In a typical experiment, we added a mixture of ^{3}H-labeled cRNA and ^{32}P-labeled Hg-cRNA in 0.2 mL of 0.1 M NaCl to 0.5 mL of sulfhydrylcellulose (packed volume) in a 1.5-mL microfuge tube. We agitated the sample with a vortex mixer for 15 s, then centrifuged it for 30 s in a microfuge, removed the supernatant fluid, and analyzed it for radioactivity. To the pellet we added 1 mL of 0.1 M NaCl and repeated the process until the level of radioactivity in the 0.1 M NaCl wash was negligible. We then repeated the procedure with wash buffer and finally with elution buffer.

This method produced an elution profile identical with column chromatography profiles of an identical sample (see Figure 2), with the same volumes of wash and elution buffers needed to remove nonmercurated RNA and to elute mercurated RNA, respectively. This application should prove useful for large samples of RNA, since sizable amounts of sulfhydrylcellulose can be processed rapidly in a tabletop centrifuge.

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References

Cuatrecasas, P., & Anfinsen, C. B. (1971) *Methods Enzymol. 21*, 345-378.
Dale, R. M. K., Martin, E., Livingston, D. C., & Ward, D. C. (1975) *Biochemistry 14*, 2447.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Konkel, D. A., & Ingram, V. M. (1977) *Nucleic Acids Res. 4*, 1979.
Lindstrom, D. M., & Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1517.
Mory, Y., & Gefter, M. (1978) *Nucleic Acids Res. 5*, 3899.
Smith, M. M., & Huang, R. C. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 775.
Yang, V. W., Binger, M.-H., & Flint, S. J. (1980) *J. Biol. Chem. 255*, 2097.
Zasloff, M., & Felsenfeld, G. (1977) *Biochemistry 16*, 5135.

Anion-Dependent Sodium Ion Conductance of Platelet Plasma Membranes†

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ABSTRACT: External anions stimulate Na^+ efflux from platelet plasma membrane vesicles. Efflux is apparently electrogenic since K^+ diffusion potentials induced with valinomycin (interior positive) accelerate and potentials of the opposite polarity (interior negative) inhibit. In the presence of stimulatory anions, voltage-dependent Na^+ efflux is much faster than Na^+–Na^+ exchange in the absence of an induced membrane potential. Anions stimulate voltage-dependent efflux in the following order: $SCN^- > I^- > NO_3^- > Br^- \sim$ acetate $\sim Cl^- > F^- \sim SO_4^{2-} > HPO_4^{2-}$, gluconate, and isethionate. Thiocyanate, the most stimulatory anion, increases Na^+ efflux 20-fold in the presence of a membrane potential (interior positive). Stimulation of efflux by Cl^- is a saturable phenomenon with a $K_{0.5}$ of 41 mM and a maximal 2–3-fold stimulation over the basal level of efflux. Neither basal nor valinomycin-stimulated efflux was influenced by the presence of the platelet-aggregating agents thrombin, epinephrine, or ADP in the presence of fibrinogen.

Plasma membrane vesicles isolated from osmotically lysed platelets have proven extremely useful in the study of platelet transport phenomena (Rudnick, 1977; Nelson & Rudnick, 1979; Talvenheimo et al., 1979). These vesicles are functionally sealed as evidenced by their ability to maintain high internal concentrations of serotonin and lipophilic cations (Rudnick & Nelson, 1978; Nelson & Rudnick, 1979). Preliminary experiments measuring exposure of sialic acid residues suggest that greater than 90% of the vesicles have the same orientation as that of the intact platelet. The primary driving forces for serotonin accumulation in plasma membrane vesicles are experimentally imposed transmembrane gradients of Na^+ and K^+ (Nelson & Rudnick, 1979). Even though these gradients are not maintained by ongoing energy metabolism, the driving forces for serotonin transport in vesicles are apparently stable for at least 10 min. Thus, plasma membrane vesicles provide an experimental model system in which to study the ion permeability of platelet plasma membranes.

