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Role of Phospholipids in the Binding of Bumetanide to the Rabbit Parotid Na/K/Cl Cotransporter

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Summary. It was recently reported (Turner, R.J., George, J.N., 1990, *J. Membrane Biol.* **113**:203–210) that the high affinity bumetanide binding site of the rabbit parotid Na/K/Cl cotransporter could be extracted from a basolateral membrane preparation from this gland using relatively low concentrations of the non-ionic detergent Triton X-100. At the detergent : protein ratios required for complete membrane solubilization bumetanide binding activity in this extract was lost but could be recovered by the addition of crude soybean lipids. In the present paper the ability of various purified lipids to restore high affinity bumetanide binding activity in detergent solubilized rabbit parotid basolateral membranes is studied. We show that the effect of exogenous lipid on the detergent-inactivated bumetanide binding site is to increase the affinity of binding without affecting the number of binding sites. Of the 11 lipid species tested, several relatively minor, negatively charged membrane phospholipids are the most effective in restoring binding activity (phosphatidylserine ≈ phosphatidylglycerol > phosphatidylinositol > cardiolipin), while the major mammalian plasma membrane lipid components phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol are without effect. In addition, we show that in the presence of these minor lipids the affinity of bumetanide binding is considerably increased over that observed in the native membrane (e.g., $K_d \approx 0.06$ μM in membranes extracted with 0.3% Triton and treated with 0.15% wt/vol phosphatidylserine, *vs.* $K_d \approx 3$ μM in native basolateral membranes). This dramatic dependence of bumetanide binding affinity on the presence of certain lipid species suggests that the properties of the bumetanide binding protein *in situ* may be quite dependent on the minor lipid content of the plasma membrane. This effect may account for the relatively large variations in bumetanide binding affinity observed from tissue to tissue.

Key Words loop diuretics · exocrine gland · fluid secretion · lipid · acinar cell · ion transport · chloride secretion · detergent

Introduction

Considerable recent experimental effort has been devoted to the characterization of Na/K/Cl cotransport systems [9, 19]. These transporters are responsible for driving salt and water movements in many absorptive and secretory epithelia [6, 8, 16, 19, 20, 32] and are also involved in volume regulatory ion fluxes in a number of other cell types [9]. Clinically, the Na/K/Cl cotransporter is the renal site of action of the commonly used loop diuretic furosemide and its more potent and specific analogue bumetanide.

A series of recent papers have demonstrated that the rabbit parotid is a convenient and relatively abundant source of the Na/K/Cl cotransporter [25–27]. Basolateral membrane vesicles prepared from this tissue show high levels of KCl-dependent ^{22}Na uptake [27] and Na/K/Cl-dependent [^{3}H]-bumetanide binding activity [25; 70–100 pmol of binding site/mg protein]. The properties of this bumetanide binding site correlate well with those of the inhibitory bumetanide site on the Na/K/Cl cotransporter [25], indicating that bumetanide binding can serve as a marker for the transport molecule, or at least a major part of it. The solubilization and partial purification of the rabbit parotid bumetanide binding site has been described [26]. In these studies it was found that the bumetanide binding site could be extracted from a parotid basolateral membrane preparation using a relatively low concentration of the non-ionic detergent Triton X-100 (0.07% wt/vol at 1 mg membrane protein/ml). This extracted site still exhibited Na/K/Cl-dependent, high affinity bumetanide binding. At higher detergent : protein ratios (>2 : 1, wt : wt) bumetanide binding activity was lost; however, it was found that this loss of binding could be prevented or (partially) reversed by the addition of crude soybean lipids [26]. The lipid-stabilized bumetanide binding site sedemented as a single band with $S_{20,w} \approx 9$ S in a 5–25% sucrose gradient [26]. This corresponds to a mol wt ~ 200 kDa for the bumetanide binding protein-detergent-lipid complex, in reasonably good agreement with estimates of the molecular weights of Na/K/Cl cotransporters in several other tissues [10, 11, 21].

