Evidence for Tryptophan Residues in the Cation Transport Path of the Na^+,K^+-ATPase†

Guillermo A. Yudowski,‡ Meirav Bar Shimon,§ Daniel M. Tal,§ Rodolfo M. González-Lebrero,|| Rolando C. Rossi,|| Patricio J. Garrahan,|| Luis A. Beaugé,‡ and Steven J. D. Karlish*,§

Laboratorio de Biofísica, Instituto M. y M. Ferreyra, INIMEC-CONICET, 5000 Córdoba, Argentina, Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, and Departamento de Química Biológica, IQUIFIB, Facultad de Farmacia y Bioquímica, UBA, 1113 Buenos Aires, Argentina

Received February 18, 2003; Revised Manuscript Received July 4, 2003

ABSTRACT: A family of aryl isothiouronium derivatives was designed as probes for cation binding sites of Na^+,K^+-ATPase. Previous work showed that 1-bromo-2,4,6-tris(methylisothiouronium)benzene (Br-TITU) acts as a competitive blocker of Na^+ or K^+ occlusion. In addition to a high-affinity cytoplasmic site (K_D < 1 μM), a low-affinity site ($K_D \sim 10$ μM) was detected, presumably extracellular. Here we describe properties of Br-TITU as a blocker at the extracellular surface. In human red blood cells Br-TITU inhibits ouabain-sensitive Na^+ transport ($K_D \sim 30$ μM) in a manner antagonistic with respect to extracellular Na^+. In addition, Br-TITU impairs K^+-stimulated dephosphorylation and Rb^+ occlusion from phosphorylated enzyme of renal Na^+,K^+-ATPase, consistent with binding to an extracellular site. Incubation of renal Na^+,K^+-ATPase with Br-TITU at pH 9 irreversibly inactivates Na^+,K^+-ATPase activity and Rb^+ occlusion. Rb^+ or Na^+ ions protect. Preincubation of Br-TITU with red cells in a K^+-free medium at pH 9 irreversibly inactivates ouabain-sensitive $^{22}Na^+$ efflux, showing that inactivation occurs at an extracellular site. K^+, Cs^+, and Li^+ ions protect against this effect, but the apparent affinity for K^+, Cs^+, or Li^+ is similar ($K_D \sim 5$ mM) despite their different affinities for external activation of the Na^+ pump. Br-TITU quenches tryptophan fluorescence of renal Na^+,K^+-ATPase or of digested "19 kDa membranes". After incubation at pH 9 irreversible loss of tryptophan fluorescence is observed and Rb^+ or Na^+ ions protect. The Br-TITU appears to interact strongly with tryptophan residue(s) within the lipid or at the extracellular membrane−water interface and interfere with cation occlusion and Na^+,K^+-ATPase activity.

P-type ATPases are a group of enzymes that transport ions across the plasma membrane using the energy released from ATP hydrolysis (*1*). The Na^+,K^+-ATPase is a type II P-type ATPase, others of which include the gastric H^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase (*2*, *3*). Na^+,K^+-ATPase consists of an α subunit (MW ∼112 kDa) with 10 transmembrane segments, as found for all type II ATPases, and a β subunit with a single transmembrane segment (MW ∼35 kDa protein plus ∼15 kDa sugars) (*3*). The α subunits contain the functional sites for ATP and transported cations, while the β subunit is required for stabilization of the α subunit and transit from the endoplasmic reticulum to the cell membrane (*4*). A third regulatory subunit, including the γ subunit of the renal Na^+,K^+-ATPase or other members of the FXYD family, is expressed in a tissue-specific fashion but is not essential for pump function (*5*).

Structure−function relations of ATP and cation sites of P-type ATPases have been studied extensively by chemical modification and site-directed mutagenesis (*6*). Recently, the crystal structures of the Ca^{2+}-ATPase at atomic resolution have become available, in both E_1Ca and E_2 conformations (*7*, *8*). Due to the sequence homologies of the different P-type ATPases, these structures serve as a paradigm for assessing the correctness of conclusions based on lower resolution techniques and predicting new experiments for all P-type ATPases. For example, it was shown that cation occlusion sites of Ca^{2+}-ATPase are located within transmembrane segments M4, M5, M6, and M8, confirming the conclusions of many mutagenesis experiments (*9*). Most of the residues found in the two Ca^{2+} occlusion sites of Ca^{2+}-ATPase are conserved in Na^+,K^+-ATPase, and extensive mutagenesis work shows them to be involved in interactions of Na^+ and K^+ with their sites (*6*). In the case of Na^+,K^+-ATPase an additional third Na^+ site is required to explain the classical 3:2 Na^+:K^+ stoichiometry. Recent homology models of the transmembrane segments of Na^+,K^+-ATPase make specific suggestions which can be tested by further mutagenesis work (*6*, *10*). Although the crystal structures of Ca^{2+}-ATPase are invaluable for explanatory and predictive purposes, they cannot explain all features of other pumps, and a variety of different and complementary approaches are required. As an example, the Ca^{2+}-ATPase structures do not provide direct information on access pathways of cations to their occlusion

† This work was supported by a grant to S.J.D.K., L.A.B., P.J.G., and R.C.R. from the Fundación Antorchas for Argentina−Weizmann Institute collaboration. G.A.Y. thanks the Fundacion Antorchas for a fellowship.

* To whom correspondence should be addressed. Tel: 972 8 934 2278. Fax: 972 8 934 4118. E-mail: steven.karlish@weizmann.ac.il.

‡ Instituto M. y M. Ferreyra, INIMEC-CONICET.

§ Weizmann Institute of Science.

|| IQUIFIBB, Facultad de Farmacia y Bioquímica, UBA.

10.1021/bi0342721 CCC: $25.00
Published on Web 08/06/2003

sites in Na^+,K^+-ATPase, which have been inferred from studies of charge movements, or on "gates", which control access to or exit from occlusion sites (*11, 12*).

As one approach for characterization of cation entry pathways, we synthesized a number of positively charged aromatic guanidinium and isothiouronium derivatives (Br-TITU[1] and Br_2-TITU among others) and used them to probe cation binding sites (*13*−*16*). Br-TITU and Br_2-TITU act as high-affinity competitive blockers of K^+ (Rb^+) and Na^+ at the cytoplasmic surface. Although Br-TITU and Br_2-TITU compete with Rb^+ or Na^+ in occlusion assays, there is good evidence that these probes are not themselves occluded (*15*, *17*). Therefore, the site of action appears to be at an entrance port at the cytoplasmic surface which is recognized either by Na^+ and K^+ ions or by cation blockers, while only the occluded cations are then able to move into occlusion sites within transmembrane segments. One can infer that occlusion of transported cations from the cytoplasmic surface requires at least two separate steps (*16*). Recently, we have proposed that negatively charged residues within the cytoplasmic loop between transmembrane segments 6 and 7 of the α subunit (L6/7) bind Br-TITU and thus serve as an entrance port (*17*). A similar suggestion has been made in regard to sarcoplasmic reticulum Ca^{2+}-ATPase (*18, 19*).

The previous experiments with Br-TITU revealed the existence of both a high-affinity, Na^+-like effect and a lower affinity, K^+-like effect on the E1/E2 conformational equilibrium (*15*). The lower affinity site was proposed to be extracellular, although no direct evidence was available. We also reported, in a preliminary way, that incubation of the renal Na^+,K^+-ATPase with Br-TITU or Br_2-TITU at pH 9 causes irreversible inactivation of Na^+,K^+-ATPase activity. This finding suggests that the compound could chemically modify the protein (*15*). The present work examines extracellular effects of Br-TITU as a reversible cation blocker and also characterizes the irreversible inactivation. As a preliminary indication we observed recently that Br-TITU quenches tryptophan fluorescence of sarcoplasmic reticulum Ca^{2+}-ATPase (*20*). It turns out that irreversible inactivation of Na^+,K^+-ATPase occurs also at the extracellular surface but does not involve chemical modification of the protein. It appears to involve tight interactions of Br-TITU with tryptophan residues. Thus, the focus of this work is the cation access path at the extracellular surface and the possible role of tryptophan residues.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Na^+,K^+-ATPase was partially purified from pig kidney red outer medulla following the procedure described by Jørgensen (*21*). Immediately before use the enzyme was washed twice (1:10 v/v) and resuspended in a solution containing 30 mM Tris-HCl and the indicated NaCl concentrations (pH 7.5 at 20 °C). Protein was determined by the modified method of Lowry (*22*). ATPase activity was determined as described before (*23*). The specific activity of the purified enzyme was in the range of 12−18 μmol of P_i/(min·mg of protein).

19 kDa Membranes. 19 kDa membranes were prepared as described before (*24*). Dialyzed Na^+,K^+-ATPase (1.5 mg/mL) was suspended in a medium containing 25 mM imidazole, 1.5 mM EDTA, and 10 mM RbCl, pH 7.5, and incubated for 60 min at 37 °C with TPCK−trypsin (10:1 w/w). Soybean trypsin inhibitor was added (5:1 w/w). The mixture was diluted to 25 mL with 25 mM imidazole, 1.5 mM EDTA, and 2 mM RbCl, pH 7.5 (buffer A), and incubated for another 10 min at 37 °C. The membranes were collected by centrifugation at 250000*g* for 60 min. The pellet was hand-homogenized in buffer A, and the procedure of dilution, warming, and centrifugation was repeated twice to remove traces of trypsin from the membranes. The tryptic "19 kDa membranes" were resuspended at 2−5 mg/mL in buffer A and stored in ice.

