Biochimica et Biophysica Acta, 774 (1984) 103–109
Elsevier

BBA 72148

THE SPONTANEOUS ACTIVATION OF A POTASSIUM CHANNEL DURING THE PREPARATION OF RESEALED HUMAN ERYTHROCYTE GHOSTS

P.G. WOOD

Max-Planck Institute for Biophysics, Department of Cell Physiology, D-6000 Frankfurt/Main 71 (F.R.G.)

(Received September 20th, 1983)
(Revised manuscript received 2nd March, 1984)

Key words: Ca^{2+}-activated channel; Gardos effect; K^+ channel; (Human erythrocyte ghost)

Resealed erythrocyte ghosts prepared under conditions which deplete the cell of its endogenous chelators and metabolites are found to be selectively permeable to potassium. The net efflux of potassium is stimulated by low concentrations of external potassium and can be inhibited by oligomycin. The effect is not expressed when resealed ghosts are formed by hemolysis in the presence of chelators or magnesium. The spontaneously activated pathway is actually the calcium-activated potassium channel, first discovered by Gardos in 1958. In the intact cell, the combined actions of the calcium pump and endogenous chelators maintain the calcium concentration below the threshold for activation. Current observations indicate that the channel is spontaneously activated by traces of calcium originating from the cell itself or from the unavoidable background of calcium found in the media. The channel in ghosts depleted of endogenous chelators exhibits its high affinity for calcium. Channel activation occurs during hemolysis and persists throughout subsequent washings.

Introduction

The overall permeability characteristics of the resealed ghost membrane depend strongly on the methods utilized in ghost preparation (for a review see Ref. 1).It was previously demonstrated that resealed ghosts may become selectively permeable to potassium under conditions which deplete the cytosol of its endogenous metal-ion chelators at hemolysis [2]. This paper compares the characteristics of the spontaneously generated potassium pathway to those of the calcium-activated potassium channel, discovered by Gardos [3] (for reviews see Refs. 4,5). The data presented indicate that the properties of the spontaneously generated pathway are remarkably similar to those of the calcium-activated potassium channel. It is suggested that either the redistribution of cellular calcium during hemolysis or trace amounts of calcium found in suspending media may be the cause for channel activation. Resealed ghosts formed under conditions which deplete the cell of endogenous metal-ion chelators, magnesium and ATP are particularily vulnerable to the effects of trace amounts of calcium, since the potassium channel is found in a high-affinity state for calcium activation [6]. Channel activation due to calcium redistribution has also been seen under conditions where proteins of the ghost membrane are partially digested by trypsin [7].

Materials and Methods

Resealed ghosts were prepared as previously described [2] from human erythrocytes, 1–6 days after collection in acid-citrate-dextrose buffer by the Hessen Red Cross Blood Bank. The cells were

Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

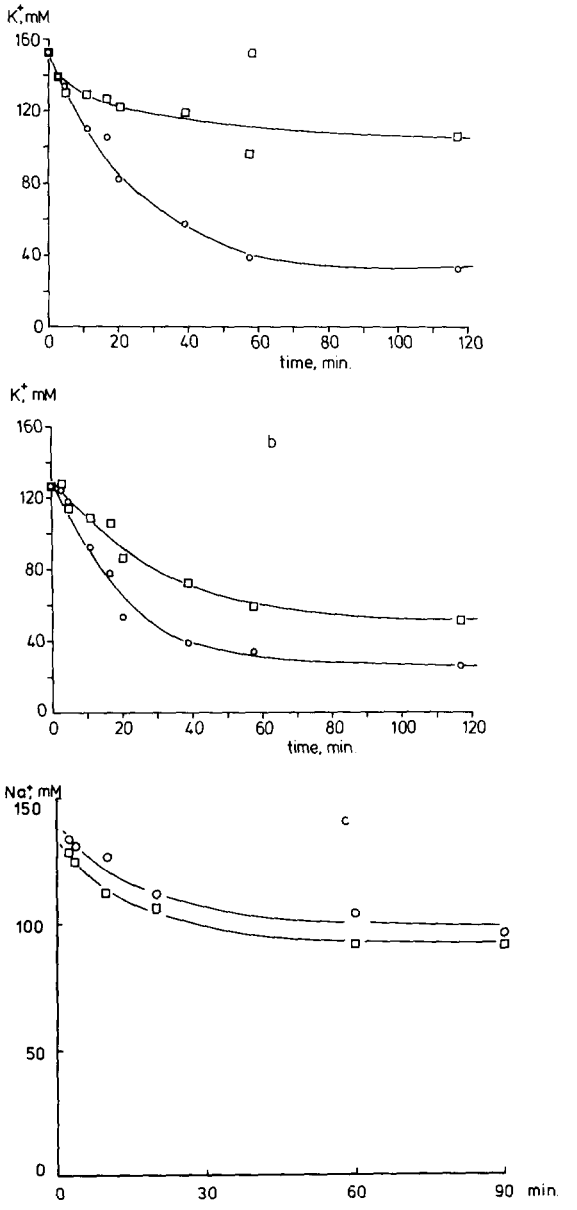

Fig. 1. (a) The efflux of potassium into choline-chloride medium from resealed ghosts. The ghosts were prepared as described in Materials and Methods. After loading with KCl and resealing, the ghosts were washed two times in a medium comprising 146 mM KCl/20 mM Pipes (pH 6.75). Thereafter, they were washed three times in a medium comprising 146 mM choline chloride/ 20 mM Pipes (pH 6.75). the efflux of potassium was measured into a medium comprising either 146 mM choline chloride/20 mM Pipes (□———□) or 145 mM choline chloride/1 mM KCl/30 mM Pipes (○———○) (pH 6.75, 37 °C, 0.5% suspension). Ordinate: mmol/l packed membranes. (b) Same conditions as in (a) except that NaCl is substituted for choline chloride. Flux medium: 146 mM NaCl/20 mM Pipes (□———□) or 145 mM NaCl/1 mM KCl/20 mM Pipes (○———○). (c) The efflux of sodium into choline chloride medium from resealed ghosts. The conditions are the same as in (a) except that potassium is replaced by sodium.

washed three times in 166 mM NaCl and resuspended in 166 mM Pipes (potassium salt, Calbiochem) (pH 6.5) 30% hematocrit. The cells were cooled to 0 °C and 1 vol. suspension was hemolysed with 20 vol. doubly quartz-distilled water at 0 °C. 5 min later the ionic strength was restored to 166 mM with 3 M KCl or NaCl (Merck, Darmstadt, suprapur grade). The membranes were resealed by incubation at 37 °C for 45 min. The only inorganic cation present in the ghost was potassium. The resealed ghosts were washed twice in a potassium medium (146 mM KCl/20 mM Pipes) and three times in either a choline chloride (Syntex, Springfield, MI) medium or NaCl medium (146 mM choline chloride or NaCl, 20 mM Pipes). Unless otherwise indicated, the concentration of total calcium was 1 μM or less in all solutions used (i.e., hemolysis media, wash media, flux media) as measured on a Perkin-Elmer Atomic Absorption spectrometer, Model 503. The washings served to adjust pH and to deplete any leaky ghosts of their potassium.

The rate of net potassium efflux into a medium which had the same composition as the last wash medium was measured at 37 °C, 0.5% cytocrit. Samples were taken at selected intervals and centrifuged. The potassium remaining in the ghosts was measured by flame photometry after hemolysis in 1 mM CsCl. Corrections were made for trapped volume and rate constants were calculated for the initial 15–20 min of efflux. Unless otherwise stated, all chemicals were P.A. grade (Merck, Darmstadt).

Results and Discussion

The time-course for the net efflux of potassium from the resealed ghost is shown in Fig. 1. The efflux of potassium into either choline (Fig. 1a) or sodium (Fig. 1b) medium at pH 6.75 is shown. In contrast, Fig. 1c shows the efflux of sodium into choline medium. The membrane is selectively permeable to potassium. Net potassium efflux is greater into sodium medium than into choline

medium. Similar observations have been reported by Knauf and co-workers [8] and Hoffman and Blum [10] for the calcium-actived channel.

