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A CALCIUM-ACTIVATED POTASSIUM CHANNEL PRESENT IN FOETAL RED CELLS OF THE SHEEP BUT ABSENT FROM RETICULOCYTES AND MATURE RED CELLS

ANTHONY M. BROWN [a], J. CLIVE ELLORY [a], JAMES D. YOUNG [b] and VIRGILIO L. LEW [a]

[a] *Physiological Laboratory, Downing Street, Cambridge, and* [b] *ARC Institute of Animal Physiology, Babraham, Cambridge (U.K.)*

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Summary

Red cells of adult sheep, like those of other ruminants, lack the calcium-activated potassium channel which is present in the membrane of human red cells. Since the activities of other transport systems in the sheep red cell are known to decrease during maturation of the cell or during development of the animal it was investigated whether the K^+ channel is present in red cells from younger animals or in reticulocytes. Using the divalent cation ionophore A23187 to increase the intracellular Ca of intact cells, it was found that the K^+-selective channel is present in foetal red cells from the foetus or newborn animal but not in reticulocytes. The presence of the channel showed no dependence on the K^+ genotype of the sheep and was not associated with either "high K^+"- or "low K^+"-type Na^+ pump. No Ca^{2+}-dependent change in K^+ permeability was found in red cells from either newborn or adult donkeys suggesting that its presence in the red cells of the foetus may not be general. The role of the K^+ channel in the mammalian red cell and the relationship between the K^+ channel and the Na^+ pump are discussed.

Introduction

The plasma membranes of many cells possess a K^+-selective channel which is activated by intracellular Ca^{2+} [1,2] and which may mediate important physiological functions of internal Ca. Although these effects appear to be associated mainly with excitable phenomena [2], this channel has also been found in some non-excitable cells, including the mature red cells of certain species which provide a convenient experimental system for the study of its properties. Whether the channel has any function in the red cell is still obscure though its

apparent absence from the red cells of some ruminants, e.g. HK (high K^+) and LK (low K^+) sheep, cows and goats [3], suggest that, at least in the adult, there is probably no evolutionary advantage associated with its presence or absence.

In these same species, however, there are several red cell membrane transport systems whose activities are markedly lower in the mature animal than in the foetus or neonate. In cattle, the $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase activities of calf red cell membranes have been shown to decrease as the animal matures [4], while in sheep the red cell glucose and nucleoside transports both decrease substantially (Mooney, N. and Young, J.D., in preparation). The Na^+ transport system in sheep red cells also changes in some animals as they develop since there is a polymorphism in the red cell K^+ levels of adult sheep which is not apparent in the foetal red cells or reticulocytes [5]. This polymorphism is expressed through genetic control of the cell Na^+ pump kinetics and in animals of the low K^+ genotype these kinetics change from the high K^+ type of the foetal red cells to the LK type as the animal matures.

We present evidence that the Ca^{2+}-activated K^+ channel also shows age-related activity changes in sheep red cells but that, unlike the HK-type Na^+ transport system, it is present only in the foetal red cells and not in the reticulocytes or red cells of either HK or LK mature animals.

Methods

All experiments were carried out with intact red cells fed with inosine or glucose. The effect of intracellular Ca^{2+} on the K^+ permeability of the cells was studied using essentially the technique of Lew and Ferreira [1] in which the level of intracellular Ca of these normally "Ca-free" cells is altered in a controlled manner by the use of the divalent cation selective ionophore A23187 [6]. Because of the limited availability of ^{42}K and the unpredictable supply of blood from foetal and newborn animals, ^{86}Rb, in tracer amounts, was generally used instead of ^{42}K in measurements of K^+ permeability. It has been shown in human red cell ghosts that the permeabilities of the Ca^{2+}-activated K^+ channel to Rb and K^+ are very similar [7]. Cell Ca, when required, was measured using ^{45}Ca as a Ca marker in the incubation medium. In experiments with ^{86}Rb, these measurements were made using parallel cell suspensions because of the difficulty in determining ^{86}Rb and ^{45}Ca in the same sample.

Table I shows the compositions of the media used in these experiments. Medium A was used in washing cells prior to density fractionation (see below) and medium B, whose Na^+, K^+ and Mg^{2+} concentrations were similar to those of plasma, was used as a storage medium for the fractionated cells. Media C and D were similar in composition to the incubation media used in the experiments, 0.1 mM EGTA being present in medium C to remove as much extracellular and externally-bound cell Ca as possible before the start of the final incubation. The basic incubation media used in all experiments were medium E (experiments with sheep cells) and medium F (experiments with donkey cells). The high K^+ concentration in these media was designed to produce an electrochemical "clamp" for K^+ and so minimise net cellular gain or loss of K^+ which could occur in the presence of a large Ca^{2+}-induced increase in the K^+ permeability [1]. 0.3 mM Mg^{2+} was present in order to minimise net movements of Mg^{2+}

TABLE I

COMPOSITION OF MEDIA USED IN THE EXPERIMENTS

Concentrations are mM.