It has been appreciated for some time that the

lipid composition of cell membranes can markedly affect the function of membrane bound proteins [1, 14, 29, 31]. In many cases the existence of specific lipid-protein interactions has been demonstrated. These interactions may involve the binding of lipids to highly specific modifier sites on the protein but are more commonly due to a more general association with a surrounding region of lipid (a so-called "lipid annulus") whose composition can be quite different from that of the rest of the membrane [3, 15, 29, 31, 33]. It is thought that the lipid annulus serves to both stabilize the protein and influence its functional properties. The characterization of these lipid-protein interactions is obviously of considerable importance since this information is essential for the success of protein purification and reconstitution studies as well as for our eventual understanding of the mechanism of action of membrane-bound proteins.

The inactivation of the rabbit parotid Na/K/Cl-dependent bumetanide binding site by Triton X-100 and its reactivation by the addition of exogenous lipid [26] is highly indicative of the involvement of membrane lipids in the functional state of this protein. In the present paper we investigate the specificity of this effect of exogenous lipid. We find that several relatively minor, negatively charged membrane lipids are the most effective in restoring bumetanide binding in detergent-treated membranes, while the major membrane lipid components tested are without effect. In addition, we show that in the presence of these minor lipids the affinity of bumetanide binding is dramatically increased over that observed in the native membrane. We suggest that this effect may account for the large variations in bumetanide binding affinity observed from tissue to tissue.

Materials and Methods

Vesicle Preparation

Basolateral membrane vesicles (BLMV) were prepared from rabbit parotid by a Percoll gradient method as previously described [27]. Relative to the starting tissue homogenate, the activity of the basolateral membrane marker K-stimulated *p*-nitrophenyl phosphatase is enriched 9–12 times in this vesicle fraction. Freshly prepared BLMV were suspended in buffer A (10 mM Tris/HEPES plus 100 mM mannitol) containing 1 mM EDTA and 100 mM KCl at a protein concentration of approximately 5 mg/ml. Aliquots (0.75 mg protein) of BLMV were fast frozen and stored above liquid nitrogen.

On the day of the experiment an appropriate number of aliquots of frozen BLMV were thawed for 30 min at room temperature, diluted 100 times with buffer A containing 1 mM EDTA (buffer A/EDTA) and centrifuged at 48,000 × *g* for 20 min. The resulting pellets were taken up in buffer A/EDTA and stored on ice until use.

Protein was measured by the method of Bradford [2] using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine gamma globulin as the standard.

Membrane Protein Extraction with Triton X-100

BLMV in buffer A/EDTA were diluted with a Triton X-100 (Sigma T-6878) stock solution to yield a mixture of the following composition: sodium gluconate, 100 mM; potassium gluconate, 95 mM; KCl, 5 mM; EDTA, 0.5 mM; Triton X-100, 0.3% (wt/vol); protein, 1 mg/ml; in buffer A. This material was left on ice for 30 min then centrifuged for 5 min at 150,000 × *g* in a Beckman Airfuge. The supernate from this spin is referred to as the "0.3% Triton extract." Unless otherwise stated, all steps involving the 0.3% Triton extract were carried out on ice.

Treatment of the 0.3% Triton Extract with Lipids

Lipids supplied as solids by the manufacturer were dissolved in chloroform (1% wt/vol). Lipids were added to the 0.3% Triton extract by evaporating a suitable volume of dissolved lipid under nitrogen at the bottom of a 10 × 75 disposable glass test tube then adding the 0.3% Triton extract. The resulting mixture was vortexed vigorously for 10 sec, sonicated for 10 sec in a Branson B12 ultrasonic cleaner, vortexed and sonicated again, and passed through a 30-g needle.

