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Intracellular Calcium: Lack of Effect on Ovine Red Cells (40086)

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Although human red cells normally have only small amounts of intracellular Ca^{2+} (ca. 20 μM), cellular Ca^{2+} concentrations may be greatly increased through incubation of human erythrocytes with the ionophore, A23187 ("Calcimycin"). A23187 allows the free passage of divalent cations, notably Ca^{2+} and Mg^{2+}, across biological membranes until equilibrium is established (1–3). Such artificial Ca^{2+} loading of human and animal erythrocytes does extensive damage as reflected by (i) marked shape changes; (ii) concomitant loss of cell water and K^+; (iii) increased mean cell hemoglobin concentration (MCHC); (iv) profound and rapid hydrolysis of intracellular adenosine triphosphate (ATP), presumably due to stimulation of membrane Ca^{2+} efflux ATPase; (v) decreased osmotic fragility; and (vi) stiffening of the red cell membrane (2–10). We have suggested that similar Ca^{2+}-induced cellular damage may underlie the formation of irreversibly sickled cells in patients with sickle cell disease (6, 7, 9, 11). These permanently deformed cells have abnormally large amounts of Ca^{2+} and possess most of the characteristics of Ca^{2+}-laden normal erythrocytes, being dehydrated, distorted and energy depleted (11–13).

We have since determined that red cells from diverse species of mammals, including rats, mice, rabbits and pigs, respond as do human erythrocytes to ionophore-induced Ca^{2+} influx. Most recently, we have investigated the effects of elevated intracellular Ca^{2+} on red cells from newborn and adult sheep. Whereas most red cells from newborn sheep are Ca^{2+} responsive, those from adult sheep are not.

Materials and methods. In all experiments reported here, heparinized blood from sheep and humans was kept on ice and used within 4 hr of venipuncture. Intracellular Ca^{2+} concentrations were measured on saline washed erythrocytes by atomic absorption spectroscopy as described earlier (11). ATP was measured by the technique of Beutler (14).

Incubations with ionophore. In all experiments, erythrocytes were washed 3 times with, and resuspended in, Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, 20 mM in Tris (tris[hydroxymethyl]aminomethane). At the beginning of each incubation, 50 μM A23187 was introduced, dissolved and diluted as previously reported (9). Incubations were conducted for 60 min at 37° with cell suspensions of hematocrit 35% (v/v). Ca^{2+}, when present, was added to a final concentration of 50 μM. Control samples had no added Ca^{2+} and contained 1 mM ethyleneglycol-bis-(α-amino-ethyl ether)N,N'-tetraacetic acid (EGTA) to chelate any residual Ca^{2+}.

Ca^{2+} ATPase assays. Ca^{2+} ATPase activity was estimated in crude lysates of thrice saline washed, packed erythrocytes, lysed by sonic disruption and by freezing and thawing once. Control experiments indicated that, although Ca^{2+} ATPase activity requires the presence of membranes, washing red cell membranes after lysis causes progressive diminuation of Ca^{2+} ATPase activity. Reaction mixtures contained 0.28 ml of crude lysate, 0.2 ml of 100 mM Tris buffer, pH 7.2, containing 2 mM $MgCl_2$, 0.02 ml of 30 mM ouabain (to inhibit Na^+/K^+ ATPase), 0.02 ml of 60 mM ATP, 0.06 ml of 500 μM A23187 (to ensure availability of Ca^{2+} to membrane ATPase sites), and either 0.02 ml of 100 mM neutralized EGTA or 0.02 ml of 1.5 mM $CaCl_2$ (total volume of reaction mixture = 0.60 ml). ATPase activity was determined by measuring the ATP content of the reaction mixtures at 0 time and after 60 min incubation at 37° (during which time the rate of ATP hydrolysis was approximately linear). Conditions of assay, including concentrations of Mg^{2+}, Ca^{2+}, EGTA, and ATP were experimentally optimized for human erythrocyte Ca^{2+} ATPase.

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Results. The effects of ionophore-induced Ca^{2+} influx upon human and adult sheep erythrocytes are strikingly discrepant. Human erythrocytes, during 60 min incubation with A23187 and Ca^{2+}, become "spheroechinocytic" in shape, lose volume, become osmotically less fragile and hydrolyze most of their intracellular ATP (Fig. 1A and 1B, Table I).

