Ligands Presumed to Label High Affinity and Low Affinity ATP Binding Sites Do Not Interact in an (αβ)2 Diprotomer in Duck Nasal Gland Na+,K+-ATPase, nor Do the Sites Coexist in Native Enzyme*

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The interaction of ligands deemed to be ATP analogues with renal Na+,K+-ATPase suggests that two ATP binding sites coexist on each functional unit. Previous studies in which fluorescein 5-isothiocyanate (FITC) was used to label the high affinity ATP site and 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) was used to probe the low affinity site suggested that the two sites coexist on the same αβ protomer. Other studies in which FITC labeled the high affinity site and erythrosin 5-isothiocyanate (ErITC) labeled the low affinity site led to the conclusion that the high and low affinity sites exist on separate interacting protomers in a functional diprotomer. We report here that at 100% inhibition of ATPase activity by FITC, each αβ protomer of duck nasal gland enzyme has a single bound FITC. Both TNP-ADP and ErITC interact with FITC-bound protomers, which unambiguously demonstrates that putative high and low affinity ATP sites coexist on the same protomer. In unlabeled nasal gland enzyme, TNP-ADP and ErITC inhibit both ATPase activity and p-nitrophenyl phosphatase activity, functions attributed to the putative high and low affinity ATP site, respectively, by interacting with a single site with characteristics of the high affinity ATP binding site. In FITC-labeled enzyme, TNP-ADP and ErITC inhibit p-nitrophenyl phosphatase activity but at much higher concentrations than with the unmodified enzyme. Low affinity sites do not exist on the unmodified enzyme but can be detected only after the high affinity site is modified by FITC.

There is much evidence that the biphasic substrate-velocity curve occurs, because the reaction mechanism between the enzyme form E2K, an E2 conformation that binds K+, and E2ATP, an E1 conformation that binds ATP, is branched (3). In one branch, E2K releases K+ and changes its conformation to E1, to which ATP binds with high apparent affinity. In the second branch, ATP binds to E2K with low apparent affinity with subsequent release of K+ and conformational change to E1 ATP. In each case, ATP binds to the same physical site, which changes conformation and function during the reaction cycle from a high affinity catalytic site to a low affinity regulatory site. This mechanism accounts in detail for many observations that have been made with the enzyme including the role of ATP in promoting deocclusion of K+ and in supporting K+–K+ exchange across the membrane (4, 5).

On the other hand, the results of studies in which ligand binding abolishes functions attributed to a high affinity ATP binding site, such as Na+-dependent ATPase activity and Na+-dependent phosphorylation from ATP, or functions, such as phosphorylation from inorganic phosphate or K+-dependent para-nitrophenyl phosphatase (pNPPase) activity, of a site at which ATP acts with low apparent affinity have been advanced as evidence for the presence of separate coexisting high and low affinity ATP binding sites in a single enzyme unit (6–8). These studies have, for the most part, been performed with enzyme with significantly less than the theoretical maximal phosphorylation capacity of 6.8 nmol of phosphate (mg of protein)−1, and the remainder of the protein is either extraneous protein or inactive αβ protomers.

We recently perfected a method for preparing Na+,K+-ATPase from duck nasal glands that reliably yields enzyme of maximal theoretical phosphorylation capacity (9). We have used this preparation to reevaluate two studies relevant to the issue of whether or not protomer interaction is involved in the manifestation of putative high and low affinity ATP binding sites. Both studies used fluorescein 5-isothiocyanate (FITC) to label and inactivate the high affinity site. In one study, 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) was used to inhibit the functions of the low affinity site. Demonstration of the inhibitory site on detergent solubilized FITC-modified enzyme shown by active enzyme centrifugation to contain only αβ monomers indicated that both the high and low affinity site coexist on the same αβ protomer (10). In the second study, erythrosin 5-isothiocyanate (ErITC) was used to label and inactivate the low affinity site. Fluorescence resonance

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energy transfer measurements were used to show that the probes resided on separate αβ protomers (11). Our results show that, in the nasal gland enzyme, FITC and TNP-ADP or ErITC react with sites on a single αβ protomer. We also found that putative low affinity sites do not exist on unmodified enzyme but are a product of FITC modification.