Binding Measurements

Equilibrium bumetanide binding was measured using a nitrocellulose filtration assay as previously described [25, 26]. Briefly, a 20 μl aliquot of sample was combined with the same volume of incubation medium consisting of buffer A containing 100 mM sodium gluconate, 95 mM potassium gluconate, 5 mM KCl and appropriate concentrations of [^{3}H]-bumetanide (1.0 μM unless otherwise stated). In previous experiments we have established that these concentrations of sodium, potassium and chloride yield near optimal conditions for high affinity bumetanide binding to the rabbit parotid Na/K/Cl cotransporter [25, 26]. After 60 min of incubation at 25°C the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (buffer A containing 100 mM NaCl plus 100 mM KCl) followed by Millipore filtration (HAWP 0.45 μm). The filter was then washed with a further 6.0 ml of stop solution, placed in a scintillation vial with 10 ml of ACS (Aqueous Counting Scintillant, Amersham, Arlington Heights, IL) containing 1% glacial acetic acid (vol/vol) and counted for radioactivity along with samples of the incubation medium and appropriate standards.

The validity of the above rapid filtration assay for the measurement of [^{3}H]-bumetanide binding to both native and detergent-treated BLMV has been previously established [26].

All data have been corrected for nonspecific binding and retention of [^{3}H]-bumetanide by subtracting the binding observed in the presence of 0.1 or 1.0 mM unlabeled bumetanide (3.82 ± 0.64 pmol/mg protein/μM, n = 24). All binding studies were carried out in triplicate. The errors shown in the Figures and

quoted in the text are standard deviations. Unless otherwise indicated, the results of single experiments representative of three or more studies carried out under similar experimental conditions are shown.

Materials

Crude soybean lipid was from Sigma Chemical (P-5638). The following purified lipids were from Avanti Polar Lipids (Birmingham, AL): PS[1] (#840032, from bovine brain), PG (#831138, from egg), PI (#840042, from bovine liver), PI (#830044, from soybean), CL (#830012, from bovine heart), PA (#840101, from egg), PC (#830054, from soybean), PE (#840024, from soybean), Ly-PC (#830071, from egg). SM (from bovine brain) was from United States Biochemical Corp. (Cleveland OH) and cholesterol was from Sigma (C-7402). Unlabeled bumetanide was a gift from Hoffman-LaRoche (Nutley, NJ). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

[^{3}H]-bumetanide (66.2 Ci/mmol, radiochemical purity 98%) was custom synthesized for us by Amersham (Arlington Heights, IL) from the precursor 3-amino-4-phenoxy-5-sulfamoylbenzoic acid, generously supplied by Dr. P.W. Feit (Leo Pharmaceuticals, Ballerup, Denmark).

Results

It was recently shown that the high affinity bumetanide binding site of the rabbit parotid acinar cell could be extracted from a BLMV preparation from this gland using relatively low concentrations of the non-ionic detergent Triton X-100 (0.07% wt/vol; 1 mg membrane protein/ml). This extracted site could not be sedemented at 100,000 × g × 1 hr [26]. Bumetanide binding to this site retained the ionic characteristics of bumetanide binding to native membranes but showed a fivefold increase in binding affinity ($K_d \approx 0.6$ μM for the extract *vs.* $K_d \approx 3$ μM for native membranes). At this detergent : protein ratio (approximately 1 : 1), however, one would not expect a complete solubilization of the membrane but rather dissolution into macromolecular lipid-protein-detergent complexes with molecular weights ~ 1 million [12]. At the higher concentrations of Triton X-100 required for complete solubilization, the bumetanide binding site was inactivated and appeared to be unstable [26]. But both of these effects could be at least partially reversed by the addition of 0.2% (wt/vol) crude soybean lipid [26]. The dependence of this effect on lipid concentration

