Journal of Cellular Biochemistry 18:377-391 (1982)

Effects of Divalent Cations, Trypsin, and Phospholipases on the Passive Permeability to Sodium of Inside-Out Vesicles From Human Red Cells

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Inside-out vesicles (IOV) were prepared from human red blood cells. Steady-state uptake of ^{22}Na was observed to generally follow an exponential time course with a rate constant of 1.57 ± 0.09 h^{-1} (SE). One week of cold storage (0–4°C) increased the rate constant to 2.50 ± 0.12 h^{-1} (SE). Mg^{2+}, Ca^{2+}, or Sr^{2+} decreased the rate of ^{22}Na uptake with no observable differences between the three divalent cations when tested at concentrations of 50 μM. Mg^{2+} was shown to decrease the rate of ^{22}Na uptake at concentrations as low as 5 μM with maximal effect at 50 to 100 μM. The decrease in rate of ^{22}Na uptake induced by Mg^{2+} could be enhanced by exposure of IOV to Mg^{2+} for longer periods of time. Trypsin treatment of IOV increased the rate of uptake of ^{22}Na and was dependent on the concentration of trypsin added between 5 to 25 μg/ml (treated for 5 min at 25°C). The ability of Mg^{2+} (50 μM) to decrease the rate of ^{22}Na uptake was still observed after maximal trypsin treatment. Phospholipase A_2 or phospholipase C treatment of IOV increased the rate of ^{22}Na uptake and was dependent on the amount of phospholipase A_2 (0.1 to 1.0 units/ml) or phospholipase C (0.25 to 2.5 units/ml) added (treated for 5 min at 25°C). After phospholipase A_2 treatment, the observed decrease in the rate of ^{22}Na uptake induced by Mg^{2+} (50 μM) was generally greater than controls. After phospholipase C treatment, the observed decrease in rate of ^{22}Na uptake induced by Mg^{2+} (50 μM) was less or absent when compared with controls. Phospholipase C treatment was less effective in preventing the Mg^{2+} effect the longer IOV were exposed to Mg^{2+}. The results suggest that Mg^{2+} binds to phospholipid headgroups to reduce Na permeability perhaps by inducing a change in bilayer structure or phospholipid association.

Key words: inside-out vesicles, sodium transport, passive permeability, membrane structure

Abbreviations used: PLA_2, phospholipase A_2 (bee venom); PLC, phospholipase C (B cereus); IOV, inside-out vesicles.

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Received August 4, 1981; revised and accepted November 6, 1981.

0730-2312/82/1803-0377$04.50

This study is concerned with the mechanism of passive cation permeation of the human red cell membrane and the role of membrane structure in this process. A general concept concerning the mechanism of passive membrane permeability is the solubility-diffusion model defined in this study to be composed of three fundamental steps: 1) adsorption of the solute to the membrane surface, 2) chemical solvation of the solute in the membrane, and 3) diffusion of the solute through the membrane.

The cation permeability of liposomes has been extensively studied [1–4] and appears to be consistent with the solubility-diffusion model. However, the biological membranes have a complex mosaic structure of protein and lipid, and it is not clear what role either of these play in passive cation permeation. Passive cation permeability of intact human red cells is characterized by slow and equal penetration rates for both Na^+ and K^+ where P, the permeability coefficient, is equal to 10^{-10} cm sec^{-1} [5–7]. Red cell passive cation permeability increases when cells are suspended in nonelectrolyte solutions [8,9], an effect that can be reversed by the addition of 1–5 mM divalent cations [10]. Sulfhydryl reagents alter cation permeability in intact cells [11–13] and isolated vesicles derived from red cells [14] probably at cytoplasmic membrane sites. Trypsin does not appear to affect Na^+ and K^+ permeability of intact red cells [15,16] but pronase increases permeability to both of these cations [15].

The action of various phospholipases on human red cell phospholipids has been well characterized [17,18]; however, the resulting permeability changes have received little attention. Treatment of rabbit red cells with phospholipase A_2 from bee venom results in a twofold to fourfold increase in rate of leakage of electrolytes as measured by conductivity [19].

This study describes the action of proteases and phospholipases on inside-out vesicles (IOV) from human red cells to determine the role of cytoplasmic proteins and lipids in passive Na^+ permeability. Further, the effect of divalent cations on this permeability was examined and the results interpreted by the solubility-diffusion model.

MATERIALS AND METHODS

Experiments were performed under steady-state tracer exchange conditions in which the chemical composition inside and outside the vesicles was as close to identical as possible. This was done to eliminate the effects of electrochemical gradients and therefore to closely approximate the true passive permeability of the membrane. Furthermore, IOV were allowed to equilibrate with ouabain (0.1 mM) overnight at 0–4°C before each experiment. Preliminary experiments showed that ouabain had no effect on uptake.

Preparation of Inside-Out Vesicles and Measurement of Steady-State Uptake

IOV were prepared from human blood obtained from the Middlesex General Hospital Blood Bank,New Brunswick, NJ. Blood was used within 14 days of the date it was drawn. The method of Steck and Kant [20], without modification, was used to prepare IOV. IOV were stored and used for up to one week after preparation in 0.5 mM Na_2HPO_4 pH 8.0 at 0–4°C. All IOV preparations used in this study were greater than 75% sealed and inside-out as judged by acetylcholinesterase accessibility [20].

Steady-state uptake of ^{22}Na was measured in 0.5 mM Na_2HPO_4 pH 7.4 (incubation buffer) at 25°C using Millipore filtration technique. The incubation buffer contained 0.01 μCi/ml ^{22}Na as $^{22}NaCl$. An uptake experiment was initiated by the addition of 0.1 ml of IOV stock suspension, containing 1.0 unit acetylcholinesterase/ml, to 0.4 ml of incubation buffer. One unit of acetylcholinesterase activity is that volume of vesicles which will hydrolyze one μmole of acetylthiocholine in one minute at 25°C under standard assay conditions in the presence of 0.1% Triton X-100 [20]. Uptake was terminated by dilution of the incubation medium with 2.5 ml of ice-cold nonradioactive buffer, filtration, and further washing of the filter with 2.5 ml of ice-cold nonradioactive buffer. A zero-time measurement was obtained by adding 0.1 ml of IOV suspension to incubation medium to which 2.5 ml of ice-cold nonradioactive buffer had already been added. The mixture was immediately filtered and washed as above. This value was subtracted from counts per minute observed at all subsequent time intervals. Filters were counted in a LKB 1280 ultragamma gamma counter with a window opening of 420–880 KeV. Results are expressed as counts per minute remaining on the filter per unit of acetylcholinesterase in the incubation media.