Enzyme Phosphorylation by [γ-^{32}P]ATP. Phosphoenzyme formation was done manually for about 30 s at 0 °C (*25*). Aliquots of 50 μg of enzyme were incubated in a medium containing variable Na^+ concentrations (osmolarity was kept with choline), 30 mM imidazole (pH 7.5 at 0 °C), and 1 mM $MgCl_2$ with 10 μM [γ-^{32}P]ATP in a final volume of 100 μL. When present, Br-TITU was preincubated for 30 s. Phosphorylation was stopped by the addition of 1 mL of 20 mM CDTA, 10 mM P_i, and 1 mM ATP (final concentration). The dephosphorylation solution contained 0.1 mM KCl, final concentration. The reaction was stopped with 1 mL of 20 mM CDTA, 12% perchloric acid, 10 mM P_i, and 1 mM ATP. The $t = 0$ values for dephosphorylation were taken as those obtained by adding stopping solution after the phosphorylation periods.

Equilibrium Rb^+ Occlusion Measurements. The Rb^+ occlusion assay was performed as described (*26*, *27*). The assay is based on elution of the Rb^+− or Na^+−enzyme complex on Dowex-Tris 50W columns at 0 °C. After incubation, 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^+ was eluted with 1.0 mL of ice-cold 200 mM sucrose. $^{86}Rb^+$ was measured by Cerenkov radiation.

Rb^+ Occlusion through the Physiological Route. Occluded Rb^+ was measured as described by Rossi et al. (*28*) using $^{86}Rb^+$ (Perkin-Elmer NEN Life Sciences) as a K^+ congener. Briefly, reactions were carried out in a rapid-mixing apparatus (SFM4 from Bio-Logic) connected to a chamber that contained a Millipore filter through which an ice-cold solution of 30 mM KCl and 20 mM imidazole hydrochloride (pH 7.4 at 0 °C) was flowing at a rate of 40 mL/s. As the reaction mixture is injected into the chamber, quenching occurs due to a sudden drop in temperature and in ligand concentrations, and the enzyme is retained and washed in the Millipore filter.

All reactions took place at 25 °C in media with pH = 7.4. To measure the time course of Rb^+ occlusion starting from EP, the enzyme was first phosphorylated by mixing 1 volume of enzyme suspension (0.0737 mg of protein/mL) in a medium with 1 mM $MgCl_2$ (0.75 mM free Mg^{2+}), 150 mM NaCl, 25 mM imidazole hydrochloride, and 0.25 mM EDTA with 1 volume of the same medium with 20 μM ATP. After 2 s, the suspension with phosphorylated enzyme was mixed with a medium containing $^{86}Rb^+$, with or without Br-TITU (see legend to Figure 4A), and the resulting mixture

[1] Abbreviations: Br-TITU, 1-bromo-2,4,6-tris(methylisothiouronium)benzene; Br_2-TITU, 1,3-dibromo-2,4,6-tris(methylisothiouronium)-benzene; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HRBC, human red blood cells.

was aged for different time lengths before being squirted into the quenching and washing chamber.

The steady-state amount of occluded Rb^+ was measured after 3 s of reaction in media with the composition described in the legend to Figure 4B. To control steady-state conditions, samples with 1.03 mM Br-TITU were also incubated during 6 s, yielding results that were not significantly different from those obtained after a 3 s long incubation.

In all cases, blanks were estimated from similar experiments except that ATP was omitted during enzyme reactions.

$^{22}Na^+$ Efflux from Human Red Cells. The techniques have been described elsewhere (*29*, *30*). Briefly, fresh blood samples were taken from healthy donors, and cells were washed three times with ice-cold medium containing 140 mM choline chloride and 10 mM Tris-HCl, pH 7.5. Cells were incubated with $^{22}Na^+$ for 4−5 h at 37 °C in the same medium to load the cells with $^{22}Na^+$. Efflux was measured on the supernatant after 1 h at 37 °C. All experiments were run in triplicate.

Irreversible Inactivation of Na^+,K^+-ATPase by Br-TITU. In standard conditions, 50 μg of enzyme was suspended in 80 μL of a reaction medium containing 25 mM Tris−base, final pH 9.0, and varying concentrations of RbCl or NaCl plus choline chloride to preserve a constant ionic strength and was incubated with 0−400 μM Br-TITU at room temperature for 0−60 min as indicated. The reaction was stopped by adding a buffer containing 25 mM histidine and 1 mM EDTA, pH 5.0, to decrease the pH to a neutral value, and 100 mM RbCl. The mixtures were centrifuged in an airfuge for 20 min at 30 psi. The pellets were washed once with 10 mM Tris-HCl, 100 mM choline chloride, and 2 mM RbCl, pH 8.0, and resuspended in 85 μL of this buffer. Samples were assayed for Rb^+ occlusion or ATPase activity. In some cases protein was determined by the modified method of Lowry (*22*).

Because of the instability of the 19 kDa membranes in the absence of Rb^+ at pH 9.0, the conditions of inactivation by Br-TITU were slightly altered. 19 kDa membranes were suspended in 80 μL of reaction medium containing 25 mM Tris−base, either 125 mM RbCl or 125 mM choline chloride (to adjust the ionic strength), and 0−250 μM Br-TITU, final pH 9.0, and incubated at 10 °C in a cold water bath for 5 or 15 min. The procedure of stopping the reactions was exactly the same as for the control enzyme.

Tryptophan Fluorescence Measurements. Partially purified pig kidney renal Na^+,K^+-ATPase (50−80 μg) was suspended in 2.0 mL of 10 mM Tris-HCl, pH 9. Fluorescence measurements were performed using an ISS-K2 multifrequency phase fluorometer in the analog mode (1602 Newton Drive, Champaign, IL). Excitation and emission wavelengths were set to 295 and 325 nm, respectively. Slits widths were 8 nm, and the time constant was 0.2 s. All of the experiments were performed at room temperature.

Treatment of a Model Peptide with Br-TITU. The acetylated peptide Ac-WEWEWE (MW = 960) was dissolved in a 0.2 M Tris-HCl, pH 9, at 0.15 mM. Br-TITU (1 mg) was added (corresponding to 1.5 mM, or a 10-fold molar excess), and the contents of the tube were well mixed by vortexing for 1 h at room temperature. A sample without added Br-TITU was incubated as the control. The samples were then dialyzed overnight against 2 L of H_2O to remove excess Br-TITU and the solutions lyophilized. The concentrations of the peptide were determined by measuring the OD at 280 nm assuming $\epsilon = 16500\ M^{-1}$. The treated or untreated peptide was dissolved in 0.6 mL of 50% AcOH. Aliquots of each solution were set aside for injection into the HPLC, and aliquots were treated for 1 h with a 10-fold molar excess of Chloramine-T (added from a freshly prepared 40 mM solution). Samples of the Br-TITU-treated or control samples, or the Chloramine-T-treated samples, were injected onto a Chromolith Performance RP-18e column (100 × 4.6 mm, monolithic silica-based octadecyl reverse phase, Merck). The peptide or its reacted products were eluted at 2 mL/min with a linear gradient of acetonitrile produced by mixing solution A, 0.1% trifluoroacetic acid in water, with solution B, 0.1% trifluoroacetic acid in acetonitrile. The eluted peptides were detected by simultaneous detection of absorbance at 280 and 254 nm.

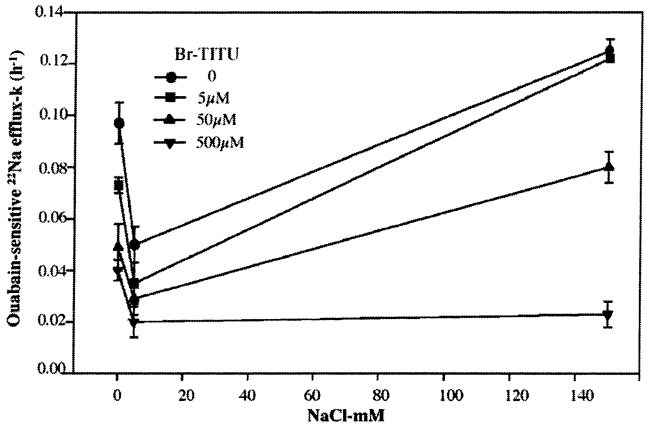

FIGURE 1: Effect of Br-TITU concentration on the Na^+-Na^+ exchange mode of the Na^+ pump in human red blood cells. HRBC were loaded with $^{22}Na^+$ for 5 h in 150 mM NaCl and 10 mM Tris-HCl at pH 7.5. After loading, cells were washed four times with cold 150 mM choline chloride and 10 mM Tris-HCl. Transport was assessed in Na^+ medium as described in the figure. Efflux was calculated from the amount of $^{22}Na^+$ lost after 1 h of incubation at 37 °C, pH 7.5.

Br-TITU, Br_2-TITU, and ^{14}C-Br-TITU synthesis was done as described previously (*14*).

The specific activity of the ^{14}C-Br-TITU was in the range of 120−180 mCi/mmol.

RESULTS

Effects of Br-TITU as a Reversible Blocker at the Extracellular Surface. (A) Br-TITU Blocks Na^+-Na^+ Exchange in Red Cells. To explore whether Br-TITU had an extracellular effect on the cation transport of the Na^+,K^+-ATPase, we looked at the Na^+-Na^+ exchange mode described before (*29*, *30*). Ouabain-sensitive $^{22}Na^+$ efflux was measured in the supernatant of the incubated red cells in the absence and in the presence of different Na^+ concentrations. As shown in Figure 1, Br-TITU inhibits the ouabain-sensitive Na^+-Na^+ exchange in the absence and in low (5 mM) or high (150 mM) [Na^+]. Br-TITU inhibition of the Na^+ transport is dose dependent, and it also has an effect in the absence of external Na^+. Upon washing the red cells free of Br-TITU in the medium, the inhibition is removed, indicating that Br-TITU is a reversible inhibitor in these incubation conditions of near neutral pH.