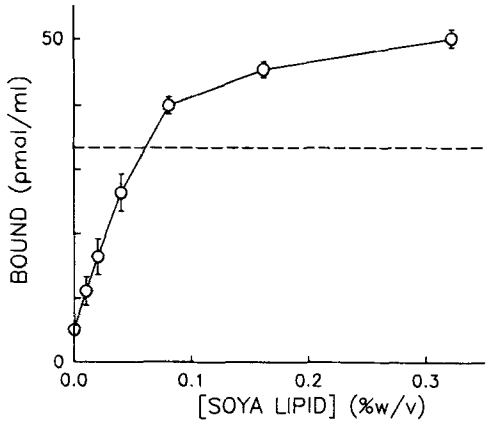

Fig. 1. Effect of various concentrations of crude soybean lipid on specific bumetanide binding to the 0.3% Triton extract of rabbit parotid BLMV. Binding is expressed per milliliter of sample in order to allow direct comparison of binding to the extract and to the original vesicles (33.4 pmol/ml at 1 mg BLMV/ml, dotted line)

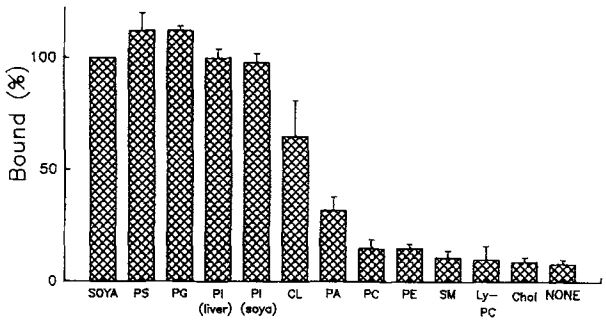

Fig. 2. Ability of various lipids to reverse the inactivation of bumetanide binding produced by 0.3% Triton X-100. Lipids were added to the 0.3% Triton extract at a concentration of 0.15% wt/vol. All results are normalized to the binding observed in the presence of 0.15% crude soybean lipid. The results of nine independent experiments were combined to produce the figure; $n \geq 3$ for each lipid. The bar labeled *NONE* represents binding in the untreated 0.3% Triton extract

is illustrated in Fig. 1. Here various amounts of crude soybean lipid were added to the 0.3% Triton extract of rabbit parotid BLMV (*see* Methods) before assaying for bumetanide binding activity. In the absence of exogenous lipid little bumetanide binding activity is detectable (2.8 ± 1.5 pmol/ml, $n = 6$); however, binding activity markedly increases with lipid concentration with a half maximal effect at ~ 0.05%.

In Fig. 2 we investigate the specificity of this effect of exogenous lipids. Here we compare the abilities of various highly purified lipids (typically > 99%) to reverse the inactivation of bumetanide binding produced by 0.3% Triton X-100. The results have been expressed relative to the binding observed

[1] *Abbreviations used:* PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Ly-PC, lysophosphatidylcholine; CL, cardiolipin; PA, phosphatidic acid; SM, sphingomyelin; BLMV, basolateral membrane vesicles.

