Biochimica et Biophysica Acta, 508 (1978) 236—245

BBA 77972

CHEMICALLY-INDUCED CATION PERMEABILITY IN RED CELL MEMBRANE VESICLES

THE SIDEDNESS OF THE RESPONSE AND THE PROTEINS INVOLVED

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(Received August 31st, 1977)

Summary

Cation fluxes were measured in right-side-out and inside-out vesicles obtained from human red cells. Rubidium, which is spontaneously released at very slow rates, can be rapidly released from both types of vesicle by addition of valinomycin. *P*-Chloromercuriphenyl sulfonic acid (PCMBS) also increases the cation permeability of the vesicles with reversal to normal after addition of dithiothreitol. The effect of PCMBS is considerably larger and appears faster in the inside-out vesicles as compared to the right-side-out vesicles, the difference being greater at low temperatures. These data indicate that the SH groups responsible for the changes in cation permeability are more accessible from the inside face of the membrane. The response to PCMBS was not diminished after selective removal of extrinsic proteins by alkaline extraction, and/or after the membranes were exposed to proteolytic enzymes. The major polypeptide component remaining in vesicles after both treatments was a 17 000-dalton transmembrane fragment derived from band 3 which might, therefore, be responsible for the permeability response. Addition of Ca^{2+} to either right-side-out or inside-out vesicles, in the presence or absence of ionophore A23187, was without effect on monovalent cation permeability, indicating that the mechanism of Ca^{2+}-induced K^+ permeation was lost or inactivated during the preparation of the vesicles.

Introduction

The maintenance of the relatively low permeability of the red cell membrane to cations requires the integrity of certain sulfhydryl groups in the membrane.

Abbreviations: PCMBS, *p*-chloromercuriphenyl sulfonic acid; PCMB, *p*-chloromercuribenzoate.

Thus, X-irradiation of red cells or ghosts, which induces the oxidation of sulfhydryl groups with formation of disulfide bonds [1], increases cation leakage and can eventually produce hemolysis [2]. Similarly, the reaction of sulfhydryl groups of erythrocyte membranes with a variety of specific reagents such as mercury and organic mercurials, results in loss of potassium, gain of sodium and hemolysis [3]. Both effects are reversible and both involve the same sulfhydryl groups. Nevertheless, the effects of X-irradiation are virtually immediate, whereas those produced by organic mercurials such as *p*-chloromercuriphenyl sulfonic acid (PCMBS) are delayed. Based on this difference and on the dependence of the delay period on the temperature and on the PCMBS concentration, it has been suggested that the critical sulfhydryl groups are isolated from the external medium in an interior compartment of the membrane [3,4]. On this basis, rapidly penetrating, lipid-soluble mercurials would be expected to enhance the cation permeability faster and to a greater extent than PCMBS. Surprisingly, the opposite has been found. The more lipid-soluble compound *p*-chloromercuribenzoate (PCMB), which permeates the membrane more than ten times faster than PCMBS at physiological pH values is considerably less effective than equal concentrations of PCMBS [5—7]. Furthermore, nonionic mercurials such as 1-bromomercuri-2-hydroxypropane that enter even more rapidly than PCMB are without effect on cation permeability [7]. The interpretation of the inverse relationship between permeation rate and effect requires assumptions about the location of the sensitive sulfhydryl sites, the pathways by which the sulfhydryl reagents penetrate, and the protective effects of hemoglobin in binding the agents as they reach the cytoplasmic surface [3,6].

To circumvent such complications, and to avoid possible differences in the reactivity of different chemical probes, we decided to compare the effects of a single reagent, the slowly penetrating PCMBS, added to either the external or to the cytoplasmic face of hemoglobin-free red cell membranes. This was achieved by preparing resealed right-side-out or inside-out membrane vesicles derived from red cells [8], loading them with radioactive rubidium (which serves as a K^+ analog for both active and passive fluxes in red cells [9,10]), and then measuring the effects of the mercurial agent on the efflux of the isotope.

