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The effect of cellular calcium on Na^+/K^+ cotransport in human red blood cells

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The increase in Ca^{2+} permeability by addition of ionophore A23187 in the presence of external Ca^{2+} did not alter the bumetanide-sensitive Na^+/K^+ effluxes in human red blood cells. An inhibition of this pathway by cellular Ca^{2+} could be observed only under conditions in which the cellular ATP content was drastically depleted.

In most cells, the ouabain-sensitive Na^+/K^+ pump is a major determinant of Na^+ and K^+ gradients across cell membranes. Recently, systematic study of ouabain-insensitive fluxes revealed the presence of a transport pathway sensitive to inhibition by derivatives of 2-sulfamoylbenzoic acid such as furosemide, bumetanide and piretanide [1,2]. While various cell types exhibit furosemide-sensitive Na^+/K^+ fluxes [3] red cells have proved to be especially suitable for their study and various investigators showed it to be mediated by the $Na^+/K^+/Cl^-$ cotransport and to be independent of other transport systems such as the Na^+/K^+ pump, Na^+-Na^+ exchange or K^+, Cl^- pathway [2,5,6]. The $Na^+/K^+/Cl^-$ cotransport pathway has been suggested to be implicated in a variety of cell functions such as epithelial transport, Cl^- accumulation or cell volume regulation [7,8]. Furthermore its activity was shown to be modulated by agents that induces a variation in cAMP or cGMP levels [8–11]. The role of another ubiquitous second messenger, the calcium ion, has not been extensively investigated. In one report, Garay [9] observed in human erythrocyte that furosemide-sensitive Na^+ efflux was markedly decreased in the presence of ionophore A23187 and extracellular Ca^{2+}. The effect of calcium in this study is not clear as Ca^{2+}-induced ATP changes were not recorded. In fact it is known that the exposure of erythrocyte to high levels of cell calcium induces a drastic ATP depletion due to the activation of the Ca^{2+}-ATPase associated with the Ca^{2+} pump [12]. On the other hand ATP depletion was reported to induce an inhibition of the Na^+/K^+ cotransport pathway [13]. In view of these arguments, the significant inhibition of the Na^+/K^+ cotransport by high levels of cellular calcium reported by Garay [9], could be consequent to ATP depletion per se. In this study we wished to clarify this point by assessing the effect of cellular calcium on the bumetanide-sensitive Na^+ and K^+ efflux, under experimental conditions in which erythrocyte ATP was kept at levels high enough not to induce an inhibition of this pathway.

Red cells from fresh heparinized blood were washed three times in a medium containing in mM: NaCl 70, KCl 80, Hepes-Na 10 (pH 7.4 at 37 °C), $MgCl_2$ 0.2. In order to study the maximal velocity of the Na^+/K^+ cotransport system, the cells were loaded to Na^+ levels of 40–50

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mmol/litre cells using the nystatin technique as described previously [13]. In brief the cells were incubated at 10% haematocrit for 20 min in an ice-cold solution containing in mM: NaCl 70, KCl 70, sucrose 52, Mops-Tris 10 (pH 7.4 at 4°C) to which nystatin (40 μg/ml) was added. The nystatin was removed by five successive washes at 37°C with the same solution (pH 7.4 at 37°C) to which 0.1% albumin was added. To remove external Na^+ contamination, the cells were further washed five times at 4°C with a choline solution containing in mM: choline chloride 150, $MgCl_2$ 0.2, Mops-Tris 10, ouabain 0.1. Bumetanide-sensitive Na^+ and K^+ effluxes were assessed as previously described [13,14]. In brief cells were suspended at 4–5% haematocrit in choline solution in the absence and presence of 0.02 mM bumetanide. 10 mM inosine was added to the medium. Triplicate samples were taken at time 0, 30 and 60 min and the supernatant Na^+ and K^+ content determined by flame photometry.

Cellular $[Ca]_i$ can be increased by raising artificially the effective Ca^{2+} permeability of the cell membrane by addition of the divalent cation ionophore A23187 [15]. Varying the ionophore concentration allows a precise control of the Ca^{2+} permeability over a wide range, up to several times the Ca^{2+} pump maximal activity [16]. In the present study, ionophore A23187 was added in nanomolar concentrations in presence of either 0.5 mM EGTA or 0.5 mM calcium. Under the latter condition the induced Ca^{2+} influx could be handled by the Ca^{2+} pump and was unable to significantly raise the Ca^{2+} content of inosine-fed cells. The increase in Ca^{2+} permeability thus had to be followed in an aliquot of cells preloaded with quin2 [17] and incubated in parallel conditions with the addition of tracer quantities of ^{45}Ca.