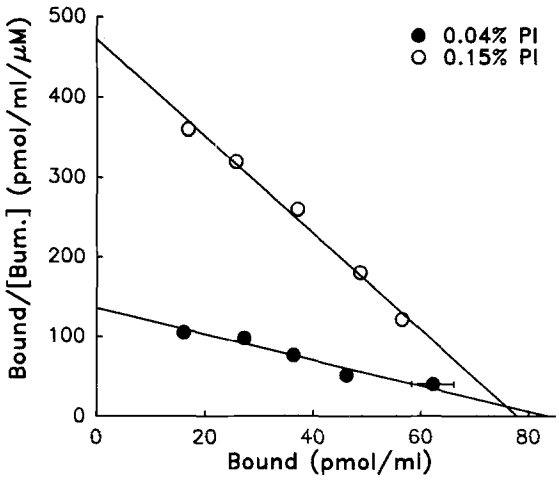

Fig. 3. The effect of PI concentration on the kinetics of high affinity bumetanide binding to the 0.3% Triton extract of rabbit parotid BLMV. Purified PI (Avanti #840042, from bovine liver) was added to the 0.3% Triton extract at the concentrations indicated (wt/vol). Binding was measured over the bumetanide concentration ranges 0.15 to 1.5 μM (●) and 0.047 to 0.47 μM (○). Linear least squares regression analysis of the data yield K_d = 0.616 ± 0.085 μM, B_{max} = 83.4 ± 6.6 pmol/ml, r = 0.973 (●) and K_d = 0.165 ± 0.009 μM, B_{max} = 77.9 ± 2.4 pmol/ml, r = 0.996 (○)

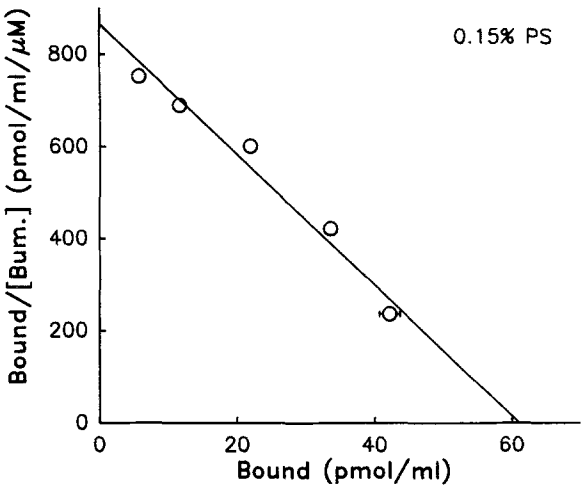

Fig. 4. The concentration dependence of bumetanide binding to the 0.3% Triton extract measured in the presence of 0.15% PS (Avanti #840032, from bovine brain). Binding was measured over the bumetanide concentration range 0.0076 to 0.179 μM. Linear least squares regression analysis of the data yield K_d = 0.0.071 ± 0.007 μM, B_{max} = 61.6 ± 4.1 pmol/ml, r = 0.985

in the presence of the same concentration (0.15%, wt/vol) of crude soybean lipid. In order of decreasing effectiveness, PS ≈ PG > PI > CL > PA resulted in a significant ($P < 0.02$) increase in specific bumetanide binding activity relative to untreated controls, while PC, PE, SM, Ly-PC and cholesterol were without significant effect.

Figure 3 shows the results of an experiment where bumetanide binding was determined as a function of bumetanide concentration in the 0.3% Triton extract in the presence of 0.04 and 0.15% PI (the effect of PI in restoring specific bumetanide binding activity is near maximal at 0.15%—*data not shown*). The effect of increasing the concentration of PI is clearly to decrease the K_d for bumetanide binding with little change in the number of binding sites. The averaged results of three experiments of this type yielded K_d's of 0.552 ± 0.056 μM at 0.04% PI and 0.158 ± 0.006 μM at 0.15% PI. The average ratio of the number of binding sites measured at 0.15% PI to that found at 0.04% PI in these experiments was 0.99 ± 0.05.

In the experiment illustrated in Fig. 4 the dependence of specific bumetanide binding on bumetanide concentration was determined in the 0.3% Triton extract in the presence of 0.15% PS. The averaged results of three experiments of this type yielded K_d = 0.064 ± 0.014 μM, a value significantly ($P <$ 0.0002) lower than that observed in the presence of the same concentration of PI (*cf.* Fig. 3) and approximately 50 times lower than that observed in native BLMV [25, 26].

Discussion

We demonstrate here that there is a dramatic difference in the ability of various purified lipids to restore high affinity bumetanide binding activity in detergent solubilized rabbit parotid BLMV. In order of decreasing effectiveness, the lipids PS ≈ PG > PI > CL > PA restored significant binding activity, while PC, PE, SM, cholesterol and Ly-PC were without effect (Fig. 2). Similar observations of a loss of membrane protein function following detergent treatment and the recovery of this function with the addition of exogenous lipids have been made for a number of membrane bound enzymes [14, 29]. The specificity of these effects for various lipid species has been shown to vary markedly from protein to protein [14, 29]. Analogous lipid requirements for membrane transport proteins can be inferred from studies which demonstrate that certain lipids or combinations of lipids are required for optimal reconstitution of transport activity [4, 24, 28, 30].

