The Interaction of Amines with the Occluded State of the Na,K-Pump*

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We have studied the effect of various amines on the rate of release of 86Rb from the occluded state of dog kidney Na,K-ATPase formed by pre-incubation of the enzyme with 86Rb. In the presence of MgPi, various amines act like K+ or Rb+ in blocking the release of 86Rb from one of two sites for occlusion (the "s" site). Of 38 amines tested, tetrapropylamine and various benzyl amines exhibit the highest affinity; the Ks for these compounds is 2–5 mM. In the presence of ATP, when 86Rb is presumably released towards the intracellular face of the pump in the normal mode of operation, 86Rb release is blocked by the presence of amine, but only if the amine is also included in a preincubation with MgPi. The data are consistent with a model in which the interaction of amine with one of the transport sites (the "f" site) prevents the Eα → Eβ transmutation that is stimulated by ATP. When 86Rb deocclusion from the f site has occurred in the presence of amine, the lone 86Rb at the s site can be released in the presence of ATP if the amine is removed from the medium. This suggests that a single 86Rb ion at the s site can be released to the intracellular face of the membrane, and therefore that transport can occur with only one K+ site occupied. The amine that blocks release of one 86Rb ion does not itself become occluded: (a) The interaction of amine and ATP is only seen when both ligands are present in the medium; (b) the effects of amines are not "remembered" after a brief exposure to a rinse medium; (c) with the vanadate-inhibited enzyme, benzyltriethylamine and tetrapropylamine are only weakly effective in blocking 86Rb release from the s site; and (d) organic cations exhibit very low affinity in competition with 86Rb for occlusion at equilibrium. Thus the results are consistent with the idea that monofunctional amines block by binding to the f site but that, unlike K+ and Rb+, they do not become occluded. In contrast, at equilibrium ethylenediamine prevents 86Rb occlusion in a competitive manner, suggesting the possibility of occlusion of the bifunctional amine.

It is now clear that in the K+ transport steps of the Na,K-pump cycle, K+ (or Rb+) ions become "occluded" within the pump molecule, inaccessible for a time to the intracellular and extracellular media (Post et al., 1972; Glynn and Richards, 1982). In the normal turnover of the pump, the K+ (or Rb+) ions are released into the intracellular medium subsequent to the binding of ATP at a low affinity site, and accompanying an Eα-Eβ conformational change in the enzyme; in the isolated Na,K-ATPase, this is manifested by a rapid release of 86Rb on exposure to ATP (Forbush, 1987a). Alternatively, if the transport cycle is partially reversed by phosphorylation of the enzyme by Pi, the occluded ions are released (Glynn et al., 1985b; Forbush, 1987b), to the extracellular face of the pump (Kenney and Kaplan, 1987; Forbush et al., 1988). In this case it has been found that the interaction of the two 86Rb ions with the extracellular medium is an ordered process, the release of 86Rb from one site ("s" site, with slow release) being blocked by occupancy of the other site ("f" site, with fast release; Forbush, 1985, 1987b; Glynn et al., 1985a, 1985b). The data are consistent with the simple geometric model in Scheme 1A. The kinetics of interaction have led us to propose that there is a barrier to the free exchange of Rb+ (or K+) between the transport sites and the extracellular medium, such that even when the enzyme is phosphorylated by Pi, a "gate" is only open briefly to allow exit of one and then the other 86Rb ion (Forbush, 1987b).

Recently the complete amino acid sequences of both the α and β subunits of the Na,K-ATPase have been deduced from cDNA clones (Shull et al., 1985, 1986; Kawakami et al., 1985). To gain an understanding of how the structure of the enzyme relates to function, it will also be necessary to identify individual amino acids involved in ligand binding and catalytic activity. Suitable probes have been prepared for the ouabain-binding site (cf. Forbush, 1983; Lowndes et al., 1984), the ATP binding site (Ohta et al., 1986; Ovchinnikov et al., 1987) and the phosphorylation site (Pi itself; Albers et al., 1983; Post et al., 1965), but as yet there is no way to identify the translocation sites for Na+ and K+. Two general approaches have been suggested: (a) the use of group-specific reagents to label amino acids presumed to be involved in ion binding, e.g. carboxylic acids and (b) affinity or photoaffinity derivatives of organic compounds that mimic the inorganic ions. If suitable compounds can be found, the latter approach has the advantage that it will be possible to ascertain that the labeled site is indeed the cation binding site, by evaluation of the behavior of the organic molecule; it may be transported or at

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1. The abbreviations used are: Eα and Eβ conformational forms of Na,K-ATPase. All substituted organic compounds are N-substituted. EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.
least occluded on a Na,K-ATPase.

It has been known for some time that the Na,K-pump is inhibited by quaternary amines such as tetraethylammonium (Sachs and Conrad, 1968) and tetrapropylammonium (Kropp and Sachs, 1977) in the extracellular medium. The inhibition is in competition with extracellular K+, in a manner generally consistent with binding of the amines to K+ transport sites (Sachs and Conrad, 1968). Tris and imidazole have also been found to interact with the pump, acting like Na+ in stabilizing the Ei form of the enzyme (Skou and Esmann, 1960) and in promoting phosphorylation (Norbry et al., 1983; Schuurmans Steckhoven et al., 1985), and it has been proposed that these cations bind at a distinct Tris site and at Na+ sites. These interactions suggest the possibility that appropriate organic cations might be used to covalently label the transport sites. Although the apparent affinities for the organic cations are quite low (the K+ of tetrapropylammonium in inhibiting transport is about 3 mM (Kropp and Sachs, 1977), the possibility of an organic cation might become “occluded” raised our hopes that a photoaffinity labeling approach would be feasible. Recently, Schuurmans Steckhoven et al. (1988) and Fukushima (1987) have reported the effects of a number of amines on phosphorylation of Na,K-ATPase. Noteworthy among the compounds tested was ethylenediamine, which inhibited phosphorylation of Na,K-ATPase with K+ < 0.1 mM (Schuurmans Steckhoven et al., 1988).