Medium	NaCl	KCl	Choline-Cl	Tris/Cl	Glucose	Inosine	$MgCl_2$	Tris-EGTA	Na_2-EDTA	pH at 22°C
A	150	—	—	—	—	—	—	—	—	—
B	140	5	—	20	5	—	2	—	0.1	7.9
C	75	75	—	10	—	—	—	0.1	—	8.3
D	75	75	—	10	—	—	—	—	—	8.3
E	75	75	—	10	10	—	0.3	—	—	8.3
F	75	75	—	10	10	10	0.3	—	—	8.3
G	—	—	150	10	—	—	—	—	—	8.3

which would otherwise result on addition of A23187 to the cell suspension. In medium F, 10 mM inosine was present in addition to 10 mM glucose because of uncertainty about the best metabolic substrate for donkey red cells.

All additions to the incubation media were made in small volumes of concentrated stock solutions. $CaCl_2$ (with tracer amounts of $^{45}CaCl_2$ where required) EGTA and quinine-HCl were added before the cells; A23187, ^{42}K and ^{86}Rb were added to the final cell suspension. A23187 was added from a 2 mM stock solution in ethanol to give a nominal concentration in the medium of 10 μM while RbCl (including $^{86}RbCl$) was present in a final concentration of 40 μM to 120 μM. ^{42}K was added as isotonic KCl and its addition increased the K^+ concentration in the medium by 3—4 mM.

(a) Preparation of cells. Blood was collected in heparinised syringes either by jugular venepuncture or, in the case of the foetal sheep, by an in utero catheter chronically implanted in the femoral vein [8]. Reticulocyte-rich blood was obtained from a sheep with reticulocytosis induced by phlebotomy [9]. In all cases cells were used within 36 h of collection. Donkey cells were kept in their own plasma at 4°C until that time, while sheep cells were processed within 1 h of collection as follows. Foetal, lamb and normal adult sheep blood was centrifuged at 2500 × *g* for 5 min, the plasma and buffy coat removed by aspiration and the red cells washed three times by centrifugation and resuspension in about 10 vols. of medium A. Cells from adult and foetal animals were then stored at 4°C at 50% haematocrit in medium B until use. Lamb cells were separated before storage into three equal volume fractions according to density by the method of Tucker and Ellory [10]. The percentage of foetal cells in each fraction was determined using the acid-elution method of Moore et al. [11] so that in the present work foetal cells are defined as those containing foetal haemoglobin. Reticulocyte-rich sheep blood was filtered twice through glass wool to remove leucocytes and platelets and the cells washed three times in medium A. A reticulocyte-enriched cell preparation was then obtained by double fractionation of the cells according to density [10], the top fraction from the first separation being again separated into three fractions of which only the least dense was used in the experiments. The percentage of reticulocytes in this fraction was determined by supravital staining with Brilliant Cresyl Blue according to the method of Archer [12].

At the time of the experiments, all cells were centrifuged at 2500 × *g* for 5 min and the storage medium and any buffy coat aspirated. The cells were then washed two or three times with medium C, twice with medium D and finally packed to about 80% haematocrit for distribution to the incubation vials.

(b) Experimental procedure. Incubations were carried out at about 10% haematocrit in magnetically stirred plastic vials at 37°C. After addition of packed cells to the incubation medium, the suspensions were allowed 10 min to equilibrate, after which ^{86}Rb or A23187 (experiment with reticulocytes) was added to begin the experiment. For measurement of cell radioactivity or cell Mg, 0.1 ml samples of the cell suspensions were taken and processed as described previously [6], the cells being separated from the medium by rapid centrifugation through a layer of oil. Samples were also taken for determination of total radioactivity and haemoglobin (see below). In experiments to measure total cell Na^+ and K^+, the cells contained in 0.25 ml samples were rapidly washed four times in 40 vols. of isotonic choline chloride (medium G).

^{86}Rb radioactivity was determined by Čerenkov counting of haemolysates and ^{42}K by Čerenkov counting of TCA extracts. ^{45}Ca was determined by liquid scintillation counting of TCA extracts using Bray's solution [13]. When ^{45}Ca and ^{42}K were determined in the sample, one week was allowed for decay of ^{42}K before ^{45}Ca was measured. No quench corrections were necessary since in all cases quenching was similar in samples for cell radioactivity and total radioactivity. Mg in the cell pellets was measured by atomic absorption spectrophotometry (Pye Unicam SP 90B) of TCA extracts and Na^+ and K^+ by flame photometry (EEL) of haemolysates using Na^+ and K^+ standards prepared from a solution containing 100 mM K^+/10 mM Na^+. Haemoglobin in the cell suspensions and haemolysates was determined by absorbance measurements at 540 nm and correlated with packed cell volume (Clay-Adams Microhaematocrit centrifuge, 10 min centrifugation) using conversion factors determined separately for each animal type.