Studies of lipid-protein interactions employing electron spin resonance, fluorescence anisotropy, differential scanning calorimetry and ^{31}P nuclear magnetic resonance indicate the presence of a motion-restricted population of lipid associated with membrane-bound proteins [1, 3, 15, 17, 23, 31].

These observations together with model calculations and more direct studies of protein-associated lipid [18] have given rise to the concept of a relatively immobile lipid "annulus," or "boundary layer," associated with most if not all membrane proteins. These lipids are thought to interact with the protein at specific amphiphile binding sites [5, 18] and to stabilize its three dimensional structure as well as affect its functional properties. Since these sites would be expected to preferentially bind some lipids over others, the composition of the lipid annulus could be quite different from that of the membrane as a whole. In this regard, there is strong evidence for a preference for negatively charged lipid at the protein-lipid interface of Na/K ATPase [3, 33].

The concept of a stabilizing annulus of bound lipid can also account for the observations cited above concerning the loss of membrane protein function with detergent treatment and the recovery of this function following the addition of certain lipids. At relatively high detergent : lipid concentration ratios one might expect detergent molecules to effectively compete with and displace lipids at their hydrophobic binding sites on proteins. The presence of amphiphilic binding sites on the sarcoplasmic reticulum Ca^{2+}-ATPase with much higher affinity for phospholipids than nonionic detergents has, in fact, been demonstrated [5]. Unless bound detergent could suitably duplicate the stabilizing effect of bound lipid, this displacement would result in protein inactivation. The addition of suitable exogenous lipid would be expected to reverse this process, as observed here for the Na/K/Cl-dependent bumetanide binding site (Fig. 1).

The effect of exogenous lipid on the detergent-inactivated bumetanide binding site is to increase the affinity of binding (Figs. 3 and 4) without affecting the number of binding sites (Fig. 3). The observation that increasing concentrations of lipid result in an increasing affinity for a single class of binding sites (Fig. 3) is consistent with the concept that the protein progressively regains its complement of associated lipids (presumably in exchange for detergent) as their availability increases. The resulting affinity of bumetanide binding is clearly dependent on lipid species as well as concentration (Figs. 3 and 4).

It is worth stressing at this point that, although our data are consistent with the concept of simple displacement and replenishment of stabilizing annular lipid according to the lipid/detergent composition of the surrounding solution, many other phenomena could be responsible for our results. For example, the high affinity Na/K/Cl-dependent bumetanide binding site could be a multimer and specific lipids might be required to preserve subunit associations. Alternatively, protein-associated lipid could actually form a part of the bumetanide binding site such that the presence of this lipid is required for high affinity binding. The exploration of these possibilities is beyond the scope of the present article.

It is particularly interesting that the major mammalian plasma membrane components PC, PE, SM and cholesterol are ineffective in restoring bumetanide binding activity, while the relatively minor negatively charged phospholipids PS, PG, PI and CL are particularly potent. This observation suggests that the properties of the bumetanide binding site and thus of the Na/K/Cl cotransporter may vary considerably with the minor lipid composition of the plasma membrane. Indeed, the protein composition of the membrane may also prove to be important in this regard, as the following calculation shows. Values of the protein : lipid ratio of plasma membranes vary widely from cell type to cell type; however, on average, a ratio of about 60 : 40 (wt/wt) is observed. This value is, in fact, approximately what is found in the parotid basolateral membrane (R.J. Turner and J.N. George, *unpublished observations*). Assuming an average protein mol wt of 100,000 and a lipid mol wt of 800, one can calculate from this ratio that there are approximately 85 lipid molecules/protein. Estimates of the amount of protein-associated (annular) lipid range from 20–80 lipid molecules/protein molecule [1]. Despite the approximate nature of these numbers it is clear that a significant proportion of the membrane lipid can be sequestered in protein boundary layers. Thus the lipid specificity of the major membrane proteins can dramatically affect the availability of minor membrane lipids to less abundant proteins such as the Na/K/Cl cotransporter (the rabbit parotid basolateral membrane expresses approximately 80 pmol of bumetanide binding sites per mg of protein; with an estimated mol wt of 160,000 Da this means that the bumetanide binding site accounts for only 1.2% of the total membrane protein).