EXPERIMENTAL PROCEDURES

Materials—FITC, ErITC, and the disodium salt of TNP-ADP were purchased from Molecular Probes, Inc. (Eugene, OR). MeSO was purchased from Aldrich and dried over activated molecular sieves type 4A (Fluidite Molsieve). ATPase was made by passing Na$_2$-ATP over a 50-ml column containing Dowex 50W (Sigma) cation exchange resin, hydrogen form, and titrating the eluant to pH 7.5 with Tris base. All other reagents were research grade.

Na$_2$-K'-ATPase Preparation and ATPase Activity Measurements—Purified, membrane-bound Na$_2$-K'-ATPase was prepared from the nasal glands of salt-adapted Pekin ducks as described previously (9). Na$_2$-K'-ATPase activity was measured using an NADH-coupled assay (9). All preparations had an initial ATPase activity of >60 enzyme units (1 enzyme unit = 1 μmol of ATP hydrolyzed per min per mg of protein at 37 °C). K'-dependent pNPPase Activity—pNPPase activity was measured at 37 °C as indicated in the figure legends, with the assay medium contained 5 mM EGTA, 40 mM KCl, 5 mM MgCl$_2$, 10 mM p-nitrophenyl phosphate, and 10 mM Tris/His, pH 7.5. The reaction was initiated by adding Na$_2$-K'-ATPase (~1 μg) to a temperature-equilibrated cuvette, and the change in absorbance at 410 nm was recorded in the dark. The suspension was diluted 10-fold in ice-cold 1% Me$_2$SO and were protected from light.

RESULTS

Labeling of Nasal Gland Enzyme with FITC—Nasal gland enzyme with close to maximal theoretical phosphorylation capacity of 6.8 nmol of phosphate (mg of protein)$^{-1}$ binds an average of 6.68 ± 0.28 nmol of FITC (mg of protein)$^{-1}$ at 100% inhibition of Na$_2$-K'-ATPase activity (Table I). There is no nonspecific FITC binding. The equivalence of the phosphorylation capacity and the binding capacity for FITC is similar to findings with ATPase preparations from other sources (15), but in those preparations phosphorylation capacity is considerably less than the maximal theoretical value. In all samples shown in Table I (see Footnote a), as in enzyme from other sources, FITC labeling spares nearly all of the K'-dependent pNPPase activity of the enzyme. In the nasal gland enzyme, at 100% inhibition of Na$_2$-K'-ATPase activity FITC must be bound to every αβ protomer, and the residual K'-dependent pNPPase activity must be a function of FITC-labeled protomers.

**Table I**

| Purified ATPase sample | FITC bound | Inhibited ATPase activity$^a$ | FITC bound/inhibited ATPase | Average FITC bound |
|------------------------|------------|-------------------------------|-----------------------------|---------------------|
| 6028                   | 5.20       | 81.8                          | 6.41                        | 6.68 ± 0.28         |
| 607                    | 5.28       | 87.4                          | 6.05                        | 6.98                |
| 1150                   | 6.91       | 99.0                          | 6.98                        | 7.28                |
| 1209                   | 7.06       | 97.0                          | 7.28                        |                     |

$^a$ Less than 5% of the pNPPase activity was inhibited in all preparations.

$^b$ Average of four measurements ± S.E.