In contrast, erythrocytes from adult sheep do not change shape (Fig. 1C and 1D), show no detectable increase in MCHC, retain their original osmotic fragility and hydrolyze relatively little of their original ATP (Table I). The lack of response of sheep red cells to Ca^{2+} influx is not due to an insensitivity of these cells to the Ca^{2+} ionophore; sheep erythrocytes, which normally have low intracellular Ca^{2+}, accumulate almost as much Ca^{2+} as do human red cells during 60 min incubation with A23187 and Ca^{2+} (Table I). In other experiments, the inclusion of as much as 10^{-3} *M* Ca^{2+} elicited no response from red cells of adult sheep in any of these parameters. Furthermore, sheep red cells previously ATP depleted by incubation with sodium fluoride do not change shape or shrink when subsequently incubated with A23187 and Ca^{2+}. Similarly ATP depleted human red cells retain their tendency to shrink and

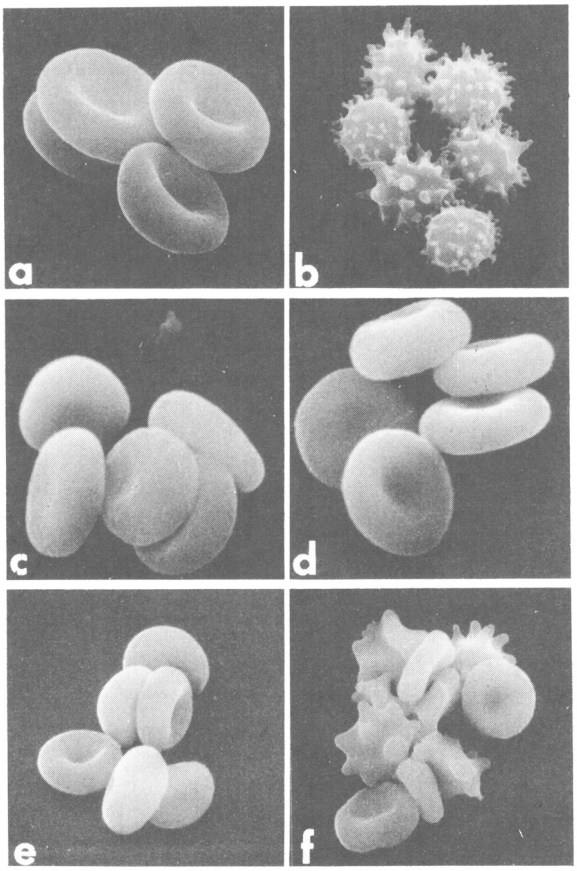

FIG. 1. Scanning electron micrographs showing morphologic effects of ionophore-induced Ca^{2+} accumulation on human, adult sheep and newborn lamb red cells. Human erythrocytes, incubated with A23187 and EGTA, remain normally shaped biconcave discs (A) (× 2900), whereas incubation with A23187 and Ca^{2+} causes the formation of spiky "spheroechinocytes" (B) (× 2500). Similarly treated adult sheep red cells remain biconcave discs whether exposed to A23187 and EGTA (C) (× 4500) or Ca^{2+} (D) (× 4200). Erythrocytes from newborn lambs, although morphologically normal in the presence of A23187 and EGTA (E) (× 3000), become spheroechinocytes when exposed to A23187 and Ca^{2+} (F) (× 2500). All cells were fixed in phosphate buffered formalin after 60 min incubation. Other experimental conditions as given in the legend to Table I.

TABLE I. CHARACTERISTICS OF HUMAN, ADULT SHEEP AND NEWBORN (24-hr OLD) LAMB RED CELLS BEFORE AND AFTER INCUBATION WITH A23187 AND EGTA OR ADDED Ca^{2+}. VALUES IN PARENTHESES REPRESENT ±1 SD.