To further characterize this inhibition, the ouabain-sensitive efflux of Na^+ from human red blood cells as a function of

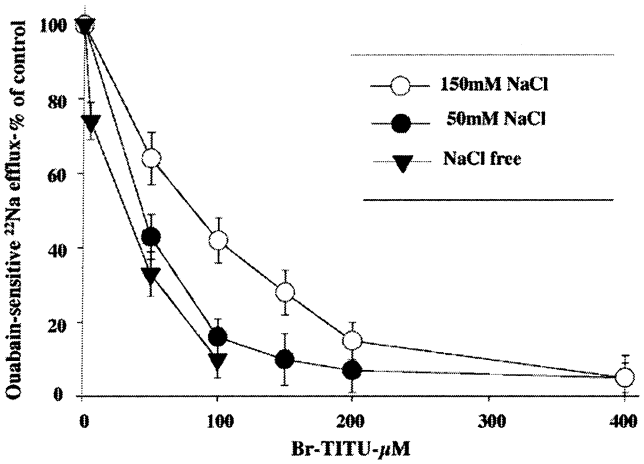

FIGURE 2: Effect of Br-TITU concentration on the ouabain-sensitive Na^+ efflux from red cells in the presence of 50 mM (filled circles) and 150 mM (open circles) extracellular Na^+. Data from another experiment performed in Na^+-free media are also included (filled triangles). The osmolarity was maintained with choline chloride. Notice that the $K_{0.5}$ for Br-TITU increases together with the external Na^+ concentration. The time for measuring $^{22}Na^+$ efflux was 1 h at 37 °C.

the concentration of TITU was followed at two concentrations of extracellular Na^+, 50 mM and 150 mM, or without extracellular Na^+. The results are illustrated in Figure 2. Within the dispersion of the values it is clear that the presence of Na^+ ions reduces the affinity for TITU inhibition. Thus, the $K_{0.5}$ for TITU is around 80 μM at 150 mM Na^+ and 40 μM at 50 mM Na^+, and data taken from Figure 1 indicate that without external Na^+ the $K_{0.5}$ for TITU is around 30 μM. Because the affinity for extracellular Na ions is low, a test for strict competition between Br-TITU and Na ions would require one to look at effects of NaCl concentrations higher than 150 mM, which is difficult experimentally. Nevertheless, the behavior of Br-TITU in Figures 1 and 2 is compatible with that of a reversible competitive blocker of Na ions at the extracellular surface.

(B) Br-TITU Blocks K^+-Catalyzed Dephosphorylation and Rb^+ Occlusion via the Physiological Route. Incubation of the Na^+,K^+-ATPase with ATP in the presence of Na^+ and Mg^{2+} ions at 0 °C phosphorylates the protein, and in the steady-state E_2P is the principal conformation present, with cation binding sites accessible to extracellular Na^+ and K^+ ions (*31*). In the absence of ions, dephosphorylation is slow. Na^+ ions somewhat accelerate and K^+ ions greatly accelerate dephosphorylation (*1*). If Br-TITU impairs the interaction of the extracellular cation with the Na^+ pump, one could expect it to inhibit effects of K^+ and Na^+ ions on the dephosphorylation rate. Partially purified renal enzyme, in the absence or presence of Br-TITU, was phosphorylated by ATP as described in Experimental Procedures, phosphorylation was stopped by addition of CDTA, ATP, and P_i, and the level of phosphoenzyme remaining after fixed times of dephosphoryation, without or with added K^+ ions, was then measured (Figure 3). In the presence of 500 μM Br-TITU and 120 mM Na^+ ions, the steady-state E−P level was about 70% of that without Br-TITU, a level which suffices for measurement of the dephosphoryation rate after arresting phosphorylation. As can be seen in Figure 3, Br-TITU diminished the dephosphorylation rate both in the absence and in the presence of K^+ ions. Thus, in the absence

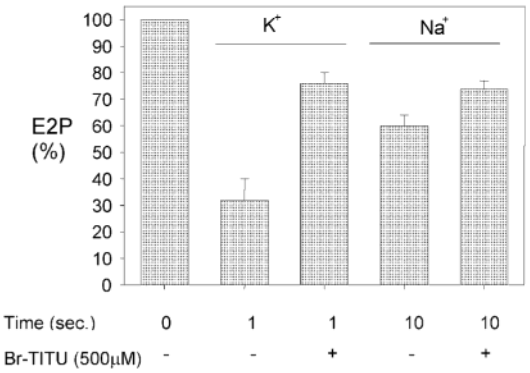

FIGURE 3: Effect of 500 μM Br-TITU on the dephosphorylation of the Na^+,K^+-ATPase. Purified phosphorylated Na^+,K^+-ATPase (50 μg) was incubated in the presence or absence of 500 μM TITU. The reaction was stopped after 1 s for KCl-dependent dephosphorylation (0.1 mM) or after 10 s for Na^+-dependent dephosphorylation. Incubation media: 120 mM NaCl, 1 mM $MgCl_2$, and 30 mM imidazole, pH 7.5. Dephosphorylating cations are indicated in the figure.

of K^+ ions, after 10 s of dephosphorylation, the amount of phosphoenzyme was 60 ± 4% (k = 0.054 ± 0.05 s^{-1}) without Br-TITU and 74 ± 3% (k = 0.036 ± 0.05 s^{-1}) with Br-TITU. When K^+ ions were present, a limiting concentration of 0.1 mM was used to moderate the rapid dephosphoryation sufficiently to allow manual estimation of the rate and allow calculation of rate constants. In this condition, the level of phosphoenzyme declined to 32 ± 8% (k = 1.24 ± 0.08 s^{-1}) after 1 s, while with Br-TITU present in the incubation media, the amount of phosphoenzyme remaining was 76 ± 4% (k = 0.31 ± 0.01 s^{-1}). The high concentration of Br-TITU required for the experiment (500 μM) is, of course, indicative of a rather low affinity at the extracellular surface in E_2−P and also competition by the monovalent cations present.

As could be expected from the blockage of K^+-stimulated dephosphorylation, Br-TITU decreased both the rate of Rb^+ occlusion from phosphorylated enzyme and the steady-state amount of occluded Rb^+ in partially purified renal Na^+,K^+-ATPase. Results are shown in Figure 4. Reactions were done at 25 °C and pH 7.4. To investigate the effect of Br-TITU on the rate of Rb^+ occlusion from E−P, the enzyme was first phosphorylated for long enough to reach a steady state (see Experimental Procedures), and then it was mixed with a solution containing $^{86}Rb^+$ with or without Br-TITU. Occluded Rb^+ was measured as a function of time, from 0.042 to 4−6 s after addition of Rb^+. Nonsaturating Rb concentrations were used in order to facilitate observation of the inhibitory effects of Br-TITU. Therefore, the amount of occluded Rb measured in the absence of Br-TITU reached a steady-state value of one-fourth to one-fifth of that obtainable at saturating concentrations of Rb. Results in Figure 4A show that addition of Br-TITU significantly slowed Rb^+ occlusion, decreasing the initial rate from 2.57 ± 0.11 to 0.718 ± 0.063 nmol/(mg of protein·s) as well as the rate coefficient for a single-exponential function of time from 2.94 ± 0.10 to 1.86 ± 0.14 s^{-1} in the absence or in the presence of 0.82 mM Br-TITU, respectively (see Figure 4 legend). The inhibition of the rate of Rb^+ occlusion caused by Br-TITU was reflected in a decrease of the steady-state amount of occluded Rb^+ (Figure 4B). Enzyme was incubated in media with ATP, Mg^{2+}, Na^+, $^{86}Rb^+$, and different

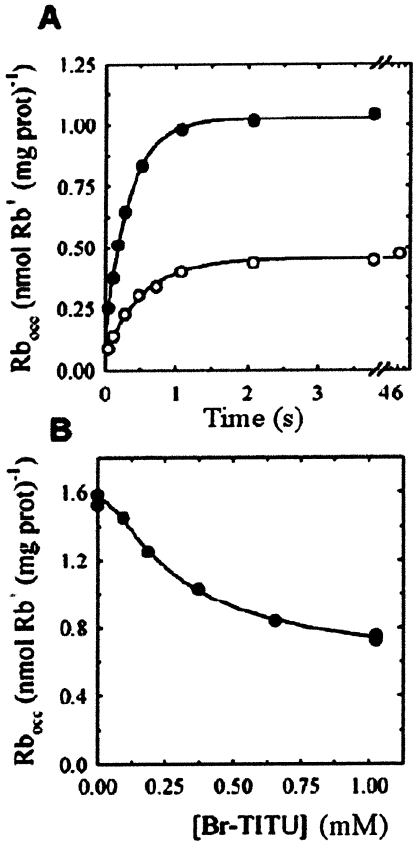

FIGURE 4: Effect of Br-TITU on Rb^+ occlusion via the physiological route. (A) Time course of Rb^+ occlusion in media with 0.0283 mg of protein/mL of enzyme, 9.6 μM ATP, 0.96 mM $MgCl_2$ (0.71 mM free Mg^{2+}), 81 μM Rb^+, 144 mM Na^+, 0.25 mM EDTA, 25 mM imidazole hydrochloride (pH 7.4 at 25 °C), and either 0 (closed circles) or 0.82 mM (open circles) Br-TITU, after addition of a solution containing $^{86}Rb^+$, without or with Br-TITU, to phosphoenzyme formed as described under Experimental Procedures. Each continuous line is a plot of the following function of time: $f(t) = A_0 + A_1[1 - \exp(-kt)]$. Best-fitting values of rate coefficients, k, and initial rates of Rb^+ occlusion, kA_1, are given in the main text. (B) Steady-state amount of occluded Rb^+ measured in media with 0.027 mg of protein/mL of enzyme, 10 μM ATP, 1 mM $MgCl_2$ (0.75 mM free Mg^{2+}), 100 μM RbCl, 150 mM NaCl, 0.25 mM EDTA, 25 mM imidazole hydrochloride (pH 7.4), and 0, 0.094, 0.188, 0.375, 0.656, or 1.03 mM Br-TITU. The continuous line is a plot of $f(x) = (N_0 + N_1x + N_2x^2)/(1 + D_1x + D_2x^2)$, where x = [Br-TITU] and whose parameters N_i and D_i were fitted to the data.