Another characteristic of the calcium-induced pathway is the stimulation produced by 0.1–3 mM external potassium [8–10]. Fig. 1a and b indicate that this is also the case for the spontaneously generated channel, the net efflux of potassium being increased by 1 mM external potassium. The relative effect of external potassium is greater in choline medium than in sodium medium, which is similar to the observations of Knauf et al. [8] and Hoffman and Blum [10] for the calcium-activated channel.

Fig. 2 summarizes five similar experiments in which the external potassium concentration was varied from 0 to 3 mM. The rate of potassium loss increases by a factor of 4 in this range. The greatest effects are seen between 0 and 0.5 mM potassium and beyond 1 mM may even decrease. The half-maximal concentration for activation of the channel by external potassium in choline medium is estimated to be between 0.1 and 0.2 mM. The spontaneous pathway has, therefore, about the same affinity for external potassium as in the calcium-activated channel [11].

It has been previously shown that, when the rate of net potassium efflux is measured between pH 6 and 8, a maximum is found near pH 6.7 [2]. In Fig. 3, the pH dependence of the spontaneously formed channel is compared in the presence and

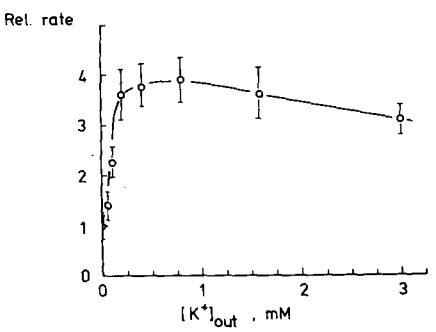

Fig. 2. The stimulating effect of external potassium on the rate of potassium loss. The ghosts were prepared as in Fig. 1a. The flux medium contained the indicated potassium concentrations. The sum of potassium and choline was kept at 146 mM. The efflux was measured at 37 °C, pH 6.75 (0.5% suspension). The curve is an average of five similar experiments and the S.E. is indicated.

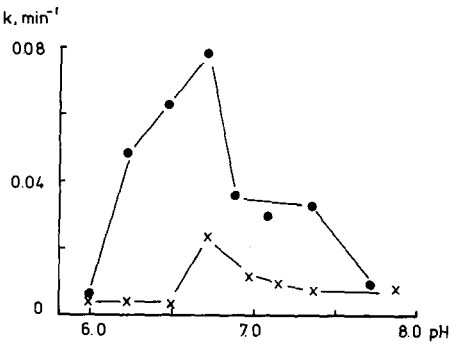

Fig. 3. The pH dependence of the spontaneously activated potassium channel in the absence and in the presence of 1 mM KCl. The ghosts were prepared as in Fig. 1a with the exception that they were washed at the indicated pH. The flux medium comprised either 146 mM choline chloride/20 mM Pipes (×——×) or 145 mM choline chloride/1 mM KCl/20 mM Pipes (●——●).

absence of external potassium. In both cases a maximum is found near pH 6.7. Unpublished observations suggest that the actual position of the maximum may be determined by the amount of residual cell content trapped at resealing. With 1 : 10 hemolysis the maximum is shifted toward pH 7.0. The appearance of a maximum for the spontaneous channel differs with the observations of Knauf et al. [8], in which it it was shown that in the case of the calcium-activated channel in ghosts, the rate of potassium loss increases with pH up to about pH 7.5 and, thereafter, forms a broad plateau.

It has been shown in several laboratories that the calcium-stimulated pathway is inhibited by the antibiotic oligomycin [9,12]. Figs. 4a and b compare the inhibitory effects of oligomycin in sodium and choline medium. In both cases the rate of potassium loss is decreased to about the same extent in the presence and absence of external potassium.