In this study we examine the interaction of various amines with the 86Rb-occluded state of the Na,K-pump. It is found that organic cations do indeed act like K+ or Rb+ at a high affinity site that is apparently the extracellular transport site; in doing so, they block both the MgATP-stimulated release of 86Rb from the s site and the ATP-stimulated release of 86Rb associated with the Ei → Es conformational change. However, the data indicate that the monofunctional amine does not themselves become occluded, but instead interact with the Na,K-ATPase to hold the gate open, as indicated in Scheme 1B, above. On the other hand, the bifunctional compound ethylenediamine appears to compete at the transport site with high affinity, and it may become occluded. Preliminary results of part of the work have been presented (Forbush, 1986).

EXPERIMENTAL PROCEDURES

Methods for the measurement of the rapid release of 86Rb from the occluded state of Na,K-ATPase have been described previously (Forbush, 1987a, 1987b, 1988a, 1988b); experiments described here were carried out during the same time period as those in these references, and the same methods were used. Briefly, a sample of dog kidney Na,K-ATPase (sodium dodecyl sulfate-washed microsomes) is incubated with 0.5 mM 82Rb for 30 s at 20 °C (and usually 10–60 min at 0 °C), diluted in imidazole/EDTA, filtered, rinsed, and transferred to a rapid filtration apparatus (Forbush, 1984) where, following exposure to a rinse solution, the release of 86Rb from the sample is followed in a test solution as a function of time. All solutions contained 25 mM imidazole, and 1 mM EDTA or EGTA pH 7.2 (or pH 6.8 for the rinse solution), unless otherwise noted. When included, “MgP” refers to 4 mM Mg2+/8 mM P, and “MgATP” refers to 5 mM Mg2+/4 mM ATP. Unless noted otherwise, included monovalent cations were 100 mM.

Reagents—Organic compounds were obtained from the following vendors: Eastman Kodak (Rochester, NY): tributylamine, tetraethylammonium, benzyltriethylamine; Fluka (Ronkonkoma, NY): benzyldimethylammonium, benzyltrimethylammonium, dibutylmethylammonium, dibutyl-ethylammonium; K & K (ICN, Plainview, NY); diethanolamine, dipropylamine, dibutylamine, butyramine, butylpropylamine, benzylhexylammonium; Mallinckrodt Chemical Works: aniline, morpholine; J. T. Baker Chemical Co.: ethylenediamine; Aldrich: dibutylamine, tetraethyamine, triethyamine, tripropylamine, dibutylhexamethylene; Sigma: benzylamine, butylamine, hexylamine, pentylamine, propylamine, t-butylamine, tetraphénylamine, tritylamine, triethylamine; CTC (CTC Organics, Atlanta, GA) benzylidyethilamine; Alfa Products (Andover, MA) benzylidyethilamine, benzyldimethylamine, benzyltrimethylamine, benzylhexylammonium, dibutylmethylammonium, butyramine, phenylpyridinium, tributyramine, trimethylamine, triethylamine, cyclohexylpropylamine. Sigma tetrapropylammonium chloride was contaminated with Na+ (10 mM Na+ in the 1 M stock solution) and was unsuitable for these experiments. In this case we used less than 0.15 mmol/mol relative to the amine (except 7.5 mmol/mol in tributylhexamethylene), and K+ was less than 0.15 mmol/mol (except 0.5 mmol/mol in tetraethylamine and 0.2 mmol/mol in dipentylamine). Limited solubility precluded testing of many other available compounds (e.g. benzylbutylamine, trimethylamine, tetraethylamine, triphenylamine). Benzylisopropylamine was obtained as the chloride and the bromide, and identical results were obtained with each. Benzylmethylphedrinium bromide and phenacylpyridinium bromide were tested; all other compounds were prepared as chloride salts.

Choline chloride was obtained from Syntax (Springfield, MO), stored frozen as a dry salt, and a fresh solution prepared before use. Decomposition is a well known difficulty with the use of choline, and we have noted that the stability of the singly occluded 86Rb ion (see “Results,” with respect to Fig. 6) appears to be sensitive to breakdown products or impurities in choline chloride. In direct comparisons of different batches of choline chloride from various suppliers, we have noted differences in the rate of 86Rb release in solutions containing 100 mM choline and MgP.

Data Presentation—Our methods of data analysis and presentation have been described previously (Forbush, 1987a, 1987b, 1988a, 1988b). In Figs. 5 and 7 we have corrected for a spontaneous background rate of release in applying the method of analysis when the amount of spontaneous release is proportional to the remaining counts; thus we have subtracted the product of the amount of remaining occluded 86Rb in the sample and the instantaneous fractional background rate of release in a control sample. In practice, compared with a simple subtraction of one curve from another, this amounts to the same correction for the early time points when all of the occluded 86Rb remains, but a much smaller correction late in the time course when there is no occluded 86Rb left to maintain a background rate.