**Inhibition of K'-dependent pNPPase Activity by TNP-ADP**—The persistence of pNPPase activity, which can be inhibited by nucleotides such as TNP-ADP and by other ligands thought to be ATP analogues in enzyme in which high affinity effects of ATP have been abolished by irreversible binding of ligands such as FITC to a supposed high affinity ATP binding site, is cited as evidence that high and low affinity ATP binding sites exist simultaneously on each functional enzyme unit. The functional enzyme unit might consist of a single αβ protomer with two binding sites or a diprotomer in which each of the interacting αβ protomers contains a single site, one of which is a high affinity site, while the site on the other protomer is locked in a low affinity state. This latter possibility was made unlikely by an experiment with renal Na$_2$-K'-ATPase with phosphorylation capacity less than the theoretical maximum; solubilization of FITC-bound enzyme to monomeric form did not increase the residual Na$_2$-K'-ATPase activity (i.e. did not expose more high affinity sites) (10). The point is reinforced by our observation that K'-dependent phosphatase activity persists in nasal gland enzyme in which all αβ protomers have bound FITC. The putative low affinity ATP binding site resides on the same α chain as the putative high affinity ATP binding site.

**Fig. 1A** is a Dixon plot of Na$_2$-K'-ATPase activity of unmodified nasal gland enzyme at low concentrations of TNP-ADP, and **Fig. 1B** is a Dixon plot of K'-dependent pNPPase activity of the same enzyme. The results shown in **Fig. 1B** are similar to those obtained in a comparable experiment with unmodified renal enzyme (10). $K_I$ for TNP-ADP calculated from **Fig. 1A** is 0.06 μM, and the $K_I$ calculated from **Fig. 1B** is 0.22 ± 0.07 μM. TNP-ADP competitively inhibits Na$_2$-K'-ATPase and K'-dependent pNPPase of unmodified nasal gland enzyme with about the same affinity.

**Fig. 2** shows a Dixon plot of K'-dependent pNPPase activity in FITC modified nasal gland Na$_2$-K'-ATPase. The results are similar to results obtained in the same experiment with solubilized FITC-labeled renal enzyme shown by active enzyme ultracentrifugation to consist only of monomeric αβ protomers.
Modification of Na\textsuperscript{+},K\textsuperscript{+}-ATPase ATP Binding Site

Fig. 1. Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and K\textsuperscript{+}-dependent pNPPase activity by TNP-ADP. Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (A) and pNPPase activity (B) were assayed in the presence of varied concentrations of substrate and varied concentrations of TNP-ADP. The units for both activity measurements were μmol/mg/min. The data are plotted as Dixon plots. A, ATP concentrations were as follows. ○, 0.5 mM; ●, 1 mM; □, 2 mM; ■, 3 mM. Total [Na\textsuperscript{+}] was kept constant. Double reciprocal plots of the data for [TNP-ADP] = 0 yielded $V_{\text{max}} = 66.7 \pm 2.2$ enzyme units and $K_{\text{m}} = 0.4 \pm 0.05$ mM (data not shown). The data yielded a $K_i = 0.21 \pm 0.06$ μM for TNP-ADP. B, pNPP concentrations were as follows. ○, 2 mM; ●, 4 mM; □, 6 mM; ■, 10 mM. Double reciprocal plots of the data for [TNP-ADP] = 0 yielded $V_{\text{max}} = 6.42 \pm 0.26$ μmol/mg/min and $K_{\text{m}} = 0.65 \pm 0.22$ mM (data not shown). The data yielded a $K_i = 0.22 \pm 0.07$ μM for TNP-ADP.

Fig. 2. Inhibition of the K\textsuperscript{+}-dependent pNPPase activity of FITC-modified Na\textsuperscript{+},K\textsuperscript{+}-ATPase by TNP-ADP. Na\textsuperscript{+},K\textsuperscript{+}-ATPase was modified in the presence of 15 mM NaCl with FITC as described under "Experimental Procedures." K\textsuperscript{+}-dependent pNPPase activity was measured in the presence of varied concentrations of pNPP and TNP-ADP. The pNPP concentrations were as follows. ■, 1 mM; □, 2 mM; ●, 4 mM; ○, 10 mM. Double reciprocal plots of the data for [TNP-ADP] = 0 yielded $V_{\text{max}} = 5.2 \pm 0.5$ μmol/mg/min and $K_{\text{m}} = 3.2 \pm 0.8$ mM (data not shown). The data yielded a $K_i = 51 \pm 7$ μM for TNP-ADP.