Reported values for the K_d of Na/K/Cl-dependent bumetanide binding, measured in isolated membranes under optimal ionic conditions for the experimental system under study, range from 0.03 [7] to 3 μM [25]. Reported IC_{50}'s for bumetanide inhibition of Na/K/Cl cotransport processes in intact cells also range over several orders of magnitude [13, 19, 22]. Although this diversity could simply be due to differences in the Na/K/Cl cotransport proteins expressed in these tissues, the results of the present paper suggest that variations in membrane minor lipid composition or availability could be responsible for these effects.

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References

1. Benga, G. 1985. Protein-lipid interactions in biological membranes. *In:* Structure and Properties of Cell Membranes. G. Benga, editor. Vol. 1, pp. 159–188. CRC, Boca Raton, FL
2. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:**248–254
3. Brotherus, J.R., Jost, P.C., Griffith, O.H., Keana, J.F.W., Hokin, L.E. 1980. Charge selectivity at the lipid-protein interface of membranous Na,K-ATPase. *Proc. Natl. Acad. Sci. USA* **77:**272–276
4. Chen, C.C., Wilson, T.H. 1984. The phospholipid requirement for activity of the lactose carrier of *Escherichia coli*. *J. Biol. Chem.* **259:**10150–10158
5. Dean, W.L., Suarez, C.P. 1981. Interactions between sarcoplasmic reticulum calcium adenosinetriphosphatase and nonionic detergents. *Biochemistry* **20:**1743–1747
6. Epstein, F.H., Stoff, J.S., Silva P. 1983. Mechanism and control of hyperosmotic NaCl-rich secretion by the rectal gland of *Squalus acanthias*. *J. Exp. Biol.* **106:**25–41
7. Forbush, B., Palfrey, H.C. 1983. [^{3}H]Bumetanide binding to membranes isolated from dog kidney outer medulla. *J. Biol. Chem.* **258:**11787–11792
8. Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:**F1–F8
9. Geck, P., Heinz, E. 1986. The Na-K-2Cl cotransport system. *J. Membrane Biol.* **91:**97–105
10. Haas, M., Forbush, B. 1987. Photolabeling of a 150-kDa (Na + K + Cl) cotransport protein from dog kidney with a bumetanide analogue. *Am. J. Physiol.* **253:**C243–C250
11. Haas, M., Forbush, B. 1988. Photoaffinity labeling of a 150-kDa (Na + K + Cl) cotransport protein from duck red blood cells with an analogue of bumetanide. *Biochim. Biophys. Acta* **939:**131–144
12. Hjelmeland, L.M., Chrambach, A. 1984. Solubilization of functional membrane proteins. *Methods Enzymol.* **104:**305–318
13. Hoffmann, E.K., Schiodt, M., Dunham, P. 1986. The number of chloride-cation cotransport sites on Ehrlich ascites cells measured with [^{3}H]bumetanide. *Am. J. Physiol.* **250:**C688–C693
14. Jain, M.K., Wagner, R. 1980. Introduction to Biological Membranes. John Wiley and Sons, New York
15. Jost, P.C., Griffith, O.H., Capaldi, R.A., Vanderkooi, G. 1973. Evidence for boundry lipid in membranes. *Proc. Natl. Acad. Sci. USA* **70:**480–484
16. Kinne, R., Hannafin, J.A., Konig, B. 1985. Role of the NaCl-KCl cotransport system in active chloride absorption and secretion. *Ann. N.Y. Acad. Sci.* **456:**198–206