Temperature—The rinse (“a”) and test (“b”) solutions in the rapid filtration apparatus were at 20 °C in all of these experiments.

RESULTS

Release of 86Rb in the Presence of Tetrapropylamine and MgP—We have previously found that when the Na,K-ATPase is phosphorylated by MgP++, and the K+ transport sites are thereby exposed to the extracellular face of the membrane, release of 86Rb ions from the sites proceeds in an ordered fashion (Forbush, 1987c). Thus the fast release of one 86Rb ion (from the f site) is unaffected by cations in the medium, but release of the second site is blocked by K+ or Mg2+ in the medium. We therefore used a new approach to see if they act like K+ in blocking 86Rb release from the s site; as before, we preincubated Na,K-ATPase with 86Rb to form the occluded state and studied the release of 86Rb in a rapid filtration apparatus (Forbush, 1984) in media containing 4 mM Mg2++, 8 mM P, 100 mM salt, 25 mM imidazole, 1 mM EGTA. Fig. 1 compares the time course of P, stimulated 86Rb release in choline chloride, RbCl, and tetrapropylammonium chloride. As indicated by the break in the curves in both the log(O) and integral (O) plots, tetrapropylamine (upper right)was found to act like Rb+ (bottom left; or K+, not shown) in blocking the release of one of the two 86Rb ions. To confirm that the 86Rb ion whose release is blocked by tetrapropylamine is the same as the ion whose release is blocked by “Rb”, i.e. the ion at the s site, we used a test solution containing RbMgP++, following a rinse solution containing tetrapropylamine-MgP++, as shown in the lower right panel of Fig. 1, in this case only a slow phase of release was observed, demonstrating that indeed the same 86Rb ion is blocked by the amine as by Rb. A similar result was obtained when the order of RbMgP++, and tetrapropylammonium-MgP++, was reversed (not shown). The remaining 86Rb ion rapidly dissociates in choline-MgP++, (dashed lines in the same panel), indicating that the effect of tetrapro-
FIG. 1. The release of $^8$Rb from an occluded state of Na,K-ATPase in the presence of MgP$_i$ and choline, Rb$^+$, or tetrapropylamine. Na,K-ATPase was incubated with 0.5 mM $^8$Rb in 3 mM Mg/imidazole/EDTA for 30 s at 20°C to form the occluded state as described previously (Forbush, 1987a). After dilution, filtration, and prerinse in imidazole/EDTA (pH 6.8) the sample was transferred to the rapid filtration apparatus where the rinse solution contained imidazole/EDTA (except lower right) and the test solution contained MgP$_i$ and indicated cations. The logarithm of the rate of release of $^8$Rb ($\bullet$) and the total amount of released $^8$Rb (●) are plotted, from duplicate runs in each experimental condition. Lower right panel, the rinse solution contained tetrapropylamine-MgPi and the test solution contained RbMgP$_i$ (●, ○) or choline-MgPi (dashed lines).

FIG. 2. The concentration dependence of block of $^8$Rb release from the s site by various amines. Time courses of $^8$Rb release were determined as in Fig. 1 in media containing MgP$_i$ and four concentrations of amine as noted. A, the time courses of $^8$Rb release. B, the rate of constant of $^8$Rb release obtained by a least squares curve fit using only the last 40% of total counts in A; each data point in this figure represents one such rate constant. The lines are least squares curve fits to a model in which the amine binds to a single site and alters the rate constant of $^8$Rb release; the values of $K_{am}$ from these and other similar experiments are presented in Table I.
pylamine is readily reversible. Thus these data demonstrate that tetrapropylamine acts like Rb+ (or K+) and does not interfere with 86Rb release from the s site, but blocks release of 86Rb from the s site.

Comparison of Various Organic Amines—In an attempt to find the most effective K+-like organic compounds and to gain insight into the structural constraints on cation interaction, we examined the concentration dependence of a number of different organic cations in blocking the release of 86Rb from the s site on Na,K-ATPase. Fig. 2A presents results for three amines. Benzyltripropylamine (left) is representative of amines which interact with high affinity to block the release from the s site (like tetrapropylamine in Fig. 1); pentyamine (center) is representative of compounds with similar action but low affinity; and propylamine (right) is representative of a number of compounds which slow the release of both 86Rb ions (see below). It is seen here that with increasing concentrations of benzyltripropylamine and pentyamine that, although the initial rate of 86Rb release is hardly affected, the rate of 86Rb release is decreased late in the time course; i.e. the curves are clearly biphasic. (Variation in the total amount of 86Rb released in 3 s reflects errors in aliquoting the sample, e.g. results with 12, 40, and 100 mM pentyamine). With propylamine the initial rate of 86Rb release is lowered as well as the late phase. To estimate the degree of change in the second phase of release, first order rate constants were obtained from a least squares curve fit of the time course of 86Rb release in Fig. 2A, utilizing only the last 40% of the total 86Rb; the values are plotted in Fig. 2B (points). These data were then fit to a model of inhibitor binding to a single site: the curves in Fig. 2B express the least squares solutions. The apparent inhibitory constants obtained from these fits, and similar data for 35 other amines are summarized in Table I (17 other experiments are summarized; typically 4–6 cations/experiment).