concentrations of Br-TITU during a time long enough as to reach steady state. Under these conditions, occluded Rb^+ is almost exclusively formed via the formation and breakdown of phosphorylated intermediates (*28*). It can be seen that an increase in [Br-TITU] from 0 to 1.03 mM decreased the amount of occluded Rb^+ along a sigmoid curve with a value of $K_{0.5}$ of about 0.85 mM. Note, again, that the high concentrations of Br-TITU required to block Rb occlusion by the physiological route reflect a low affinity for Br-TITU at the extracellular site of E_2−P, as well as competition by the Na and Rb ions present.

Taken together, the data in Figures 3 and 4 suggest strongly that Br-TITU binds with low affinity to an extracellular site of the Na^+,K^+ pump, thus preventing K^+ (Rb^+) ions from being occluded or activating dephosphorylation. This is consistent with the other data for an extracellular site presented in Figures 1 and 2.

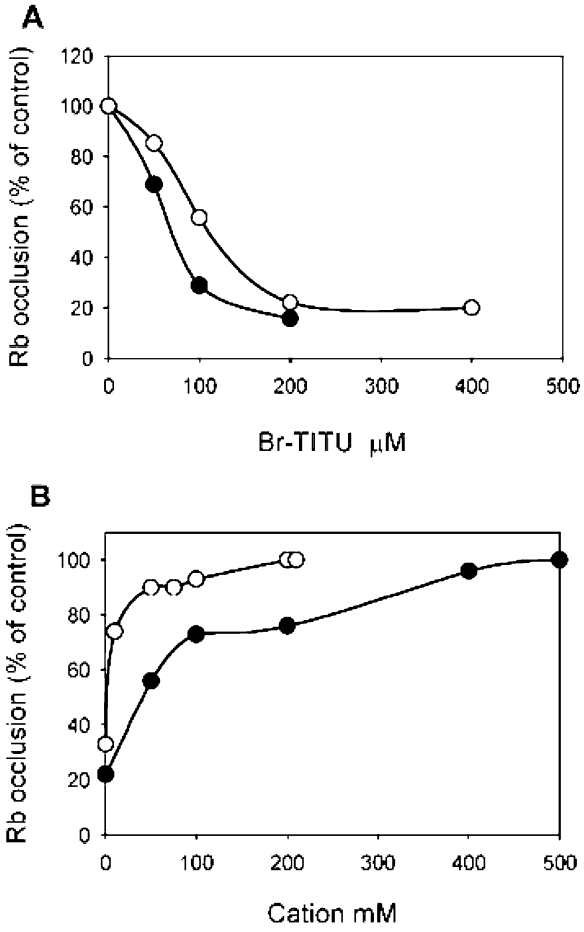

FIGURE 5: Irreversible inactivation by Br-TITU of the Rb^+ occlusion of Na^+,K^+-ATPase. (A) Native Na^+,K^+-ATPase (50 μg) was incubated for 60 min at room temperature in 25 mM Tris−base, pH 9.0, and 100 mM choline chloride medium in the presence of Br-TITU (●) or Br_2-TITU (○) (0−200 or 0−400 μM, respectively). (B) Na^+,K^+-ATPase (50 μg) was incubated for 60 min at room temperature in 80 μL of reaction medium, 0−200 mM RbCl (○), and 0−500 mM NaCl (●) in the presence of 100 μM Br-TITU, maintaining the ionic strength constant with choline chloride in each sample.

Irreversible Inactivation of Na^+,K^+-ATPase by Br-TITU at Alkaline pH. (A) Irreversible Inactivation of Rb Occlusion. By contrast with effects of Br-TITU at pHs below 8, which are fully reversed upon removal of Br-TITU, incubation of renal Na^+,K^+-ATPase with Br-TITU at pH 9 irreversibly inactivates Na^+,K^+-ATPase activity; i.e., the effect remains upon removal of the Br-TITU (*15*). The experiments in Figures 5 and 6 describe inactivation of Rb^+ occlusion. The enzyme was incubated at pH 9.0 with Br-TITU (or Br_2-TITU), the pH was then reduced to 7.0, and excess Br-TITU was removed by centrifuging the membranes. As seen in Figure 5A, the capacity for Rb^+ occlusion was irreversibly inactivated by either Br-TITU (0−200 μM) or Br_2-TITU (0−400 μM). The degree of inactivation was calculated as a percent of the control sample, incubated at pH 9 without Br-TITU, which inactivated Rb occlusion by less than 10%. Figure 5B demonstrates that the presence of either Rb^+ or Na^+ ions during the incubation at pH 9 protects fully against irreversible inactivation of Rb^+ occlusion, although rather high concentrations of the ions were required. On the basis of this finding, one could hypothesize that the

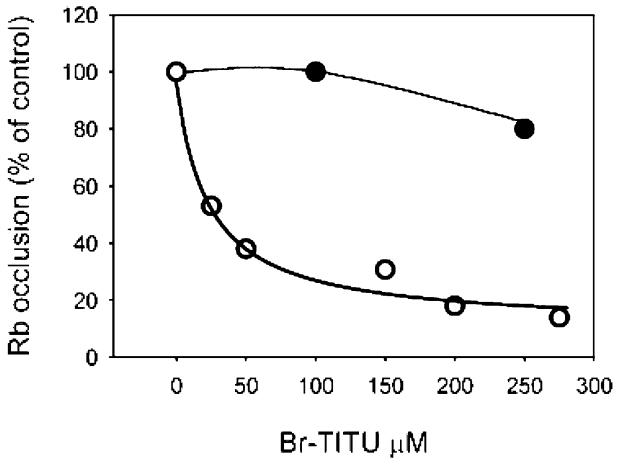

FIGURE 6: Irreversible inactivation of Rb occlusion in 19 kDa membranes. 19 kDa membranes (50 μg) were incubated for 20 min at 10 °C in 80 μL of reaction medium containing 25 mM Tris−base, pH 9.0, and 125 mM RbCl (●) or 125 mM choline chloride (○) in the presence of 0−275 μM Br-TITU. The capacity for Rb occlusion by the 19 kDa membranes was ~7 nmol/mg of protein, close to double the specific activity of the intact Na^+,K^+-ATPase from which they were prepared.

reaction occurs at residues near cation binding sites or in an access pathway to the sites.

A further indication that irreversible inactivation occurs at sites close to the membrane surface is provided by experiments using 19 kDa membranes, in which the cytoplasmic loops of the α subunit have been digested away by trypsin, leaving short cytoplasmic stalks, transmembrane segments, intact extracellular loops, and an intact or partially clipped β subunit (*24*). In this preparation cation occlusion and ouabain binding are preserved, but all ATP-dependent reactions are absent. Figure 6 shows that incubation with 250 μM Br-TITU at pH 9 completely inactivated Rb^+ occlusion, and the presence of Rb^+ ions fully protected against inactivation by Br-TITU. Inactivation was carried out at 10 °C since, in the absence of Rb^+, at pH 9.0, the 19 kDa membranes undergo rapid thermal denaturation (*16*).

(B) Sidedness of Irreversible Inactivation by Br-TITU. Irreversible inactivation of Na^+-Na^+ exchange in red blood cells has provided further evidence that this phenomenon occurs at the extracellular surface of the pump. After a short preincubation of $^{22}Na^+$-loaded red cells with Br-TITU at pH 9 or 7.5, in a medium containing 140 mM NaCl and 10 mM Tris-HCl, the cells were washed thoroughly at pH 7.5, thus removing the free Br-TITU, and ouabain-sensitive $^{22}Na^+$ efflux was measured in the Na^+-containing, K^+-free medium. As depicted in Figure 7A, a large fraction of the ouabain-sensitive $^{22}Na^+$ efflux was irreversibly inactivated in the cells preincubated with Br-TITU at pH 9, while no such inactivation occurred with cells preincubated with Br-TITU at pH 7.5. A similar effect was observed when red cells were preincubated with Br-TITU at pH 9 in the presence of 140 mM choline chloride and 10 mM Tris-HCl medium (Figure 7B). By contrast, when K^+ was the major cation in the preincubation medium (140 mM KCl, 10 mM Tris-HCl) irreversible inactivation of $^{22}Na^+$ efflux was prevented (Figure 7C). Potassium congeners, Cs^+ and Li^+, activate Na^+ efflux from red cells, but the affinities are quite different (*32*) with $K_{0.5}$ values of 0.1 mM for K^+, 0.3 mM for Cs^+, and 5 mM for Li^+ (not shown). The congeners were tested