Horne & Simons (1978a) have reported that thrombin, which stimulates platelet aggregation, depolarizes the platelet plasma membrane and proposed that Na^+ influx is responsible (Horne & Simons, 1978b). In many cell types, hormones are believed to act, in part, by altering the plasma membrane potential, presumably through changes in ion permeability (Peterson, 1974; Zierler, 1972; Korchak & Weissman, 1978; Grollman et al., 1977). In the case of platelets, thrombin stimulation was inhibited by amiloride (Horne & Simons, 1978a,b), which blocks Na^+–H^+ exchange and Na^+ channels in such diverse tissues as sea urchin eggs (Johnson et al., 1976), mouse soleus muscle (Aickin & Thomas, 1977), and rabbit renal brush border vesicles (Kinsella & Aronson, 1980). Other pathways for Na^+ movements through membranes include the voltage-sensitive channel of nerve cells (Hodgkin & Huxley, 1952), the Na^+,K^+-ATPase (Glynn, 1957), and the Na^+–K^+ cotransporter found in a variety of cells (Geck et al., 1980). In this report, we describe a Na^+ transporter in the platelet plasma membrane with properties different from those of known Na^+ transport systems.

Materials and Methods

Materials

^{22}NaCl was obtained from New England Nuclear, bumetanide from Leo Pharmaceuticals (Denmark), and nigericin from Hoffmann-La Roche Inc. Porcine blood was obtained fresh at a local slaughterhouse. All other materials were

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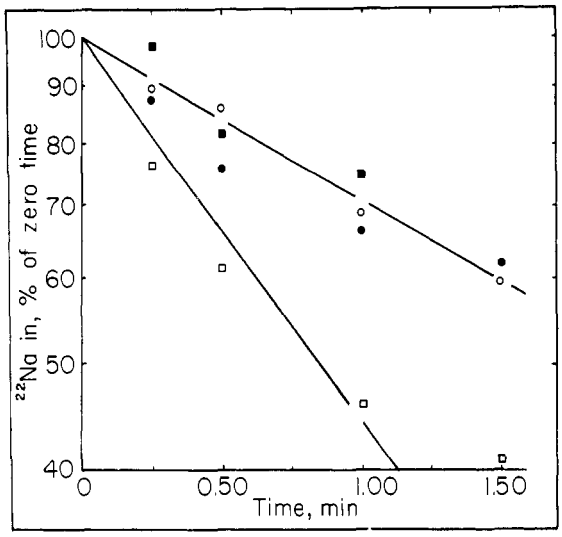

FIGURE 1: Stimulation of Na^+ efflux by a membrane potential. Vesicles preincubated with $^{22}NaCl$ in the presence (open symbols) or absence (filled symbols) of valinomycin were diluted into either 0.1 M NaCl containing 1 mM $MgSO_4$ (circles) or 0.1 M KCl containing 1 mM $MgSO_4$ (squares). The $^{22}Na^+$ content of the vesicles was monitored as described under Methods. Although straight lines are drawn through the points, it is clear that, at least in the case of valinomycin-stimulated efflux, the time course is not strictly first order. This probably reflects heterogeneity in the sizes of vesicles in the preparation.

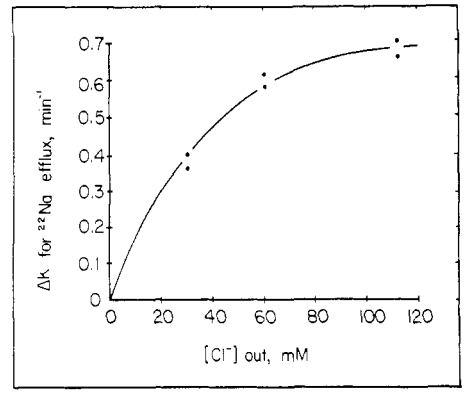

FIGURE 2: Chloride dependence of potential-dependent Na^+ efflux. Efflux of Na^+ into a medium of varying Cl^- concentration was measured as described in the legend to Figure 1. The external medium varied from 0 to 0.1 M KCl containing 1 mM $MgSO_4$ with potassium gluconate added to maintain constant K^+ concentration. The increase in the first-order rate constant (k) for efflux (as determined from $t_{1/2}$) induced by valinomycin is plotted as a function of Cl^- concentration. The maximal stimulation by Cl^- calculated from this experiment was 0.95 min^{-1}, and half-maximal stimulation was obtained at approximately 41 mequiv of Cl^-/L.

reagent grade obtained from commercial sources.