The vesicles offer another advantage for the study of cation permeability. During and after the formation of the vesicles, some of the membrane proteins can be selectively removed from the membranes by appropriate manipulation of the pH and ionic composition of the medium [8,11]. To determine whether the extracted proteins play any role in the mercurial-induced change in permeability, the vesicles formed during the extraction process can be separated and their transport properties measured. The proteins can also be dissected by proteolytic treatment from the outside (in the intact cell) or the cytoplasmic side (in inside-out vesicles) [12]. Therefore, the possible transport role of particular protein fragments can be assessed.

The low permeability of red cell membranes to some monovalent cations such as K^+ and Rb^+ can also be dramatically increased by elevating the concentration of Ca^{2+} in the cytoplasm [13—15]. The mechanism underlying this effect was studied and compared with the mercurial-induced change in permeability.

Materials and Methods

Dithiothreitol, 5,5-dithiobis-(2-nitrobenzoic acid), acetylcholine chloride and the monosodium salt of PCMBS were all purchased from Sigma. *O*-Phenanthroline was obtained from Fisher Scientific Co., ^{86}RbCl was obtained from New England Nuclear, valinomycin from Calbiochem, and A23187 was a gift from Eli Lilly Co.

Right-side-out and inside-out vesicles from human erythrocyte membranes were prepared from recently outdated blood essentially as described by Steck [8], with only minor modifications [16]. Although contamination of the vesicle preparation with leaky membranes is not expected to alter the results of the efflux experiments, the contaminating membranes could bind PCMBS or other reagents used during the assay. For this reason, leaky vesicles and membrane fragments were systematically removed by means of a Dextran density gradient [8,17] before loading the vesicles with the isotope. After this step, the vesicles were washed once in 0.5 mM sodium phosphate buffer, pH 9.0, and resuspended in a medium containing 5 mM RbCl and 0.5 mM sodium phosphate, to a final concentration of 1.5—2.5 mg protein/ml. This suspension was equilibrated overnight at 5°C with 0.2—2 μCi/ml ^{86}Rb.

For efflux determinations, the extravesicular ^{86}Rb was removed by filtering the vesicle suspensions through a 3 ml column of Dowex 50W-X8 (20—50 mesh) that had been prequilibrated with 5 mM sodium phosphate buffer, pH 8. The column was then washed with a column of the same buffer equal to the volume of the applied vesicle suspension, so that the final concentration of protein in the medium was 0.75—1.25 mg/ml. The amount of ^{86}Rb remaining in the vesicles was then determined at different time intervals by filtering 250-μl aliquots of the suspension through 1 ml columns of Dowex 50W-X8 equilibrated with 5 mM sodium phosphate (pH 8) buffer and washing the columns with 1 ml of ice-cold buffer. The filtrate was collected and counted to determine the amount of radioactivity remaining in the vesicles.

The sidedness of the vesicle preparations was determined by measuring acetylcholinesterase activity in the presence and absence of Triton X-100, as described previously [17]. Since the protein composition of right-side-out and inside-out vesicles is somewhat different [8], total membrane protein should not be used to compare the fluxes in the two systems. Therefore, the intrinsic protein acetylcholinesterase was used for quantitation in some of the experiments.

Alkaline extraction was performed by incubating the packed vesicles with 40 volumes of 10 mM NaOH and 0.1 mM EDTA on ice for 15 min. Proteolysis of the external surface of cells with chymotrypsin, and of the cytoplasmic surface of vesicles with trypsin was performed as described before [16].

Interchain disulfide bond formation was catalysed by adding 100 μM 1,10-phenanthroline and 50 μM $CuSO_4$ to the vesicle suspension at room temperature [18,19]. The concentration of PCMBS was determined spectrophotometrically, measuring the absorbance at 224 nm. Protein was measured by the method of Lowry et al. [20], using bovine serum albumin as a standard. Electrophoretic analysis was performed in 7.5% acrylamide gels with 1% SDS, following the procedure of Fairbanks et al. [21].