It is seen in Table I that almost all of the amines tested slowed the second phase of 86Rb release. The affinity for the cations varies considerably; although it is difficult to formulate a general rule, it may be noted that the effectiveness of the hydrocarbon side chains increases in the order methyl < the and < ethyl < propyl, butyl, penty, benzyl. It is seen that to be effective the organic molecule must have a sufficient bulk in the side chains, with effective compounds (e.g. Kapp < 15 mM) having at least 8–9 carbon atoms; on the other hand it appears that beyond a certain size the effectiveness decreases, since the apparent affinity of compounds with butyl chains is less than the affinity for those with propyl chains.

Among the organic cations listed in Table I (data marked with asterisk), several compounds were similar to propylamine (Fig. 2) in that they inhibited the initial rate of 86Rb dissociation as well as the late phase, generally interacting with low affinity (Kapp > 30 mM, except benzylamine). Most of these were among the smallest molecules tested. One possibility which we have not tested, is that these compounds interfere with phosphorylation of the K'-occluded form, similar to an effect of very high concentrations of NaCl (Forbush, 1987b).

The pH Dependence of Block by Amines—In a search for conditions which would increase the affinity for Na,K-ATPase for organic cation, we examined the pH dependence of the apparent affinity for benzyltriethylamine in blocking release of 86Rb from the s site. As shown in Fig. 3 (C), the apparent affinity for the amine increased about 10-fold between pH 7 and 8. Further experiments, one of which is illustrated in Fig. 3 (C), demonstrated that the dependence on pH is not unique to the interaction of amines with Na,K-

![Fig. 3. The pH dependence of block by benzyltriethylamine and 86Rb. The apparent affinity for benzyltriethylamine (C) and 86Rb (O) were determined as in Fig. 2, at the indicated pH values. Lines were drawn by eye.](image-url)
ATPase but also applies, albeit less strongly, to the block by 86Rb itself. These results suggest the possibility that protons compete directly with 86Rb or amines at the f site, although alternative explanations involving allosteric interactions can not be ruled out.

**The Interaction of Amine with the Vanadate-inhibited Na,K-ATPase**—We have previously shown that the release of 86Rb from the occluded state with vanadate bound (E$n_{p,b}$-VO$_4$) is similar to release from the phosphorylated enzyme but about 25-fold slower; importantly 86Rb$^+$ (or K$^+$) blocks release from one of the two occlusion sites (Forbush, 1987b). Simple models of block predict a decreased apparent affinity for the blocking ion if the overall rate of deocclusion is decreased, as it is in the vanadate-inhibited enzyme (Forbush, 1987b, and see “Discussion” regarding Scheme 2); thus we pointed out that high apparent affinity of the vanadate-inhibited enzyme for 86Rb implies that the re-release of the blocking ion involves a step in addition to simple dissociation, for instance a small conformational change that would be needed to expose an occluded ion.

Organic cations are very different from Rb$^+$ (or K$^+$) in their ability to block the release of one of the two 86Rb ions from E$n_{p,b}$-VO$_4$. As illustrated in Fig. 4, 100 mM Rb$^+$ effectively blocks release of half of the 86Rb (bottom left; the second phase is only about half complete on this time scale; 1 mM is sufficient, see Forbush, 1987b), whereas in 100 mM benzyltriethylamine, the biphasic nature of the curve is hardly detectable (upper right). Importantly the slope of the late phase of release is less in 200 mM (lower right) than 100 mM benzyltriethylamine, indicating that the ineffectiveness of the amine is probably a result of very low apparent affinity; this is further supported by the data in Table II, which presents the rate constants for the late phase of 86Rb release in 50, 100, and 200 mM tetrapropylamine, benzyltriethylamine, and benzyltributylamine (from the same experiment as Fig. 4). If complete block is assumed at saturating amine concentrations, then the estimated $K_{app}$ values for each of the amines in Table II is >170 mM. Thus the organic cations exhibit a much lower apparent affinity when the rate of 86Rb release is decreased by vanadate inhibition, than in the control enzyme; this behavior is consistent with rapidly reversible binding of the blocking cation, as contrasted to occlusion.

It may be noted in Fig. 4 that the initial rate of 86Rb release is higher in Rb$^+$ or benzyltriethylamine than in imidazole/EDTA alone (upper left; the rates of release at the t = 0 time point are 2.1, 2.4, and 3.4 in 100 mM 86Rb, 100, and 200 mM benzyltriethylamine, respectively, relative to a rate of 1.0 in imidazole/EDTA). We have not further investigated as to whether this is an effect of ionic strength or whether it reflects specific ion interactions. However it may be noted that in Fig. 10 of Forbush (1987b) the initial rate of 86Rb release from the vanadate-inhibited enzyme is higher in 100 mM Na$^+$ than in 100 mM Rb$^+$.

**The Effect of Amines on 86Rb Release in the Presence of ATP**—We have previously shown that the same 86Rb ions that dissociate in the presence of MgPi, are rapidly released in the presence of ATP, presumably from the intracellular face of the Na,K-pump following the conformational changes involved in ion translocation (Forbush, 1987a, 1987b). As illustrated in the top panels of Fig. 5, the release of both 86Rb ions is promoted by ATP when tetrapropylamine is present (top right), just as when 86Rb (top left) or other cation (not shown) is present. Note that the rate of release is somewhat lower when tetrapropylamine replaces Rb$^+$ in the medium; this is consistent with tetrapropylamine acting as an “inert” cation like choline or N-methylglucamine and with our previous finding of a rather nonspecific stimulation of deocclusion by Rb$^+$ and other metal cations (Forbush, 1987a).