Methods

Preparation of Membrane Vesicles. Plasma membrane vesicles were isolated from porcine platelets as described previously (Rudnick & Nelson, 1978).

Efflux Measurements. Membrane vesicles were equilibrated with 0.1 M sodium phosphate, pH 6.7, containing 1 mM $MgSO_4$ by 10-fold dilution and 5-min incubation at 37 °C. The vesicles were collected by centrifugation at 48000g for 20 min and resuspended in the same buffer at 10 mg of protein per mL. $^{22}NaCl$ was added to a final specific activity of approximately 8500 cpm/nmol and the suspension incubated at 25 °C for 2 h. For the initiation of efflux, 4–5 μL of this suspension was diluted into 0.2 mL of the indicated solution at 25 °C. At the indicated times, the samples were diluted with 2 mL of ice-cold 0.1 M NaCl, filtered through a Millipore nitrocellulose filter (HAWP), and washed with another 2 mL of ice-cold 0.1 M NaCl. Dilution, filtration, and washing routinely took less than 10 s. Valinomycin, when added, was present at a concentration of 10 nmol/mg of membrane protein in the suspension before dilution. For assessment of the amount of $^{22}Na^+$ left in the vesicles at equilibrium, some of the suspension was diluted into medium containing 1 μM nigericin, incubated for 1 h at 25 °C, and filtered as above. This equilibrium value (typically less than 10% of the initial time points) was subtracted from the raw data to obtain the data displayed in the figures. Rate constants were estimated from the times required for half of the $^{22}Na^+$ to leave the vesicles ($t_{1/2}$). Protein was determined by the method of Lowry et al. (1951).

Results

When membrane vesicles equilibrated with ^{22}Na-labeled sodium phosphate are diluted into unlabeled NaCl or KCl, $^{22}Na^+$ exits the vesicle relatively slowly. As shown in Figure 1, efflux into KCl is dramatically accelerated by addition of valinomycin, which is expected to generate an electrical potential (interior positive) across the vesicle membrane under these conditions ($[K^+]_{out} > [K^+]_{in}$). In the absence of a K^+ gradient, when the vesicles are diluted into NaCl, no such acceleration is observed, indicating that increased Na^+ efflux is not mediated directly by the ionophore.

Table I: Na^+ Efflux into Solutions of Na^+ and K^+ Salts[a]

	cation		
	K^+		
anion	control	+valinomycin	Na^+
$C_2H_5SO_4^-$	0.12	0.14	0.19
gluconate$^-$	0.13	0.16	0.173
PO_4^{2-}	0.19	0.20	0.33
F^-	0.87	1.03	0.49
SO_4^{2-}	0.43	0.65	0.33
Cl^-	0.33	0.80	0.33
CH_3COO^-	0.80	1.39	0.53
Br^-	0.36	1.33	0.63
NO_3^-	0.37	1.98	0.58
I^-	0.36	6.3	3.96
SCN^-	0.56	11.55	0.45

[a] Rates represent first-order rate constants (min^{-1}) calculated from measured half-times for Na^+ efflux, as described under Methods.

