈ 9.83; Dean, 1985). Thus the protonated form should predominate in the range of pH values 7.0–8.5. 4) The apparent affinity for competition of Br-TITU on 19-kDa membranes is about 20-fold lower than on native enzyme (see Table I). Previously we reported that by comparison with native enzyme the 19-kDa membranes display a roughly 2-fold lower affinity and higher degree of co-operativity for occlusion of sodium ions at three sites (Karlish et al., 1990). The results for Br-TITU (and also pXBG, Or et al., 1993) are in line with these previous findings, but the difference of affinity be-

### Table II

| Addition                | % of control | Na/K-ATPase |
|-------------------------|--------------|------------|
| DCCD                    | 56           | 65         |
| DCCD + Rb               | 94           | 99         |
| DCCD + Br-TITU          | 87           | 104        |
| DCCD + Rb + Br-TITU     | 94           | 104        |

*Fig. 4. Br-TITU does not protect a 19-kDa fragment of the α subunit against tryptic digestion.* Na/K-ATPase was digested as described by Capasso et al. (1992) in the presence of 10 mM RbCl or 10 mM choline chloride plus 200 μM Br-TITU. 25 μg of protein was applied to a 10% Tricine Mini-gel as indicated.

*Fig. 5. Model with simultaneous binding of two molecules of Na+ antagonists.* See text for explanation.
formation, but at higher concentrations it occupies simulta-
neously both high and low affinity sites and stabilizes the E₂
conformation. Stabilization of the E₂ state is reminiscent of
effects of ouabain on the Na/K pump (see Forbush, 1983) or
the substituted pyridyl(1,2a)imidazole K⁺ competitor, SCH28080,
on the gastric H/K pump (Wallmark et al., 1987; Munson and
Sachs, 1988). Possibly, Br-TITU at high concentrations, like
ouabain or SCH28080, binds to the extracellular surface. Si-
multaneous binding of Br-TITU at opposite surfaces might
appear paradoxical in view of the accepted view that Na⁺ and
K⁺ are transported in a consecutive fashion (Glynn, 1985;
Sachs, 1991). Note, however, that we are not dealing with a
transported cation but with a cation antagonist. It is conceivable
that elements of the cation-path are fixed and co-exist at
two surfaces of the membrane (see also Karlish and Stein
(1985)).

Irreversible Inactivation of Cation Occlusion at pH 9.0—At
alkaline pH, isothiouronium derivatives are able to react with
lysine or cysteine residues and chemically modify them (Reid,
1958). In preliminary unpublished experiments it was found that
incubation of the native enzyme or 19-kDa membranes at
pH 9.0 with TITU derivatives at rather high concentrations
(200–700 μM), inactivates occlusion irreversibly. The presence
of Na⁺ or K⁺ ions protects against this inactivation. The con-
centration dependence, and structure-activity relations of the
various derivatives are different from those of the reversible
competitive effects described in this paper. The latter proper-
ties imply that this chemical modification is not occurring from
within cytoplasmic sites to which the derivatives bind with
high affinity, i.e. this is not affinity labeling but a separate
phenomenon. Chemical modification by the isothiouronium
derivatives is now the subject of further study.2

Potential Applications of the Isothiouronium Derivatives—(a)
The high affinity of Br-TITU and Br₂-TITU in competing for
Na⁺ or Rb⁺ overcomes one of the principal problems in the
development of an effective affinity or photoaffinity label of the
cation binding region. Therefore these compounds are candi-
dates for conversion to reactive derivatives. Benzophenone de-
nation binding region. Therefore these compounds are candi-
dates for development of an effective affinity or photoaffinity label of the
Na/K-ATPase with high affinity, the isothiouronium moiety does not
appear to be highly selective for Na⁺ binding sites on the pump.
This conclusion is compatible with the concept that the isothe-
iiouronium moiety interacts at the entrance of the cation sites on
the Na/K-ATPase. The isothiouronium derivatives may become
useful probes for other cation pumps, co-transporters, ex-
change diffusion systems, or cation channels.

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