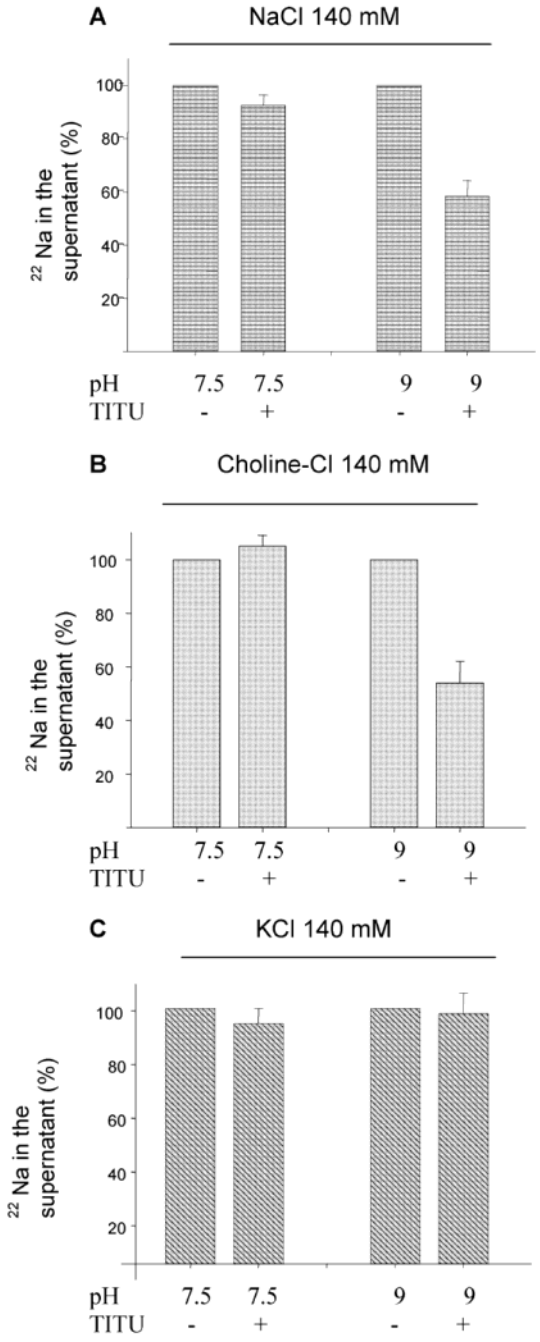

FIGURE 7: Irreversible inactivation of ouabain-sensitive Na^+ efflux by TITU. HRBC were washed with 140 mM choline chloride and 10 mM Tris-HCl, pH 7.5. Cells were loaded with $^{22}Na^+$ and washed, and preincubations were made at 37 °C with or without TITU (200 μM) for 30 s at pH 7 or 9. After preincubation the cells were washed free of Br-TITU, and the $^{22}Na^+$ efflux was measured in a medium containing 140 mM NaCl and 10 mM Tris-HCl, pH 7.5. Preincubation conditions: (A) 140 mM NaCl, 10 mM Tris-HCl; (B) 140 mM choline chloride, 10 mM Tris-HCl; (C) 140 mM KCl, 10 mM Tris-HCl.

for their ability to prevent irreversible inhibition by Br-TITU. When present during the preincubation, K^+, Cs^+, and Li^+ prevented inhibition. It is noteworthy that the apparent affinity for all three cations for this protective effect was similar ($K_D \sim 5$ mM) (Figure 8), despite the fact that Cs^+, Li^+, and K^+ have completely different affinities for the external activation effect of the Na^+ pump (see also ref *32* and the Discussion).

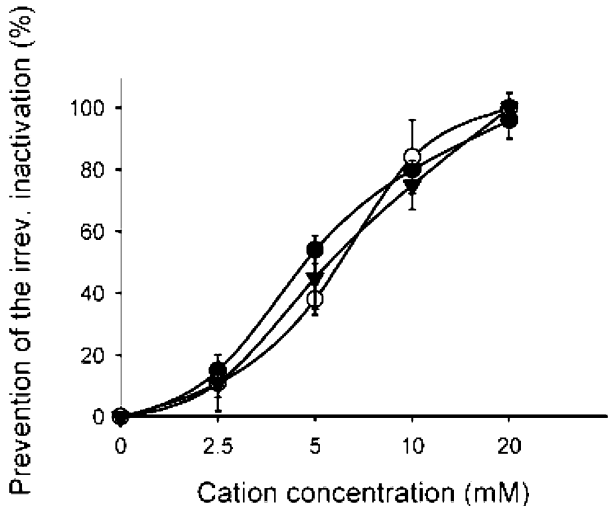

FIGURE 8: Protection by different cations against irreversible inactivation by Br-TITU. HRBC loaded with $^{22}Na^+$ were preincubated for 30 s in KCl (●), CsCl (▼), or LiCl (○) medium supplemented with choline chloride (up to a 140 mM), 10 mM Tris-HCl, pH 9, and 200 μM Br-TITU. After preincubation, cells were washed in cold 140 mM choline chloride and 10 mM Tris-HCl, pH 7.5, and $^{22}Na^+$ efflux was measured after 1 h incubation in 140 mM NaCl and 10 mM Tris-HCl, pH 7.5, at 37 °C.

Nature of Residues Interacting with Br-TITU at Alkaline pH. Our initial hypothesis was that, at pH 9, Br-TITU chemically modifies lysine or cysteine, or perhaps residues such as serine and threonine, by nucleophilic substitution of the side chain on the carbon atom of the isothiouronium moiety, thus leading to covalent incorporation of the uronium moiety into the protein and irreversible inactivation of the Na^+,K^+-ATPase activity (see mechanism in Figure 12A). Accordingly, a ^{14}C-labeled derivative of Br-TITU (^{14}C in the isothiouronium moiety) was prepared and incubated with the enzyme in conditions of irreversible inactivation of Rb^+ occlusion without and with Rb ions (results not shown). Substantial covalent labeling was observed, amounting to about 10 mol of ITU moieties/mol of enzyme, which was reduced to about 2 mol of ITU/mol of enzyme after treatment with β-mercaptoethanol, which should remove the ITU moieties from cysteine residues. However, Rb occlusion was not restored by the β-mercaptoethanol treatment. Furthermore, the covalent labeling remaining after β-mercaptoethanol treatment was not protected against by the presence of Rb ions, by contrast to irreversible inactivation of Rb occlusion, which is strongly protected against. Thus, the covalent labeling is unspecific, and contrary to the initial expectation, inactivation of Rb occlusion cannot be explained by specific chemical modification of cysteine, lysine, or any other nucleophilic side chains (Figure 12A). Thus, an alternative hypothesis is required.

Our recent observation that Br-TITU quenches tryptophan fluorescence of sarcoplasmic reticulum Ca^{2+}-ATPase (*20*) suggests that Br-TITU might also interact with tryptophan residues in Na^+,K^+-ATPase. Figure 9A shows that Br-TITU quenches the fluorescence of the free amino acid tryptophan at pH 9. This quenching occurs rapidly upon addition of Br-TITU. A similar rapid quenching is observed at pH 7. Figure 9B shows the excitation and emission spectra of partially purified Na^+,K^+-ATPase in the presence and absence of Br-TITU at pH 9. The emission spectra were substantially

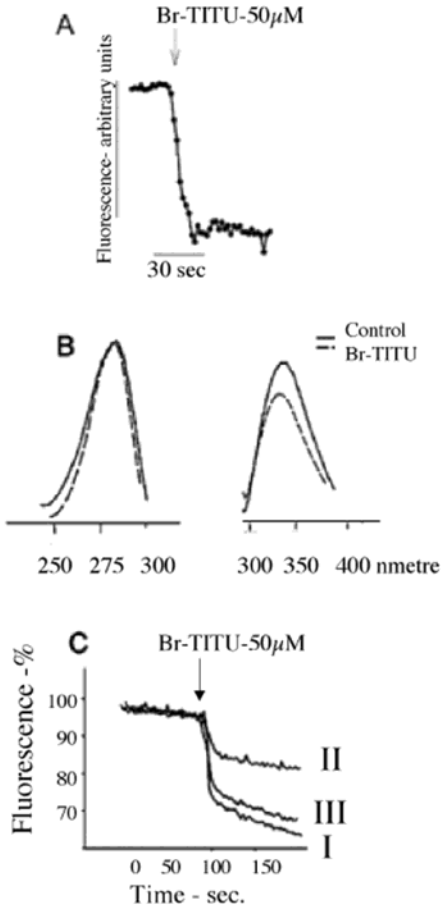

FIGURE 9: Quenching of tryptophan fluorescence by Br-TITU. (A) 100 μM Trp was suspended in 2.0 mL of 10 mM Tris-HCl, pH 9. Addition of 50 μM Br-TITU is shown. (B) Emission (right) and excitation (left) spectra of intrinsic fluorescence of Trp in Na^+,K^+-ATPase. 60 μg of enzyme was suspended in 2.0 mL of 100 mM choline chloride and 10 mM Tris-HCl, pH 8.1, at 20 °C. 200 μM TITU was added and changes were recorded. (C) (Trace I) Na^+,K^+-ATPase (50−80 μg) was suspended in 2.0 mL of 10 mM Tris-HCl, pH 9. Emission wavelength at 340 nm was recorded showing the effect of Br-TITU (50 μM). (Trace II) Same as trace I but 20 mM KCl was present in the medium. (Trace III) Same as trace I but preincubated with 10^{-4} M ouabain, 1 mM $MgCl_2$, and 1 mM P_i.

diminished by Br-TITU when compared with the control. This quenching was fast and constant in time, demonstrating an effect of Br-TITU on the renal enzyme. Figure 9C shows that the rapid Br-TITU-induced quenching of the tryptophan fluorescence occurs regardless of the initial conformational state of the pump, E_1 (trace I) or E_2 (trace III). Interestingly, the presence of K^+ in the incubation medium partially protected against this quenching (trace II). It is important to note that the rapid quenching at pH 7 is reversed by removal of Br-TITU by dilution or centrifugation.