Table I shows the effect of increasing concentrations of ionophore A23187 on bumetanide-sensitive Na^+ and K^+ effluxes. It can be seen that in presence of 0.5 mM $[Ca^{2+}]_o$, the addition of 20–200 nM A23187 had no effect on the bumetanide-sensitive Na^+ and K^+ effluxes. Similarly, no effet was observed on either the ouabain-resistant or the bumetanide-resistant Na^+

TABLE I

THE EFFECT OF INCREASING THE Ca^{2+} PERMEABILITY ON Na^+ AND K^+ EFFLUX FROM Na^+-LOADED CELLS

Na^+ and K^+ effluxes ± S.D. were calculated by linear regression analysis [14]. Na^+-loaded cells ($[Na^+]_i$ = 43 mmol/litre cells) were suspended at 4% HTc [14] in choline media + 10 mM inosine. Cell ATP content was assessed by luciferin-luciferase assay using a Lumac luminometer. Total calcium content (Ca_i^T) was assessed in parallel conditions in quin2-loaded cells [17] in the presence of tracer amounts of $^{45}Ca^{2+}$. After 60 min of incubation, triplicate aliquots of cell suspension were centrifuged through dibutylphtalate oil layer and the total $^{45}Ca^{2+}$ content (Ca_i^T) assessed after protein precipitation with 7% trichloroacetic acid. Free Ca^{2+} contents of cells were estimated following the procedure first described by Lew et al. [23] and modified by Waller et al. [20]. Similar results were observed in erythrocytes from four other donors.

	Efflux (mmol/l cells per h)						ATP content (mmol/l cells)	Ca_i^T (quin2)-loaded cells (μmol/l cells)	Free Ca^{2+} content (nM)
	Ouabain-resistant		Bumetanide-resistant		Bumetanide-sensitive				
	Na^+ efflux	K^+ efflux	Na^+ efflux	K^+ efflux	Na^+ efflux	K^+ efflux			
Control									
A23187 (100 nM) + EGTA(500 μM)	1.25 ± 0.01	2.19 ± 0.06	0.42 ± 0.03	1.07 ± 0.01	0.84 ± 0.03	1.13 ± 0.06	1.50	20	36
$[Ca^{2+}]_o$ (500 μM) + A23187 (nM)									
20	1.23 ± 0.04	2.17 ± 0.06	0.40 ± 0.01	0.98 ± 0.03	0.83 ± 0.04	1.18 ± 0.07	1.28	30	54
60	1.20 ± 0.01	2.29 ± 0.06	0.42 ± 0.0	1.05 ± 0.0	0.78 ± 0.01	1.24 ± 0.05	1.37	70	126
100	1.28 ± 0.01	2.21 ± 0.0	0.47 ± 0.01	1.18 ± 0.04	0.81 ± 0.01	1.02 ± 0.04	1.32	110	196
150	1.27 ± 0.01	2.34 ± 0.01	0.41 ± 0.02	1.08 ± 0.01	0.86 ± 0.02	1.26 ± 0.04	1.27	140	252
200	1.32 ± 0.03	2.16 ± 0.03	0.42 ± 0.0	1.09 ± 0.01	0.90 ± 0.03	1.06 ± 0.06	1.11	160	282

and K^+ fluxes suggesting no stimulation of the Ca^{2+}-sensitive Na^+ or K^+ pathways, in agreement with previous reports [18,19]. Cellular ATP content was assessed at the end of the incubation period and remained above 1 mmol/litre cell, a concentration high enough not to alter the Na^+/K^+ cotransport system. In fact we previously reported [13] that erythrocyte ATP levels had to be reduced below 0.15 mmol/litre cell to induce an inhibition of Na^+/K^+ fluxes mediated by this pathway. Free $[Ca^{2+}]_i$ content assessed in parallel conditions in quin2-loaded cells increased from 36 to 282 nM. Thus increasing the Ca^{2+}-content up to about eight times that of control cells does not inhibit the Na^+/K^+ cotransport pathway.

It could be argued that micromolar levels of free $[Ca^{2+}]_i$ rather than nanomolar levels are necessary to inhibit cotransport fluxes. Thus experimental conditions similar to those used by Garay [9] were mimicked by addition of a high concentration of A23187 (10 μM) in the presence of 50–200 μM $[Ca^{2+}]_o$. Under these conditions, the Ca^{2+}-induced influx swamps the activity of the Ca^{2+} pump and Ca^{2+} distribution across cell membrane is in 'near equilibrium' conditions [21].

To prevent excessive ATP depletion due to maximal activity of the Ca^{2+}-ATPase, the ATP content of Na^+-loaded cells was enriched by incubation in a high phosphate medium as previously described [12,27]. Briefly, red blood cells were incubated for 4 h at 15% Htc in a medium containing 60 mM NaCl, 40 mM KCl, 50 mM sodium phosphate, 10 mM adenosine, 10 mM pyruvate, and 10 mM Tris-Mops (pH 7.4 at 37 °C). The cells were then washed twice and incubated for 30 min in phosphate-free Na^+, K^+ medium to which 10 mM pyruvate and 10 mM adenosine were added. Cells were then washed five times with choline medium and incubated at 4–5% HTc in the same medium containing $[Ca^{2+}]_o$ 50–200 μM (or 200 μM EGTA) and 0.4 mM $MgCl_2$, carbocyanine derivative diS-C_3 0.1 mM and amiloride 1 mM were added to the medium to inhibit respectively the Ca^{2+}-dependent K^+ channel [22] and the Ca^{2+}-dependent Na^+ fluxes [18].