(c) Calculations. Rate constants for ^{86}Rb and ^{42}K uptake were calculated using a two compartment analysis assuming that ^{86}Rb, when at equilibrium, would distribute between cells and medium in the same way as K^+, i.e., $[Rb]_{medium}/[Rb]_{cells} = [K]_{medium}/[K]_{cells}$.

^{86}Rb uptake data obtained either before addition of A23187 or between 10 and 40 min after were generally well fitted by a single exponential rate constant and it is these values that have been used in this paper. They are given in the text and tables as the mean slope ± standard error of the slope of the regression line drawn through the experimental points. In cases where ionophore addition resulted in an increase in the rate of ^{86}Rb uptake, the uptake in the first 10 min was faster than during the subsequent period. This transient was always small and the rate constants determined for the period between 10 and 40 min reflect qualitatively the changes in ^{86}Rb uptake rate constant resulting from ionophore addition. The complex nature of the ^{86}Rb uptake means that caution must be exercised when drawing conclusions from comparisons of the numerical values of the ionophore-increased rate constants. In the experiment with reticulocytes, ^{42}K uptake curves were well fitted by single exponentials over 40 min following ^{42}K addition, except in the conditions noted.

Typical measured values for cell K^+, used in the calculations of the rate con-

stants for ^{42}K and ^{86}Rb uptake, were as follows (mean ± S.E. in mmol/l original cells): adult sheep (HK), 69.7; lambs (newborn), 101.7 ± 1.2; sheep reticulocytes (HK sheep), 86.8 ± 0.3; adult donkey, 100.5 ± 0.4; donkey foal, 96.5 ± 0.5. These values were calculated assuming values of 291 and 337 for the packed cell absorbance at 540 nm, 1-cm light path for sheep and donkey red cells, respectively.

Cell Ca content was calculated from the ^{45}Ca activity in the cell pellet and the specific activity of ^{45}Ca in the suspension. Ca contamination was assumed to be negligibly small compared with the amount of added calcium. The external Ca^{2+} concentration following addition of ionophore was calculated by subtracting cell Ca from the total Ca added to the cell suspension. The ionised intracellular Ca, Ca_i^{2+}, was estimated by assuming equilibrium with extracellular Ca^{2+} and a membrane potential of 8 mV, inside negative, at 37°C. Though this assumption is not strictly correct because of the activity of the Ca^{2+} pump [6], the ionophore concentration used was so high that, at least in the human red cell under these conditions, the Ca^{2+}-pump flux would have been negligible compared with the ionophore-induced flux.

It should perhaps be noted that A23187 is a carboxylic ionophore which equilibrates Ca^{2+} and H^+ activity ratios regardless of the membrane potential [14]. However, in the particular case of the red cell, because of the anion shunt and the relatively high anion permeability, pH and membrane potential (in mV at 37°C) are linked by the equation $V = 61.5\ (pH_i - pH_o)$ and, thus, the ionophore-mediated Ca^{2+} equilibria are effectively potential dependent.

Results

Fig. 1 shows the uptake of ^{86}Rb by a newborn and an adult sheep's red cells incubated in a Ca^{2+}-containing medium. It is seen that while addition of A23187 to the cell suspension had no effect on the rate of ^{86}Rb uptake in the adult's cells, it caused a rapid increase in this rate in the cells from the newborn animal. Table II summarises the results from several such experiments in which the rate constant for ^{86}Rb uptake was determined in the top, middle and bot-

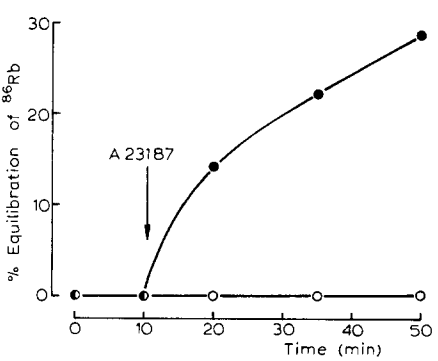

Fig. 1. Time course of ^{86}Rb uptake by red cells from a newborn lamb and adult sheep in the presence of external Ca^{2+}, before and after addition of the ionophore. Medium E (Table I); ionophore concentration, 10 μM; Ca^{2+} concentration, 0.7 mM. ●———●, newborn lamb; ○———○, adult sheep.