Inhibition of K\textsuperscript{+}-dependent pNPPase Activity by ErITC—Renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase with about half-maximal theoretical phosphorylation capacity binds about 0.5 FITC molecules per αβ protomer and then binds an additional 0.5 molecule of ErITC per αβ protomer to the FITC-labeled enzyme with inhibition of the residual K\textsuperscript{+}-dependent pNPPase activity (11). In this calculation, the entire protein mass of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase preparation was taken to consist of both α and β protomers although only half could be phosphorylated. Fluorescence resonance energy transfer measurements between bound FITC and bound ErITC indicated that the two probes were too far apart to be bound to the same protomer (11). ATP at high concentration prevented the binding of ErITC to the FITC-labeled enzyme (11).

Fig. 3 shows the results of an experiment in which unmodified nasal gland enzyme was incubated in the presence of the indicated concentrations of ErITC. The incubation took place in solutions containing Na\textsuperscript{+}, which promotes the E\textsubscript{1} conformation, and in solutions containing K\textsuperscript{+}, which promotes the E\textsubscript{2} conformation (16). After incubation for 1 h at 37 °C, samples were removed and used for the measurement of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and K\textsuperscript{+}-dependent pNPPase activity. The fitted curves indicated that half-maximal inhibition of ATPase activity occurred at 56 nM ErITC after incubation in Na\textsuperscript{+} solution and at 144 nM after incubation in K\textsuperscript{+} solution. Half-maximal inhibition of K\textsuperscript{+}-dependent pNPPase activity occurred at 107 nM ErITC after incubation in Na\textsuperscript{+} solution and at 301 nM after incubation in K\textsuperscript{+} solution. The data indicate that half-maximal inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and pNPPase activity occurred at comparable but not identical low concentrations of ErITC in the preincubation solution whether the solution contained Na\textsuperscript{+} or K\textsuperscript{+}. The size of the experiments required that measurement of ATPase and pNPPase activity take place on separate days,
and uncontrolled differences in the ErITC preincubation conditions may have contributed to the discrepancies. However, for either activity, inhibition was more effective when incubation with ErITC took place in a Na⁺ solution in which the E₁ conformation is favored than in a K⁺ solution, which favors the E₂ conformation (16).

Fig. 4 shows another, more focused, experiment in which unmodified enzyme was preincubated with ErITC in Na⁺ solution, and Na⁺,K⁺-ATPase and K⁺-dependent pNPPase activity was determined immediately after the preincubation. In this case, the plots of ATPase activity and pNPPase activity versus the concentration of ErITC in the preincubation solution are nearly superimposable.

Fig. 5 shows the effect of ATP in the preincubation solution on inhibition of the activities of enzyme preincubated with ErITC. ATP protects Na⁺,K⁺-ATPase activity against inhibition with high affinity (half-maximal protection at 0.94 μM) and protects K⁺-dependent pNPPase activity from inhibition with a similar high affinity (half-maximal protection at 1.48 μM). In these studies, preincubation with ATP took place in sodium solution so that protection cannot be attributed to selection of the E₁ conformation by ATP.

ErITC inhibits K⁺-dependent pNPPase activity of FITC-modified renal enzyme, and ATP protects against inhibition (11). Fig. 6 shows that ErITC inhibits K⁺-dependent pNPPase activity of FITC-labeled nasal gland enzyme as well, but at a higher concentration than with renal enzyme and at a higher concentration than with the unmodified enzyme. As with the unmodified enzyme, half-maximal inhibition occurred at a lower concentration of ErITC in Na⁺ solution (2.6 μM) than in K⁺ solution (5.5 μM).

For the experiment shown in Fig. 7, FITC-labeled nasal gland enzyme was incubated at 2 μM ErITC (the concentration that half-maximally inhibited pNPPase activity in Na⁺ solution in the experiment shown in Fig. 6) and the indicated concentrations of ATP. ATP does not protect against inhibition by ErITC of the FITC-modified nasal gland enzyme even at very high ATP concentrations.