We also examined the rate of release of the 86Rb ion that remains in the s site after exposure to RbMgPi, a procedure which results in replacement of 86Rb at the f site by unlabeled rubidium (Forbush, 1987b). As shown in the middle panels of Fig. 5, this ion is released in the presence of tetrapropylamine.

**TABLE II**

| Concentration | Tetrapropylamine | Benzyltriethylamine | Benzyltributylamine |
|---------------|------------------|---------------------|---------------------|
| mM            |                  |                     |                     |
| 50            | 0.123            | 0.104               | 0.129*              |
| 100           | 0.104            | 0.072               | 0.098               |
| 200           | 0.084            | 0.068               | 0.079               |

**Fig. 4. The effect of benzyltriethylamine on 86Rb dissociation from vanadate-inhibited Na,K-ATPase.** The vanadate-inhibited occluded state was formed by incubation of Na,K-ATPase with 0.5 mM 86Rb, 2.7 mM Mg$^{2+}$, 110 μM vanadate for 30 min at 20 °C (Forbush, 1987b); after rinsing in imidazole/EDTA, 86Rb release was monitored in imidazole/EDTA or MgPi, with the indicated cations. Duplicate runs are presented.
MgATP, albeit more slowly than with RbMgATP. This result is in agreement with the above finding that both \(^{86}\)Rb ions are released under these conditions (top panels).

A remarkable finding is illustrated in the lower panels of Fig. 5: in these samples pre-release of \(^{86}\)Rb from the f site was brought about by pre-exposure to benzyltriethylamine-MgP, (as in Fig. 1, lower right), rather than RbMgP. In this case the ATP-stimulated release of \(^{86}\)Rb from the s site was completely blocked by benzyltriethylamine in the medium (lower right panel; compare to lower left panel). Although other explanations will be considered below, the most plausible explanation is that continued occupancy of one cation site by amine (presumably the external f site for K') prevents the \(E_2 \rightarrow E_1\) conformational change. This would account for the strong inhibitory effect of the quaternary amines on the overall pump activity (Kropp and Sachs, 1977).

**Release of \(^{86}\)Rb from the "Singly Occupied" Transport Sites**

It was just shown that in order for an organic cation to prevent the release of \(^{86}\)Rb from the f site into an ATP-containing medium, it is necessary that the amine be present in the ATP-containing medium as well as during preexposure to MgP, (Fig. 5; compare upper and lower middle panels). This indicates that upon removal of amine from the medium, the amine dissociates rapidly from Na,K-ATPase; additional arguments for this conclusion will be presented below. Thus after exposure to amine-MgP, to allow release of \(^{86}\)Rb from the f site, and then removal of amine, the transport sites must be occupied by a single \(^{86}\)Rb ion (and possibly a proton). We found that the "spontaneous" release of this lone \(^{86}\)Rb ion was quite rapid compared with the rate of spontaneous release when both sites were occupied. For instance, in 100 mM choline the rate of release from the singly occupied transport sites was typically ~5 s\(^{-1}\) compared with ~0.2 s\(^{-1}\) in control samples (cf. Forbush, 1987a). The instability of the single ion was especially marked at low pH, as shown in Fig. 6 (C), increasing to a spontaneous deocclusion rate of ~15 s\(^{-1}\) at pH 6.5. The direction of the variation with pH is also just the reverse of that when both sites are occupied (O). Note that two different scale factors are used in this figure, the rate of \(^{86}\)Rb release from the singly occupied Na,K-ATPase being 20-1000-fold more rapid than from the fully occupied enzyme.

Two uncertainties are associated with this set of observations. First, the spontaneous release of the single \(^{86}\)Rb ion was sometimes much lower than in the experiment summarized in Fig. 6 (e.g. ~1 s\(^{-1}\) at pH 7.2). Although variation in Na,K-ATPase preparations may be part of the explanation (the experiments were conducted over a 2-year span), it is also possible that impurities in the choline chloride (see "Experimental Procedures") contribute to the high rate of spontaneous release in a Na"-like manner (see Forbush, 1986; Forbush et al., 1988). Second, in the course of these experiments we noted that not all of the remaining \(^{86}\)Rb was rapidly released into choline alone. Compared with the amount of \(^{86}\)Rb release measured in solutions containing ATP (below) or into Na" (Forbush et al., 1988), only 68 ± 8% of bound \(^{86}\)Rb (14 pairs of duplicates at various pH values in three experiments) dissociated spontaneously in 2-3 s in choline with no ATP. This does not appear to be an artifact of the experimental procedure. For instance, it is not due to incomplete emptying of the f site during the rinse, which would leave some fraction of the \(^{86}\)Rb in the doubly occupied state: this fraction would be released rapidly into amine-MgATP, but it is not seen in Fig. 5 (lower right) or Fig. 7 (lower middle). At present we have no explanation for this observation.