Results and Discussion

Considerable scattering was found in the amount of Rb^+ trapped per unit of acetylcholinesterase when different batches of vesicles were compared. However, no differences were noted in the amount of Rb^+ trapped by inside-out and right-side-out vesicles from the same batch of ghosts, as illustrated by the fact that the zero time intercepts in Fig. 1 are similar. This figure shows a typical experiment in which the time course of Rb^+ efflux from both kinds of vesicles was measured. The data can be fitted by two slopes, the initial component lasting for about 20 min. This may be due to heterogeneity in the size of the vesicles. For convenience, in the following experiments a 20 min interval was allowed between the filtration of the vesicles through the first column to remove extracellular Rb^+, and the first flux determination (time 0 in Figs. 2—4, and 6). To allow valid comparisons between different batches of vesicles, the results are expressed as the percentage of the radioactivity remaining in the vesicles after this interval. As seen in Fig. 1, addition of the cation ionophore valinomycin (1 μM) to either type of vesicles results in the immediate release of more than 95% of the trapped radioactivity, indicating that most of the Rb^+ is exchangeable. Similar results were obtained when the ionophore was introduced at different times during the course of similar experiments.

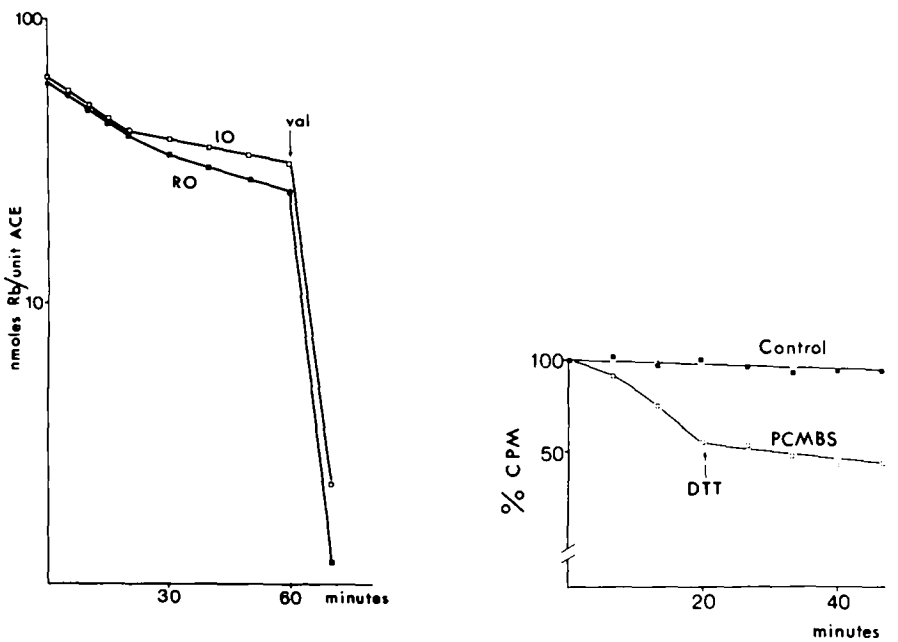

Fig. 1. Time course of the efflux of Rb^+ from right-side-out and inside-out vesicles. The addition of 1 μM valinomycin is indicated by the arrow. Temperature, 21°C. The right-side-out vesicles were 97% pure and the inside-out vesicles were 81% pure. Abscissa: Time in minutes. Ordinate: Rb^+ content of the vesicles per unit acetylcholinesterase (ACE). One unit acetylcholinesterase = 1 μmol/min.

Fig. 2. Effect of PCMBS on the efflux of Rb^+ from inside-out vesicles. $5 \cdot 10^{-4}$ M PCMBS was added to the experimental vesicles (empty symbols) at 0 time. The addition of $2 \cdot 10^{-3}$ M dithiothreitol (DTT) is indicated by the arrow. Temperature, 0°C. Abscissa: Time in minutes. Ordinate: Logarithm of the percent radioactivity remaining in the vesicles.

Fig. 2 shows the effect of PCMBS on the efflux of Rb^+ from inside-out vesicles. The mercurial produced an increased efflux which was detectable at the earliest sampling times (6 min). This increase in permeability is not due to the breakdown of the vesicles since addition of an excess of dithiothreitol (a sulfyhydryl-containing compound that will complex PCMBS) to the medium returned the permeability to normal values.