Trypsin, Phospholipase A_2, or Phospholipase C Treatment

Trypsin was 3× crystallized (Worthington). The IOV stock suspension (1.0 unit acetylcholinesterase/ml) was treated with various concentrations of trypsin for 5 min at 25°C. Trypsin treatment was stopped by addition of soy bean trypsin inhibitor (Sigma Type 1–S) at 10 × trypsin concentration.

Phospholipase A_2 (PLA_2) was from bee venom (Sigma) and phospholipase C (PLC) was from Bacillus cereus (Sigma Type III). The IOV stock suspension (1.0 unit acetylcholinesterase/ml) was treated with various amounts of PLA_2 or PLC for 5 min at 25°C. The activity measurements used are those of Sigma. The treatment with PLA_2 or PLC was stopped by addition of 5 ml of ice-cold buffer (the IOV stock suspension contained 2 ml) and centrifugation at 27,000g (max) in an SM-24 rotor in a Sorval RC2 refrigerated centrifuge for 15 min. The supernatant was removed and the vesicles were resuspended to their original volume and kept on ice until they were used. Trypsin, trypsin inhibitor, PLA_2, and PLC were prepared fresh in ice-cold buffer just before use.

Reproducibility of the Results

Hanahan [21], in his review on variability in red cell membrane preparations, emphasized the deleterious effect of very low buffer concentrations (< 30 mOSM) and low ionic strength treatment of ghosts, leading to fragmentation and significant losses of protein. IOV formation requires the treatment of membranes at low ionic strength. Because of this effect, it is impossible to normalize data from different IOV preparations on a per mg protein basis. Normalizing data per unit acetylcholinesterase allowed reasonable comparisons of different IOV preparations to be made but data from different IOV preparations still could not be pooled.

It should be understood that while the results of single experiments are presented, similar findings were obtained in at least two and in most cases several different IOV preparations. Each point is the average of triplicate determinations and each experiment has its own control. Data were subjected to analysis of variance across two different vesicle preparations. The results of these analyses are presented in the appendix.

RESULTS

The Effect of Divalent Cations on Sodium Permeability

Uptake of ^{22}Na by IOV always deviated from a single first order process at early time periods (up to 5 min) with a much faster rate of uptake than in subsequent time periods (up to 40 min), where a single first order rate was observed (Fig. 1).

The mean value of α (the first order rate constant for the uptake process) for freshly prepared IOV (determined within 48 h from start of vesicle preparation) was $1.57 \pm 0.09\ h^{-1}$ SE ($n=5$). The rate constant increased significantly when vesicles were stored at 0–4°C. After one week of cold storage, the value of α increased to $2.50 \pm 0.12\ h.^{-1}$ SE ($n=3$). No significant effect of preparation age upon the results reported in this study was observed.

Experiments were performed to determine the relative effectiveness of various divalent cations in reducing the rate of uptake of ^{22}Na by IOV. When Mg^{2+}, Ca^{2+}, or Sr^{2+} (50 μM) was added to the IOV stock suspension just before the incubation with tracer began, a decrease in the rate of ^{22}Na uptake was observed (Fig. 2). Because there was no observable difference between the effects of the three divalent cations tested, addition of Mg^{2+} was used as representative of the divalent cation effect. The results show that Ca^{2+} was not more effective than other divalent cations in reducing Na^+ permeability of IOV under the conditions studied.

To further characterize the effect of Mg^{2+}, experiments were performed in which counts per minute associated with IOV at equilibrium was determined at

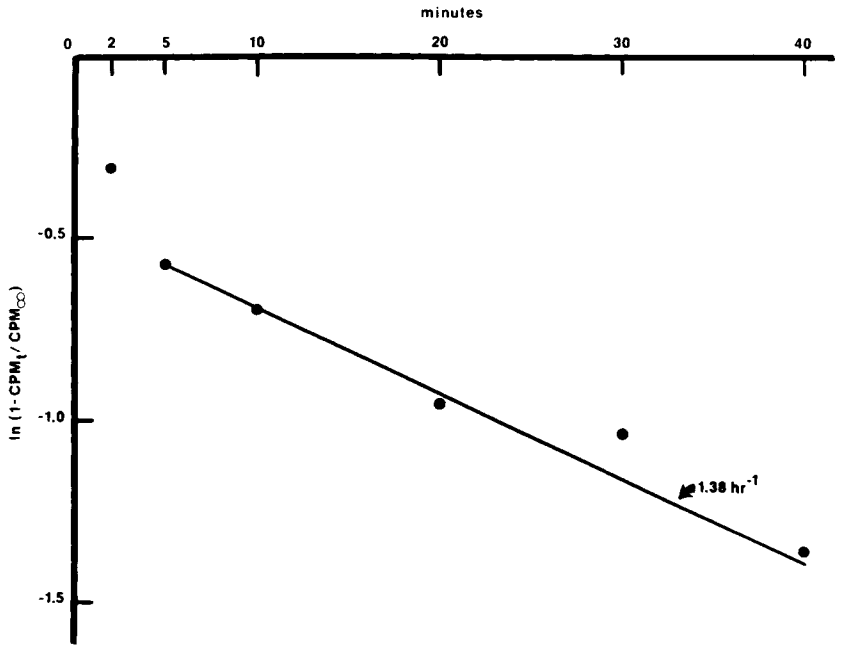

Fig. 1. Determination of rate constant (α) for ^{22}Na uptake by a typical preparation of inside-out vesicles under control conditions (ie, 0.5 mM Na_2HPO_4 pH 7.4, 25°C), where CPM_t is the counts observed on the filter after a given time of incubation and CPM_∞ is the counts observed on the filter after isotopic equilibrium has occurred. Isotopic equilibrium usually occurred within 6 h at 25°C.

various concentrations of Mg^{2+} so that the rate constants (α) could be calculated. The concentration dependence of the effect of Mg^{2+} on the rate constant α for ^{22}Na uptake is shown in Figure 3. The results show that Mg^{2+} did decrease α and, therefore, permeability to Na^+. The effect could be observed at concentrations as low as 5 μM. A maximum effect was observed between 50 and 100 μM.