Initial work on the spontaneous pathway indicated that metal ions were responsible for activating the pathway [2]. It was shown that the addition of EDTA and EGTA to the hemolysis medium or to the flux medium inhibited the potassium pathway. Since the properties of the spontaneous pathway and the calcium-induced pathway are very similar, the activating metal ion must be calcium. The low permeability state of the membrane to cations is preserved when either magnesium or a

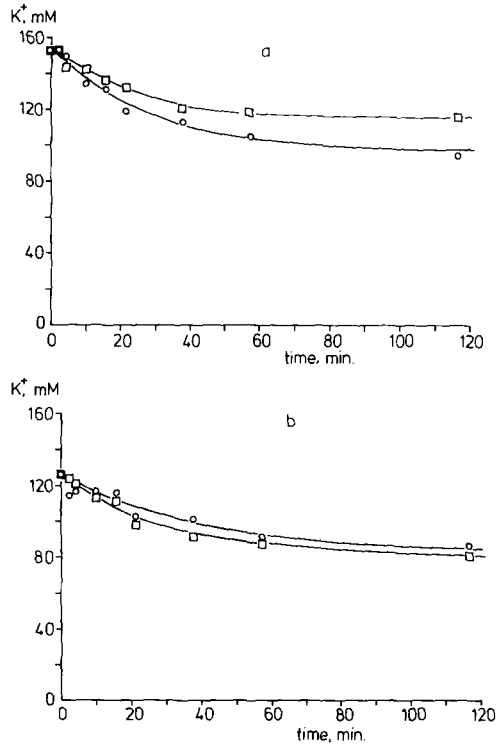

Fig. 4. The effect of 10 μg/ml oligomycin on the spontaneously activated channel. The ghosts were prepared as in Fig. 1. The efflux of potassium was measured at pH 6.75, 37 °C (0.5% suspension). (a) The flux medium comprised either 146 mM choline chloride/20 mM Pipes (□———□) or 145 mM choline chloride/1 mM KCl/20 mM Pipes (○———○). (b) The flux medium comprised either 146 mM NaCl/20 mM Pipes (□———□) or 145 mM NaCl/1 mM KCl/20 mM Pipes (○———○).

chelator is present at hemolysis. Fig. 5 compares the effectiveness of various chelators and magnesium. EDTA and EGTA are the most effective, with half-maximal action near 2 and 3 μM, respectively, followed by ATP, whereas magnesium is about 500-times less effective than EGTA. Since the potencies of EDTA and EGTA are about the same, this can be taken as further evidence that calcium is involved in the spontaneously activated potassium channel. The stability constants for EDTA and EGTA are of the same order of magnitude for calcium, while differing by nearly 4 orders of magnitude for magnesium [13]. The EDTA and EGTA dose-response curves may be considered as calcium titration curves at hemolysis and suggest that on the removal of 2–3 μM of calcium from the hemolysate, the potassium channel is inactivated. Simons [14] and Heinz and Passow [11] have reported half-maximal activation of the channel by about 1–2 μM intracellular calcium.

The results described above indicate that in the preparation of resealed ghosts, type 2 in the nomenclature of Bodemann and Passow [15], under conditions in which the intracellular contents are extensively diluted during hemolysis, a potassium channel becomes activated. Under these conditions cellular ATP, magnesium and endogenous chelators have been depleted. The potassium channel is in a high-affinity state for calcium [6]. The properties of the spontaneously generated channel are quite similar to those of the calcium-activated channel. The only apparent difference seems to be in pH dependence for potassium loss. It is not immediately apparent what may be the cause of this difference. In the work of Knauf et al. [8] the ghosts were prepared against a background of magnesium. In metal-ion buffer systems, the use of an excess of weakly binding metal ions in the buffer, removes the pH dependence of the metal-ion buffer by displacing the protons on the metal ion binding ligands [16]. In ghosts containing magnesium, the pH dependence of net potassium efflux represents the response of the activated channel. However, the pH dependence of the spontaneously induced channel, includes a superposition of the pH dependence of the channel and the pH

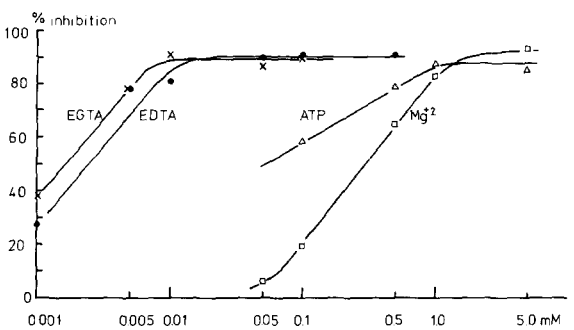

Fig. 5. The inhibitory effects of chelators and magnesium at hemolysis on the magnitude of the spontaneously activated potassium channel. The resealed ghosts were prepared as in Fig. 1b with the exception that the hemolysing medium contained, additionally, the indicated compound. The subsequent washings were carried out in the absence of the components. The flux medium comprised 146 mM NaCl/20 mM Pipes. Efflux was measured at 37 °C, pH 6.75.

dependence of the various metal-ion binding sites of the membrane. Overall, the basic characteristics of the spontaneously induced channel indicate calcium activation.

