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Induction of a Ca^{2+}-activated K^+ channel in human erythrocytes by mechanical stress

Robert M. Johnson and Ke Tang

Department of Biochemistry, Wayne State Medical School, Detroit, MI (USA)

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Mechanical deformation of normal ATP-replete human erythrocytes increased their permeability to Ca^{2+} sufficiently to turn on the Ca^{2+}-activated K^+ channel (the Gardos channel). When Ca^{2+} is absent, mechanical deformation of normal erythrocytes induces an equivalent increase the permeability of both Na^+ and K^+. In the presence of 0.1 to 1 mM Ca^{2+}, a further increase in the K^+ efflux rate was seen. There was no increase in Na^+ flux above that induced by deformation itself. The involvement of the Ca^{2+}-activated H channel was verified by showing the specific inhibitors of the channel, quinine and charybdotoxin, prevent the Ca^{2+}-induced increase in K^+ efflux. These results are consistent with a model of sickle cell dehydration proposed by Bookchin et al. ((1987) Prog. Clin. Biol. Res. 240, 193–200). The estimated rate of Ca^{2+} entry under these conditions (37 °C, 1000 dyne/cm^2, and laminar shear) was about 1 mmol/loc per h.

Some sickle erythrocytes, primarily those with low HbF content [1], rapidly lose cell water after emerging from the bone marrow, attaining MCHC values ranging up to 45 or 50 gHb/l. The number of dense cells is positively correlated with the hemolytic rate [2] and conjunctival blood vessel anomalies [3]. An understanding of the mechanism of dehydration may suggest methods to prevent the production of dense sickle cells and their associated pathology. Since cell hydration is controlled by monovalent cation movements in the erythrocyte, proposed explanations for dehydration have focussed on Na and K permeabilities [4–11]. A current model postulates the operation of a Ca-activated K channel in the red cell, first described by Gardos in 1956 (Ref. 12; reviewed in Ref. 13). This Gardos channel can be activated by Ca in cells that have been depleted of ATP to inhibit the Ca-ATPase [12,13], or exposed to the Ca ionophore, A23187 [13,14]. Activation allows the exit of K^+ without a balancing uptake of other cations; the loss of chloride and water that follows dehydrates the cell. Upon deoxygenation, sickle cells admit Ca at a increased rate [15–18], and Bookchin et al. [9] suggested that with a sufficiently rapid uptake, a steady state level of Ca may be attained that is high enough to activate the K^+ channel, even in the face of a normal Ca pump activity. The resulting loss of K triggers dehydration. Recently, a monovalent cation exchange was shown to occur in normal erythrocytes when they are deformed by shear [19–21], which had many of the characteristics of the deoxygenation induced exchange of Na and K that occurs in sickle cells. We here extend these observations to show that deformation induced by mechanical stress can also admit Ca into erythrocytes at a rate sufficient to trigger the Ca-dependent K channel.

Correspondence to: R.M. Johnson, Department of Biochemistry, Wayne State Medical School, 540 E. Canfield, Detroit, MI 48201, USA.

Materials and Methods

The procedures used were as described earlier [19], with minor changes. Red cells were obtained in heparin from normal volunteers, and were washed three times in phosphate-buffered saline (PBS): 9 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5 mM glucose, 150 mM NaCl. For subsequent dilutions, the cells were resuspended in PBS at a hematocrit of exactly 47.

Stress conditions. For the experiments, flux buffer was prepared, which was 10 mM sodium phosphate and 1 mM EDTA, with polyvinylpyrrolidone (PVP K-90, average molecular weight 300000, GAF Corporation) added to about 9% in order to increase the solution viscosity. The pH was adjusted to 7.4 and sufficient NaCl was added to raise the osmolality to 290 msM as determined by a vapor pressure osmome-

ter (Wescor, Logan, UT). On the day of use, 5 mM glucose was added. The red cell suspension and the flux buffer, both pre-warmed to 37°C, were mixed at a 1:5 ratio to make the final hematocrit equal to 8 and the final PVP concentration approx. 7.2%. The red cell-PVP suspension was loaded into a cylindrical Couette viscometer with a radius of 5 cm and a gap between the cylinders of 0.5 mm. The cylinders were wrapped in heating coils to maintain the internal temperature at 37°C, monitored with a thermocouple inserted through the outer cylinder wall. Samples were removed at appropriate intervals through a port drilled in the cylinder wall midway between the top and bottom of the cylinder. Care was taken to obtain samples only from regions of undisturbed flow and uniform shear rates. Unstressed controls were obtained by performing the entire experiment with the same cell suspension in the same buffer, but with a cylinder rotation speed of 5 rpm. PVP concentrations in the viscometer were about 7–7.5%, and the viscosity at 37°C was 70–85 cP. The shear rate was 1300/s, and shear stress (shear rate × viscosity in poise) was in the range of 1000–1300 dyne/cm^2 [22].