Fig. 8 shows the results of an experiment in which ErITC inhibition of K⁺-dependent pNPPase activity of FITC-treated nasal gland enzyme was measured as a function of time in the presence and absence of Na⁺ and ATP. The experiment was similar to one previously reported using renal enzyme (11). ATP protected against inhibition of the renal enzyme but not of the nasal gland enzyme. In fact, in Na⁺-free solution, incubation with ATP led to about the same level of inhibition by ErITC as in Na⁺ solution, and in both cases inhibition was greater than in Na⁺-, K⁺-, and ATP-free solution. Both ATP and Na⁺ promote E₁ conformations.

**DISCUSSION**

The Oligomeric Structure of the Functional Enzyme Unit—We have examined the effect of the two ligands that inhibit pNPPase activity of FITC-labeled renal enzyme on duck nasal gland enzyme, which has nearly maximal phosphorylation capacity (9) and FITC binding capacity. All protomers in the nasal gland enzyme bind FITC, which inhibits functions of the putative high affinity site but spares functions of the low affinity site, so that residual K⁺-dependent phosphatase activity is certainly a property of FITC-modified protomers. Since
incubated at 37 °C, and K

enzyme was subsequently divided into four suspensions, which received described under "Experimental Procedures." The modified enzyme was divided into two suspensions, which received varied amounts of Tris-ATP. In addition, one suspension received 2 μM ErITC (□), and the other was a control (○). The suspensions were incubated for 1 h at 37 °C and assayed for K

-dependent pNPPase activity. The units of activity are μmol/mg/min. The data were fit to a linear regression.

TNP-ADP and ErITC inhibit pNPPase activity of FITC-modified nasal gland enzyme, they must do so by binding to FITC-modified protomers. Our results unambiguously exclude the possibility that high affinity and low affinity ATP binding sites that react with these ligands exist simultaneously on separate protomers in nasal gland enzyme.

Our findings are consistent with the results of similar studies with TNP-ADP using renal enzyme (10) but are inconsistent with studies of ErITC inhibition in renal enzyme (11). Although the findings with ErITC in renal and nasal gland enzyme differ, in both renal and nasal gland enzyme the binding sites for FITC and TNP-ADP coexist on the same protomer. Moreover, recent measurements in renal enzyme of fluorescence resonance energy transfer between FITC and Co(NH₃)₄ ATP, which labels the proposed low affinity ATP binding site, show that the two ligands are close enough to be bound to the same αβ protomer (17). Nasal gland enzyme is fully active, with a turnover rate even greater than that of renal enzyme. It is inconceivable that Na⁺ ,K⁺-ATPase, which is highly conserved, operates by radically different reaction mechanisms in enzyme from the two sources.

The Putative ATP Binding Sites—Both TNP-ADP and ErITC inhibit Na⁺ ,K⁺-ATPase activity and K⁺-dependent pNPPase activity in unmodified nasal gland enzyme. Both activities are equally sensitive to inhibition as evidenced by the Kᵢ values for TNP-ADP (Fig. 1) and the EC₅₀ values for ErITC (Fig. 4). For ErITC, the relation between ATPase activity and inhibitor concentration is the same as the relation between pNPPase activity and inhibitor concentration. ATP at micromolar concentrations protects both ATPase activity and pNPPase activity against ErITC inhibition, and the concentration of ATP necessary for half-maximal protection is the same for both activities. The very low concentration of TNP-ADP that inhibits ATPase and pNPPase activity, and the very low concentration of ATP that protects against ErITC inhibition, are characteristic of the concentrations at which these ligands interact with the high affinity ATP binding site in the E₁ conformation of the enzyme. The half-maximal concentration of ErITC that inhibits the enzyme activities is lower in Na⁺ solutions, which promote E₂ conformations, than in K⁺ solutions, which promote the E₁ conformation. Equivalence of the conditions of ErITC incubation that inhibit Na⁺ ,K⁺-ATPase activity with those that inhibit pNPPase activity in the unmodified enzyme and equivalence of the concentration of ATP that protects against inhibition lead to the conclusion that inhibition of the two activities in the unmodified enzyme occurs as a result of binding of the inhibitor to a single site, and that site is the high affinity ATP binding site in the E₁ conformation. There is no reason to believe that there is more than one ATP binding site in unmodified nasal gland enzyme. Since it is unmodified enzyme that exchanges cations, the conclusion that coexisting low and high affinity ATP binding sites are not involved in the reaction mechanism is inescapable. Specifically, there is no K⁺-dependent pNPPase activity left that could be inhibited by binding of ErITC to a lower affinity site.

