The effects of various anions on calcium transport and calcium-stimulated magnesium-ATPase in human erythrocyte inside-out vesicles (IOV) was investigated. Chloride, sulfate, acetate, and phosphate stimulated both calcium transport and (Ca$^{2+}$-Mg$^{2+}$)-ATPase. However, a specific anion did not stimulate calcium transport and (Ca$^{2+}$-Mg$^{2+}$)-ATPase to a similar degree. As a consequence, the ratio of calcium transported to ATP hydrolyzed was found to vary from 0.87 in the case of phosphate to 0.25 in the case of chloride.

The molar ratios of calcium to anion transported were 0.6, 2.0, and 1.5, for chloride, acetate, and sulfate, respectively, suggesting that calcium uptake proceeds by an electroneutral mechanism.

The stimulation of calcium transport by the anions was additive. Conversely, uptake of subsaturating phosphate was inhibited by the presence of other anions. This suggests that phosphate, sulfate, chloride, and acetate activate calcium transport by a similar mechanism and are transported into IOV by a similar pathway, presumably band III.

Approximately 4.0 gluconate molecules were taken up per calcium transported. However, the inability of gluconate to inhibit phosphate transport or to stimulate calcium transport additively with other anions suggests that gluconate may move into IOV as an uncharged species.

These results suggest that the pumping of calcium by the human erythrocyte membrane may proceed by a mechanism involving calcium/anion transport. A role for band III as the anion transporter is suggested.

In the preceding reports in this series (1, 2), we demonstrated that calcium transport by inside-out vesicles of human red blood cells is stimulated by the presence of inorganic phosphate, and that phosphate, when present, is co-transported with calcium. Furthermore, our data indicated that the P$_i$ transport was secondary to an electrogenic calcium pump, and that band III protein, known to be an anion exchange protein, served as the carrier for unidirectional P$_i$ transport in response to this electrogenic calcium pump.

The purpose of the present studies was to explore the effects of other anions on calcium transport, and to determine whether anions other than P$_i$ could stimulate Ca$^{2+}$ transport. The results indicate that a number of anions support a concentration-dependent stimulation of calcium transport. Evidence is presented that all anions penetrate the membrane via band III, but the ratio of calcium transported to ATP hydrolyzed depends on the anionic species. It is also suggested that under physiological conditions, it is likely that P$_i$ is the preferred counter ion for Ca$^{2+}$ transport.

**MATERIALS AND METHODS**

Methods for preparation of human erythrocyte inside-out vesicles (IOV), purification of bovine brain calmodulin, and measurement of Ca$^{2+}$-stimulated Mg$^{2+}$-ATPase have been described previously (1). Measurement of ion uptake was carried out as described previously (1). The standard incubation media contained 40 mM sodium gluconate 7.5 mM potassium gluconate, 3.0 mM magnesium gluconate, 20 mM glycylglycine (pH 7.1), 10 mM $\beta$-mercaptoethanol and, where indicated, 0.125 mM calcium gluconate and 1.45 mg/ml of purified bovine brain calmodulin. After a 5-min preincubation (37°C), the reaction was initiated by the addition of 0.9 mM ATP. In experiments where anion uptake or anion stimulation was measured, sodium gluconate was omitted from the incubation media. Typical concentrations of anions were: sodium chloride, sodium acetate (pH 7.1), and sodium sulfate (pH 7.1), 100 mM; sodium phosphate (pH 7.1), 10 mM.

**RESULTS**

**Anion Stimulation of Calcium Transport**—The results shown in Fig. 1 indicate that a variety of anions, other than P$_i$, stimulate calcium uptake into human erythrocyte IOV when compared to the rates seen with gluconate (100 mM) alone. Also shown in this figure is the fact that addition of the calcium ionophore A23187, after a 30-min incubation, led to a prompt and complete efflux of calcium from the IOV regardless of anionic species. The only exception to this behavior was the case of P$_i$, in which case A23187 caused the release of only 80 to 90% of the accumulated calcium. This suggests that in contrast to the other anions tested, the accumulated calcium and P$_i$ within the vesicles exist in part in nonionic form. Of the anions tested, phosphate was the most potent; 10 mM phosphate was more effective than 100 mM chloride and only slightly less effective than 100 mM sulfate or acetate in stimulating calcium uptake.