Ca, quinine and charybdotoxin were added from stock solutions dissolved in PBS immediately before the application of stress. The total volume of PBS was the same in all samples to ensure that viscosity was identical in all experimental runs.

Intracellular Na and K concentrations. Triplicate 0.2 ml samples from the viscometer side port were placed in a chilled 1.5 ml centrifuge tube containing ice-cold 10 mM Tris-HCl, 107 mM $MgCl_2$ (pH 7.4). The cells were immediately pelleted by a 90 s spin in a chilled Eppendorf microcentrifuge. The supernatant was removed and an aliquot saved for hemoglobin determination with Drabkin's reagent to estimate hemolysis. As was reported previously [19], hemolysis was minimal (1–3%) at all shear stresses, provided that the viscometer was carefully cleaned before each experiment.

The pelleted red cells were washed a second time in 10 mM Tris-HCl, 107 mM $MgCl_2$ (pH 7.4), which was sufficient to reduce external Na and K to negligible levels. The cells were lysed in 1.0 ml of distilled water. Hemoglobin was determined with Drabkin's reagent, and Na and K were determined by flame photometry.

Calculation of rates. As reported earlier [19], cation release and uptake are linear for 15–20 min. Triplicate data points were taken at zero and 10 min. Rates were calculated from the slopes of least-squares fits, and the standard error of the slope was calculated [23]. Rates are reported as micromoles of K released or Na taken up per gHb per min.

Red cell deformability. To ascertain that the various treatments did not alter cation flux rates by affecting cell flexibility, which would in turn affect the extent of membrane stress induced by deformation, erythrocyte deformability was determined before and after the stress runs with osmotic scan ektacytometry [24]. The ektacytometer (Technicon Instruments, Tarrytown, NY), a combined visco-diffractometric device [22,24] determines the extent of deformation of red cells subjected to a defined laminar shear stress. For the osmotic scan, 0.4 ml of the red cell suspension in flux buffer was mixed with 2.6 ml of osmoscan buffer (9 mM NaP_i (pH 7.4), 1 mM EDTA, 40 μg/ml sodium aside, 3% PVP K-90), adjusted to 290 mosM with NaCl. The red cells were then added to a continuous gradient of osmoscan buffer in which the osmolality was varied from 40 to 700 mosM, before entering the ektacytometer for the deformability measurement.

Other methods. ATP assays were performed with the linked enzyme assay kit from Sigma Chemical Company. Erythrocyte calcium was determined as previously described [25]. Red cell parameters were determined by standard methods using the Coulter counter Zf with a Channelyzer attachment for cell size and number, and Drabkin's reagent to determine hemoglobin. Charybdotoxin was a product of the Peptide Institute, Osaka, Japan. Quinine and all other compounds were obtained from Sigma Chemical Company.

Results

Ca activates K efflux from stressed erythrocytes

We [19] and others [20] have reported that when normal human red cells are sheared, there is an increase in the rate of Na influx and K efflux. The cells do not hemolyze during this procedure, and they remain discocytes after the stress is released. The flux rate increases are equal for both cations, leaving the total intracellular cation concentrations unchanged. When cells were sheared in the presence of 10^{-4} to 10^{-3} M Ca, however, there was a selective increase in K efflux, above that induced by mechanical stress itself (Fig. 1). There was little or no effect on the deformation induced Na flux. Cation release and uptake remained linear for the 15 min required for the measurement of flux rates. Results at concentrations up to 3 mM are shown in Fig. 1, which is one of seven similar experiments which all demonstrated the selective increase in K efflux. Additional experimental runs were performed at 10 and 30 μM, which are not shown as they were identical to controls. At 1 mM or above, Na influx was inhibited. Significantly, Bookchin et al. [11] have noted that 1.2 mM Ca also inhibited the influx of Na into deoxygenated sickle cells. At 5 mM or higher Ca, both Na and K movement appeared to be inhibited, but hemolysis was 6–10%, and the data are therefore not shown. Blood samples for these experiments were obtained from four normal individuals. The extent to which the deformation-induced K efflux could be activated by Ca appeared to vary among donors,

with increases ranging from 20 to 50% above the control rate without Ca.

Osmoscans (Fig. 2) of cells after the stress experiments showed no change in deformability in the range 0–100 μM Ca. At higher Ca concentrations, there was a leftward shift of the curve, as expected [24] for cells that had an imbalanced loss of K and consequent dehydration. This resulted in a slight loss of deformability at these concentrations. Addition of Ca, without the application of stress, had no effect on cell deformability, at least up to 5 mM Ca.