An obvious question concerns the source of the calcium which actives the potassium channel. One possible source of calcium is the erythrocyte membrane itself. Included are the charged phospholipids and cytoskeleton of the inner surface which can weakly bind calcium. The migration of calcium from nonspecific binding sites to the activating sites of the potassium channel during hemolysis cannot be excluded. The intact cell contains sufficient amounts of calcium to activate itself [17,18]. Porzig [17] has shown that with the addition of propranolol to resealed ghosts, a potassium pathway is activated. He reasoned that the effect was due to to a release of membrane-bound calcium to the inner surface. Sufficient calcium may be positioned near the activating site of the channel. In the presence of endogenous chelators and a functioning calcium pump, the channel remains inactive.

Another obvious source of calcium is the water and reagents which are used to wash and suspend the cells and ghosts. Preliminary experiments have shown no significant effects when the purity of the inorganic salts is improved. To investigate further the possibility that the solutions rather than the membrane are the source of activating calcium, the following experiments were conducted. Resealed ghosts were prepared on a gel-filtration column [1,19,20] at pH 6.5 from cells which had been extensively washed at pH 7.6 previously in the presence of the calcium-ionophore, A23187 (Calbiochem, calcium-free) and EDTA to deplete the cells of metal ions. Thereafter, the rate of potassium loss was measured from resealed ghosts which had been again washed in the presence of ionophore and EDTA and compared to ghosts which had not been rewashed.

Fig. 6 shows that the ghosts prepared from metal-ion-depleted cells also show a high rate of potassium loss. However, when the ghosts are again depleted of metal ions after resealing, the rate of potassium loss is substantially reduced. Furthermore, the ghosts, when exposed to the ionophore alone, after depletion do not show an increased rate of potassium loss. The response to 5 μM

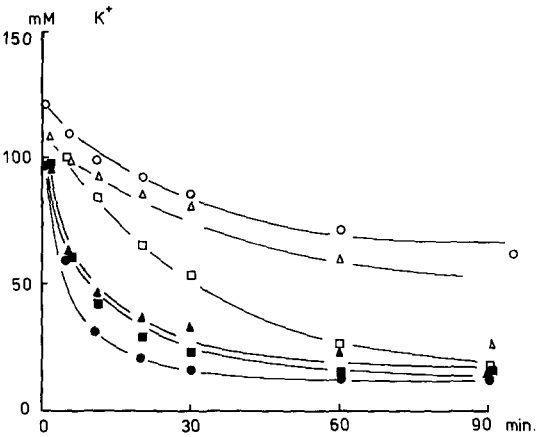

Fig. 6. The effects of metal ion depletion on erythrocytes in ghost preparation. Cells were washed two times in a medium comprising 146 mM NaCl/20 mM Hepes (pH 7.6) and two times in a medium comprising 146 mM NaCl/10 mM Hepes/10 mM EDTA (pH 7.6). Thereafter, the cells were washed four times in the same medium plus 0.1 μM ionophore A23187. Finally, the cells were washed five times in the same medium in the absence of the ionophore at room temperature and at 10% cell density. Resealed ghosts were prepared from the metal-ion-depleted cell by hemolysis on a gel filtration column [1,19,20]. The column was pre-equilibrated with a buffer comprising 10 mM Pipes (sodium salt)/5 mM KCl (pH 6.5). (Agarose A50m gel, Bio-Rad, column temp., 0 °C). After elution of the membranes at 0 °C, the membranes were restored to 155 mM KCl and resealed by incubation at 37 °C for 45 min. The efflux of potassium was measured from ghosts that were either washed nine times in a medium comprising 146 mM KCl/20 mM Hepes (pH 7.4) or depleted of metal ions by washing two times in the above medium, three times in a medium comprising 146 mM KCl/10 mM Hepes/10 mM EDTA/0.1 μM ionophore 146 mM KCl/20 mM Hepes (pH 7.4). Efflux of potassium was measured at 37 °C, 0.5% cytocrit into a medium comprising 145 mM Choline chloride/20 mM Hepes/1 mM KCl (pH 7.4). Ghosts from metal-ion-depleted cells (●———●); same ghosts in the presence of 0.1 μM ionophore A23187 (▲———▲); same ghosts in the presence of 0.1 μM ionophore A23187 and 5 μM CaCl (■———■). Metal-ion-depleted ghosts from metal-ion-depleted cells (○———○); same ghosts in the presence of 0.1 μM ionophore A23187 (△———△), same ghosts in the presence of 0.1 μM ionophore A23187 and 5 μM CaCl (□———□).