We asked whether the single occupancy enzyme could undergo a conformational change and release \(^{86}\)Rb in the presence of ATP. Fig. 7 illustrates the result of an experiment in which the effect of ATP was examined following preexposure to benzyltriethylamine-MgP. The experiment was performed at pH 8.2 where the spontaneous release of \(^{86}\)Rb is low (above). The first two columns in Fig. 7 present data similar to those in Fig. 5, but at higher pH and using benzyltriethylamine; as in Fig. 5, it is seen that when amine is present both during the preexposure to MgP, and during the test with ATP, \(^{86}\)Rb release from the s site is prevented (lower middle). When the test solution contained choline (right column), \(^{86}\)Rb was rapidly released both from the control

![Fig. 5. The effect of amines on the release of \(^{86}\)Rb in the presence of ATP. In the rapid filtration apparatus the rinse solution contained imidazole/EDTA (top), RbMgP, (middle) or tetrapropylamine MgP, (bottom); and the test solution contained RhMgATP or tetrapropylamine-MgATP. The time course of the amount of release of \(^{86}\)Rb is plotted, after correction for the background rate of release in the absence of ATP (see "Experimental Procedures").](image)

![Fig. 6. The pH dependence of spontaneous \(^{86}\)Rb release. The spontaneous release of \(^{86}\)Rb into choline/imidazole/EGTA was measured, and the rate constant of release was obtained from the least square curve fits. ◆, control, after rinsing in imidazole/EDTA. ○, release following exposure to tetrapropylamine-MgP, to cause dissociation of \(^{86}\)Rb from the f site (from a separate experiment). Lines were drawn by eye.](image)
sample (upper right) and from the sample in which $^{86}$Rb remained only in the s site. This suggests that when only a single $^{86}$Rb occupies the transport sites, the translocation process involving an ATP-induced conformational change can take place.

**Competition of Amines with $^{86}$Rb in the Equilibrium Level of Occlusion**—The results presented above demonstrate that amines can interact with Na,K-ATPase, probably at one of two $^{86}$Rb transport sites. The process of ion occlusion is clearly a multistep process, involving both binding of the ions to an accessible site and a conformational change in the protein that renders the site inaccessible (Forbush, 1987b). If other ions interact directly with these sites, any manifestation of competition will depend on a number of parameters of the process which we still do not know (e.g. the binding and occlusion equilibrium constants and whether the singly occupied site can become occluded). However, it can be readily appreciated that in a model in which the equilibrium is displaced in the direction of occluded states from states in which the binding sites are exposed to the medium (as in the flickering gate model, in which the gate only opens briefly), an ion that interacts only with the nonoccluded states will have little effect on the apparent affinity for the overall process of occlusion of $^{86}$Rb. On the other hand, an ion that interacts with both sites, and itself becomes occluded, will compete effectively with $^{86}$Rb for occlusion. Therefore, we examined the effect of several amines on the apparent affinity for $^{86}$Rb at equilibrium.

The results in Fig. 8 illustrate a decrease in the apparent affinity for $^{86}$Rb in the presence of tetrapropylamine; the data are fit by a competitive interaction of $^{86}$Rb and tetrapropylamine with one set of sites on Na,K-ATPase, with $K_s$ values 0.06 mM and 52 mM, respectively (a similar result was obtained with benzyltriethylamine, not shown). The apparent affinity for tetrapropylamine in competing with $^{86}$Rb for occlusion is at least an order of magnitude lower than the apparent affinity reported above (Table I) for the effect of this same amine in blocking release of $^{86}$Rb from the s site. However similar results were also obtained with N-methylglucamine (the apparent affinity for $^{86}$Rb was reduced by N-methylglucamine with $K_{app} = 30$ mM), an inert cation with little effect on the apparent affinity for the overall process. If other ions interact directly with these sites, any manifestation of competition will depend on a number of parameters of the process which we still do not know (e.g. the binding and occlusion equilibrium constants and whether the singly occupied site can become occluded).

In these experiments, $^{86}$Rb occlusion reaches an equilibrium level during the incubation period. While the determination of the amount of the occluded ion is made under disequilibrium conditions (after dilution and rinsing), the determination faithfully reflects the amount of $^{86}$Rb occluded under the conditions of the incubation, since the rate of spontaneous deocclusion is very slow at 0 °C (Forbush, 1987a).

![Amines and the Occluded State of the Na,K-Pump](image_url)

**Fig. 7.** The release of $^{86}$Rb from the singly occupied occluded state in the presence of ATP. In the rapid filtration apparatus the rinse solution contained imidazole/EDTA (top) or benzyltriethylamine-MgP (bottom); and the test solution contained RbMgATP (left), benzyltriethylamine-MgATP (middle), or choline-MgATP (right). The data are plotted after correction for the background rate of release in the absence of ATP (see "Experimental Procedures"). Duplicate runs are presented; part of one run was lost (top right). A small "bump" in the curves at $t \approx 0.4$ s is an artifact due to a warm spot in the test solution line (see Forbush, 1987b).

![Fig. 8. The effect of tetrapropylamine and ethylenediamine on $^{86}$Rb occlusion by Na,K-ATPase](image_url)
regard to \(^{86}\)Rb release (Table I), suggesting that this phenomenon may be an ionic strength effect or reflect interaction at low affinity sites other than the K\(^{+}\) transport sites. This result contrasts sharply to the case with Rb\(^{+}\) (or K\(^{+}\)), where the apparent affinity for Rb\(^{+}\) in blocking release from the s site is essentially the same as the affinity seen for \(^{86}\)Rb in occlusion (Forbush, 1987b). The result is therefore strongly suggestive that, unlike Rb\(^{+}\) or K\(^{+}\), the monovalent amines do not themselves become occluded.