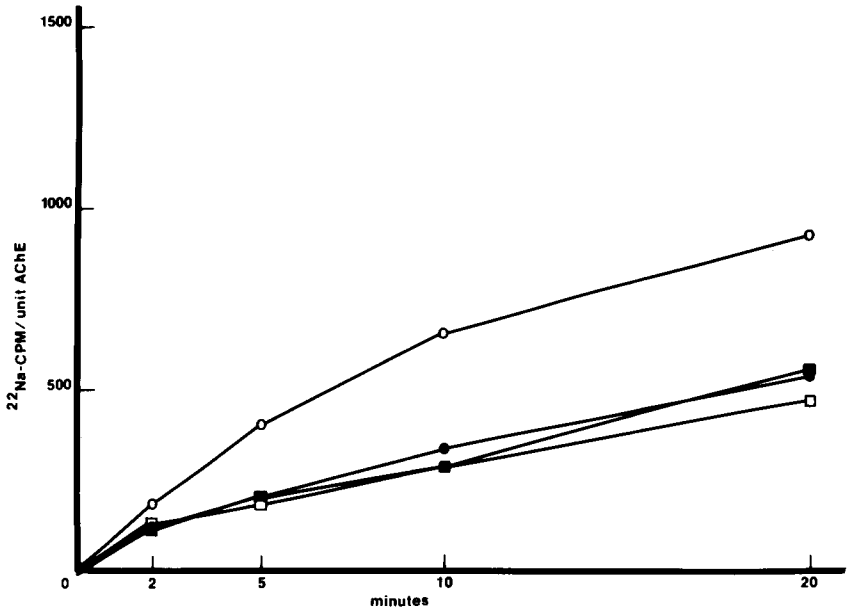

Fig. 2. Steady-state uptake of ^{22}Na was measured after addition of $MgCl_2$, ●—●; $CaCl_2$, □—□ or $SrCl_2$, ■—■, to the stock IOV suspension, to a concentration of 50 μM; control, ○—○ (no additions).

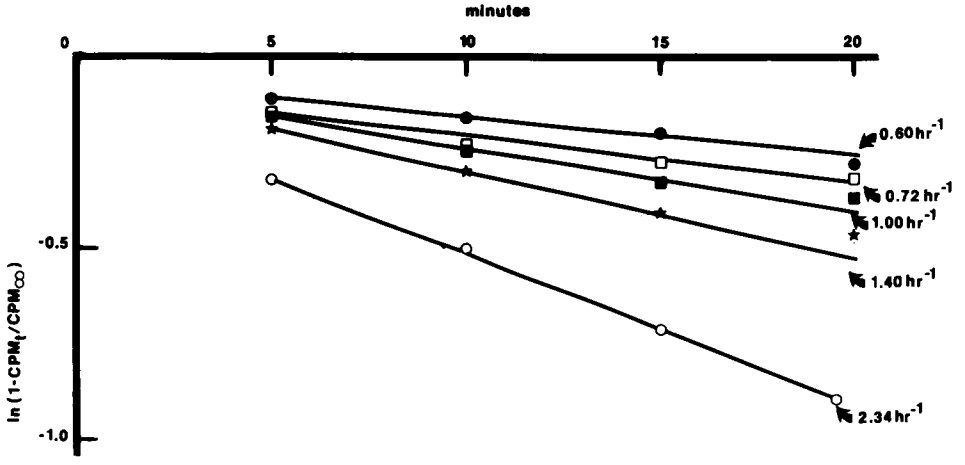

Fig. 3. The rate constant (α) for ^{22}Na uptake was determined after stock IOV suspensions of vesicles were allowed to equilibrate overnight with the following concentrations of $MgCl_2$: ●—●, 50 μM; □—□, 20 μM; ■—■, 10 μM; ★—★, 5 μM; control, ○—○ (no additions).

Studies on the effects of divalent cations on Na^+ permeability of intact red cells [10] show that divalent cations are effective when added externally. Therefore, experiments were performed to determine the effectiveness of Mg^{2+} after it had an opportunity to diffuse to transmembrane sites (ie, sites that may be exposed externally in the intact red cell). Under conditions where Mg^{2+} was allowed to equilibrate with the IOV stock suspension for 48 h at 0–4°C, the resulting decrease in ^{22}Na uptake was greater than when Mg^{2+} was added to the IOV stock suspension just before the uptake assay began (Fig. 4).

The Effect of Trypsin on Sodium Permeability

To determine the role of proteins exposed at the cytoplasmic membrane surface in passive permeability, IOV were treated with trypsin for 5 min at 25°C. After treatment, an increase in the rate of uptake of ^{22}Na was observed that was dependent on the concentration of trypsin added (Fig. 5). The effect was observed at concentrations as low as 5 μg/ml and a maximum effect was observed at 20 μg/ml.

To analyze the molecular mechanism of the divalent cation effect, experiments were performed to determine what effect protease treatment would have on the action of Mg^{2+} on ^{22}Na uptake. When the IOV stock suspension was treated with trypsin (20 μg/ml, 5 min) the ability of Mg^{2+} (50 μM) to decrease ^{22}Na uptake was still evident, and the magnitude of the effect was qualitatively the same as observed in untreated vesicles (Fig. 6). Mg^{2+} was added to the IOV stock suspension after trypsin treatment, just before the uptake assay began.