Characteristic	Human (n = 12)	Adult sheep (n = 12)	Newborn sheep (n = 5)
MCHC (gm Hb/dl RBC)			
A. Unincubated[a]	35.1 (±1.9)	34.7 (±1.2)	33.7 (±1.9)
B. A23187 + EGTA[b]	34.4 (±1.2)	34.7 (±1.3)	33.5 (±0.7)
C. A23187 + Ca^{++}[c]	44.6 (±3.1)	34.5 (±1.1)	41.2 (±3.0)
ATP (μM/gm Hb)			
A. Unincubated	3.85 (±0.51)	2.43 (±0.42)	2.15 (±0.30)
B. A23187 + EGTA	3.70 (±0.60)	2.15 (±0.64)	1.78 (±0.58)
C. A23187 + Ca^{++}	0.60 (±0.53)	1.84 (±0.53)	0.51 (±0.32)
Osmotic fragility (%NaCl @ 50% hemolysis)			
A. Unincubated	0.43 (±0.02)	0.62 (±0.03)	—
B. A23187 + EGTA	0.43 (±0.04)	0.61 (±0.04)	—
C. A23187 + Ca^{++}	0.28 (±0.04)	0.64 (±0.05)	—
Intracellular Ca^{++} (μM/l RBC)			
A. Unincubated	24.3 (±9.1)	29.5 (±6.5)	31.1 (±8.4)
B. A23187 + EGTA	<10	<10	<10
C. A23187 + Ca^{++}	160.2 (±27.1)	138.3 (±21.1)	—
Predominant cell shape			
A. Unincubated	biconcave discs	biconcave discs	biconcave discs
B. A23187 + EGTA	biconcave discs	biconcave discs	biconcave discs
C. A23187 + Ca^{++}	spheroechinocytes	biconcave discs	spheroechinocytes (≃70%)

[a] Fresh red cells.
[b] Red cells incubated 60 min with A23187 and EGTA.
[c] Red cells incubated 60 min with A23187 and Ca^{2+}.

change shape when Ca^{2+} loaded (9). Fluoride-induced energy depletion alone has no effect upon the shape of either human or sheep erythrocytes. Therefore, the insensitivity of sheep red cells to intracellular Ca^{2+} is not explained by a failure of these cells to accumulated Ca^{2+}, or by insufficient Ca^{2+} concentrations in the incubation medium, or simply by their failure to become ATP depleted.

Suspension of human erythrocytes in isotonic KCl during ionophore-induced Ca^{2+} loading will block both cellular shrinkage and shape change (but not ATP hydrolysis) (9). This is presumably due to inhibition of passive cellular K^+ loss by the high extracellular K^+ concentrations. Sheep are polymorphic with regard to red cell K^+ content, and some have high Na^+ and low K^+ erythrocytes (15, 16), apparently due to a deficiency of membrane $Na^+ - K^+$ ATPase (17, 18). Using red cells from 3 high K^+ and 3 low K^+ ewes, we sought to determine whether this ionic peculiarity might explain the lack of responsiveness to Ca^{2+} influx. First, erythrocytes from both high K^+ and low K^+ sheep were exposed to ionophore and Ca^{2+}. All parameters shown in Table I were measured and neither cell type responded detectably. Second, we suspended high K^+ and low K^+ sheep erythrocytes in both isotonic NaCl and isotonic KCl and, again, observed no response to calcium influx (results not shown). These two experiments indicate that the inherited variation in monovalent cation content of sheep erythrocytes is not directly responsible for the lack of response to Ca^{2+} influx.

Because red cells from adult sheep hydrolyze little intracellular ATP when exposed to A23187 and Ca^{2+}, we measured the Ca^{2+} ATPase activity of crude lysates of both human and adult sheep erythrocytes. Human erythrocytes display a very active Ca^{2+} ATPase in such preparations, consuming almost as much ATP as do whole cells under similar conditions (Table II). However, lysates of adult sheep erythrocytes have very little Ca^{2+} ATPase activity, in agreement with the lack of Ca^{2+}-induced ATP hydrolysis by whole sheep erythrocytes (Tables I and II). The stability of ATP in sheep erythrocytes incubated with A23187 and Ca^{2+}, therefore, appears to be due to a congenital absence of

TABLE II.[a]

	n	ATP hydrolyzed (μM/g Hb/hour)		
		+EGTA	$+Ca^{2+}$	Ca^{2+} ATPase Activity
Human	7	1.95 (±0.54)	5.04 (±0.46)	3.09 (±0.82)
Adult Sheep	5	2.86 (±0.61)	2.97 (±0.58)	0.11 (±0.18)
Newborn Lambs	10	3.35 (±1.06)	6.54 (±1.13)	3.19 (±0.90)

[a] Ca^{2+} ATPase activity of crude lysates of human, adult sheep and newborn (24-hr old) lamb red cells. Values in parentheses represent ±1 standard deviation. Ca^{2+} ATPase activity is expressed as μM ATP hydrolyzed/g Hb/hour as a result of the addition of Ca^{2+} to the assay mixture. It represents the value obtained by subtraction of the ATP hydrolysis in Ca^{2+}-free samples (+EGTA) from the ATP hydrolysis in Ca^{2+}-containing samples ($+Ca^{2+}$).

an active membrane Ca^{2+} efflux ATPase.