Alternative Explanations for the Effect of Ligands on pNPPase Activity in Modified Enzyme—FITC modification of nasal gland enzyme eliminates Na⁺ ,K⁺-ATPase activity, but K⁺-dependent pNPPase activity is preserved. TNP-ADP inhibits pNPPase activity in FITC-modified nasal gland enzyme, but half-maximal inhibition occurs at a concentration of inhibitor 200 times higher than the concentration at which it inhibits pNPPase activity in unmodified enzyme. Similarly, ErITC inhibits pNPPase activity in FITC-modified nasal gland enzyme but at a concentration more than 50-fold higher than with unmodified enzyme. Half-maximal inhibition of pNPPase activity by ErITC in FITC-modified enzyme occurs at a lower concentration in Na⁺ solution than in K⁺ solution. ATP even at very high concentration does not protect pNPPase activity of FITC-modified enzyme against inhibition by ErITC. If there is a specific site at which the ligands inhibit pNPPase activity, it must be induced in nasal gland enzyme by FITC. But more likely explanations for the findings that do not involve coexisting high affinity and low affinity ATP binding sites can be advanced.

Inhibition of ATPase and pNPPase activity by ATP analogues is frequently attributed to steric hindrance. In the experiments reported here, TNP-ADP and ErITC (ErITC is a significantly larger molecule than FITC) may inhibit ATPase and pNPPase activities of the unmodified enzyme by binding to the enzyme in the E₁ conformation at the high affinity ATP binding site and blocking access of the substrates ATP and pNPP to the hydrolysis site. FITC, on the other hand, may block access of ATP to its binding site but only partially impair access of pNPP to its hydrolysis site. By binding to a portion of
the ATP binding site not occupied by FITC, other ligands could impair access of pNPP to the hydrolysis site. It has been suggested that ligands can inhibit pNPPase activity in FITC-modified enzyme because FITC occupies less than the entire ATP binding site, leaving enough of the site available to bind other ligands (although at low affinity) (18). Such a mechanism could account for some of the observations we report here. In unmodified enzyme, TNP-ADP and ErITC may occupy enough of the high affinity ATP binding site to block access of pNPP as well as ATP to the hydrolysis site. Since the site that these ligands occupy in the unmodified enzyme is the high affinity ATP binding site, and the high affinity site is a property of the E₁ conformation, it would not be surprising that the effect of ErITC in FITC-modified enzyme is greater in Na⁺ and stabilize an E₁ conformation that does not hydrolyze two ligands interact nonspecifically with FITC-labeled enzyme (15), and we found that ErITC inhibition of ability of ATP to prevent inhibition by ErITC of p

not hydrolyze tration bind nonspecifically (16). Both TNP-ADP (22) and ErITC (23) at high concentration bind nonspecifically (i.e. at sites not protected by high concentrations of ATP) to unmodified renal enzyme. This non-specific binding most likely occurs to FITC-modified enzyme too. FITC-labeled enzyme is known to undergo conformational changes in response to changes in Na⁺ and K⁺ concentration (15), and we found that ErITC inhibition of pNPPase activity is more effective in Na⁺ solution (E₁ conformation) than in K⁺ solution (E₂ conformation). Perhaps at high concentration, the two ligands interact nonspecifically with FITC-labeled enzyme and stabilize an E₁ conformation that does not hydrolyze pNPP.