The data in Figure 2 demonstrate that Cl^- is required for the acceleration of Na^+ efflux due to imposition of a membrane potential. As shown in this figure, the stimulation of efflux increases with Cl^- concentration, from 0.033 min^{-1} with no added Cl^- to 0.68 min^{-1} at 112 mM Cl^-. The process saturates with increasing Cl^- and is half-maximal at approximately 41 mM Cl^-. Other, less physiological anions also stimulate, to widely varying degrees (Table I). Changing the external anion from isethionate to thiocyanate increases Na^+ efflux almost 2 orders of magnitude in the presence of a membrane potential (interior positive). In the absence of an imposed potential, the change is much less dramatic, only 2-fold in the absence of cation gradients and 5-fold in the absence of valinomycin. Of the anions tested, only I^- specifically increased Na^+ efflux into the Na medium relative to K^+. Although I^- concomitantly increased both electroneutral Na^+–Na^+ exchange and electrogenic Na^+ efflux, other stimulatory anions accelerated only electrogenic efflux, suggesting that I^- might have a different mode of action. Since Cl^- is the most physiological anion of

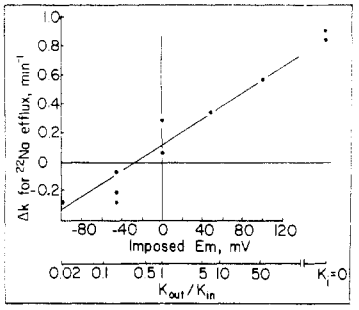

FIGURE 3: Dependence of Na^+ efflux on the K^+ diffusion potential. K^+ concentration gradients were generated by diluting vesicles equilibrated in 0–50 mM potassium phosphate into medium containing 62–0 mM KCl. The remainder (up to 224 mosM) of both solutions consisted of the corresponding Na salt and 1 mM $MgSO_4$. The increase in efflux induced by valinomycin is plotted as a function of the imposed chemical potential difference for K^+ and the calculated membrane potential expected with valinomycin addition.

those tested, we will concentrate on those results obtained with Cl^-.

The voltage dependence of Cl^--stimulated Na^+ efflux was approached by imposing various K^+ concentration gradients across the vesicle membrane in the presence of valinomycin. This ionophore is expected to make the membrane behave as a K^+ electrode, where the electrical potential across the membrane is related to the ratio of external to internal K^+ concentrations by the Nernst equation:

$$E_m = \frac{RT}{F} \ln \frac{[K^+]_{out}}{[K^+]_{in}}$$

where E_m is the membrane potential (interior positive), R is the gas constant, T is the absolute temperature, and F is the Faraday constant. As the ratio of external to internal K^+ concentration is varied from 50:1 to 1:50 (out:in), the membrane potential is expected to vary approximately from 100 mV (interior positive) to −100 mV (interior negative). The data in Figure 3 show how such a potential change influences $^{22}Na^+$ efflux into a KCl medium. Chloride ion stimulated Na^+ efflux increases linearly with increasingly positive (inside) potentials. When no potential was imposed ($[K^+]_{in} = [K^+]_{out}$), valinomycin stimulated Na^+ efflux only 15% as well as in the presence of a maximal K^+ gradient. Only when the K^+ concentration was slightly higher inside than outside did valinomycin fail to stimulate efflux. One possible explanation for these results is that the internal K^+ concentration is not as high as expected due to incomplete equilibration. The results of Figure 3 can be viewed as a crude current–voltage relationship, where the voltage applied by a given K^+ gradient determines the Na^+ current across the vesicle membrane. Although the data do not allow a clear interpretation, upward curvature of the current–voltage curve would suggest a diffusion process with Na^+ going from high to low concentration. However, such an analysis assumes that Na^+ flux directly represents a current and that the vesicles behave as ideal K^+ electrodes, neither of which has been established.

A variety of inhibitors were incubated with plasma membrane vesicles to test the possibility that the observed Na^+ flux or anion stimulation is mediated by known transport pathways. Tetrodotoxin blocks voltage-dependent Na^+ channels in nerve and muscle (Narahashi et al., 1964), and amiloride inhibits epithelial Na^+ channels and Na^+–H^+ countertransporters (Biber & Curran, 1970; Kinsella & Aronson, 1980). Bumetanide is one of the most potent known inhibitors of anion-dependent Na^+–K^+ cotransport (Palfrey et al., 1980), and ouabain inhibits the Na^+,K^+-ATPase (Aledort et al., 1966).