Ethynediamine has been reported to have an effective inhibitor of Na,K-ATPase activity, with a K\(_d\) < 0.1 mM (Schuurmans Stekhoven et al., 1988); as shown in Fig. 8B, ethynediamine competes with Rb\(^{+}\) with high affinity (0.03 mM in this experiment, 0.014, 0.07, 0.12 mM in three others; the reason for the wide spread is unclear). Thus, although ethynediamine interacts weakly if at all with the f site to block the monovalent amines do not effectively compete with Rb\(^{+}\) in occlusion, possibly by binding to both transport sites and becoming occluded.

**DISCUSSION**

We have found that many organic amines interact with the Na,K-ATPase, in a manner very similar to Rb\(^{+}\) or K\(^{+}\). The action of these compounds on the occluded state of the enzyme is to block the release of \(^{86}\)Rb from one of two transport sites, without affecting the release of the other \(^{86}\)Rb ion. It is release of \(^{86}\)Rb from the s site that is blocked by amines, just as it is by K\(^{+}\) or Rb\(^{+}\) (Fig. 1), so it is reasonable to propose that the organic molecule binds to the same site as K\(^{+}\) or Rb\(^{+}\), that is to the other transport site (the f site).

Since the benzyl amines are among the most effective in blocking release of \(^{86}\)Rb from Na,K-ATPase, and since the specificity is very broad, it is likely that suitable photoaffinity reagents (e.g. nitroazidobenzyl amines) could be designed which would interact with the Na,K-ATPase in the same way. As shown here (Fig. 2 and Table I), the apparent affinity for the organic cations is quite low (K\(_{app}\)  > 1 mM), and there is no indication from the data that a monofunctional amine can be found with a substantially greater affinity for the transport site. If this reflects the true K\(_d\) for the amines (see below) it is well out of the range in which a photoaffinity approach would be useful because of nonspecific binding and photolabeling. However, if it were found that the organic cation became "occluded," it might be possible to wash away most of the nonspecifically bound compound prior to photolabeling.

Unfortunately, several lines of evidence demonstrate that the monovalent amines do not become occluded. 1) The inhibition of \(^{86}\)Rb dissociation from the s site in the presence of ATP requires the continued presence of amine in solution (Figs. 5 and 7). When the overall process of deocclusion is slowed by vanadate inhibition, the apparent affinity for amine decreases by more than an order of magnitude compared with the uninhibited enzyme (Fig. 4 and Table II) as predicted for a rapidly reversible inhibitor (see discussion of Scheme 2, below), in contrast to an increase in apparent affinity for Rb\(^{+}\) in similar experiments (Forbush, 1987b). 2) The monovalent amines compete poorly with Rb\(^{+}\) for formation of the occluded state (Fig. 8A). 4) In experiments to be presented elsewhere (Forbush et al., 1988; see also Forbush, 1986), we have found a dramatic effect of Na\(^{+}\) in driving the release of the lone \(^{86}\)Rb ion from the s site; quaternary amines are fully competitive with Na\(^{+}\) and stabilize the \(^{86}\)Rb ion, but the amine is effective only as long as it is kept in the medium. 5) We have performed a number of experiments in search of direct evidence that tetrapropylammonium ion or benzyltriaethylammonium ions can themselves become occluded on Na,K-ATPase in a manner similar to K\(^{+}\) or Rb\(^{+}\) (results not shown here). Generally, we looked for continued stabilization of \(^{86}\)Rb at the s site after removing tetrapropylammonium from the medium, in the presence or absence of Mg\(^{2+}\) and P\(_i\). In a typical experiment, \(^{86}\)Rb was bound to Na,K-ATPase, exposed to benzyltriaethylammonium-MgPi, during the dilution-filtration-pre-rinse procedure prior to transfer to the rapid filtration apparatus, rinsed with imidazole/histidine in the first solution, and tested with amine-ATP (or Na\(^{+}\); see Forbush et al., 1988) in the second solution. In none of these experiments did we obtain any evidence that the effect of the organic cation could be "remembered" after exposure to the rinse solution. Thus, we conclude that the organic cation that blocks the release of one Rb\(^{+}\) ion cannot itself become occluded.

We have found it convenient to think of the action of Rb\(^{+}\), its congeners, and organic cations in terms of a geometrical model indicated in Scheme 1 in the Introduction (Forbush, 1985a; Glynn et al., 1985a; Forbush, 1985b). It is proposed that release to the extracellular medium of "J Rb from the innermost site (s) is blocked by occupancy of the f site by a K\(^{+}\) or Rb\(^{+}\) ion. Importantly, interaction of extracellular ions with the f site is limited by some slow step, as would be expected of a small conformational change associated with opening of a gate (Forbush, 1987b). In this context the organic cations appear to be able to interact like Rb\(^{+}\) or K\(^{+}\) (hydroxymethyl at the f site), but somehow, possibly due to steric hindrance, it appears that the gate is unable to close on the organic cations (Scheme 1B). However alluring this notion, it should be stressed that kinetic data cannot prove such models. We have previously noted that, although the data are fairly compelling that the blocking Rb\(^{+}\) ion is bound at the occlusion site, it is not possible to prove by kinetic evidence alone that the constraint on release from the s site is simple steric hindrance, as opposed to an allosteric interaction. With regard to the organic cations, the idea that these bind to the transport sites is supported by analogy to the blocking action of K\(^{+}\) or Rb\(^{+}\), but because the monovalent amines do not become occluded, we lack direct evidence to prove this point. Although it appears unlikely, it is possible that amines bind to another site (not the f transport site) and by an allosteric effect prevent release from only one of the two K\(^{+}\) transport sites.