calcium and ionophore is greater than to ionophore alone. However, in both cases, the rates of potassium loss are much less than the spontaneously induced rate. Thus, channel activation probably took place at some time during hemolysis while the membranes were exposed to the low ionic strength conditions. The response of the metal-ion-depleted resealed ghosts to an increasing

concentration of calcium is shown in Fig. 7. The metal-ion-depleted ghosts were suspended in solutions containing the indicated calcium concentrations in the presence of ionophore A23187. Under these conditions, the half-maximal response (as determined from the initial rates of potassium loss) is found at more than 20 μM calcium.

In comparison, Fig. 7b shows the calcium dose-response curve of the untreated ghosts. Added calcium produces only a small increase in the efflux of potassium, suggesting that the channel is nearly fully activated. Comparing Fig. 7A and b, one can estimated the amount of calcium the

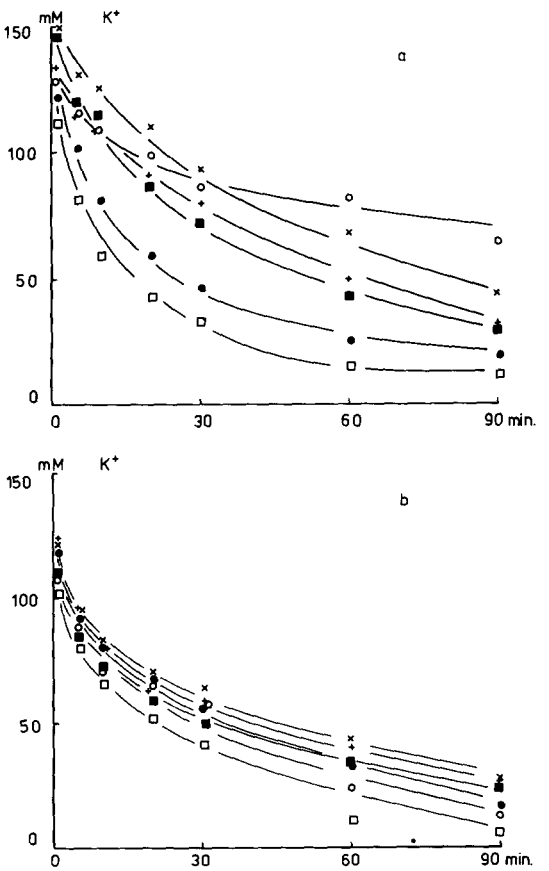

Fig. 7. Cells and ghosts were treated as in Fig. 6. Efflux of potassium was measured in the presence of the indicated concentration of CaCl and 145 mM Choline chloride, 20 mM Hepes, 1 mM KCl and 0.1 μM ionophore A23187. Calcium was added to a final concentration (μM) of: 0 (○——○); 5 (×——×); 10 (+——+); 20 (■——■); 50 (●——●); 1000 (□——□). (a) Metal-ion-depleted ghosts from metal-ion-depleted cells. (b) Untreated ghosts from metal-ion-depleted cells.

untreated ghosts apparently encountered during preparation. The ghosts act as if they had been in contact with solutions containing more than 50 μM calcium, presumably during hemolysis. That is a calcium level that is too high to have originated solely from solutions contaminated with calcium. The high values would suggest that substantial amounts of calcium must bind to the membrane before the channel receptor can be activated, even under conditions in which endogenous chelators have been maximally depleted. It is, therefore, difficult to understand why during hyemolysis trace calcium should activate the channel of the metal-ion-depleted cells. Perhaps calcium can migrate during hemolysis into regions on the membrane that are normally shielded from the cytosol. The channel is apparently shielded from the cytosol by a network of membrane proteins. Recently it has been shown that the properties of the channel cannot be altered by the action of incorporated trypsin until the proteins residing at the inner surface are partially digested [7]. Thus, the channel is not directly exposed to the cytosol.