Table II: Effect of Inhibitors on Na^+ Flux[a]

inhibitor	% of control rate −valinomycin	% of control rate +valinomycin
amiloride, 100 μM	93	100
bumetanide, 10 μM	100	109
ouabain, 100 μM	103	106
SITS, 100 μM	105	81
tetrodotoxin, 1 μM	100	120

[a] Rates of Na^+ efflux from vesicles diluted into 0.1 M KCl containing 1 mM $MgSO_4$ were determined in the presence and absence of indicated concentrations of the above inhibitors as described under Methods.

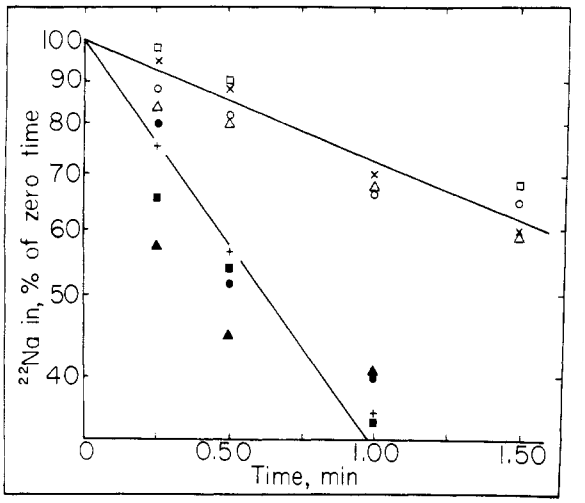

FIGURE 4: Lack of effect of aggregating agents on Na^+ efflux. Efflux was measured as described in the legend to Figure 1 in the presence and absence of aggregating agents. (○) Control; (△) 0.6 unit/mL human thrombin; (□) 10 μM ADP + 1 μM porcine fibrinogen; (×) 10 μM L-epinephrine. Filled symbols represent vesicles pretreated with valinomycin; (+) epinephrine + valinomycin.

While not an inhibitor of Na^+ transport directly, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), an inhibitor of anion transport in red cells and other systems (Cabantchik et al., 1978), was also tested. As shown by the data in Table II, none of these agents inhibited Cl^--stimulated, voltage-dependent Na^+ efflux from platelet plasma membrane vesicles.

A wide variety of agents stimulate platelets to undergo a morphological transformation, aggregate, and release the contents of their intracellular storage organelles. Of these, the most effective agents include ADP (in the presence of fibrinogen), epinephrine, and thrombin. The data in Figure 4 demonstrate that none of these agents influence the rate of Cl^--dependent Na^+ efflux, either in the presence or in the absence of an imposed membrane potential (interior positive). This observation argues against the Na^+ conductance as a direct effect of platelet-aggregating agents. If, however, these agents increase Na^+ permeability in intact platelets, then coupling between their receptors and the transport pathway may have been disrupted in the process of preparing plasma membrane vesicles.

Discussion

The electrogenic, anion-dependent Na^+ permeability of platelet plasma membranes, described above, differs from known Na^+ transport systems. The insensitivity of Na^+ efflux to inhibitors of Na^+,K^+-ATPase, Na^+ channels, Na^+–H^+ countertransport, and Na^+–K^+ cotransport suggests that this flux reflects the action either of a novel transport system or

of a nonspecific leak pathway. Moreover, the stimulation of efflux by external anions is uncharacteristic of known Na^+ transporters and is also unexpected of a nonspecific leak.

The mechanism of this Na^+ transporter is unknown. Its dependence on K^+ diffusion potentials (Figure 3) indicates that net positive charge crosses the membrane with Na^+, as expected for either a Na^+ uniport or a Na^+–anion countertransport. If anions are not countertransported with Na^+, another role for anions must be invoked to explain their striking stimulation of transport. This might involve a regulatory site which must be occupied by an appropriate anion for transport to occur. Alternatively, Na^+ may leak through the membrane upon anion-induced denaturation of some intrinsic membrane protein. In this regard, it is noteworthy that the order of activity for anions roughly follows the Hofmeister or lyotropic series (Jencks, 1969) for dissociation and denaturation of proteins. It must also be noted, however, that the anion concentrations used here are lower than those which show marked effects on protein structure.