We suggest these possible explanations for our results that do not involve ATP binding sites since it is clear that low affinity and high affinity ATP binding sites do not coexist in unmodified enzyme. In fact, selecting a unique model for these data is quite difficult, and a functional or binding experiment that would unambiguously select among the two possibilities we discuss above has not occurred to us. A final resolution of the question of whether two ATP sites coexist on a functional enzyme unit may require a high resolution structure obtained by diffraction methods (7). But since the phenomena are essentially artifacts in no way related to the reaction mechanism of the enzyme, the need for an explanation does not seem urgent.

In the meantime, our results rule out the possibility that the ligand pairs we tested bind to separate protomers in an interacting diprotomer and exclude the possibility that high and low affinity ATP binding sites coexist in the unmodified enzyme. Since it is unlikely that multiple high affinity and low affinity sites exist, we doubt that other ligand pairs will yield results supporting the presence of two coexisting binding sites. If such findings are made with renal enzyme, it would be prudent to evaluate the results with a fully active enzyme such as the nasal gland preparation or red cell membranes (24).

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REFERENCES

1. Czerwinski, A., Gitelman, H. J., and Welt, L. G. (1967) Am. J. Physiol. 213, 786–792
2. Neufeld, A. H., and Levy, H. M. (1969) J. Biol. Chem. 244, 6493–6497
3. Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2357–2366
4. Post, R. L., Hegvary, C., and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540
5. Glyn, I. M., and Karlish, S. J. D. (1982) in Membranes and Transport, Vol. 1 (Martonosi, A. N., ed) pp. 529–536, Plenum, New York
6. Buchbaum, E., Serpersu, E. H., Antalovic, R., Hamer, E., Willeke, M., and Schoner, W. (1991) in The Sodium Pump: Recent Developments (Kaplan, J. H., and DeWeer, P., eds) pp. 405–408, Rockefeller University Press, New York
7. Scheiner-Bobis, G., Antonipillai, A., and Farley, R. F. (1993) Biochemistry 32, 9592–9599
8. Schoner, W., Thönges, D., Hamer, E., Antalovic, R., Buchbaum, E., Willeke, M., Serpersu, E. H., and Scheiner-Bobis, G. (1994) in The Sodium Pump (Bamberger, E., and Schoen, W., eds) pp. 332–341, Steinhoff, Darmstadt, Germany
9. Martin, D. W., and Sachs, J. R. (1999) Biochemistry 38, 7485–7497
10. Ward, D. G., and Cavieiras, J. D. (1996) J. Biol. Chem. 271, 12317–12321
11. Linnertz, H., Urbanova, P., Ohsil, T., Hermann, P., Amler, E., and Schoner, W. (1998) J. Biol. Chem. 273, 28813–28821
12. Carilli, C. T., Farley, R. A., Perlman, D. M., and Cantley, L. C. (1982) J. Biol. Chem. 257, 5601–5606
13. Xu, K. Y. (1989) Biochemistry 28, 5764–5772
14. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356
15. Karlish, S. J. D. (1980) J. Bioenerg. Biomembr. 12, 113–116
16. Jargensen, P. L. (1974) Biochem. Biophys. Acta 247, 2357–2366
17. Faller, L. D., Kasho, V. N., Smirnova, I. N., Lin, S.-H., and Farley, R. A. (2000) Biophys. J. 79, 77 (abstr.)
18. Davis, R. L., and Robinson, J. D. (1988) Biochem. Biophys. Acta 953, 26–36
19. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biol. Sci. 23, 127–129
20. Zhang, P., Toyoshima, C., Yonehura, K., Green, N. M., and Stokes, D. L. (1998) Nature 392, 835–839
21. Stokes, D. L., and Green, N. M. (2000) Biochem. J. 370, 1765–1776
22. Hellen, E. H., and Pratap, P. R. (1997) Biophys. Chem. 69, 197–214
23. Amler, E., Abbott, A., and Ball, W. J., Jr. (1992) Biochem. J. 281, 553–568
24. Sachs, J. R. (1994) Biochim. Biophys. Acta 1193, 199–211