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A Ca^{2+}-REFRACTORY STATE OF THE Ca-SENSITIVE K^+ PERMEABILITY MECHANISM IN SICKLE CELL ANAEMIA RED CELLS

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Summary

Simultaneous measurements of Ca content and $^{42}K^+$ influx in sickle cell anaemia red cells confirm predictions from earlier data in the literature that the increased Ca content of sickle cell anaemia cells which are not metabolically depleted does not cause a quinine-sensitive increase in K^+ permeability.

It is shown that the ionophore, A23187, can cause the Ca contained inside sickle cell anaemia cells to activate the quinine-sensitive K^+-permeability mechanism. This demonstrates the existence of a Ca^{2+}-refractory state of the K^+ channel in sickle cell anaemia cells and a direct stimulatory effect of the ionophore A23187 on its Ca sensitivity.

Earlier observations by Tosteson et al. [1] and by Glader and Nathan [2] had shown that sickling induces K^+ loss and an almost compensating Na^+ gain in sickle cell anaemia red cells with little or no fall in mean corpuscular volume [2] or cell water [1].

Sickle cell anaemia cells, whatever their metabolic state, are known to gain Ca when they sickle [3—5] and, like normal red cells [6—10], possess a Ca-sensitive K^+-selective channel [2, 11]. Yet, in the experiments of Glader and Nathan [2], only those cells which had been substantially depleted of ATP showed Ca-dependent activation of their K^+ permeability. Bookchin and Lew [12] recently established that the fraction of ionized Ca within fed sickle cell anaemia cells is not less than a fifth of their total Ca content, a fraction similar to that found in normal red cells [13]. If the apparent Ca^{2+} affinity of the K^+-permeability mechanism had been in the 0.4—1 μM range

found in ATP-depleted cells [14] and resealed ghosts [15—17], the intracellular Ca^{2+} concentration found in the sickle cell anaemia cells which, with normal Ca^{2+} buffering, could be estimated to be higher than 5 μM [5, 12, 18], should have sufficed for substantial activation of the K^+ permeability.

The main purpose of the experiments reported here was (a) to establish whether the apparent failure of intracellular Ca^{2+} to activate the K^+ permeability in substrate-fed sickle cell anaemia cells could be observed when Ca content and K^+ fluxes were measured simultaneously in the presence and absence of quinine, a well known inhibitor of the Ca-dependent K^+ fluxes [10] and (b) to explore whether the lack of activation was due to a low Ca-sensitivity state of the K^+ channel which could be converted to a high-sensitivity state by the use of the ionophore A23187, as had been suggested by Lew and Ferreira [19] for normal red cells.

The experiments of Figs. 1 and 3 (control period) confirm the earlier conclusion from more indirect data in the literature [1—3, 5, 12, 18] that the Ca contained or gained experimentally by fed sickle cell anaemia cells is not associated with a measurable Ca-dependent quinine-sensitive increase in K^+ permeability.