Horne & Simons (1978b) reported that thrombin-induced changes in platelet aggregation and cyanine dye fluorescence are inhibited by amiloride and concluded that thrombin increased the Na^+ permeability of platelets. It is unlikely that the Na^+ permeability described here was responsible for their results. In plasma membrane vesicles, thrombin has no effect on either the resting Na^+ permeability or the increased Na^+ flux observed when a K^+ diffusion potential is imposed in the presence of a stimulatory anion (Figure 4). Moreover, Na^+ flux from membrane vesicles is insensitive to amiloride (Table II). It is possible, therefore, that Na^+ influx in intact platelets is not a consequence of thrombin action and that the inhibitory effects of amiloride on intact platelets are independent of its ability to block Na^+ transport. Although ADP, monensin, nigericin, and ouabain all increase platelet Na^+ levels (Feinberg et al., 1977; Feinstein et al., 1977; Mason & Saba, 1969), only ATP also aggregates platelets, suggesting that Na^+ influx and aggregation are not obligatorily coupled.

References

Aickin, C. C., & Thomas, R. C. (1977) *J. Physiol. (London) 273*, 295–316.

Aledort, L. M., Troup, S. B., & Weed, R. I. (1966) *J. Clin. Invest. 45*, 980.

Biber, T. U. L., & Curran, P. (1970) *J. Gen. Physiol. 56*, 83–99.

Cabantchik, Z. I., Knauf, P. A., & Rothstein, A. (1978) *Biochim. Biophys. Acta 515*, 239–302.

Feinberg, H., Sandler, W. C., Scorer, M., LeBreton, G. C., Grossman, B., & Born, G. V. R. (1977) *Biochim. Biophys. Acta 470*, 317–324.

Feinstein, M. B., Henderson, E. G., & Sha'afi, R. I. (1977) *Biochim. Biophys. Acta 468*, 284–295.

Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B., & Heinz, E. (1980) *Biochim. Biophys. Acta 600*, 432–447.

Glynn, I. M. (1957) *J. Physiol. (London) 136*, 148–173.

Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Heldolesi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., & Kohn, L. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2352–2356.

Hodgkin, A. L., & Huxley, A. F. (1952) *J. Physiol. (London) 117*, 500–544.

Horne, W. C., & Simons, E. R. (1978a) *Blood 51*, 741–749.

Horne, W. C., & Simons, E. R. (1978b) *Thromb. Res. 13*, 599–607.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 358–363, McGraw-Hill, New York.

Johnson, J. D., Epel, D., & Paul, M. (1976) *Nature (London) 262*, 661–664.

Kinsella, J. L., & Aronson, P. S. (1980) *Am. J. Physiol. 238*, F461–F469.

Korchak, H. M., & Weissman, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3818–3822.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mason, R. G., & Saba, S. R. (1969) *Am. J. Pathol. 55*, 215–223.

Narahashi, T., Moore, J. W., & Scott, W. R. (1964) *J. Gen. Physiol. 47*, 965–974.

Nelson, P. J., & Rudnick, G. (1979) *J. Biol. Chem. 254*, 10084–10089.

Palfrey, H. C., Felt, P. W., & Greengard, P. (1980) *Am. J. Physiol. 238*, C139–C148.

Peterson, O. H. (1974) *J. Physiol. (London) 239*, 647–656.

Rudnick, G. (1977) *J. Biol. Chem. 252*, 2170–2174.

Rudnick, G., & Nelson, P. J. (1978) *Biochemistry 17*, 4739–4742.

Talvenheimo, J., Nelson, P. J., & Rudnick, G. (1979) *J. Biol. Chem. 254*, 4631–4635.

Zierler, K. L. (1972) *Handb. Physiol., Sect. 7: Endocrinol.*, 347–368.