**Anion Stimulation of (Ca$^{2+}$-Mg$^{2+}$)-ATPase**—The (Ca$^{2+}$-Mg$^{2+}$)-ATPase activity is considered as a biochemical measure of the calcium pump activity in the human erythrocyte membrane. Because of this, it was of interest to examine the effects of these various anions on the activity of this ATPase activity, and to compare these results to those shown in Fig. 1. As shown in Fig. 2, the various anions tested stimulated the ATPase activity as compared to that seen in the presence of P$_i$.

The abbreviations used are: IOV, inside-out vesicles; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)N,N,N',N'-tetraacetic acid.
mulation of both anions were linear for 30 min. Furthermore, both anions were released by treatment of the IOV with A23187.

When acetate served as substrate (Fig. 5), the rate of acetate uptake was linear over time, and during the early time periods, the ratio of acetate to \( \text{Ca}^{2+} \) uptake was 2.1. However, after 15 min, the rate of calcium accumulation reached a plateau whereas that of acetate uptake did not. At 100 mM acetate or chloride, calcium uptake was linear for 30 min (Fig. 1).

When chloride served as anion (Fig. 6), the ratio of \( \text{Cl}^- \) to

**CONCENTRATION DEPENDENCE OF ANION EFFECTS**—In the preceding paper (1), the stimulation of calcium uptake by \( \text{P}_i \), was shown to be enhanced by calmodulin. The results shown in Fig. 3 indicate that this is also true when sulfate serves as the major anion. In the absence of calmodulin, the stimulatory effect of sulfate is quite limited, but in its presence, calcium uptake is a nearly hyperbolic function of sulfate concentration. Similar results were obtained when either acetate or chloride served as anion. From these results, the apparent \( K_a \) values for anion-activated, calmodulin-supported calcium uptake were calculated. These values are shown in Table I. The apparent \( K_a \) values for chloride, sulfate, and acetate were in the range of 10 to 20 mM, whereas that for phosphate was 1.2 mM (2).

**CALMODULIN-STIMULATED ANION UPTAKE**—In the preceding paper (2), the suggestion was made that phosphate entered the IOV via the anion exchange protein, band III, in order to neutralize the membrane potential generated by the electrogenic calcium pump. This anion exchange protein has a relatively broad specificity for anions, so it seemed quite possible that anions other than phosphate could enter via this carrier. This would account for their ability to stimulate calcium transport (Fig. 1). The results shown in Figs. 4 to 6 demonstrate that there is an ATP-dependent, calmodulin-stimulated, calcium-dependent uptake of sulfate, acetate, and chloride when any of these anions is present as the predominant anion (40 mM). Also plotted in these figures are the simultaneous rates of \( \text{Ca}^{2+} \) uptake under each of these circumstances. When sulfate serves as anion (Fig. 4), the ratio of sulfate to \( \text{Ca}^{2+} \) uptake was approximately 1.5:1.0, and the rates of ac-
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Ca\(^{2+}\) transported was 0.6, and both Cl\(^{-}\) uptake and Ca\(^{2+}\) uptake reached plateaus between 20 and 25 min.

Competitive Anion Studies—The data shown in Fig. 7 show that when P\(_i\) is present at a concentration of 5 mM, addition of another anion to 100 mM has varying effects depending upon the anion tested. Gluconate had essentially no effect. Sulfate and acetate stimulated P\(_i\) uptake slightly, and chloride suppressed it. On the other hand, at 5 mM phosphate, calcium transport was not significantly stimulated by any of these anions (data not shown). When a lower concentration of P\(_i\), (1 mM) was present, addition of other anions affected both phosphate (Fig. 8) and calcium (Fig. 9) uptake. Again, gluconate had little effect on phosphate uptake and produced a slight inhibition of calcium uptake. Acetate and sulfate stimulated the uptake of calcium and markedly inhibited phosphate transport. Chloride appeared to modify calcium and phosphate transport differentially, as this anion is the most potent inhibitor of phosphate transport but the least effective stimulator of calcium transport. These results demonstrate the additive nature of anion stimulation of calcium transport. However, at similar anion concentrations, the potency of the additive effect is anion-dependent. On the other hand, sulfate, chloride, and acetate compete with and inhibit phosphate transport.