The effect of Br-TITU on the tryptophan fluorescence of the Na^+,K^+-ATPase was then measured on samples pretreated with Br-TITU at pH 9, 37 °C, and up to 60 min (Figure 10), conditions which should be accompanied by irreversible inactivation of occlusion or Na^+,K^+-ATPase activity (*15*). Br-TITU was then removed from the medium by centrifugation or by dilution, and fluorescence was measured. Figure 10 (upper panel) shows that, in these conditions, the tryptophan fluorescence is progressively and irreversibly lost and the presence of Rb^+ ions partially

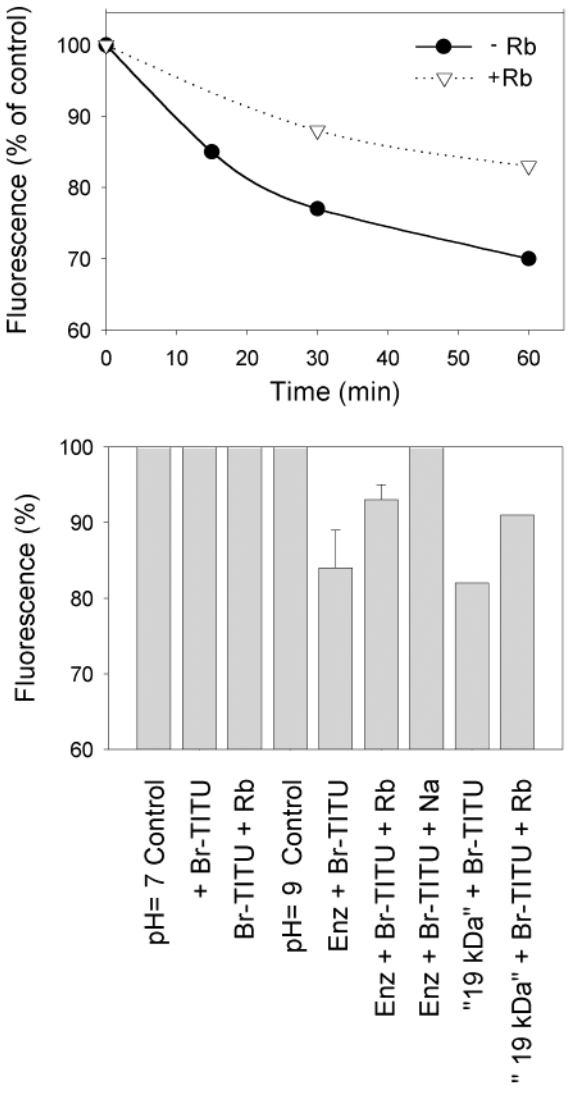

FIGURE 10: Irreversible loss of tryptophan fluorescence of Na^+,K^+-ATPase induced by incubation with Br-TITU at pH 9. Upper panel: Na^+,K^+-ATPase (1 mg/mL) was incubated for 0−60 min at 37 °C in media containing 25 mM Tris-HCl, pH 9.0, and 200 mM choline chloride or 200 mM RbCl in the presence of 400 μM Br-TITU. Lower panel: Na^+,K^+-ATPase (1 mg/mL) was incubated for 45 min at 37 °C in media containing 25 mM Tris-HCl, pH 7.0 or 9.0, and either 200 mM choline chloride, 200 mM RbCl, or 500 mM NaCl in the presence of 400 μM Br-TITU. 19 kDa-membranes (1 mg/mL) were incubated for 20 min at 10 °C in media containing 25 mM Tris-HCl, pH 9.0, with 100 mM choline chloride or 100 mM RbCl in the presence of 200 μM Br-TITU. After incubation, the membranes were diluted >200-fold into the fluorescence cell containing 25 mM Tris-HCl, pH 7.0, plus 200 mM choline chloride (upper), or the membranes were first pelleted in a Beckman TLX centrifuge before resuspension in the same medium (lower). The tryptophan fluorescence amplitude of 40 μg of protein was then measured: emission 335 nm and excitation 295 nm.

protects. The bar graph in Figure 10 (lower panel) shows that no loss of fluorescence occurred if the preincubation with Br-TITU was done at pH 7 rather than pH 9. In Figure 10 (lower panel), preincubation at pH 9.0 led to an average loss of fluorescence of about 15% of the total signal by comparison with control, incubated at pH 9, without Br-TITU, which did not affect the tryptophan fluorescence. The irreversible loss of fluorescence was partially prevented by the presence of Rb^+ or fully prevented by Na^+ ions. Similarly, the preincubation of Br-TITU at pH 9 with 19 kDa membranes produced an irreversible loss of fluorescence, which was partially protected by the presence of Rb^+ ions (Figure 10B). This loss of fluorescence at pH 9 was not reversed by removing the Br-TITU by centrifugation and resuspension in a medium at pH 7.4, even after an incubation of several hours. Thus, overall, the time course, pH dependence, and protection by the monovalent cations are similar to the irreversible inactivation of Rb^+ occlusion or Na^+,K^+-ATPase activity.

Tryptophan residues are frequently found at membrane−water interfaces of integral membrane proteins (*33*). In the present context, a relevant piece of information, obtained with the ^{14}C-Br-TITU derivative, is that the 1-octanol:water distribution coefficient is 1:350 at pH 7 but it rises steeply to 1:1 at pH 9 (result not shown). The 1-octanol:water distribution coefficient provides a good estimate of the lipid:water distribution coefficient in biological membranes, which determines passive permeation (*34*). The p*K* of the isothiouronium group is 9.8. At pH 9, a sufficient fraction of Br-TITU must be unprotonated and uncharged and able to enter the lipid, while at pH 7 it is triply positively charged and excluded from the lipid. Thus, it is likely that the relevant tryptophan residue(s) is (are) within the lipid or at the lipid−water interface.

Does Br-TITU Chemically Modify Tryptophan Residues? Because the loss of fluorescence at pH 9 is irreversible, one could hypothesize that Br-TITU acts as a chemical modifying reagent for tryptophan. Br-TITU contains three benzylic carbon atoms, like the well-known tryptophan-specific Koshland's reagent, 2-hydroxy-5-nitrobenzyl bromide (*35*). Incubation of the renal enzyme with Koshland's reagent lead to loss of both Na^+,K^+-ATPase activity and tryptophan fluorescence but no protection by Rb^+ ions, implying a nonselective effect of the tryptophan-specific reagent (result not shown). A more direct test for the possibility that Br-TITU acts as a tryptophan-modifying reagent utilized a model peptide containing three tryptophan residues, Ac-WEWEWE. The peptide was incubated with or without Br-TITU at pH 9 for 1 h, and after dialysis to remove excess Br-TITU, samples were injected on a C18 reverse-phase HPLC column or first oxidized with Chloramine-T and then injected onto the column. If Br-TITU reacts with the tryptophans of Ac-WEWEWE, similarly to Koshland's reagent, the indole ring could become alkylated at the 2 position (hypothetically as in Figure 12B). The product of such a reaction should run differently on HPLC compared to the unreacted peptide. Chloramine-T oxidizes indoles at the 2 position (as in Figure 12C) (*36*). If the Br-TITU does indeed alkylate the indole ring, the reacted peptide should be protected against Chloramine-T. The Br-TITU-treated and control Ac-WEWEWE elute at precisely the same position (Figure 11, upper panels) as do both the Chloramine-T-treated peptides (Figure 11, lower panels). The Chloramine-T-treated samples showed two peaks, identical for the Br-TITU-treated or control samples. The peak at 3.35 mL is a reduced product of Chloramine-T, as shown in separate control experiments. Thus the peak at 11.5−11.8 mL is presumed to be the oxidized peptide. This is supported by the fact that the elution position corresponds to a more hydrophilic peptide than the Ac-WEWEWE itself and the fact that the absorption at 280 nm is almost destroyed while that at 254 nm was intact, as expected for oxidation as in

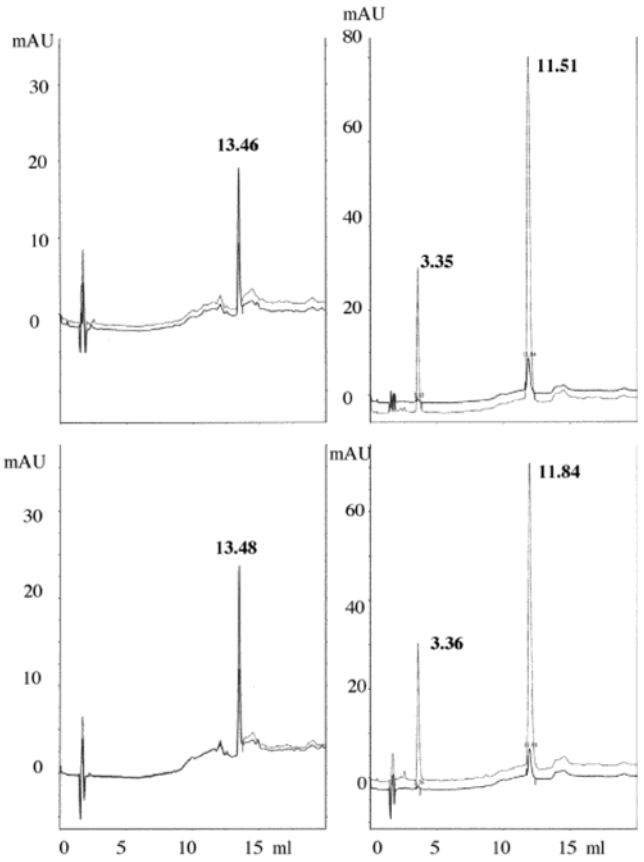

FIGURE 11: HPLC of Ac-WEWEWE, treated or not treated with Br-TITU at pH 9, and the Chloramine-T oxidized peptides: absorption at 280 nm, continuous lines; absorption at 254 nm, dotted lines.

the mechanism in Figure 12C (*36*). Overall, the HPLC results in Figure 11 show conclusively that the Br-TITU did not chemically modify the peptide.