In contrast to erythrocytes from adult sheep, those from all newborn lambs have high levels of intracellular K^+ and have an active Na^+-K^+ ATPase, regardless of the ultimate red cell K^+ phenotype of the offspring (18, 19). We therefore investigated the Ca^{2+} responsiveness of red cells drawn from lambs less than 24 hr old. Surprisingly, we found that newborn lamb red cells respond to Ca^{2+} influx precisely as do human red cells. That is, red cells from newborn lambs shrink, change shape, and hydrolyze large amounts of intracellular ATP (Fig. 1E and 1F, Table I). Lysates of lamb erythrocytes also have a very active Ca^{2+} ATPase, similar to that found in normal human red cells (Table II).

As might be expected from the presence of a certain number of adult red cells, erythrocytes from newborn lambs are mosaic in their response to Ca^{2+} influx; approximately 70–80% of these cells shrink and become spheroechinocytes, whereas 20–30% are morphologically unaffected (Fig. 1F). The fraction of Ca^{2+}-responsive cells gradually declines as the lamb matures, and all red cells from 4-month-old lambs are morphologically unaffected by Ca^{2+} influx. It appears that, in contrast to red cells from adult sheep, those from newborn lambs have a multitude of unique characteristics, including increased Na^+-K^+ ATPase activity, fetal hemoglobin and, as we have shown here, very active Ca^{2+} ATPase and Ca^{2+}-responsive red cell membranes. All these properties appear to change in concert as the animal matures.

Discussion. The lack of responsiveness of adult sheep erythrocytes to intracellular Ca^{2+} presents at least two puzzling questions. First, we are hard-put to explain the maintenance of low levels of intracellular Ca^{2+} *in vivo* in the face of an almost total lack of membrane Ca^{2+} efflux ATPase activity. This may be explained either by an impermeability of the sheep red cell to external Ca^{2+} or by the presence of a membrane Ca^{2+} ATPase which has a very low V_{max} but a high Km for Ca^{2+}. Equally puzzling is the fact that polyacrylamide gel electrophoresis of membranes from adult sheep erythrocytes reveals the presence of proteins similar to both spectrin and actin. These two proteins are thought to be the major Ca^{2+}-responsive proteins in the erythrocyte membrane (20–22). Indeed, in irreversibly sickled cells from patients with sickle cell disease, these two proteins form a meshwork, insoluble in Triton X-100, which retains the shape of the intact cell (23). However, adult sheep erythrocytes, despite the presence of these two major membrane proteins, do not respond to Ca^{2+} influx. This indicates that other proteins, not yet identified, may be a necessary part of the Ca^{2+}-reactive apparatus of the erythrocyte membrane.

The dichotomous responses of adult sheep and newborn lamb red cells to intracellular Ca^{2+} may represent a promising model for the further elucidation of the mechanisms of Ca^{2+}-induced damage to red cells and of the ways in which erythrocytes respond, metabolically and morphologically, to increased intracellular Ca^{2+}.

Summary. Normal mammalian erythrocytes contain very small amounts of Ca^{2+}. When erythrocytes from most species of mammal are subjected to ionophore-induced Ca^{2+} accumulation, extensive morphologic and metabolic damage occurs. This is not true of red cells from adult sheep; these cells appear to be totally unresponsive to sudden increases in intracellular Ca^{2+}. In contrast, red cells from new born lambs are responsive to Ca^{2+} influx and gradually become unre-

sponsive during maturation of the animal. Although the precise reasons for the lack of response of adult sheep erythrocytes to Ca^{2+} accumulation is unknown, this system may provide a valuable model for the dissection of the various effects of Ca^{2+} upon cells and cell membranes.

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