While, in the absence of calcium buffers, the depleted ghosts seem less responsive to calcium than might be expected, this may be partially explained by the buffering capacity of the membrane itself. Unpublished observations indicate that 1–2 μM calcium may be in the solutions and that the half-maximal response to calcium lies near 5 μM calcium when calcium buffers are used to overcome the buffering capacity of the membrane. The value is comparable to the level of calcium found in the solutions. In such experiments the resealed ghosts were hemolysed in 5 mM magnesium and the calcium concentration adjusted using the metal-ion buffer, nitrilotriacetic acid (NTA).

In conclusion, conditions which allow the high affinity of the channel for calcium to be exposed permit activation by endogenous calcium or by traces of calcium in the solutions used. Regardless of the source of calcium, activation seems likely to occur at some time during hemolysis and persists throughout subsequent washings.

Acknowledgments

The author thanks Professor H. Passow and Dr. W. Schwarz for reading and discussing the paper

and Professor W. Pusch for the use of the atomic absorption spectrometer and Heidi Mueller for making the measurements. In addition, he thanks Ursula Rempel and Hannelore Mueller for their fine and untiring technical assistance and Brigitte Lehmann for the photography.

References

1 Wood, P.G. and Passow, H. (1981) in Techniques in Cellular Physiology, Vol. P1/I(P112) (Baker, P.F., ed.), pp. 1–43, Elsevier/North-Holland, Shannon
2 Wood, P.G. and Rossleben, U. (1979) Biochim. Biophys. Acta 553, 320–325
3 Gardos, G. (1958) Biochim. Biophys. Acta 30, 653–54
4 Schwarz, W. and Passow, H. (1983) Annu. Rev. Physiol. 45, 359–374
5 Sarkadi, B. and Gardos, G. (1984) in The Enzymes of Biological Membranes, Plenum Press, New York, in the press
6 Lew, V.L. and Ferreira, H.G. (1976) Nature 263, 336–338
7 Wood, P.G. and Mueller, H. (1984) Eur. J. Biochem., 141, 91–95
8 Knauf, P.A., Riordan, J.H., Schuhmann, B., Wood-Guth, I. and Passow, H. (1975) J. Membrane Biol. 25, 1–22
9 Blum, R.M. and Hoffman, J.F. (1971) J. Membrane Biol. 6, 315–328
10 Hoffman, J.F., and Blum, R.M. (1977) in Membrane Toxicity (Miller, M.W. and Shamoo, A.E., eds.), pp. 381–405, Plenum Press, New York
11 Heinz, A. and Passow, H. (1980) J. Membrane Biol. 57, 119–131
12 Riordan, J.H. and Passow, H. (1971) Biochim. Biophys. Acta 249, 601–605
13 Smith, R.M. and Martell, A.E. (1975) Critical Stability Constants, Vol. 3, pp. 204–272, Plenum Press, New York
14 Simons, T.J.B. (1976) J. Physiol. (London) 256, 227–244
15 Bodemann, H. and Passow, H. (1972) J. Membrane Biol. 8, 1–26
16 Perrin, D.D. and Dempsey, B. (1974) Buffers for pH and Metal Ion Control, Ch. 7, pp. 94–101, Chapman and Hall, London
17 Porzig, H. (1975) J. Membrane Biol. 31, 317–349
18 Szasz, I., Sarkardi, B. and Gardos, G. (1977) J. Membrane Biol. 35, 75–93
19 Wood, P.G. (1975) Fed. Proc. 34, 249–249
20 Wood, P.G. (1982) in Protides of the Biological Fluids, XXIXth Colloquium (Peeters, H., ed.), pp. 283–286, Pergamon Press, Oxford