DISCUSSION

Br-TITU Acts as an Extracellular Cation Blocker with Low Affinity. Evidence that Br-TITU can act as a reversible blocker of cation effects at the extracellular surface was obtained from observations of inhibition in red cells of the Na^+-Na^+ exchange and uncoupled Na^+ efflux modes of the pump. At zero concentration of extracellular Na^+ ions and pH 7.5, the observed $K_{0.5}$ was $\approx$30 μM. This affinity of Br-TITU is much lower than that of Br-TITU at its intracellular site, described previously ($K_D \approx 1$ μM at pH 7.5) (*15*). The inhibitory effects of Br-TITU on the rates of both the K^+-dependent hydrolysis of phosphoenzyme and the occlusion of Rb^+ from EP, obtained here using unsided preparations of renal Na^+,K^+-ATPase, also exhibited low affinity and are consistent with Br-TITU acting at the extracellular surface. In addition, the sigmoid shape of the curve for the steady-state amount of occluded Rb^+ vs [Br-TITU] indicates that the mechanism for inhibition involves more than one pathway in the reaction cycle. The current data fit well with the findings in Hoving et al. (*15*), which suggested the simultaneous existence of two sites for Br-TITU, a high-affinity cytoplasmic site and an additional low-affinity site, and show that the low-affinity site is extracellular.

Irreversible Inactivation by Br-TITU from the Extracellular Surface. After preincubation of the renal Na^+,K^+-ATPase or trypsinized 19 kDa membranes with Br-TITU at pH 9 and then removal of the Br-TITU, Rb^+ occlusion is irreversibly inactivated. In both instances the presence of K^+ (Rb^+) or Na^+ ions at high concentrations protects against this inactivation (Figure 5). Inactivation of Na^+ efflux from red cells after preincubation with Br-TITU at pH >8 in a K-free medium (i.e., containing either NaCl or choline chloride) (Figure 7A) indicates that the irreversible inactivation occurs at an extracellular location. The presence of K^+ or two of its congeners, Cs^+ and Li^+, during the preincubation protected against irreversible inactivation. The latter result has two important implications. First, protection by K^+ ions strongly supports extracellular access to the Br-TITU-interactive residues. As mentioned above the p*K* of the isothiouronium moiety is about 9.8, and the Br-TITU appears to be able to penetrate the lipid at pH 9. However, the concentration of Br-TITU is unlikely to reach that outside the cells, and the presence of about 140 mM K^+ inside the cells should protect strongly against irreversible inactivation at the cytoplasmic surface. The second important point is that K^+, Cs^+, and Li^+ protect against the Br-TITU with a similar affinity, $\sim$5 mM. As discussed below, this finding is significant because these cations display quite different apparent affinities for external activation of the Na^+ pump [K^+, 0.1 mM; Cs^+, 0.3 mM; Li^+, 5 mM (*32*)].

Is There an Extracellular Cation Entrance Site? At near neutral pH the Br-TITU binds reversibly and blocks access of K^+ and Na^+ to their occlusion site within the transmembrane segments. This may be similar to the effect of a variety of bulky amines which block access of K^+ to extracellular sites, albeit with a much lower affinity than Br-TITU (*37*, *38*). The protection by the monovalent cations against irreversible inactivation is of rather low affinity (Figure 5), and there is no selectivity between K^+, Cs^+, and Li^+ (Figure 8), which is quite different from the order found for transport. The implication is that the Br-TITU is interacting with residues in the entrance region located prior to the occlusion sites, and the cations protect by binding to this region in a less selective fashion than for binding to the occlusion sites within transmembrane segments. The accumulated data on both the reversible and irreversible effects of Br-TITU and protection by monovalent cations are consistent with the existence of an extracellular entrance site, which is recognized either by the cation blocker, Br-TITU, or by transported cations. Overall, the current and previous evidence using Br-TITU (*15*) indicates that entrance sites recognizing either cationic blockers or transported cations exist at both surfaces.

Br-TITU Interacts with Tryptophan Residues. As inferred from the data in Figure 9, chemical modification by nucleophilic attack on the isothiouronium moiety of Br-TITU by lysine, cysteine, or any other nucleophilic side chains (Figure 12A) cannot explain irreversible inactivation of Rb occlusion. Neither is the hypothesis of chemical modification of tryptophan via the benzylic CH_2 groups at Br-TITU (Figure 12B) supported by experiments with the model peptide Ac-WEWEWE (Figure 11). In addition, the pH 9 dependence of the irreversible inactivation is not an expected feature of chemical modification of tryptophans.

The mechanism of irreversible inactivation appears to involve a strong but noncovalent interaction of Br-TITU with tryptophan(s) which interfere(s) with cation occlusion and Na^+,K^+-ATPase activity. The finding that Br-TITU is able

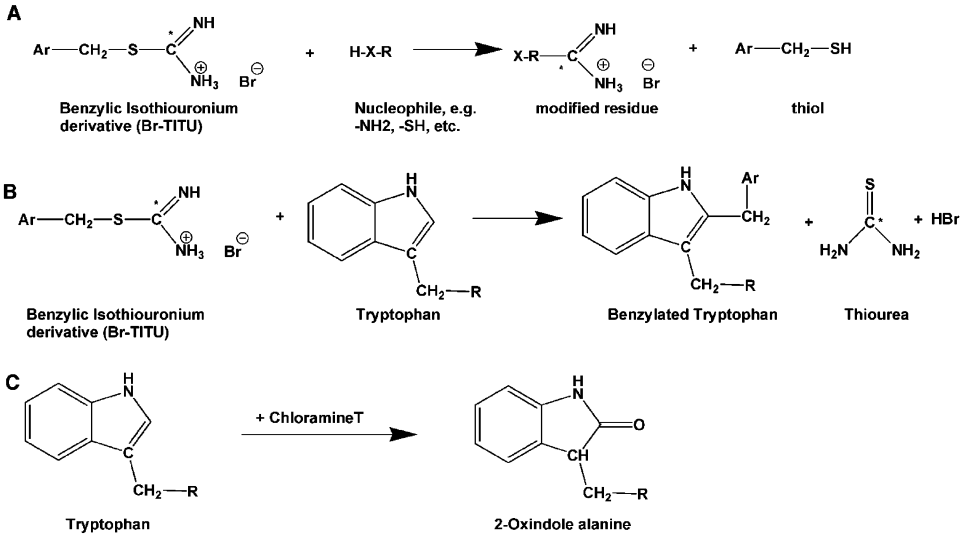

FIGURE 12: Hypothetical reaction mechanisms for chemical modifications by Br-TITU.

to penetrate the lipid at pH 9 but not at pH 7 implies that the relevant tryptophan residues are located within the lipid. In principle, Br-TITU could enter the lipid bilayer from either side of the membrane, but the experiment of Figure 7 shows that irreversible inactivation occurs at the extracellular surface. Thus one can infer that the tryptophan residue(s) is within the lipid bilayer near the extracellular membrane−water interface. Strong interactions within the lipid could account for the fact that they are not reversed by removing excess Br-TITU from the medium. As seen in Figure 9, Br-TITU quenched the fluorescence of free tryptophan and also the intrinsic fluorescence of the renal Na^+,K^+-ATPase (Figure 9A), demonstrating a direct interaction between tryptophan and Br-TITU. About 30% of the total fluorescence of the enzyme is quenched. At pH 9 the quenching is rapid ($t_{1/2} \sim 1$ s), and K^+ in the incubation media partially prevented quenching. These effects are consistent with the notion that Br-TITU interacts rapidly with accessible tryptophan residues and quenches their fluorescence. This quenching takes place when the enzyme is present initially in either an E_1 (trace I) or E_2P (trace III) conformation. Since the pig α subunit contains 12 tryptophans and the β subunit 4 tryptophan residues, one could infer that only a fraction are accessible to the Br-TITU, say five residues (30% of the total), and of these residues, K^+ ions interfere with the interaction of Br-TITU to one or two residues (trace II). The conditions of Figure 10, namely, a 45−60 min incubation with Br-TITU at pH 9 and then removal of Br-TITU, led to irreversible loss of about 20−30% of the fluorescence. Rb^+ ions protect partially and Na^+ ions appear to fully protect against the irreversible loss of 18% of the fluorescence (Figure 10, lower panel). One could propose that only 1 or 2 of the 12 residues in the α subunit are involved in the strong but noncovalent interactions with tryptophans, presumably via $\pi-\pi$ interactions. These residues, as we infer, are within the lipid or at the extracellular membrane−water interface. It is interesting to note that of the 12 tryptophans in the α subunit, the majority are either within the transmembrane segments (W98 in M1, W924 in M8, W980 and W981 in M10) or in extracellular loops (W310 in L3/4, W883, W887, W899 in L7/8), and these are well conserved in the α subunit.