In order to investigate the location of the reactive -SH groups in the membrane, various concentrations of PCMBS were added to both right-side-out and inside-out vesicles. For a given concentration of the mercurial, the reaction is expected to be larger and to appear earlier in the right-side-out vesicles if the group is more accessible from the external face of the membrane. Conversely, a faster and more pronounced response in the inside-out vesicles would favour the relevant -SH group as being more accessible from the cytoplasmic surface. The results of these experiments are depicted in Fig. 3. Clearly, identical concentrations of PCMBS produce larger increases in permeability in inside-out than in right-side-out vesicles. Moreover, certain concentrations of the mercurial which cause a substantial enhancement of the efflux from inside-out vesicles, produce little or no change in the right-side-out vesicles within the period of time studied. The difference in the responsiveness of inside-out and right-side-out vesicles to PCMBS is more clearly illustrated in Fig. 4 in which the data of Fig. 3 are presented as a dose vs. response curve. The maximal rate of efflux detected during the course of the experiment is plotted for each concentration of PCMBS.

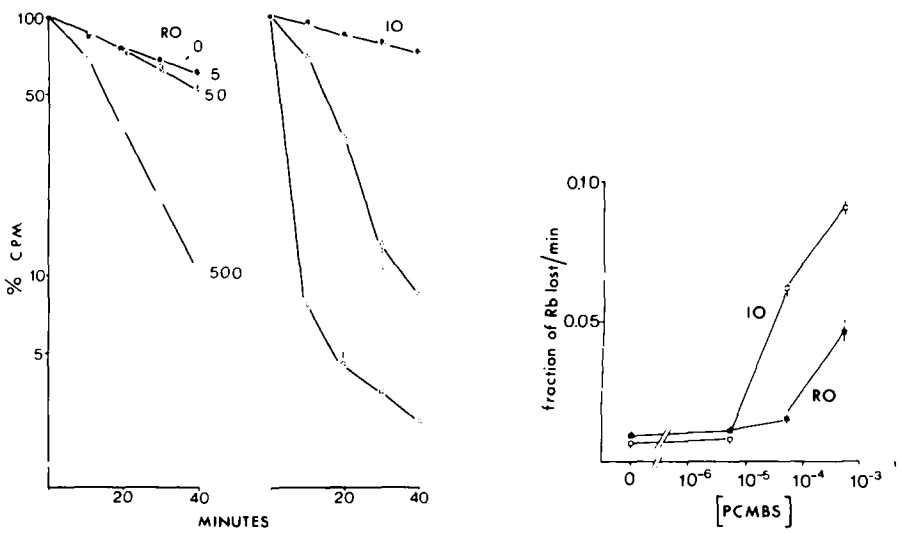

Fig. 3. Comparison of the effects of different concentrations of PCMBS on Rb^+ efflux from right-side-out and inside-out vesicles. The results are the mean of four experiments. The bars indicate ± 1 S.E. The numbers indicate the concentration of the mercurial in μmol/l. Temperature, 21°C. The right-side-out vesicles were 98% pure whereas the inside-out vesicles were 79% pure. Abscissa and ordinate as in Fig. 2.

Fig. 4. Dose vs. response curve of the effects of PCMBS on right-side-out and inside-out vesicles. The data of Fig. 3 were replotted using the maximal rate of efflux. Abscissa: Concentration of PCMBS in mol/l (logarithmic scale). Ordinate: Fraction of Rb^+ lost per min. The data are the mean of four determinations and the bars represent ± 1 S.E.

Certain features of the efflux curves in Fig. 3 can be noted: First, after most (about 90%) of the isotope has been lost from inside-out vesicles at high PCMBS concentrations, the apparent rate of efflux is notably decreased. This could be due to the presence of: (a) a small fraction of contaminating right-side-out vesicles, which are less sensitive to PCMBS or (b) a small amount of slowly exchanging membrane-bound Rb. Second, a considerable delay is observed between the application of 50 μM PCMBS to the right-side-out vesicles and the onset of the permeability change. This delay is reminiscent of that observed in intact cells [4,5], and is similarly shortened by increasing the concentration of the mercurial to 500 μM (Fig. 3).