Control experiments showed that A23187, diS-C_3 and amiloride had no significant effect on bumetanide-sensitive Na^+ effluxes. Ouabain-resistant, bumetanide-resistant and bumetanide-sensitive Na^+ effluxes were increased in ATP-enriched cells compared to fresh cells (Table II),

TABLE II

THE EFFECT OF INCREASING THE Ca^{2+} PERMEABILITY ON Na^+ EFFLUX IN ATP-ENRICHED RED BLOOD CELLS

After nystatin treatment, Na^+-loaded cells ($[Na^+]_i$ 40 mmol/l cells) were divided in two batches. One aliquot of cells was enriched with ATP by incubation in a high sodium phosphate medium (see text). The other aliquot normal ATP cells was incubated in phosphate-free Na^+, K^+ medium for the same period of time. Cells were then washed with choline medium and incubated at 5% Htc in presence of 0.1 mM diS-C_3 and 1 mM amiloride. When needed bumetanide 0.02 mM was added to the choline medium. Na^+ efflux ± S.D. was calculated by linear-regression analysis [14]. Total calcium content (Ca_i^T) was assessed by addition of tracer quantities of $^{45}Ca^{2+}$ to an aliquot of cells in parallel conditions. Free calcium content ($[Ca_i^{2+}]$) was estimated as previously reported [12] assuming a fraction of ionized calcium (α) of 0.30 for normal ATP cells and 0.23 for ATP-enriched cells [12]. Similar results were observed in erythrocytes from three other donors.

	Na^+ efflux (mmol/l cells per h)			ATP content (mmol/l cells)	Ca_i^T content (mmol/l cells)	Free Ca^{2+} content (mM)
	Ouabain-resistant	Bumetanide-resistant	Bumetanide-sensitive			
Normal ATP cells						
A23187 (10 μM) + EGTA (200 μM)	1.65 ± 0.02	0.59 ± 0.01	1.06 ± 0.02	1.47	–	–
A23187 (10 μM) + $[Ca^{2+}]_o$ (100 μM)	1.42 ± 0.03	0.72 ± 0.01	0.70 ± 0.03	0.07	0.38	0.11
ATP-enriched cells						
Control	2.72 ± 0.07	1.42 ± 0.02	1.30 ± 0.07	2.81	–	–
A23187 (10 μM) + EGTA (200 μM)	2.41 ± 0.03	1.16 ± 0.01	1.25 ± 0.03	2.70	–	–
A23187 (10 μM) + $[Ca^{2+}]_o$ (100 μM)	2.16 ± 0.04	1.14 ± 0.03	1.02 ± 0.05	1.22	1.52	0.34
A23187 (10 μM) + $[Ca^{2+}]_o$ (200 μM)	2.77 ± 0.04	1.43 ± 0.02	1.34 ± 0.04	1.18	2.12	0.49

confirming previous reports [18]. High-ATP cells are characterized by a lower pH and a higher membrane potential compared to fresh cells [12]. The relation between these parameters and the activation of Na^+ fluxes is not clear and deserves further investigation.

As shown in Table II, increasing cellular calcium content of fresh cells by addition of A23187 in presence of $[Ca^{2+}]_o$ induces a 30% inhibition of bumetanide-sensitive Na^+ efflux, concomittant with a drastic reduction in ATP levels to 0.070 mmol/litre cell. This is in agreement with the previous observation made by Garay (Fig. 5 in Ref. 9). On the other hand, the addition of A23187 in presence of $[Ca^{2+}]_o$ to ATP-enriched cells decreases ATP levels to 1.2 mmol/litre cells without affecting butamine-sensitive Na^+ efflux. These results suggest that cotransport inhibition in fresh cells is due to ATP depletion rather than to an increase in Ca^{2+} content.

Cellular Ca^{2+} and cAMP are known to be a modulator of monovalent cation transport across cell membrane [18,19,24]. Recently, activation of Na^+/K^+ cotransport pathway was found to be mediated by a cAMP dependent protein kinase [8,25]. It was thus of interest to understand the role of cytoplasmic Ca^{2+} in the regulation of cotransport fluxes and several studies attempted to identify it. In the human erythrocyte, where an inhibition of furosemide sensitive fluxes by cAMP has been reported [9], the present study shows that increasing the Ca^{2+} content does not alter the Na^+/K^+ cotransport pathway. On the other hand, a stimulation of cotransport fluxes by Ca^{2+} was observed in bovine and porcine endothelial cells [11,26] while an inhibition of Cl^- absorption was reported in flounder intestine [8]. These observations are in agreement with a previous suggestion by Palfrey and Rao [8] that the effect of cellular messengers on cotransport pathway are tissue specific [8].

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