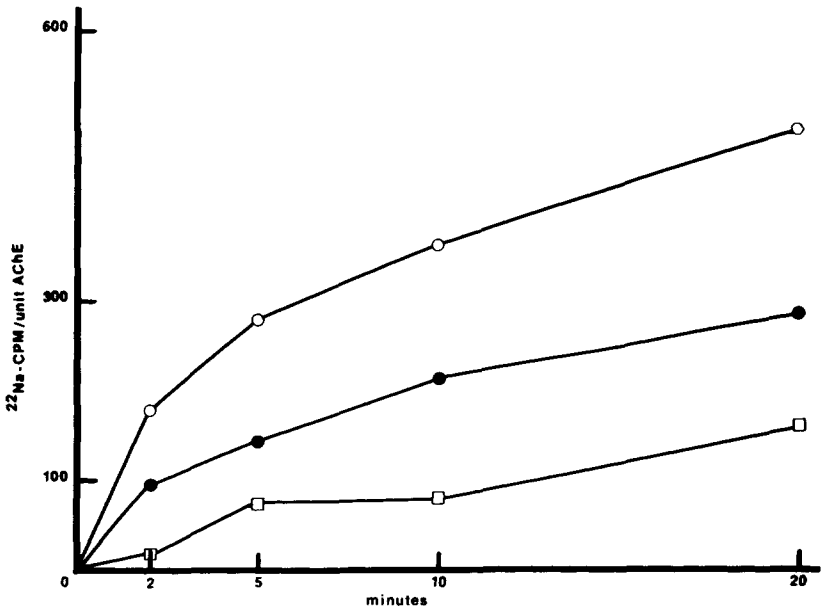

Fig. 4. Steady-state uptake of ^{22}Na was measured after the stock IOV suspension was allowed to equilibrate with $MgCl_2$ for 48 h (at 0–4 °C), □—□; or $MgCl_2$ was added to the stock IOV suspension just before the uptake assay began, ●—●. Both were at concentrations of 50 μM. Control, ○—○, no additions to the stock IOV suspension.

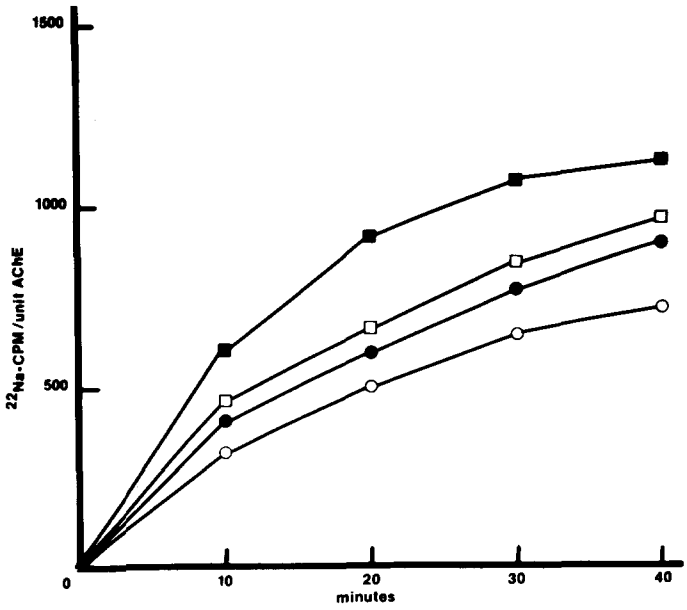

Fig. 5. Steady-state uptake of ^{22}Na was measured after treatment of the IOV stock suspension with trypsin for 5 min at 25°C, followed by 10× soybean trypsin inhibitor. Then IOV were added to incubation buffer containing tracer. Legend: 5 μg/ml, ●—●; 10 μg/ml, □—□; 20 μg/ml, ■—■; control, ○—○ (10× soybean trypsin inhibitor then trypsin [20 μg/ml]).

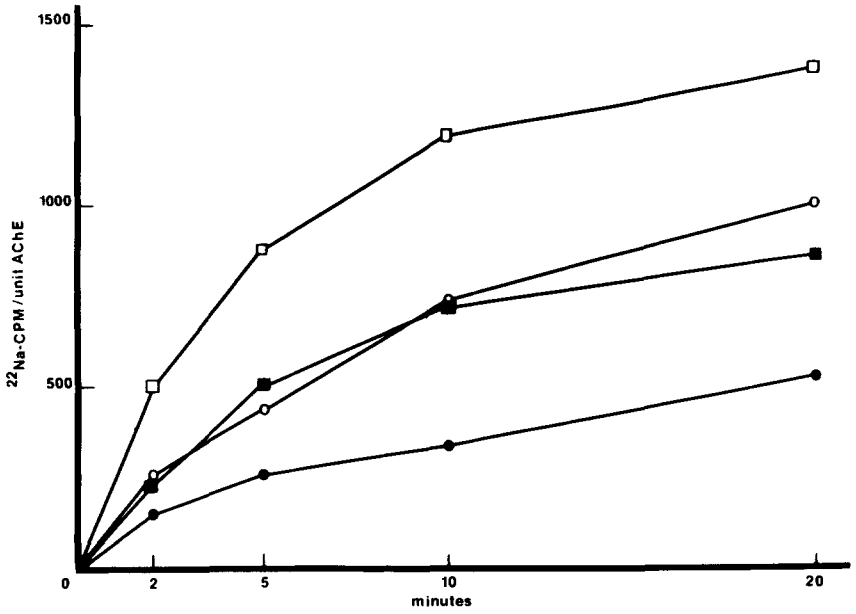

Fig. 6. Steady-state uptake of ^{22}Na was measured after treatment of the stock IOV suspension with trypsin (20 μg/ml, 5 min), □—□; trypsin (as above) followed immediately by addition of $MgCl_2$ to the stock IOV suspension (50 μM), ■—■; or $MgCl_2$ added to the stock IOV suspension (50 μM) without trypsin treatment, ●—●; control, ○—○ (trypsin inhibitor then trypsin 20 μg/ml). Then IOV were added to incubator buffer containing tracer.