ATP levels. There was no loss of ATP during the mechanical stress. Assays of intracellular ATP before and after stress were unchanged (data not shown). This indicates that 5 mM glucose was adequate to maintain normal red cell metabolism, even though the Ca-ATPase must have been activated. Any concentration of intracellular calcium high enough to induce the Ca-activated K channel is also high enough to activate the Ca pump [26,27]. There was no detectible increase in red cell calcium as a consequence of mechanical stress in the presence of Ca.

Inhibitors of the Ca activated K channel. In order to verify that the increased K efflux seen in the presence of Ca was in fact due to channel activation rather than some non-specific effect on the cell membrane, two specific inhibitors of the Ca-activated K channel were tested for their ability to inhibit the imbalanced K loss (Fig. 3). For these experiments, a Ca concentration of 750 μM was selected, since inspection of the data of

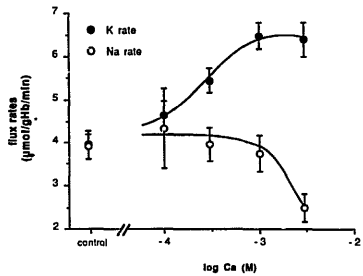

Fig. 1. The effect of Ca on stress-induced monovalent cation movements. Normal red cells were washed and mixed with PVP as described. Immediately before applying shear, Ca was added. The red cell suspension (viscosity = 94 cP) was placed in the viscometer and triplicate aliquots were taken at 0 time and after 10 min of shear. Rates were calculated as the slopes of least-squares regression lines, and error bars indicate the standard deviation of the slope [23]. 'Control' indicates the stress-induced rates without added Ca. Additional experimental runs were performed at 10 and 30 μM, which are not shown as they were identical to controls.

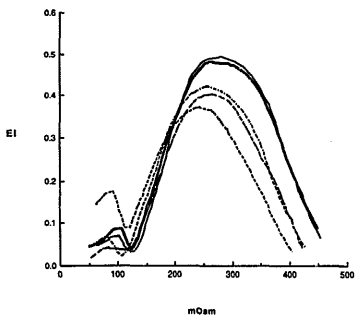

Fig. 2. Ektacytometry. After being sheared in the presence of Ca, the deformability of the red cells was determined with an osmotic gradient. Ca concentrations were: ———, 0 μM; — — —, 100 μM; –·–·–, 300 μM; — — —, 1000 μM; - - - - -, 3000 μM.

Fig. 1 suggested that this concentration increased K efflux over controls without inhibiting Na influx. Quinine was the first compound used, as it is an effective inhibitor of the Gardos channel [13], although it lacks specificity and also affects other functions of the red cell [13]. It was found that 1 mM quinine inhibited the Ca and stress induced loss of K from the cell (Fig. 3). This suggested that the extra K efflux was mediated by the Ca-activated K channel. Quinine was not an ideal inhibitor, however, because of its lack of specificity, and also because it significantly reduced cell deformability (Fig. 4A). Charybdotoxin is a highly specific inhibitor of Ca-induced K channels in red cells [28] as in many other tissues [29], and it completely inhibited the Ca induced loss of potassium at a concentration of 40 nM (Fig. 3). Moreover, the osmotic gradient scans of red cells were unaffected by exposure to charybdotoxin (Fig. 4B), showing that it had no effect on cell deformability.

Discussion

The key findings of this work are the demonstration that mechanical stress in the presence of 0.1 to 1 mM Ca caused an imbalanced increase in K efflux without augmenting Na influx, and that the highly specific Ca-activated K channel inhibitor, charybdotoxin, prevented this increase in K permeability with a K_i of 40 nM. Another less specific inhibitor, quinine, had a similar effect. This is clear evidence that the deforming stress applied to the red cells has increased their permeability to Ca.

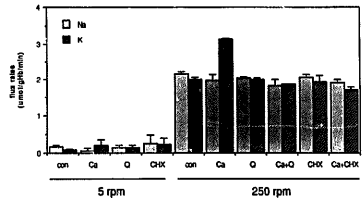

Fig. 3. Inhibitors of the Ca-activated K channel. Ca, quinine (Sigma Chemical Company, St. Louis, MO) and charybdotoxin (Peptide Institute Inc, Osaka, Japan) were added from stock solutions dissolved in PBS immediately before the application of stress. The total volume of PBS was the same in all samples to ensure that viscosity was identical throughout. Erythrocytes were sheared at 5 rpm (unstressed controls) or 250 rpm. Additions are indicated as follows: con = none; Ca = 750 μM Ca; Q = 1 mM quinine; CHX = 40 nM charybdotoxin. The error bars represent the ranges of four experiments with quinine and two with charybdotoxin.