TABLE II

EFFECT OF Ca^{2+} IN THE PRESENCE OF IONOPHORE ON THE ^{86}Rb UPTAKE RATE CONSTANT OF SHEEP RED CELLS

Cells from newborn and 5-week-old animals were separated into three equal volume fractions according to density [10] and these are designated 'top', 'middle' and 'bottom' in columns 4—9. Foetal and adult cells were not fractionated. Medium E (Table I); ionophore concentration, 10 μM; Ca^{2+} concentrations shown are the external concentrations before addition of ionophore.

Code number of animal	Age (days)	Ca^{2+} (mM)	^{86}Rb uptake rate constant (h^{-1})			Foetal cell count (%)		
			Top	Middle	Bottom	Top	Middle	Bottom
285	Foetal (—10 days)	0.7		0.726 ±0.026			n.m. *	
211	3	0.7	0.573 ±0.033	0.710 ±0.012	0.707 ±0.012	>99	>99	>99
	36	0.7	0.021 ±0.001	0.014 ±0.000	0.025 ±0.001	1.8	2.6	3.8
212	3	0.7	0.385 ±0.004	0.658	0.580 ±0.007	>99	>99	>99
	36	0.7	0.023 ±0.008	0.024 ±0.002	0.027 ±0.003	6.0	7.6	8.6
85	1	0.7	0.370 ±0.006	0.457 ±0.040	0.433 ±0.039	>99	>99	>99
	34	0.7	0.015 ±0.002	0.024 ±0.001	0.084 ±0.000	2.0	3.8	17.0
38	Adult	0.7		0.020 ±0.009			n.m.	
11	Adult	1.5		0.001 ±0.004			n.m.	

* n.m., not measured.

tom fractions of red cells from foetal, newborn, 34—36-days-old and adult sheep. In these experiments, the cells were first incubated without ionophore for 20 min and in all cell fractions little ^{86}Rb uptake occurred during this period (rate constants less than 0.02 h^{-1}). A23187 was then added to give a final concentration in the medium of 10 μM and so create conditions which produce a definite stimulation of the Ca^{2+}-activated K^+ channel in human red cells [1]. It is seen that both in cells from foetal sheep and in all cell fractions from newborn sheep the rate constant for ^{86}Rb uptake was considerably larger than that in the adult red cells. By the time the animals had matured to 34—36 days, however, only one cell sample, the bottom fraction from sheep 85, showed an increased rate constant.

A possible explanation for this age-related disappearance of the ^{86}Rb (K^+) channel from the sheep red cell population is that it is associated with a loss of foetal red cells from the circulation of the maturing sheep. This possibility is supported by the data in the right hand columns of Table II which show the percentage of foetal cells in each red cell fraction. It is seen that in the newborn animal, and so, presumably, in the foetus, the red cell population was almost entirely foetal, while in the blood from the 34—36-days-old sheep there was less than 10% foetal cells in all but the bottom fraction from sheep 85. This fraction was also the only one from these 5-week-old animals which still showed increased ^{86}Rb permeability on addition of A23187. Thus, although

intermediate time points are lacking, there appears to be a correlation between loss of the ^{86}Rb channel and the loss of foetal cells, suggesting that the channel is active primarily in the foetal cells. It is of interest to note that measurement of the K^+ content of the red cells of these animals at 6 months showed that animals 211 and 212 had LK-type red cells, while animal 85 had HK-type cells. The channel is, therefore, apparently absent from both HK- and LK-type adult red cells, as has been shown previously [3], and its presence in the foetal-type cells is independent of the genotype of the animal.

The effect of A23187 on the ^{86}Rb permeability of sheep foetal red cells suggested that these cells might have a Ca^{2+}-activated K^+ channel similar to that found in human red cells [1]. That the response of the cells to A23187 does indeed depend on the presence of Ca^{2+} was shown by an experiment in which red cells from a newborn lamb were incubated with 0.7 mM initial external Ca^{2+} in the presence and absence of 1 mM EGTA. It was found that while addition of A23187 produced an increase in the rate of uptake of ^{86}Rb by the cells in the absence of EGTA (rate constant, $0.354 \pm 0.028\ h^{-1}$), it had virtually no effect when EGTA was present, the rate constant ($0.031 \pm 0.002\ h^{-1}$) being similar to that in control cells incubated with 1 mM EGTA and no added Ca^{2+} ($0.023 \pm 0.002\ h^{-1}$). In view of the similar levels of Ca and Mg in the cell suspension and the much higher affinity of EGTA for Ca than Mg it is clear that Ca is the active component in this system. This is supported by the data of the lowest curve of Fig. 2 which shows that cell Mg changed very little on addition of A23187 under these conditions.