The Effect of Phospholipase A_2 and Phospholipase C on Sodium Permeability

The effects of phospholipases on Na^+ permeability were determined because of the fundamental importance of phospholipids and their organization in the membrane to the solubility-diffusion model.

Treatment of the IOV stock suspension with PLA_2 for 5 min at 25°C resulted in an increase in the rate of ^{22}Na uptake. The increase in uptake was dependent on the amount of PLA_2 added. The effect was observed at amounts as low as 0.1 unit/ml. A maximum effect was observed at 1.0 unit/ml. At the two highest amounts of PLA_2 used, there was an unloading of ^{22}Na from the vesicles after 20 min of incubation with tracer. This suggests that PLA_2-treated vesicles lysed during prolonged incubation and were unloading ^{22}Na previously accumulated during the incubation with tracer (Fig. 7).

Similarly, treatment of the IOV stock suspension with PLC for 5 min at 25°C resulted in an increase in the rate of ^{22}Na uptake. The increase in uptake was dependent on the amount of PLC added. The effect could be observed at amounts as low as 0.25 unit/ml. A maximal effect was observed at 2.5 unit/ml (Fig. 8). There was no observed lysis of vesicles after PLC treatment comparable to that observed with PLA_2 treatment.

Next, experiments were performed to determine if phospholipase treatment of IOV in any way affected the action of Mg^{2+} on Na^+ permeability. Intermediate amounts of PLA_2 and PLC were used to avoid complications resulting from lysis of vesicles. After PLA_2 treatment (0.5 unit/ml, 5 min) of the IOV stock suspension,

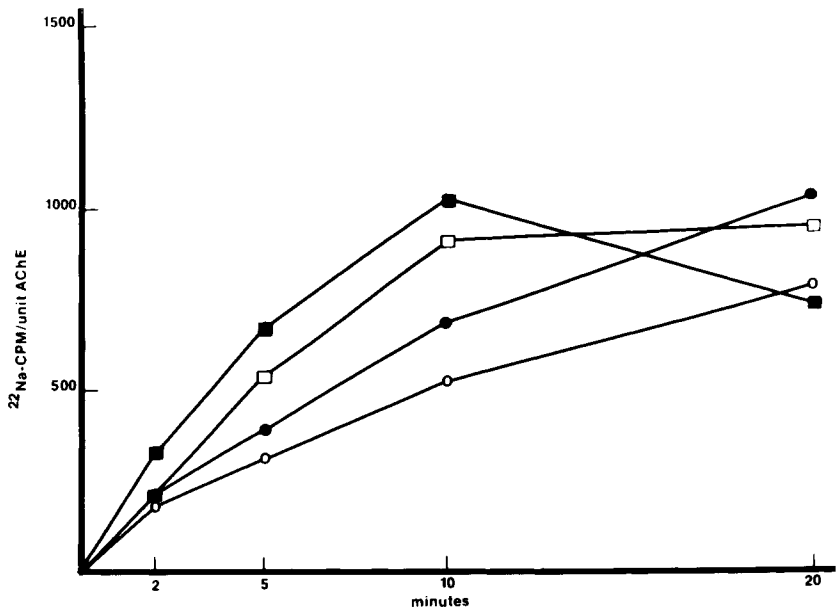

Fig. 7. Steady-state uptake of ^{22}Na was measured after treatment of the stock IOV suspension with PLA_2 for 5 min at 25°C. Then IOV were added to incubation buffer containing tracer. Legend: 0.1 unit/ml, ●—●; 0.5 unit/ml, □—□; 1.0 unit/ml, ■—■; control, ○—○ (no additions).

the observed decrease in ^{22}Na uptake induced by Mg^{2+} (50 μM) was generally the same or greater in all experiments performed. Mg^{2+} was added to the IOV stock suspension after PLA_2 treatment just before an uptake assay was begun (Fig. 9).

The effect of Mg^{2+} after PLC treatment (1.25 unit/ml, 5 min) was more involved. When Mg^{2+} was added to the IOV stock suspension after PLC treatment just before an uptake assay was begun (the usual protocol), the effect of Mg^{2+} on ^{22}Na uptake was less than the effect of Mg^{2+} on ^{22}Na uptake in control vesicles (Fig. 10). It should be noted that, under the protocol used above to measure ^{22}Na uptake, each time point was determined separately. This required storing the IOV stock solution on ice for up to an hour in the presence of Mg^{2+} until they were used. It has been demonstrated in this paper that Mg^{2+} is more effective in reducing the rate of ^{22}Na uptake the longer IOV are exposed to Mg^{2+}, presumably because Mg^{2+} was able to reach transmembrane sites. Since PLC treatment would only be expected to disrupt sites on the vesicle exterior [27 (Discussion)] it was of interest to determine the effect of Mg^{2+} when it was only exposed to the vesicle exterior. This was accomplished by adding Mg^{2+} to the incubation buffer instead of adding Mg^{2+} to the IOV stock suspension, as was usually done, so that IOV would only be exposed to Mg^{2+} during the incubation with tracer, hopefully minimizing transmembrane effects. Under these conditions, the Mg^{2+} effect was absent (Fig. 11). After a 20-min incubation with tracer, however, a Mg^{2+} effect was observed suggesting Mg^{2+} had diffused to transmembrane sites to exert its effect. PLA_2-treated IOV were also tested for Mg^{2+} effect with Mg^{2+} in the incubation media, and the Mg^{2+} effect was still evident (data not shown).