In cells, the flow of PCMBS through the membrane is drastically reduced when the temperature is lowered [6]. If the responsive sites are located near the inner surface of the membrane, the appearance of the increased Rb^+ flux in right-side-out vesicles should therefore be markedly delayed at reduced temperatures, whereas that of inside-out vesicles should be less affected. An experiment carried out to test this prediction is presented in Fig. 5. Indeed, at 0°C the highest concentration of PCMBS (500 μM) had no effect on right-side-out vesicles for 15—20 min and a minimal effect thereafter, whereas the response of inside-out vesicles was substantial (50% loss of Rb^+ in 20 min). Even though the right-side-out vesicles respond to temperature changes to a much greater degree than the inside-out vesicles (compare Fig. 3 and Fig. 5), the effect on the latter is nevertheless substantial. Although this may be due to a slower rate of association of the mercurial with sulfhydryl groups, it seems more likely that the -SH groups involved in cation permeation are more accessible to but not on the cytoplasmic surface of the membrane, so that the PCMBS must still traverse a diffusion barrier to reach them. This interpretation is consistent with the downward curvature of the PCMBS curve in Fig. 5, demonstrating a delay in the appearance of the maximal effect, suggesting a diffusion limited reaction. It

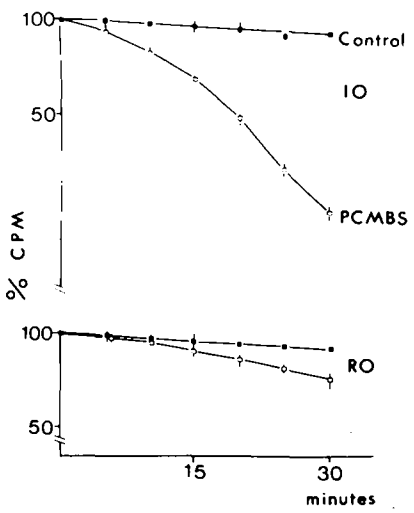

Fig. 5. Effect of PCMBS on Rb^+ efflux from right-side-out and inside-out vesicles at 0°C. The results are the mean of four experiments. The bars indicate ± 1 S.E. Full symbols, control vesicles. Empty symbols, PCMBS-treated vesicles. Abscissa and ordinate as in Fig. 2. Temperature, 0°C.

is in agreement with the previous suggestion that the cation-controlling sulfhydryl sites are located within an aqueous compartment of the membrane [1], presumably water-filled transmembrane protein channels [6].

It could be argued that the differences in responses of right-side-out and inside-out vesicles are not determined by the accessibility of the cation-controlling groups, but rather that a large number of unspecific -SH groups in the right-side-out vesicles react with PCMBS and deplete the mercurial in the medium. To rule out this possibility, an experiment similar to that in Fig. 5 was performed; right-side-out and inside-out vesicles were incubated in 500 μM PCMBS at 0°C for 15 min and immediately centrifuged at 48 000 $\times g$ for 15 min. The supernatant was then removed and the amount of free PCMBS was measured. More than 90% of the PCMBS originally added was still unreacted, indicating that depletion of the mercurial is not an important factor in those experiments where high PCMBS concentrations were used.

PCMBS is highly specific for sulfhydryl groups, indicating that its effects on permeability are mediated by membrane proteins [3]. Many proteins, however,