In order to determine how similar the Ca^{2+}-activated channel in sheep foetal red cells is to that in human red cells, the specificity of the channel for K^+ and the effect of quinine on the Ca^{2+}-induced permeability increase were examined. The upper curves in Fig. 2 show the changes in cell Na^+ and K^+ on addition of

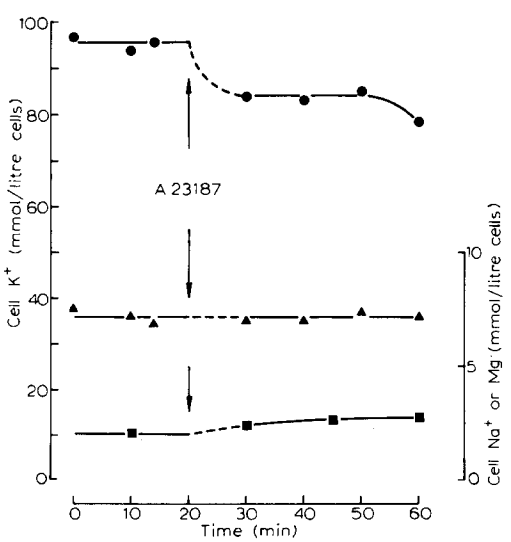

Fig. 2. Changes in cell Na^+, K^+ and Mg content in the bottom fraction of red cells from a 7-day-old lamb on addition of ionophore in the presence of external Ca^{2+}. Medium E (Table I); ionophore concentration, 10 μM; Ca^{2+} concentration, 0.7 mM. ●———●, cell K^+; ▲———▲, cell Na^+; ■———■, cell Mg.

TABLE III

EFFECT OF QUININE AND Ca^{2+} IN THE PRESENCE OF IONOPHORE ON THE ^{86}Rb UPTAKE RATE CONSTANTS OF RED CELLS FROM 1-DAY-OLD LAMBS

Medium E (Table I); ionophore concentration, 10 μM; Ca^{2+} concentration shown is external concentration before addition of ionophore.

Condition	^{86}Rb uptake rate constant (h^{-1})	
	Lamb 445	Lamb 446
EGTA (100 μM)	0.011 ± 0.003	0.015 ± 0.013
EGTA (100 μM) + quinine (1.5 mM)	0.021 ± 0.002	0.011 ± 0.001
Ca^{2+} (1.2 mM)	0.326 ± 0.014	0.556 ± 0.001
Ca^{2+} (1.2 mM) + quinine (1.5 mM)	0.007 ± 0.003	0.009 ± 0.003

A23187 to a bottom fraction of cells from a 7-day-old lamb. It is seen that cell K^+ rapidly attained a new, slightly lower, steady-state level on addition of the ionophore, indicating that the permeability to K^+ was increased and that the electrochemical "clamp" was not quite perfect. Cell Na^+, on the other hand, remained unchanged throughout the incubation even in the presence of a significant net inward electrochemical gradient. Thus, as in human red cells, the Ca^{2+}-activated channel in these cells is highly specific for K^+ relative to Na^+.

Table III shows the effect of 1.5 mM quinine-HCl on the Ca^{2+}-activated increase in ^{86}Rb uptake in red cells from two newborn lambs. It has been shown previously that the Ca^{2+}-activated K^+ channel in human red cells is specifically inhibited by quinine [15] and it is seen from the data of Table III that its effect on sheep foetal red cells is similar, the permeability increase being almost fully inhibited in the two blood samples tested. It seems, therefore, that in terms of ionic specificity and reactivity to quinine the Ca^{2+}-activated K^+ channels in sheep foetal and human red cells are similar.

Since activation of the K^+ (Rb) channel in the sheep foetal red cells required both external Ca and a divalent cation ionophore, it was concluded that intracellular Ca^{2+} was the activating agent. To establish more definitely the absence of the channel from the adult red cells, it was therefore necessary to check that the observed absence was not due merely to failure of A23187 to increase the level of ionized Ca in these cells. In order to do this, the uptake of Ca by the foetal and adult cells was measured in the presence of A23187 at three different external Ca^{2+} concentrations. Fig. 3a shows the time course of cell Ca^{2+}, Ca_i^T, in red cells from a newborn lamb, the results with adult cells being qualitatively similar. Fig. 3b shows the Ca buffering curves for the two sets of cells estimated as described in Methods from the data of Fig. 3a and from the equivalent data for the adult cells. In Fig. 3a the apparent decrease in cell calcium which is seen at later times, particularly at the higher Ca^{2+} concentrations, was mainly due to visible haemolysis in the incubation vial and the consequent decrease in the amount of cells in the analysed cell pellets. The data in Fig. 3b were therefore calculated using peak values for Ca_i^T. It is seen from Fig. 3a that at 0.7 mM and 1.2 mM initial extracellular Ca^{2+} (the levels used in most of the experiments summarized in Tables II, III and Figs. 1, 2) considerable Ca uptake occurred following A23187 addition, while Fig. 3b shows that the Ca