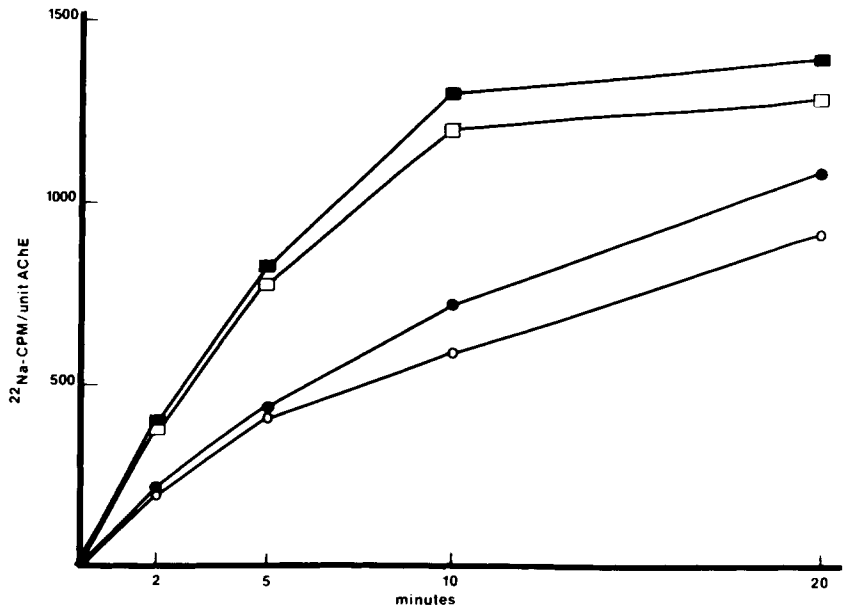

Fig. 8. Steady-state uptake of ^{22}Na was measured after treatment of the stock IOV suspension with PLC for 5 min at 25°C. Then IOV were added to incubation buffer containing tracer. Legend: 0.25 unit/ml, ●—●; 1.25 unit/ml, □—□; 2.5 unit/ml, ■—■; control, ○—○ (no additions).

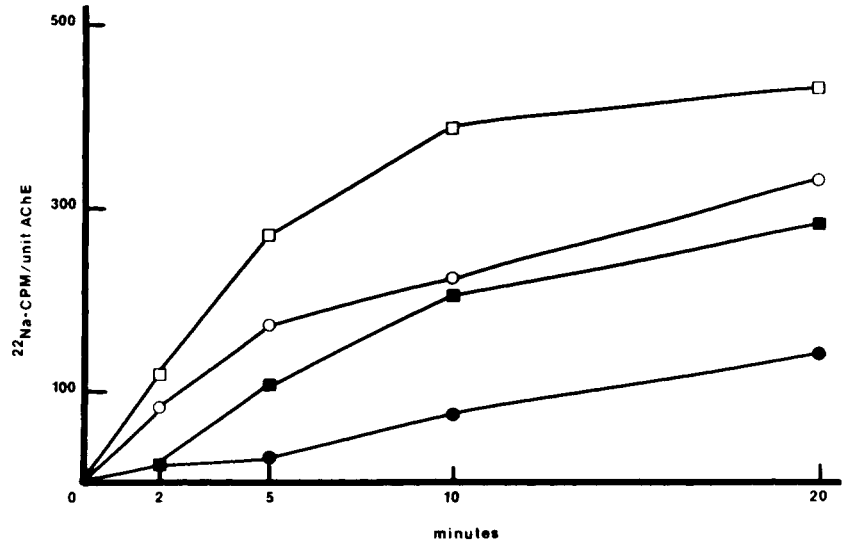

Fig. 9. Steady-state uptake of ^{22}Na was measured after treatment of the stock IOV suspension with PLA_2 (0.5 unit/ml, 5 min), □—□; PLA_2 (as above) followed immediately by addition of $MgCl_2$ (50 μM) to the stock IOV suspension, ■—■; or $MgCl_2$ (50 μM) added to the stock IOV suspension without PLA_2 treatment, ●—●; control, ○—○ (no additions). Then IOV were added to incubation buffer containing tracer.

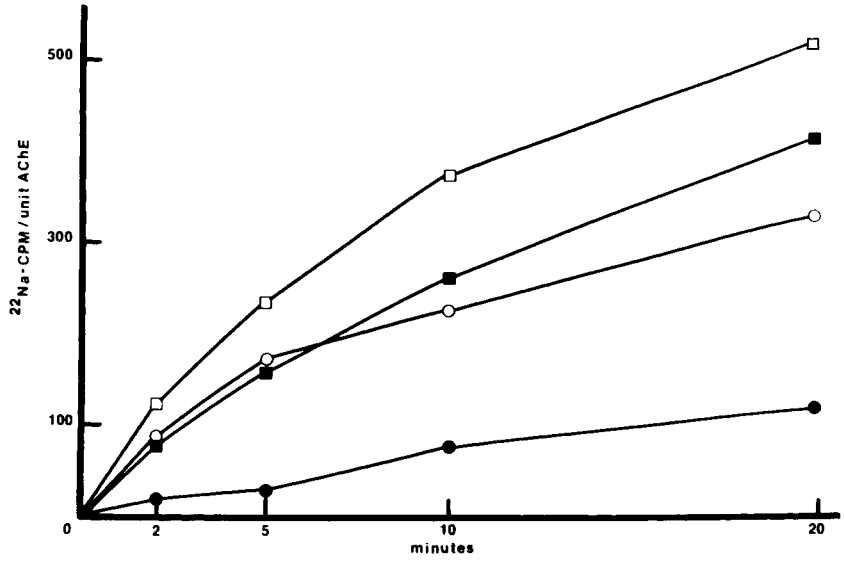

Fig. 10. Steady-state uptake of ^{22}Na measured after treatment of the stock IOV suspension with PLC (1.25 unit/ml, 5 min), □—□; PLC (as above) followed immediately by addition of $MgCl_2$ to the stock IOV suspension (50 μM), ■—■; or $MgCl_2$ (50 μM) added to the stock IOV suspension without PLC treatment, ●—●; control, ○—○ (no additions). Then IOV were added to the incubation buffer containing tracer.