It is likely that the apparent affinities for monovalent amines interacting at the f site are lower than the apparent affinities indicated by the K\(_{app}\) values in Table I. This is a multistep reaction sequence, and in such cases the apparent dissociation constant for a substrate (K\(_d\)) or for an inhibitor (K\(_i\)) is generally different than the intrinsic dissociation constant characteristic of the binding site (cf. Stein, 1986). In the proposed model for block by organic cations depicted in Scheme 2, amines bind to the f site only when the gate is open. We measure the rate of the release of the second of the two occluded Rb\(^{+}\) ions. In our flickering gate model (see Forbush, 1987b, 1988c) the sequence of events involves opening of the gate (slow) and dissociation of the first ion (very fast; k\(_c\)) followed by closing of the gate (k\(_{bc}\) fast) and finally jump of the ion within the pocket, reopening of the gate (slow), and dissociation of \(^{86}\)Rb (k\(_{cd}\)). In the absence of amine, the rate determining step in release of the second Rb\(^{+}\) ion is the reopening of the gate (k\(_{bc}\) >> k\(_{cd}\)). Amine reduces the concentration of the intermediate B, and thereby reduces the rate of B → C; the overall rate of B → C is reduced by half (K\(_{app}\) = [amine]) when B → C is as slow as C → D, that is when (B/(B + B'))k\(_{bc}\) = k\(_{cd}\). On simplification (assuming that amine binding is in rapid equilibrium, B'B' = K\(_d\) [amine]) we have K\(_{app}\) = K\(_{bc}\)(k\(_{bc}\) + k\(_{cd}\))/k\(_{cd}\), or since k\(_{bc}\) >> k\(_{cd}\), K\(_{app}\) = K\(_{bc}\)k\(_{bc}\)/k\(_{cd}\). Restated, the apparent dissociation constant for binding of amine will be larger than the intrinsic dissociation...
The simplest explanation is that these compounds do not become attached to the Na,K-ATPase but for this type of model to fit available data, under the conditions in Fig. 2, $k_a$ must be at least 3-fold greater than $k_o$ and possibly much more. Thus, we estimate that the $K_o$ for monovalent amines such as tetrapropylamine or benzyltriethylamine is $<1 \text{mM}$ (possibly $<0.1 \text{mM}$). Even so, since the intermediate to which the amines bind is present only transiently, it will be difficult to exploit photoaffinity derivatives of these compounds.

We have shown that when organic cations occupy the $s$ site, the release of $^{86}\text{Rb}$ from the $s$ site is not stimulated by ATP (Figs. 2 and 7), indicating that Na,K-ATPase is probably unable to undergo the $E_2 \rightarrow E_1$ conformational change under this condition. Since a distinguishing feature of the organic cation interaction is that these compounds do not become occluded, we propose (a) that the $E_1 \rightarrow E_2$ conformational change cannot take place as long as the gate regulating exposure of $K^+$ sites to the extracellular medium remains open. There are other alternative possibilities: (b) We have found that the rate of deocclusion in the presence of ATP depends upon the nature of the cation sharing the pocket with a single $^{86}\text{Rb}$ ion (Forbush, 1987b); it may be that organic cations are an extreme case, in which the conformational change is prohibited altogether. This is subtly different from the proposal in (a), in that it is the nature of the ion in the pocket rather than the state of the gate which affects deocclusion. (c) The action of organic cation is blocking deocclusion with ATP might be mediated at another site altogether. However this seems very unlikely, since the inhibition only occurs in the case when the enzyme has also been preexposed to the organic cation under conditions in which the $f$ site is presumed to become occupied by that cation.

We have shown here that ATP stimulates deocclusion when only one $^{86}\text{Rb}$ ion occupies the occlusion sites (Fig. 7). The simplest explanation is that this ion is released to the intracellular face of the membrane, and therefore that a single $^{86}\text{Rb}$ ion could be transported in one pump cycle. However, for this to take place during transport would also require that the singly occupied occluded state be formed in preference to the doubly occupied state at some concentration of extracellular $K^+$ (or $^{86}\text{Rb}$), and we do not yet have sufficient information to know whether this occurs. Furthermore, it should be noted that we cannot be certain that the ATP-stimulated $^{86}\text{Rb}$ release is in the intracellular direction, since we have presented arguments that ATP may also modulate release in the extracellular direction in the presence of vanadate or $P_i$ (Forbush, 1987b).

Ethylene diamine does not interact strongly with Na,K-ATPase to slow dissociation of either of the $^{86}\text{Rb}$ ions from the occluded state (Table I, $K_{app} > 100 \text{mM}$). On the other hand, this bifunctional amine competes effectively with $^{86}\text{Rb}$ in preventing formation of the occluded state, indicating that ethylenediamine complexes with the Na,K-ATPase in a state parallel to the $^{86}\text{Rb}$-occluded form. This conclusion is also supported by the finding that ethylenediamine inhibits phosphorylation (Schuurmans Stekhoven et al., 1987). Therefore it seems likely that ethylenediamine acts like $K^+$ or $^{86}\text{Rb}$ in that it can occupy $K^+$ transport sites and become occluded; if so, suitable derivatives may be useful in labeling the ion translocation sites on Na,K-ATPase.

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