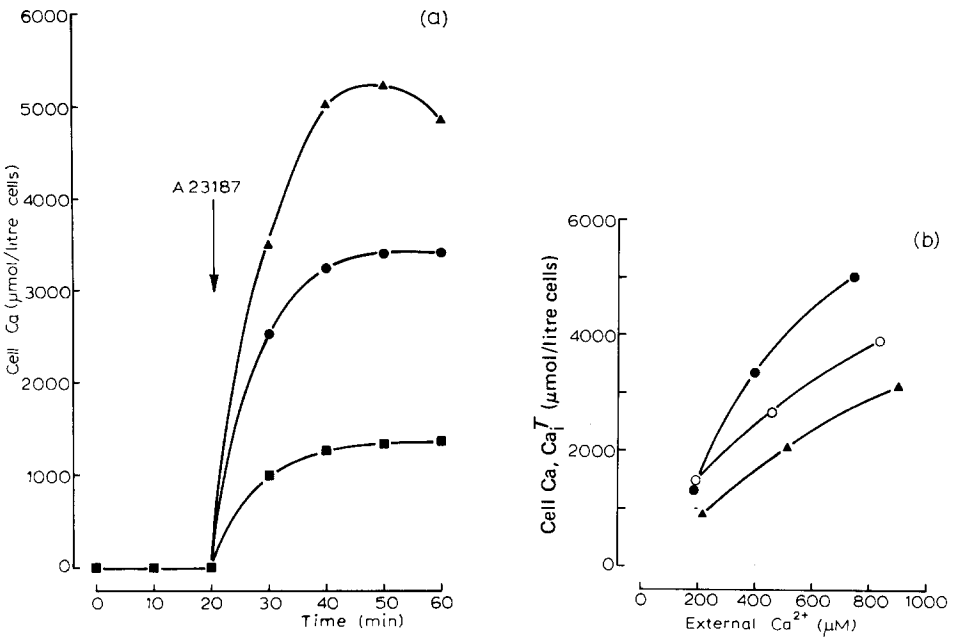

Fig. 3. (a) Changes in Ca content of red cells from a 1-day-old lamb on addition of ionophore in the presence of three external Ca^{2+} concentrations. Medium E (Table I); ionophore concentration, 10 μM; external Ca^{2+} concentrations before addition of ionophore as follows: ■———■, 0.3 mM; ●———●, 0.7 mM; ▲———▲, 1.2 mM. (b) Ca buffering curves for red cells from a 1-day old and an adult sheep and for a reticulocyte preparation from an adult HK sheep. Values were calculated from the data of (a) and from similar data for the adult mature cells and for the reticulocyte preparation, as described in Methods. Medium E (Table I); ionophore concentration, 10 μM. ●———●, lamb cells; ▲———▲, adult mature cells; ○———○, reticulocyte preparation (74% reticulocytes).

buffering in the adult cells was lower than in the foetal cells. This means that for a given haematocrit and initial extracellular Ca^{2+} concentration, Ca_i^{2+} was higher in the adult cells than in the foetal cells and it is estimated that in the experiments of Table II, Ca_i^{2+} would have been at least 900 μM and 700 μM in the adult and foetal cells, respectively. Clearly then, in these experiments the apparent lack of a Ca^{2+}-activated K^+ channel cannot have been due to a lack of Ca entry into the cells and so must reflect absence, gross insensitivity to Ca^{2+} or, perhaps, a different type of inactivation of the channel in these cells.

The data presented so far have shown that a Ca^{2+}-activated K^+ (Rb) channel is present in the foetal red cells of LK and HK sheep, but apparently absent from the mature cells of the adults of both genotype. The controversial suggestion has been made that the Ca^{2+}-activated K^+ channel and the Na^+ pump may be two aspects of the same mechanism [16,17] and it was, therefore, of interest to examine the correlation between the presence (or absence) of the K^+ channel and the characteristics of the Na^+ pump in red cells from adult and foetal sheep. The Na^+ pump in these cells has already been studied in some detail (for references see Ellory [5]) and since the studies include data for reticulocytes, measurements of Ca^{2+}-activated K^+ channel activity were extended to include these cells. Table IV shows the effects of intracellular and extracellular Ca^{2+} and of ouabain and quinine on the rate constant for ^{42}K uptake by a reticu-

TABLE IV

^{42}K UPTAKE RATE CONSTANTS FOR A RETICULOCYTE-RICH RED CELL PREPARATION FROM AN ADULT HK SHEEP

The reticulocyte count was 74%. ^{42}K was added after the cells had been incubated for 16 min at 37°C for Ca^{2+} distribution to reach steady state. In conditions C and D, the ^{42}K uptake did not follow a single exponential time course and the rate constants for both phases of the uptake are shown. Medium E (Table I); Ca^{2+} concentrations shown are the external concentrations before addition of ionophore. No ionophore was present in conditions (E) — (L).