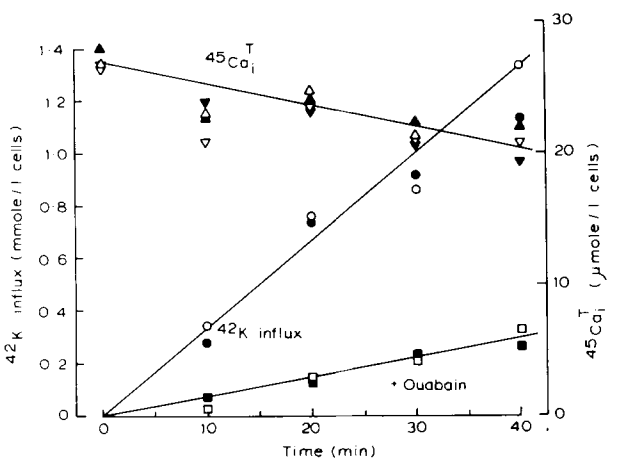

Fig. 1. The effect of quinine on the $^{42}K^+$ influx and ^{45}Ca content of sickle cell anaemia red cells preloaded with ^{45}Ca during a sickling pulse. Sickle cells anaemia red cells from freshly drawn blood were washed and resuspended at a haematocrit of about 20% in a medium containing (mM): NaCl, 150; KCl, 5; Hepes-Na (pH 7.4), 10; and $^{45}CaCl_2$, 1. The cell suspension was incubated for 3 h at 37°C in 100% argon. About 80% of the cells were sickled after 90 min. The cells were then reoxygenated, washed four times and resuspended at a haematocrit of about 10% in a similar medium containing 5 mM $^{42}K^+$ and 1 mM Tris-EGTA instead of 1 mM $CaCl_2$. When present, the concentration of quinine was 1 mM and that of ouabain, 0.1 mM. The ^{42}K and ^{45}Ca content of the cells was measured simultaneously in the same cells from samples washed twice in 15 vols. of ice-cold saline, and lysed in 10 vols. of distilled water; the protein was precipitated with 5% trichloroacetic acid and aliquots of the trichloroacetic acid supernatant used for counting. The ^{45}Ca and ^{42}K content of the cells was calculated by dividing the radioactivity per unit cell volume by the external specific activity of the isotope. Although the ^{45}Ca content thus calculated underestimates the true Ca content of the cells, it reflects the behaviour of intracellular free Ca pools during the efflux period [12]. The effect of ouabain is included in this figure to illustrate the normal magnitude of the $^{42}K^+$ influx, largely an ouabain-sensitive flux through the Na^+ pump, much smaller than that expected from any activation of the Ca-sensitive K^+ permeability (Fig. 2). In other experiments (not shown), quinine was found to induce an erratic K^+ leak in the presence of ouabain. The quinine effects will, therefore, be reported only for the ouabain-free conditions in the experiment of Fig. 3. (△, ▽) ^{45}Ca content of the cells, without and with ouabain; (○, □) ^{42}K content of the cells, without and with ouabain. Open symbols, controls; full symbols, plus quinine.

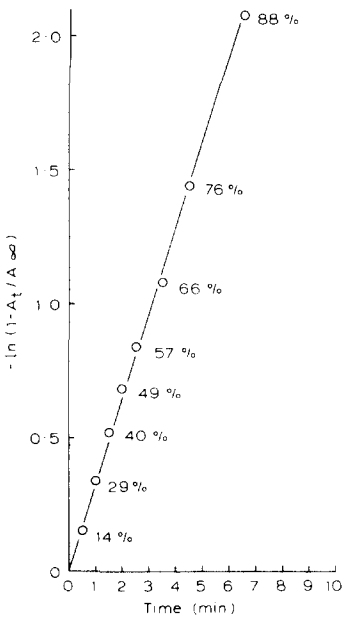

Fig. 2. The effect of intracellular Ca on the $^{42}K^+$-tracer equilibration rate of sickle cell anaemia red cells in the presence of a high concentration of the ionophore A23187. Washed sickle cell anaemia cells were resuspended at a haematocrit of about 10% in a medium containing (mM): NaCl, 75; ^{42}KCl, 75; Hepes-Na, 10; $MgCl_2$, 0.2; glucose, 10 and $^{45}CaCl_2$, 0.02. The ionophore A23187 was added to the cell suspension at 37° C under vigorous magnetic stirring from a 1 mg/ml solution in absolute ethanol to give a final concentration of 10 μM. The ionophore-induced redistribution of ^{45}Ca was completed in less than 30 s. The total Ca content of the cells at equilibrium was 100.2 ± 1.3 (S.E.) μmol/l cells and the fraction of Ca^{2+}, estimated from the equilibrium distribution of ^{45}Ca [10] was 0.22. The external Ca^{2+} concentration at equilibrium was 11.9 μM. The rate of $^{42}K^+$ uptake was followed as described before [19]. The percentual figures to the right of each of the experimental points indicate the level of $^{42}K^+$-tracer equilibration with the cell K^+ pool (70 mmol/l cells, equivalent to about 105 mmol/l cell water). The rate of $^{42}K^+$-tracer equilibration, calculated from the logarithmic slope, is 19.2 ± 0.4 (S.E.) h^{-1}. A_t and A_∞ are the ^{42}K radioactivities contained within equal volumes of cells at time t and at equilibrium, respectively.

We considered the possibility that all the cell-associated Ca was contained within a small fraction of the cells which either lacked the K^+ channel, or represented too small a proportion of the total population to produce a measurable increase in K^+ influx. The experiment of Fig. 2, however, shows that when sickle cell anaemia cells were loaded with Ca in the presence of a high concentration of the divalent cation ionophore A23187, all the intracellular K^+ could participate in the $^{42}K^+$-tracer equilibration process, and was therefore fully accessible to Ca-activatable channels. The $^{42}K^+$-tracer equilibration rate was so high that any gradient-aided $^{42}K^+$ influx from low-K^+ media, whether through countertransport or potential-coupling [20—23], would have been easily detectable in the experiments of Figs. 1 and 3 producing, say, a 50% increase over the normal influx rates observed even if only 0.5% of the cells had exhibited a Ca-saturated increase in their K^+ permeability.