This indirect method of demonstrating Ca uptake was chosen since we wished to demonstrate increased permeability to Ca in mechanically stressed but metabolically normal ATP-replete cells, where any net uptake of Ca during stress would be undetectible. The method chosen gives the additional significant information that the stress-induced Ca influx is adequate to activate the K channel and to produce dehydration, even in cells with normal ATP levels. That dehydration had occurred was shown by the ektacytometric curves from these samples (Fig. 2). These observations are supportive of the model of dense sickle cell formation formulated by Bookchin, Lew and co-workers (9–11). In their proposed mechanism, the sickling deformation increases Ca permeability transiently, raising the steady-state level of $[Ca]_{in}$ sufficiently to trigger the Ca-activated K channel, leading to net efflux of K with an accompanying anion (ordinarily Cl) and an obligate water loss. Recent modifications [10,11] to the model include an explicit role for the pH-sensitive (K, Cl)-cotransport system, which amplifies the rate of dehydration in reticulocytes. The work reported here shows that the triggering event of their model, dehydration caused by the Ca-activated K channel, can be made to operate in normal erythrocytes as a consequence of stress induced deformation.

Sickle cell membranes have increased permeability to Ca [30] which is increased by deoxygenation [15–18]. It has not been explicitly proven that the deoxygenation induced Ca uptake is dependent on sickle cell deformation rather than hemoglobin polymerization per se (as has been shown for monovalent cation uptake [31]), but it is probable that membrane deformation is the essential element. As discussed previously [19], there are significant differences in the applied stress in our experimental setup and in deoxygenated sickle cells. Membranes in sickled erythrocytes experience severe static deformations which would appear to be localized to the ends of the spicules, whereas in our experimental arrangement, erythrocytes tank-tread and elongate uniformly, with an increase in tension throughout the membrane. Nevertheless, there are many resemblances between the induced cation fluxes in both situations, including equality of rates [19,20,32, 33], pH dependence [19,33], reversibility [19–21,33], and sensitivity to DIDS inhibition (Ref. 34, unpublished data). An additional similarity is the inhibition of induced Na influx observed in both deoxygenated sickle cells [11] and mechanically stressed normal cells (Fig. 1). Cation permeability appears to respond to large increases in membrane tension, without a re-

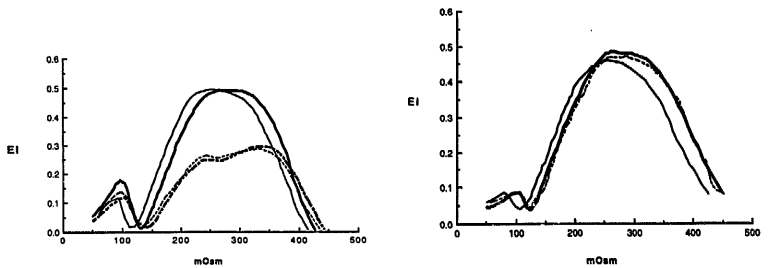

Fig. 4. Ektacytometry after stress of the same cells used in the experiments of Fig. 3. (A) ——, 0 μM Ca; ——, 750 μM Ca; (·--·), 0 μM Ca with 1 mM quinine; ·--·, 750 μM Ca with 1 mM quinine. (B) ——, 0 μM Ca; ——, 750 μM Ca; ·····, 750 μM Ca with 40 nM charybdotoxin.

quirement for any particular geometry. Normal erythrocytes need to be deformed to better than 96% of their maximal elongation before stress-induced flux can been seen [19,21]. After treatment with oxidizing agents, however, K flux can be detected at less than maximal elongation [21]. Since sickle membranes have suffered significant oxidant damage [35], their susceptibility to Ca-induced dehydration may be greater than normal.

A few previous studies have suggested that normal erythrocyte membranes can become permeable to Ca under stress. Larsen et al. [36] observed uptake of ^{45}Ca into sheared, ATP-depleted, erythrocytes. Bowlder et al. [37] reported that erythrocytes subjected to osmotic stress preferentially lose K when Ca is present in the hypotonic solutions. This was interpreted as evidence for an uptake of Ca in the swollen cells that activated the K channel.

It has been demonstrated many times that introduction of Ca into the red cell with the ionophore A23187 activates K efflux and cell dehydration. In an analysis of the process, Tiffert et al. [38] showed that an increase of $[Ca]_{in}$ from the physiological level of 10–30 nM to 40–60 nM was sufficient to induce dehydration. In their work, the Ca pump was not inhibited, and the increased steady-state calcium level required an ionophore-mediated Ca influx of about 1 mmol/loc per h. Our experiments are analogous to those of Tiffert et al., except that the Ca permeability was increased by a deforming stress rather than an ionophore. The Ca influx rate when red cells are stressed in the presence of 0.1 to 1 mM Ca is likely therefore to approximate 1 mmol/loc per h. This is considerably less than the monovalent cation rates of 20–40 mmol/loc per h under the same degree of mechanically induced deformation.

Acknowledgements

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