Condition	^{42}K Uptake rate constant (h^{-1})
(A) 0.1 mM EGTA + 10 μM A23187	0.082 ± 0.002
(B) 0.3 mM Ca^{2+} + 10 μM A23187	0.025 ± 0.002
(C) 0.7 mM Ca^{2+} + 10 μM A23187	
(a) 0—10 min	0.158 ± 0.005
(b) 10—40 min	0.034 ± 0.004
(D) 1.2 mM Ca^{2+} + 10 μM A23187	
(a) 0—10 min	0.134 ± 0.001
(b) 10—40 min	−0.001 ± 0.004
(E) 0.1 mM EGTA	0.093 ± 0.001
(F) 0.7 mM Ca^{2+}	0.072 ± 0.002
(G) 0.1 mM EGTA + 1.5 mM quinine	0.135 ± 0.002
(H) 0.7 mM Ca^{2+} + 1.5 mM quinine	0.129 ± 0.003
(K) 0.1 mM EGTA + 1 mM ouabain	0.052 ± 0.001
(L) 0.7 mM Ca^{2+} + 1 mM ouabain	0.036 ± 0.001

locyte-rich red cell preparation from an HK sheep. It is seen that in the presence of A23187 and either 0.7 mM or 1.2 mM initial external Ca^{2+}, ^{42}K uptake was initially slightly stimulated and then considerably inhibited compared with control (condition A). The stimulation of K^+ influx was small compared with that produced in sheep foetal red cells under the same conditions or in human red cells [1]. In the presence of A23187 and 0.3 mM initial external Ca^{2+}, ^{42}K uptake was inhibited over the whole 40 min incubation, while in the absence of A23187 Ca^{2+} had little effect on the ^{42}K uptake rate constant. It should be noted that although the rate constant for ^{42}K uptake in the absence of Ca^{2+} or ionophore was slightly higher than that in mature adult or foetal red cells (0.01—0.03 h^{-1}, see above and Table III) this was not due to activation of a K^+ channel by intracellular Ca^{2+} in the absence of ionophore since 1.5 mM quinine did not inhibit the uptake of ^{42}K. Instead, quinine increased ^{42}K uptake slightly, probably by a non-specific effect on cation permeability [2]. The data with ouabain show that between 45 and 50% of the ^{42}K uptake was ouabain-inhibitable, and the magnitude of the ouabain-sensitive K^+ flux is consistent with the findings of Tucker and Ellory [10]. It seems likely that the inhibitory effect of Ca^{2+} on the K^+ influx is due, at least in part, to inhibition of this ouabain-sensitive flux by intracellular Ca^{2+} (ref. 18 and Brown, A.M. and Lew, V.L., unpublished). Measurements of cell Ca^{2+} showed that cells gained Ca^{2+} only in the presence of A23187 and that, in all cases, initial transients were over before addition of ^{42}K. The Ca buffering curve derived from these data is shown on the same Figure as the buffering curves for the mature adult and foetal red cells (Fig. 3b). For the 0.7 mM initial external Ca^{2+} conditions, Ca_i^{2+} in the reticulocyte preparation was about 800 μM, indicating that the apparent

TABLE V

EFFECT OF Ca^{2+} IN THE PRESENCE OF IONOPHORE ON THE ^{86}Rb UPTAKE RATE CONSTANTS OF RED CELLS FROM NEWBORN AND ADULT DONKEYS

Medium F (Table I); ionophore concentration, 10 μM; Ca^{2+} concentrations shown are the external concentrations before addition of ionophore.

Code number of animal	Age	^{86}Rb uptake rate constant (h^{-1})		
		EGTA, 1 mM	Ca^{2+}, 0.7 mM	Ca^{2+}, 1.2 mM
B	Adult	0.019 ± 0.002	0.029 ± 0.002	—
BF	1 day	0.014 ± 0.002	0.025 ± 0.001	—
E	Adult	0.031 ± 0.000	—	0.022 ± 0.001
EF	6 h	0.033 ± 0.002	—	0.062 ± 0.002

absence of K^+ channel activity in these cells was not the result of low intracellular Ca or excessive cytoplasmic buffering.

These findings show that, like the mature adult red cell, the sheep reticulocyte apparently lacks a Ca^{2+}-activated K^+ channel, at least in the HK sheep. The association between the K^+ channel and the Na^+ pump will be discussed further later on. It should be noted that this finding also shows that the elevated reticulocyte levels found in lambs particularly in the first 30 days after birth [19] could not have contributed to the A23187-induced increase in ^{86}Rb uptake by cells from newborn animals, and thus supports the conclusion that the K^+ channel is a characteristic of the foetal red cells.