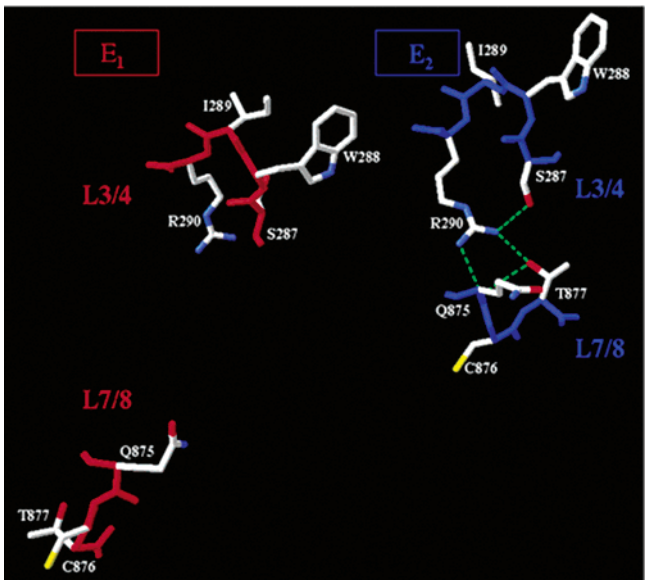

FIGURE 13: Model of L3/4 and L7/8 of Ca^{2+}-ATPase in E_1 and E_2 conformations. Ca^{2+}-ATPase structures in E1 (1eul.pdb, red backbone) and E2 (1iwo.pdb, blue backbone) conformations were visualized using the Swisspdb Viewer.

The conclusion on the location of the relevant tryptophan(s) is consistent with a known tendency of tryptophans to be located at the boundary between hydrophilic and hydrophobic regions of transmembrane segments of membrane proteins (*33*). It is also known that cations can interact with the π electron cloud of the aromatic ring (*39*, *40*). Computer calculations (*41*), mass spectrometric studies (*42*), and the development of synthetic receptors incorporating the aromatic side chains of phenylalanine, tyrosine, and tryptophan (*43*) have demonstrated crystallization of these structures in the presence of K^+ ions, showing the π system of aromatic rings to coordinate the cation. Another speculative possibility is suggested by comparing the atomic structures of the Ca^{2+}-ATPase in E_1 and E_2 conformations (*8*). Figure 13 shows the L3/4 and L7/8 loops of Ca^{2+}-ATPase in the E_1 (red backbone) and the E_2 conformations (blue backbone). Whereas in the E_1 conformation the loops are well separated, in the E_2 structure there are close interactions,

including several H-bonds. It was suggested that the closed L3/4 L7/8 loop structure could serve as a "gate" to prevent access of cations to and exit from the occlusion sites, assuming that the structure represents a state with occluded protons, i.e., E_2·(2H) (*8*). The interacting sequences of Ca^{2+}-ATPase are ^{287}SWLR in L3/4 and ^{875}QCT in L7/8. The counterparts in the Na^+,K^+-ATPase are ^{309}TWLE and ^{898}QWT. If either of these tryptophans interacted with Br-TITU, this could have the effect of destroying the gate and inactivating cation occlusion and thus Na^+,K^+-ATPase activity or transport activities such as Na^+-Na^+ exchange. A similar explanation could apply to inhibition of Ca^{2+}-ATPase by Br-TITU (*20*).

The test of these different hypotheses concerning tryptophan residues is to mutate the different tryptophan residues and examine functional effects (e.g., cation occlusion) and sensitivity to Br-TITU inactivation. Such experiments are being planned, using pig kidney Na^+,K^+-ATPase expressed in the yeast *Pichia pastoris* (D. Strugatsky and S. J. D. Karlish, unpublished work).

ACKNOWLEDGMENT

G.A.Y. thanks Sebastian Marun for help at the Hospital Privado, Córdoba, Argentina. We are grateful to Prof. Yoram Shechter, Department of Biological Chemistry, for suggesting the experiments and providing a sample of the model peptide, Ac-WEWEWE.

REFERENCES

1. Glynn, I. M. (1993) *J. Physiol. 462*, 1−30.
2. Moller, J. V., Juul, B., and le Maire, M. (1996) *Biochim. Biophys. Acta 1286*, 1−51.
3. Axelsen, K. B., and Palmgren, M. G. (1998) *J. Mol. Evol. 46*, 84−101.
4. Geering, K. (2001) *J. Bioenerg. Biomembr. 33*, 425−438.
5. Sweadner, K. J., and Rael, E. (2000) *Genomics 68*, 41−56.
6. Jørgensen, P. L., Hakansson, K. O., and Karlish, S. J. D. (2003) *Annu. Rev. Physiol. 65*, 817−849.
7. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000)- *Nature 405*, 647−655.
8. Toyoshima, C., and Nomura, H. (2002) *Nature 418*, 598−599.
9. Andersen, J. P., and Vilsen, B. (1998) *Acta Physiol. Scand., Suppl. 643*, 45−54.
10. Ogawa, H., and Toyoshima, C. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 15977−15982.
11. Rakowski, R. F., Gadsby, D. C., and De Weer, P. (1997) *J. Membr. Biol. 155*, 105−112.
12. Apell, H. J., and Karlish, S. J. D. (2001) *J. Membr. Biol. 180*, 1−9.
13. David, P., Mayan, H., Cohen, H., Tal, D. M., and Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1141−1149.
14. Tal, D. M., and Karlish, S. J. D. (1995) *Tetrahedron 51*, 3823−3830.
15. Hoving, S., Bar-Shimon, M., Tijmes, J. J., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1995) *J. Biol. Chem. 270*, 29788−29793.
16. Or, E., David, P., Shainskaya, A., Tal, D. M., and Karlish, S. J. D. (1993) *J. Biol. Chem. 268*, 16929−16937.
17. Shainskaya, A., Schneeberger, A., Apell, H. J., and Karlish, S. J. D. (2000) *J. Biol. Chem. 275*, 2019−2028.
18. Menguy, T., Corre, F., Bouneau, L., Deschamps, S., Moller, J. V., Champeil, P., le Maire, M., and Falson, P. (1998) *J. Biol. Chem. 273*, 20134−20143.
19. Menguy, T., Corre, F., Juul, B., Bouneau, L., Lafitte, D., Derrick, P. J., Sharma, P. S., Falson, P., Levine, B. A., Moller, J. V., and le Maire, M. (2002) *J. Biol. Chem. 277*, 13016−13028.
20. Berman, M. C., and Karlish, S. J. D. (2003) *Biochemistry 42*, 3556−3566.
21. Jørgensen, P. (1974) *Biochim. Biophys. Acta 256*, 36−52.
22. Markwell, M. A. K., Hass, S. M., Bier, L. L., and Tolbert, N. E. A. (1978) *Anal. Biochem. 269*, 206−210.
23. Beaugé, L. A., and Campos, M. A. (1986) *Biochim. Biophys. Acta 729*, 137−149.
24. Capasso, J. M., Hoving, S., Tal, D. M., Goldshleger, R., and Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1150−1158.
25. Beaugé, L. A. (2001) *Eur. J. Biochem. 268*, 5627−5632.
26. Shani, M., Goldshleger, R., and Karlish, S. J. D. (1987) *Biochim. Biophys. Acta 904*, 13−21.
27. Shani-Sekler, M., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1988) *J. Biol. Chem. 263*, 19331−19341.
28. Rossi, R. C., Kaufman, S. B., González-Lebrero, R. M., Nørby, J. G., and Garrahan, P. J. (1999) *Anal. Biochem. 270*, 276−285.
29. Garrahan, P. J., and Glynn, I. M. (1967) *J. Physiol. 192*, 159−174.
30. Garrahan, P. J., and Glynn, I. M. (1967) *J. Physiol. 192*, 189−216.
31. Beaugé, L. A., and Glynn, I. M. (1979) *J. Physiol. 289*, 7−31.
32. Robinson, J. D. (1975) *Biochim. Biophys. Acta 384*, 250−264.
33. Popot, J.-L., and Engelman, D. M. (2000) *Annu. Rev. Biochem. 69*, 881−922.
34. Stein, W. D. (1986) in *Transport and Diffusion across Cell Membranes*, Chapter 2, pp 86−93, Academic Press, Orlando, FL.
35. Means, G. E., and Feeney, R. F. (1971) *Chemical Modification of Proteins*, p 123, Holden-Day, New York.
36. Schechter, Y., Burstein, Y., and Patchornik, A. (1975) *Biochemistry 14*, 4497−4503.
37. Kropp, D. L., and Sachs, J. R. (1977) *J. Physiol. (London) 264*, 471−487.
38. Forbush, B. (1988) *J. Biol. Chem. 263*, 7979−7988.
39. Hu, J., Barbour, L. J., and Gokel, G. W. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5121−5126.
40. Lane, D. J., Grell, E., Bamberg, E., and Clarke, R. J. (1998) *Biochemistry 37*, 4581−4591.
41. Ma, J. C., and Dougherty, D. A. (1997) *Chem. Rev. 97*, 1303−1324.
42. Ryzhov, V., Dunbar, R. C., Cerda, B., and Wesdemiotis, C. (2000) *J. Am. Soc. Mass Spectrom. 11*, 1037−1046.
43. De Wall, S. L., Meadows, E. S., Barbour, L. J., and Gokel, G. W. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6271−6276.

BI0342721