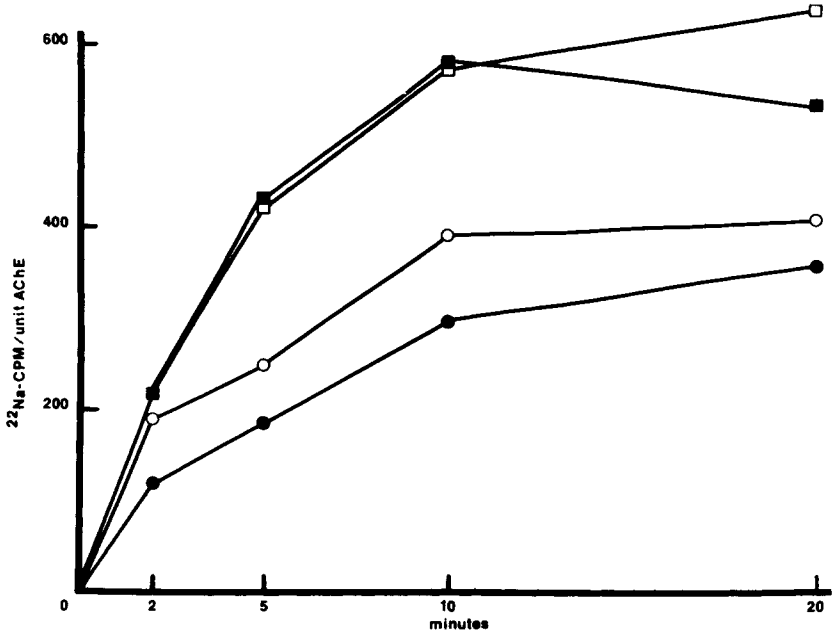

Fig. 11. Steady-state uptake of ^{22}Na was measured after treatment of the stock IOV suspension with PLC (1.25 unit/ml, 5 min), which was then added to $MgCl_2$-free incubation media containing tracer, □—□; PLC treatment of the stock IOV suspension (as above), which was then added to $MgCl_2$ containing (50 μM) incubation medium containing tracer, ■—■; or IOV was added to $MgCl_2$-containing (50 μM) incubation medium containing tracer without previous PLC treatment, ●—●; control, ○—○, placed in $MgCl_2$-free incubation medium containing tracer without previous PLC treatment of the stock IOV suspension.

DISCUSSION

Initial observations showed that addition of physiological concentrations of salts resulted in irreversible changes in vesicle preparations. These included vesicle shrinkage and reduction in the amount of sealed vesicles. Because of these changes, studies were carried out at low ionic strength and low Na^+ concentrations. Tracer assays made in the same low ionic strength buffer (0.5 mM Na_2HPO_4) used to prepare vesicles provided additional assurance that solutions inside and outside the vesicles were identical. Similar experiments with solutions of physiological ionic strength were not performed.

Under conditions of low Na^+ concentrations, one would expect relatively greater nonspecific binding to occur. However, preliminary results showed that Gramicidin D (1 μg/ml) was able to unload all but 5–10% of the ^{22}Na counts accumulated during the experiment, an amount not considered large. Nonspecific binding of ^{22}Na could account for the failure of IOV to follow a single first-order rate of uptake.

In this study, three divalent cations (Ca^{2+}, Mg^{2+}, and Sr^+) were shown to reduce ^{22}Na permeability of IOV, and all three had similar effects at a concentration of 50 μM. Ca^{2+} was not found to be more effective as has been reported for intact cells [10]. In addition, the concentration dependence of the divalent cation effect

was different for IOV when compared with intact cells. Divalent cation concentrations in the micromolar range reduced ^{22}Na permeability of IOV, but in intact cells, this occurs only with millimolar concentrations [10]. A likely reason for the differences observed in the divalent cation effect studied in intact cells and IOV is the large differences in ionic strength under which the studies were performed.

Mg^{2+} was found to be more effective in reducing the Na^+ permeability of IOV the longer IOV were exposed to Mg^{2+}. A similar observation is made with intact red cells [11]. The simplest explanation of this phenomenon is that Mg^{2+} diffuses to transmembrane sites and is most effective in reducing Na^+ permeability when exposed to both sides of the membrane simultaneously. This conclusion could be tested by loading/washing experiments and/or using right-side-out vesicles.

The ability of trypsin treatment of IOV to increase Na^+ permeability in a concentration-dependent manner is consistent with observations of Rothstein and co-workers [11–14], which indicate that sulfhydryl group reagents act at cytoplasmic membrane sites to increase Na^+ permeability of intact red cells. Preliminary results have shown that trypsin treatment of IOV also increases ^{14}C-choline uptake indicating that protease treatment of IOV resulted in a nonspecific permeability increase. Rothstein's work signifies that SH group modification is specific in the sense that it does not increase choline permeability [13]. However, much more extensive modification of polypeptides exposed at the cytoplasmic surface would be expected after protease treatment and could account for the differences in specificity observed with the two different treatments.

It can not be concluded if the permeability increase that resulted from trypsin treatment of IOV is related to that resulting from pronase treatment of intact cells [15]. Different polypeptides are no doubt affected by the pronase treatment because the proteins of the red cell membrane are distributed asymmetrically across the bilayer. However, it is also possible that modification of cytoplasmic membrane polpeptides could lead to a transmembrane effect, altering the conformation of polypeptides at the transmembrane surface. Such an effect is observed for band 3 reaction with chemical probes [22].

Treatment of IOV with either PLA_2 or PLC resulted in an increase in Na^+ permeability. The products of PLC digestion are diglycerides and water soluble phosphorylcholine, phosphorylethanolamine, and phosphorylserine. Diglycerides are thought to be completely inert, and the water-soluble products of the reaction are readily removed by washing the membranes with dilute solutions following PLC treatment [23]. PLA_2 treatment causes the liberation of fatty acids and lysophosphatides. Removal of fatty acids and lysophosphatides may be accomplished by washing membranes with 1% bovine serum albumin [23,24]. However, washing IOV with 1% bovine serum albumin caused severe shrinkage of vesicles and could not be used. Interpretation of the results of PLA_2 treatment are complicated by this fact. Lysolecithin, a product of PLA_2 digestion, has been shown to increase the cation permeability of red cells in a concentration-dependent manner at sub-lytic doses [25,26]. The unloading of ^{22}Na from IOV after PLA_2 treatment with longer incubation periods is probably due to the action of lysophosphatides causing eventual lysis of the vesicles.