Effects of Gluconate—The gluconate buffer system was chosen originally because it was considered that the mem-
brane was impermeable to this anion (Fig. 4). However, as shown in the previous report (2), Ca\(^{2+}\) transport proceeds in the presence of gluconate. Nonetheless, the data shown in Figs. 7 to 9 show that gluconate has a small effect upon either phosphate or calcium transport, suggesting that gluconate is not transported via band III. Three possible explanations for Ca\(^{2+}\) uptake in the presence of gluconate were considered: 1) uptake was due to gluconate entering the IOV by a means other than band III; 2) uptake was driven by cation exchange (internal H\(^+\) or K\(^+\) for external Ca\(^{2+}\)); or 3) uptake was supported by the P\(_i\) generated by ATP hydrolysis.

Simply on the basis of the fact that Ca\(^{2+}\) uptake was linear in the presence of gluconate, the second possibility was considered unlikely. The results shown in Fig. 10 show that gluconate enhances calmodulin-stimulated transport when no other anion is present. Of interest is the fact that gluconate addition suppressed calcium uptake slightly in the absence of calmodulin. However, as shown in Figure 12, gluconate uptake is both calcium- and calmodulin-dependent. Approximately 3.7 gluconate molecules are taken up per Ca\(^{2+}\) ion under stimulated conditions when 50 mM gluconate is present. Of note is the fact that gluconate is taken up in the absence of calmodulin, even though (Fig. 10) gluconate does not appear to enhance significantly the rate of calcium uptake under these circumstances. Also of interest is that addition of A23187 caused only a slow release of labeled gluconate, even though Ca\(^{2+}\) release was prompt (Fig. 12).

**DISCUSSION**

In the second paper of this series (2), it was suggested that the calcium translocation process might involve coupling of calcium and phosphate transport. Based on the action of anion channel blockers specific for band III, it was also suggested that net phosphate transport was mediated by the band III protein. In the present study, we have examined the possible role of other anions in the calcium translocation process. The data in Figs. 1 and 2 suggest that while chloride, sulfate, acetate, and phosphate stimulate both calcium transport and (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activities, this stimulation var-

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**Fig. 9.** Additive stimulation of calcium transport by phosphate and anions. Calcium uptake was measured (1) in the presence of 1.45 \(\mu\)g/ml of calmodulin, 0.125 mM calcium gluconate, 1.0 mM sodium phosphate (■), and 100 mM sodium acetate (■), sodium chloride (□), sodium sulfate (△), or sodium gluconate (△). Brackets indicate the addition of 1.0 \(\mu\)M A23187. \(\mu\)ACE, unit of acetylcholinesterase.

**Fig. 10.** Stimulation of calcium transport by sodium gluconate. Calcium transport into erythrocyte IOV was measured (1) in the presence (□) or absence (■) of 1.45 \(\mu\)g/ml of calmodulin. The reaction media, in the absence of added gluconate, contained 7.5 mM potassium gluconate, 3.0 mM magnesium gluconate, and 0.125 mM calcium gluconate. \(\mu\)ACE, unit of acetylcholinesterase.

**Fig. 11.** Transport of phosphate from ATP hydrolysis into IOV. Phosphate uptake into erythrocyte IOV was measured (2) in the presence of 1.45 \(\mu\)g/ml of calmodulin, 0.125 mM calcium gluconate, and 100 mM sodium acetate (■), sodium chloride (□), sodium sulfate (△), or sodium gluconate (△). The reaction was initiated by addition of 0.9 mM \(\gamma\)\(^{32}\)P\(_{\text{ATP}}\). Brackets indicate the addition of 1.0 \(\mu\)M A23187 after 30 min. \(\mu\)ACE, unit of acetylcholinesterase.

**Fig. 12.** Time course of sodium gluconate accumulation into IOV. Gluconate transport and gluconate-stimulated calcium transport were assayed under identical conditions. Gluconate transport was measured in the presence of 1.45 \(\mu\)g/ml of calmodulin and the following: 0.125 mM calcium gluconate (■); 0.5 mM EGTA (△); or in the absence of added ATP (□). Gluconate-stimulated calcium uptake was measured (1) in the presence of 1.45 \(\mu\)g/ml of calmodulin and 0.125 mM calcium gluconate (■). The total concentration of gluconate in the assay media was 50 mM. Brackets indicate the addition of 1 \(\mu\)M A23187 after 30 min. \(\mu\)ACE, unit of acetylcholinesterase.
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ies from anion to anion. Furthermore, a single anion does not stimulate calcium transport and \( \text{Ca}^{2+}-\text{Mg}^{2+} \)-ATPase activity to a similar degree. This situation is reflected in the dependence of calcium transported on ATP hydrolyzed. With phosphate present, the ratio of \( \text{Ca}^{2+} \) to ATP is about 0.9:1.0, while in the presence of chloride, this ratio is about 0.25:1.0. Therefore, the efficiency of the calcium pump is not only affected by the presence or absence of calmodulin (1) but also by the particular anion available for co-transport. Implicit in these findings is the possibility that anions stimulate calcium uptake by neutralizing the positive potential (inside positive) due to calcium pumping. This stimulation appears to be a component specific for each anion since the \( \text{Ca}^{2+} \)-anion transport ratio varies.