An alternative explanation of the failure of Ca to activate the K^+ permeability of sickle cells is that the K^+ channels are in a state of low Ca sensitivity [19, 24—26]. Lew and Ferreira [19] found that when the intracellular Ca concentration of normal red cells was controlled with the use of the ionophore A23187, the Ca-sensitivity of the K^+ permeability appeared lower, the

lower the ionophore concentration. Those findings suggested that in the intact, undisturbed cells the K^+ channel was relatively Ca-resistant, and that exposure of the cells to ionophore somehow facilitated Ca activation of the channel.

The experiment of Fig. 3 demonstrates such an effect of the ionophore and shows that the Ca originally contained inside sickle cells was capable of activating a quinine-sensitive K^+-permeability pathway. Sickle cells preincubated with ^{45}Ca were washed and resuspended with Ca^{2+}-free, EGTA-containing media and the influx of $^{42}K^+$ was measured in the presence and absence of quinine. After a control period, the ionophore A23187 was added to the cell suspensions and the $^{42}K^+$ influx followed at closer time intervals. The rationale behind this procedure was that if the ionophore could increase the

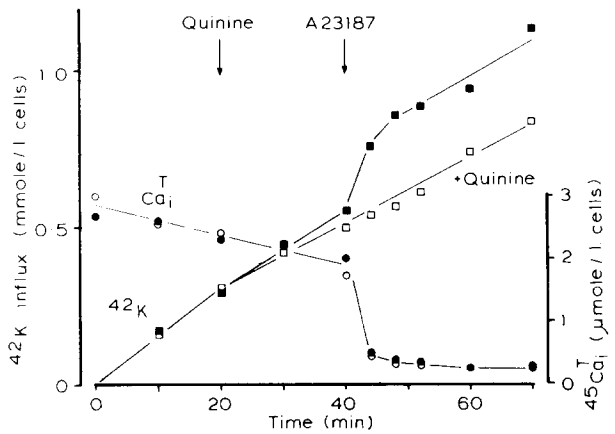

Fig. 3. The effect of the ionophore A23187 on the ^{45}Ca content and quinine-sensitive $^{42}K^+$ uptake of Ca-containing sickle cell anaemia red cells. The experimental protocol was identical to that of the experiment of Fig. 1 except for the preincubation, which was performed under air. ^{45}Ca content, therefore, represents mostly exchange of external ^{45}Ca for intracellular ^{40}Ca [12, 29, 30] during the preincubation, rather than net Ca gain. During the final incubation, the ionophore A23187 was added at the indicated time, to give a final concentration in the cell suspension of 1 μM. (●, ○) ^{45}Ca content of the cells; (■, □) ^{42}K content of the cells; full symbols, controls; open symbols, plus 1 mM quinine hydrochloride.

apparent Ca affinity of the quinine-sensitive K^+ channels before complete Ca depletion, a transient increase in the $^{42}K^+$ uptake by the Ca-containing cells should be detectable. It was important to keep the outside medium strictly Ca^{2+}-free so that a positive result could not be attributed to Ca other than that contained originally inside the cells. Preliminary trials indicated that an ionophore concentration of 1 μM resulted in an effective balance between opposing effects of increasing the Ca sensitivity of the K^+ channel on the one hand, and increasing the Ca permeability and therefore its rate of release from the cells, on the other.

Before ionophore addition, the $^{42}K^+$ influx was within normal values and was not inhibited by quinine. Ionophore addition produced a sharp but transient quinine-sensitive increase in $^{42}K^+$ influx, while the intracellular Ca^{2+} concentration fell sharply to levels of about 10^{-7} M. Upon Ca depletion, the quinine-sensitive $^{42}K^+$ influx became negligible.

These results show (a) that Ca must have been contained inside cells, of

which the quinine-sensitive K^+ channels had become substantially activated only after ionophore addition, and (b) that the ionophore A23187 could somehow facilitate the reaction between intracellular Ca^{2+} and the K^+-gating mechanism, perhaps through interactions at the lipid-protein interface of the K^+ channel [27].

The low Ca-sensitivity state of the K^+ channel in the sickle cells may help explain the relatively extended survival ($T_{1/2} \cong 2$ days) [28] of irreversibly sickled cells in vivo, for if these cells contain most of the cell-associated Ca, the rapid shrinkage and reduced cell viability expected from full activation of the K^+ channels must be considerably delayed.

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