The results of PLA_2 and PLC treatment of IOV on Na^+ permeability are consistent with the solubility-diffusion model. Van Meer et al [27] observed the preservation of bilayer structure of phospholipids and lysophospholipids remaining after

either PLA_2 or PLC treatment; therefore, effects observed in this study cannot be due to a disruption of bilayer structure. The effects of PLA_2 and PLC are best explained by concepts derived from permeability studies using liposomes [1–4]. PLA_2 action probably disrupted the molecular packing of fatty acyl chains in the bilayer. This effect would increase fatty acyl chain mobility and fluidity, resulting in an increased rate of diffusion for Na^+ through the bilayer. PLC action probably removed phospholipid headgroups, which would significantly alter remaining headgroup position, orientation, mobility, and charge. The rate of Na^+ permeation would be increased by an increased rate of Na^+ dehydration and solvation into the bilayer.

The molecular mechanism of the divalent cation effect on Na^+ permeability is not understood. The results of this study suggesting that divalent cations bind to the membrane to exert their effect on Na^+ permeability is supported by the observation that EDTA is able to completely reverse the divalent cation effect (data not shown) and that on red cell ghost membranes two high-affinity binding sites for Ca^{2+} with dissociation constants or 10^{-6} and 10^{-7} M have been found [28]. The effects of phospholipases and proteases reported here indicate the probable involvement of phospholipids rather than proteins in the divalent cation effect. The ability of Mg^{2+} to reduce Na^+ permeability was not affected by extensive tryptic digestion of the cytoplasmic membrane surface, which indicates minimum protein involvement.

The divalent cation effect remained after PLA_2 treatment (this study). Although the results would be less equivocal with a bovine serum albumin wash to remove released fatty acids, the results do suggest that the fatty acyl chains are not directly involved in the action of divalent cations.

After PLC treatment the effect of Mg^{2+} is only observed after IOV have been exposed to Mg^{2+} for a period of time (20 min at 25°C) when Mg^{2+} has probably diffused to transmembrane binding sites. This delayed effect of Mg^{2+} after PLC treatment is reduced compared with the effect of Mg^{2+} on control vesicles. Because bilayer structure is preserved after PLC treatment [27], phospholipids facing the vesicle interior would not be digested. Most likely, this is where Mg^{2+} exerts its effect on Mg^{2+} permeability after PLC treatment. The results of PLC treatment on the action of Mg^{2+} suggest that the phospholipid headgroups are the site where Mg^{2+} reduces Na^+ permeability.

The interaction and binding of divalent cations with headgroups of acidic phospholipids (especially phosphatidylserine) has been extensively studied [29–37]. Ca^{2+}, and to a lesser extent Mg^{2+} , induce phase transition shifts, phase separation and formation of Ca^{2+} phosphatidylserine aggregates in mixed lipid systems, and phosphatidylserine headgroup immobilization. However, the above effects induced by divalent cations on the physical state of phospholipid bilayers probably do not mediate the divalent cation effect on the Na^+ permeability observed in this study. Ca^{2+} at millimolar concentrations induces observable changes in the physical state of phospholipids characterized by large increases in liposome permeability to Na^+ due to instabilities produced in the bilayer [4,38].

Probably, the effects of divalent cations on Na^+ permeability reported here are the result of a more subtle and general structural change in the bilayer. The effect may be mediated by binding of divalent cations to the phosphoryl group of phospholipids. Divalent cations could act as a bridge to hold the phosphate headgroups more rigid or in an orientation that makes solvation of cations into the bilayer more difficult. If divalent cations bound to IOV decreased negative membrane surface

charge, a significant decrease in Na^+ permeation could occur. It is also likely that part of the divalent cation effect could be exerted through binding to headgroups causing tighter packing of phospholipids and less mobility in the fatty acyl chains, resulting in a decreased diffusion rate through the bilayer.

In summary, the results of this study are consistent with a solubility-diffusion model to explain Na^+ permeation of the red cell membrane. The results suggest that the solvation and diffusion steps are most important in determining the rate of Na^+ permeation and are very sensitive to the organization of proteins and lipids in the membrane as evidenced by the effects of trypsin and phospholipases on permeability. Divalent cations appear to bind to phospholipid headgroups with high affinity, resulting in a decrease in Na^+ permeability. The decrease is enhanced with longer exposure of divalent cations to the membrane.

ACKNOWLEDGMENTS

We would like to thank Dr. M. Friedman, who advised us on the statistical analysis of data, and Dr. R. Blostein, who read and contributed helpful suggestions. This research was supported by the Bureau of Biological Research, Rutgers University, New Brunswick, NJ 08903.

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APPENDIX: STATISTICAL ANALYSIS OF DATA

Analysis of variance was performed for a three factor experiment as described on page 361 of Snedecor and Cochran, "Statistical Methods" [39]. The three factors analyzed were 1) vesicle preparation, 2) experimental condition, and 3) time. The F value is termed the variance ratio and is defined as:

$$F = \text{Treatments mean square/error mean square.}$$

The F value is similar to the t value in the Student's t-test, and analogously an F distribution has been obtained and an F test can be performed to determine the probability (P) value for F. When $P < .05$ the effect being tested is indeed significant. All the data were analyzed for two different vesicle preparations.

Condition	Figure	F value	P
The Mg^{2+} effect	2	396.7	.0001
Mg^{2+} or Ca^{2+} effect	2	12.6	.0012
Mg^{2+} added overnight	4	61.3	.0001
Trypsin effect on ^{22}Na uptake	5	699.5	.0001
Mg^{2+} effect after trypsin treatment	7	1639.4	.0001
PLA_2 effect on ^{22}Na uptake	8	259.9	.0001
PLC effect on ^{22}Na uptake	9	362.8	.0001
Mg^{2+} effect after PLA_2 treatment	10	283.7	.0001
Mg^{2+} effect after PLC treatment	12	2.0	.1706