In the previous article (2), the stoichiometry of phosphate and calcium transport was reported to be equimolar. There, the following ratios were reported: \( \text{SO}_{4}^{2-}:\text{Ca}^{2+} \), 1.5:1; \( \text{Cl}^{-}:\text{Ca}^{2+} \), 2:1. This would suggest that anion-mediated calcium transport is electroneutral. In the case of chloride, the ratio of \( \text{Cl}^{-} \) to \( \text{Ca}^{2+} \) was 0.6. This discrepancy might be due to the experimental conditions, i.e. the chloride carrier may not be saturated at 40 mM chloride. Unfortunately, measurement of chloride at 100 mM presents experimental difficulties due to high background and incomplete release of chloride by A23187.

Phosphate transport has been suggested to be mediated by band III (2). Figs. 7 and 8 present curves of the inhibition of phosphate transport by chloride, sulfate, and acetate. The inhibition of phosphate uptake by these anions and the observation that phosphate blocks sulfate uptake into IOV (data not shown) suggest that these anions may be competing at a common transport site, i.e. band III. This notion is consistent with the preliminary observations that \( N-(\text{4-azido-2-nitrophenyl})-2\text{-aminoethylsulfonate} \) blocks chloride uptake into the IOV. However, more detailed kinetic analysis of anion inhibition is needed to resolve this question.

The competitive stimulation of calcium transport by the anions was also examined. In the presence of saturating phosphate, chloride, sulfate, and acetate do not stimulate calcium uptake (data not shown). At suboptimal phosphate (1 mM), these anions stimulate calcium transport to a degree unique to each anion. These results provide tentative evidence that the anions phosphate, chloride, sulfate, and acetate stimulate calcium transport by a similar mechanism. Two components to the action of the anions may exist, the binding of anion to the transport protein band III, and the transport of these bound anions through the membrane. The fact that chloride is the poorest stimulator of calcium transport (1 mM phosphate; Fig. 9) but the best inhibitor of phosphate transport (1 mM phosphate; Fig. 8) suggests that binding of anion to band III and translocation of the anion may be kinetically distinct. Clearly, however, detailed analysis is required to resolve this question.

In a previous communication (3) we reported that chloride and acetate were not significantly transported into IOV during calcium pumping. These experiments were performed in the presence of phosphate, which blocked uptake of these anions. In the absence of phosphate, these anions are accumulated (Figs. 5 and 6).

Glucuronate was originally chosen as an impermeant anion (4). However, glucuronate is transported into IOV by a calcium-dependent, calmodulin-stimulated process. The fact that glucuronate does not significantly inhibit phosphate transport or stimulate calcium transport at suboptimal phosphate (Figs. 8 and 9) suggests that glucuronate does not enter the IOV by band III, and that glucuronate does not move into the vesicles as a charged anionic species. These results are further supported by the observation that during calcium pumping in the presence of glucuronate, a membrane potential is produced.2

The net movement of ions through band III appears to be mechanistically distinct from the well characterized equilibrium exchange of anions. Under equilibrium exchange conditions, the \( K_{\text{m}} \) values of phosphate and chloride are similar (50 mM) and the time course of chloride exchange is about 10 times faster than phosphate exchange (5). Under our assay conditions, net transport of phosphate has a \( K_{\text{m}} \) of 0.6 mM and transport of phosphate is faster than is transport of chloride.

In summary, it appears that ion accumulation in human erythrocyte inside-out vesicles proceeds by a primary electrogenic calcium pump which drives a secondary accumulation of anions. Band III is probably the anion transport component. Phosphate is the most potent anion and may, in fact, be the anion transported with calcium under physiological conditions.

REFERENCES


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