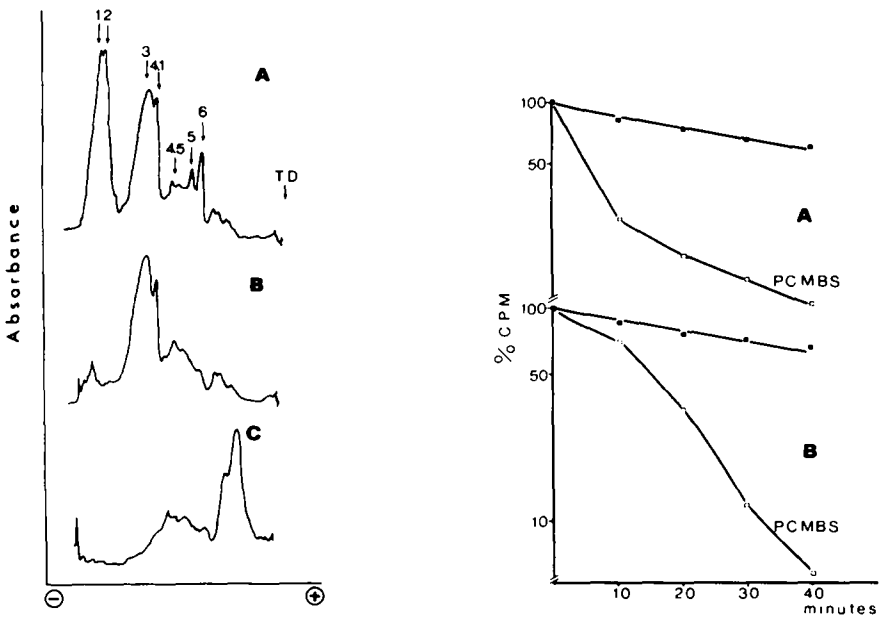

Fig. 6. Effects of alkaline extraction and proteolysis on the protein composition of red cell membranes. Sodium dodecyl sulfate (SDS) (1%) -acrylamide (7.5%) gels were run as described in Methods and stained with Coomassie Blue. The gels were scanned at 570 nm in a Beckman Acta CII spectrophotometer with a RIIC scanner. A, red cell ghosts; B, NaOH-EDTA-extracted inside-out vesicles; C, trypsin-treated and NaOH-EDTA-extracted inisde-out vesicles obtained from cells previously incubated with chymotrypsin. TD indicates the position of the tracking dye, pyronin Y. Some of the proteins are labelled according to the nomenclature of Fairbanks et al. [21].

Fig. 7. (A) Rb^+ fluces in inside-out vesicles stripped with NaOH-EDTA solutions. The concentration of PCMBS was $5 \cdot 10^{-4}$ M. Full symbols, control vesicles. Empty symbols, PCMBS-treated vesicles. Abscissa and ordinate as in Fig. 2. Temperature, 21°C. (B) Rb^+ fluxes in proteolysed and extracted inside-out vesicles. Vesicles were obtained from chymotrypsin-treated cells, and digested with trypsin as described in Methods. After stopping the proteolysis, the vesicles were extracted with NaOH-EDTA and washed. The concentration of PCMBS was $5 \cdot 10^{-4}$ M. Abscissa and ordinate as in Fig. 2. Temperature, 21°C.

contain sulfhydryl groups so that binding of sulfhydryl agents would be an insufficient criterion for the identification of the cation permeability-controlling protein. In an attempt to identify this particular protein, a selective removal of the extrinsic proteins of the vesicles was accomplished by means of an alkaline extraction in a low ionic strength medium [12]. The results of this extraction are shown in Fig. 6. Treatment of the vesicles with NaOH and EDTA largely removed bands 1, 2 and 5 and considerably depleted band 6 (Fig. 6B). ^{86}Rb efflux was measured in the extracted vesicles as described for the normal inside-out vesicles, and the results are shown in Fig. 7A. Although the alkali treatment rendered the vesicles considerably leakier than the normal inside-out vesicles ($t_{1/2}$ was 54 min for the former and 89 min for the latter), the effect of PCMBS could still be clearly observed. The mercurial immediately increased the efflux of Rb^+ several-fold, and the efflux rate tended to decrease afterwards, in a manner analogous to that observed in normal inside-out vesicles (Fig. 3). Thus the permeability response can be attributed to the intrinsic proteins that are not extracted by the described procedure.

The predominant intrinsic proteins of the red cell membrane are the major glycoprotein (glycophorin) and band 3 [22]. The former contains no sulfhydryl groups, so that it cannot be directly involved in the PCMBS response [23]. Band 3, on the other hand, contains a relatively large amount of half cystine residues [24]. One of these can form intermolecular crosslinks in the presence of the *O*-phenanthroline · $CuSO_4$ complex [18,19]. This -SH group might be involved in cation permeability regulation since it is located on the cytoplasmic portion of the protein [12]. To investigate this possibility. Inside-out vesicles were treated with *O*-phenanthroline · $CuSO_4$ under conditions where most of the band 3 molecules are dimerized [18], and the efflux of Rb^+ was measured as described above. Crosslinking of the cytoplasmic -SH groups of band 3 had no effects on either the resting cation fluxes or their activation by PCMBS.