17. Lentz, B.R., Clubb, K.W., Barrow, D.A., Meissner, G. 1983. Ordered and disordered phospholipid domains coexist in membranes containing the calcium pump protein of sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **80:**2917–2921
18. McIntosh, D.B., Ross, D.C. 1985. Role of phospholipid and protein-protein associations in activation and stabilization of soluble Ca^{2+}-ATPase of sarcoplasmic reticulum. *Biochemistry* **24:**1244–1252
19. O'Grady, S.M., Palfrey, H.C., Field, M. 1987. Characteristics and functions of Na-K-Cl cotransport in epithelial tissues. *Am. J. Physiol.* **253:**C177–C192
20. Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature (London)* **307:**693–696
21. Pewitt, B., Palfrey, H.C., Burnham, C.E. 1988. Affinity purification of a bumetanide binding protein possibly related to the Na/K/Cl cotransporter. *FASEB J.* **2:**A1725 (*Abstr.*)
22. Sands, J.M., Knepper, M.A., Spring, K.R., 1986. Na-K-Cl cotransport in apical membrane of rabbit renal papillary surface epithelium. *Am. J. Physiol.* **251:**F475–F484
23. Selinsky, B.S., Yeagle, P.L. 1984. Two populations of phospholipids exist in sarcoplasmic reticulum and in recombined membranes containing Ca-ATPase. *Biochemistry* **23:**2281–2288
24. Tefft, R.E., Carruthers, A., Melchior, D.L. 1986. Reconstituted human erythrocyte sugar transporter activity is determined by bilayer lipid head groups. *Biochemistry* **25:**3709–3718
25. Turner, R.J., George, J.N. 1988. Ionic dependence of bumetanide binding to the rabbit parotid Na/K/Cl cotransporter. *J. Membrane Biol.* **102:**71–77
26. Turner, R.J., George, J.N. 1990. Solubilization and partial purification of the rabbit parotid Na/K/Cl-dependent bumetanide binding site. *J. Membrane Biol.* **113:**203–210
27. Turner, R.J., George, J.N., Baum, B.J. 1986. Evidence for a $Na^+/K^+/Cl^-$ cotransport system in basolateral membrane vesicles from the rabbit parotid. *J. Membrane Biol.* **94:**143–152
28. Uratani, Y., Aiyama, A. 1986. Effect of phospholipid composition on activity of sodium-dependent leucine transport system in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **261:**5450–5454
29. Wrigglesworth, J.M. 1985. Lipid dependence of membrane enzymes. *In*: Structure and Properties of Cell Membranes. G. Benga, editor. Vol. 1, pp. 137–157. CRC, Boca Raton, FL
30. Xie, X.S., Tsai, S.J., Stone, D.K. 1986. Lipid requirements for reconstitution of the protein-translocating complex of clathrin-coated vesicles. *Proc. Natl. Acad. Sci. USA* **83:**8913–8917
31. Yeagle, P.L. 1989. Lipid regulation of cell membrane structure and function. *FASEB J.* **3:**1833–1842
32. Young, J.A., Cook, D.I., vanLennep, E.W., Roberts, M.L. 1987. Secretion by the major salivary glands. pp. 773–815. *In*: Physiology of the Gastrointestinal Tract. (2nd ed.) L. Johnson et al., editors. Raven, New York
33. Zachowski, A., Devaux, P.F. 1983. Non-uniform distribution of phospholipids in (Na + K)-ATPase-rich membranes from *Torpedo marmorata* electric organ evidenced by spin-spin interactions between spin-labeled phospholipids. *FEBS Lett.* **163:**245–249

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