In view of the finding that in sheep, a Ca^{2+}-activated K^+ channel is present only in the foetal red cells, it is tempting to speculate that this channel might play an important role in the red cells of the mammalian foetus. The availability of blood from donkey mares and newborn foals enabled us to test the validity of this generalisation. Table V summarises the results of experiments to measure the effect of Ca^{2+} in the presence of ionophore on the rate constant for ^{86}Rb uptake in cells from two donkey foal-mother pairs. In no case did significant ^{86}Rb uptake occur either before or after ionophore addition. Measurement of cell Ca showed that this lack of response was not due to maintenance of low intracellular Ca following addition of A23187. In the presence of 0.3—1.2 mM initial external Ca^{2+}, the ionophore caused a large and rapid uptake of Ca by the cells with time course qualitatively similar to those for newborn sheep shown in Fig. 3a and with calculated steady-state levels of Ca_i^T as high as 1100 μM and 800 μM being reached in the foal and mother cells, respectively. Thus, as in the adult sheep red cells, the Ca^{2+}-activated K^+ channel must be either absent or inactive in the red cells from both the adult and newborn donkey. It should be noted, however, that because of the similarity between equine foetal and adult haemoglobins [20] it is not possible to indentify a population of foetal cells in equine blood using standard techniques, so the percentage of pre-natal red cells in the newborn foal blood could not be measured. It is conceivable, therefore, that a high percentage of the red cells of the newborn were not of the "foetal" type with respect to their membrane properties, so the possibility that "earlier" cells from the foetus proper might have had the Ca^{2+}-activated K^+ channel cannot be excluded.

Discussion

It has been shown that in the red cells of the foetal and newborn sheep, there is a K^+-selective permeability pathway activated by intracellular Ca^{2+} and inhibited by quinine. This pathway was not evident in either mature red cells or reticulocytes from the adult sheep and its disappearance from the blood of the maturing lamb paralleled the disappearance of foetal red cells from the blood. In contrast to the findings in sheep, high levels of intracellular Ca were found to have no effect on the K^+ permeability of red cells from newborn or adult donkeys.

Mentioned earlier was the idea that the Ca^{2+}-activated K^+ channel might be just an operational state of the Na^+ pump [16,17]. This idea would gain support if it were found that changes in one transport system were paralleled by changes in the other and the sheep red cell seems to be a suitable cell in which to check whether this is so in view of its Na^+ pump characteristics which show variations both with the age of the cell and with the age and genotype of the animal. It has been shown previously that the Na^+ transport systems in foetal red cells and reticulocytes from LK and HK sheep are similar to that in the mature HK red cell, i.e., the cell Na^+ and K^+ contents are similar [21] and the $(Na^+ + K^+)$-ATPases have "HK-type" kinetics [5]. The Ca^{2+}-activated K^+ channel, however, has been found only in the foetal red cells of this species and its presence therefore does not parallel that of either the HK- or the LK-type Na^+ pump. The channel also appears to be independent of the LK-HK genotype of the animal. It is absent from the mature red cells of both HK and LK animals and its presence or absence seems to be unrelated to that of the L antigen [10,19], which is found in LK adult mature red cells and reticulocytes and is thought to be involved in the expression of the LK gene through the Na^+ pump [22,23]. These data add to earlier observations in K^+ polymorphic animals (sheep, cows and goats) that increased intracellular Ca^{2+} had no effect on the K^+ permeability of red cells from either HK- or LK-type animals and that addition of anti-L antibody to LK goat red cells caused the LK Na^+ pump to operate more like an HK pump, but had no effect on the Ca^{2+}-activated K^+ channel [3]. Thus, there appears to be no correlation between changes in the Na^+ pump and changes in the K^+ channel in these cells and no evidence that genetic control of the Na^+ pump is reflected in the properties of the K^+ channel. While these findings need not be inconsistent with the concept of the Na^+ pump and K^+ channel as two aspects of a single mechanism, they do somewhat reduce its credibility. It should perhaps be noted that although the data for the absence of a K^+ channel from reticulocytes was obtained with cells from an HK animal only, these conclusions would be unaffected by the results from LK reticulocytes since neither presence nor absence of the K^+ channel could improve the correlation between the Na^+ pump and the K^+ channel.

With respect to the physiological role of the K^+ channel in the red cell, it now seems that the presence or absence of the channel is not essential for any of the fundamental functions of the foetal or adult red cell or reticulocyte since there are well documented examples of its presence and absence in all these cells in various species [3,24]. This excludes the possibility that the channel plays an important part in O_2 or CO_2 transport, anion exchange and phys-

iological catheresis [25], all of which proceed with or without the channel present in the membrane. That does not mean, though, that when present it cannot affect or alter the mode in which these functions are performed but this would be only a by-product of its presence. Thus, a positive role for the Ca^{2+}-activated K^+ channel in red cells is still lacking.

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