A further purification of the component bearing the active -SH group was achieved by combining alkaline extraction with the use of proteolytic enzymes to digest portions of the protein exposed on the outer and inner surfaces of the membrane. The intact cells were treated with chymotrypsin before their membranes were converted to inside-out vesicles. The latter were then treated with trypsin and extracted with an alkaline, low ionic strength solution. As previously reported [12,16], only a fraction (about 30%) of the original membrane protein remains in the membrane. The major component is a 17 000 dalton, transmembrane segment derived from band 3 after loss of a 35 000 dalton segment from the outside, and a 40 000 dalton segment from the inside. Small amounts of other peptides are, however, also present (Fig. 6C). As was the case for the non-digested vesicles, the alkaline extraction rendered the vesicles more permeable to Rb^+ (Fig. 7B). However, a substantial effect of PCMBS was still observed. The response was delayed and slightly smaller in these digested vesicles than in intact ones, but more than 95% of the Rb^+ was liberated from them by PCMBS, thus ruling out the possibility of having two or more populations of vesicles with different protein composition and a different sensitivity to the mercurial agent. It can be concluded, therefore, that peptides remaining in the membrane after the proteolysis and alkaline extraction can respond

to sulfhydryl agents with a change in Rb^+ permeability. The 17 000 dalton fragment of band 3 as the predominant component is, therefore, a logical candidate. As a transmembrane polypeptide [12], it could provide an aqueous protein channel through which the Rb^+ might flow. On the other hand, because the number of sites involved is not known, it cannot be rigorously excluded that one of the minor components still present in the 'stripped' vesicles is responsible.

The K^+ permeability of red cells and ghosts can also be increased by elevating the levels of cytoplasmic Ca^{2+} [10,13—15]. It was, therefore, of interest to determine whether such effects were mediated by the same membrane constituents as the PCMBS effect. The addition to the medium of 0.1 mM Ca^{2+}, a concentration that has supramaximal effects on resealed ghosts [10,15], did not alter the efflux of Rb^+ from inside-out vesicles. Similar negative results were obtained when Ca^{2+} was added to resealed right-side-out vesicles, either in the presence or in the absence of the Ca^{2+} ionophore A23187 (5 μM). The latter was added to facilitate the entrance of Ca^{2+} into the intravesicular (cytoplasmic) medium, since the permeability of the membrane to Ca^{2+} is extremely low. In some experiments, the ionic composition of the assay medium was changed to 150 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM Tris · HCl (pH 7.4) in an attempt to mimic the conditions present inside the cell. Again, the response to Ca^{2+} was absent. These results suggest that the membrane lipids and the intrinsic proteins of the vesicles alone are not sufficient for the Ca^{2+}-mediated flux. At least one component of the mechanism seems to have been lost or inactivated during the preparation of the vesicles. The procedure for making vesicles from ghosts is mild, involving manipulation of ionic strength and salt composition [8]. Losses of extrinsic proteins such as bands 1,2 (spectrin) and 5 occur, but no particular band has been noted as being absent. Thus the loss of the Ca^{2+}-mediated permeability response cannot at this time be attributed to any particular protein.

From the above considerations, there is no basis for concluding that any common element is involved in the PCMBS- and Ca^{2+}-mediated effects. The PCMBS effect on cation permeability is mediated by an intrinsic protein, most probably the 17 000 dalton transmembrane segment of band 3, that could provide an aqueous channel for $K^+(Rb^+)$ permeation. The sulfhydryl sites involved are more accessible from the inner face of the membrane than from the outer face. The Ca^{2+} effect involves a different, more complicated mechanism. An extrinsic factor that is lost of inactivated when vesicles are formed from ghosts seems to be essential. Presumably an intrinsic component is also necessary to provide a permeation pathway through the membrane.

Acknowledgements

The authors are indebted to Dr. P. Knauf for valuable discussions relating to the study and to the manuscript. This study was supported by Medical Research Council (Canada) Grant No. MT 4665. S.G. is the recipient of a Medical Research Council (Canada) Post-doctoral Fellowship.

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