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Metabolic Control of the K^+ Channel of Human Red Cells

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Summary. The effects of cAMP, ATP and GTP on the Ca^{2+}-dependent K^+ channel of fresh (1–2 days) or cold-stored (28–36 days) human red cells were studied using atomic absorption flame photometry of Ca^{2+}-EGTA loaded ghosts which had been resealed to monovalent cations in dextran solutions. When high-K^+ ghosts were incubated in an isotonic Na^+ medium, the rate constant of Ca^{2+}-dependent K^+ efflux was reduced by a half on increasing the theophylline concentration to 40 mM. This effect was observed in ghosts from both fresh and stored cells, but only if they were previously loaded with ATP. The inhibition was more marked when Mg^{2+} was added together with ATP, and it was abolished by raising free Ca^{2+} to the micromolar level. Like theophylline, isobutyl methylxanthine (10 mM) also affected K^+ efflux. cAMP (0.2–0.5 mM), added both internally and externally (as free salt, dibutyryl or bromide derivatives), had no significant effect on K^+ loss when the ghost free-Ca^{2+} level was below 1 μM, but it was slightly inhibitory at higher concentrations. The combined presence of cAMP (0.2 mM) plus either theophylline (10 mM), or isobutyl methylxanthine (0.5 mM), was more effective than cAMP alone. This inhibition showed a strict requirement for ATP plus Mg^{2+} and it was not overcome by raising internal Ca^{2+}. Ghosts from stored cells seemed more sensitive than those from fresh cells, to the combined action of cAMP and methylxanthines. Loading ATP into ghosts from fresh or stored cells markedly decreased K^+ loss. Although this effect was observed in the absence of added Mg^{2+} (0.5 mM EDTA present), it was potentiated upon adding 2 mM Mg^{2+}. The K^+ efflux from ATP-loaded ghosts was not altered by dithio-*bis*-nitrobenzoic acid (10 mM) or acridine orange (100 μM), while it was increased two- to fourfold by incubating with MgF_2 (10 mM), or MgF_2 (10 mM) + theophylline (40 mM), respectively. By contrast, a marked efflux reduction was obtained by incorporating 0.5 mM GTP into ATP-containing ghosts. The degree of phosphorylation obtained by incubating membranes with (γ-^{32}P)ATP under various conditions affecting K^+ channel activity, was in direct correspondence to their effect on K^+ efflux. The results suggest that the K^+ channel of red cells is under complex metabolic control, via cAMP-mediated and nonmediated mechanisms, some which require ATP and presumably, involve phosphorylation of the channel proteins.

Key Words cAMP and K^+ channel · red cell K^+ channel · ghost K^+ permeability · metabolic control K^+ channel

Introduction

The Ca^{2+}-dependent K^+ channel of human erythrocytes exhibits a variable Ca^{2+} sensitivity, depending on the cellular ATP content and age [11, 13, 21, 31, 35, 37, 39]. Such characteristic may arise from either an all-or-none behavior of K^+ channels, predominant in an heterogeneous cell population [2, 10, 11], a variable channel activity among the different cellular subpopulations or both.

As the magnitude of the Ca^{2+}-dependent K^+ efflux is markedly affected by both the NAD^+/NADH ratio [1] and the ATP/ADP ratio at a constant internal free Ca^{2+} in ghosts [32], the possibility arises of a metabolic control of channel activity.

Recent work has shown modulation of ionic channels by a number of factors, including cyclic nucleotide-mediated protein phosphorylations [20, 43], GTP binding to G proteins [18, 25, 28], or direct ATP-channel interactions [4, 19].

On the other hand, a wide variety of protein kinases has been described in the human erythrocyte [16, 22, 29, 38]. The possibility then arises as to whether the Ca^{2+}-dependent K^+ channel of red cells is regulated by one or more of the above mechanisms.

We have studied the effects of cAMP, ATP and GTP on the K^+ channel of human red cells. Ghosts, which had been resealed to monovalent cations in dextran solutions, were employed as they retained a large fraction of their original cytosolic protein content. In addition, as some of the characteristics of the K^+ channel alter during aging in vitro [32], we have investigated the influence of various treatments on cold-stored cells.

It was found that the K^+ channel was affected by both ATP and conditions leading to a raised cellular cAMP level, and that channel activity was paralleled by membrane phosphorylation.

Materials and Methods

Analytical quality reagents were used wherever possible. Dextran (M_r 80,000), ATP, cAMP, GTP, adenine, inosine and EGTA were purchased from Sigma.[1] All other reagents were obtained from BDH, England. (γ-^{32}P)ATP (specific activity = 0.25×10^{10} cpm/μmol) was a generous gift of Dr. F. Proverbio.

Human blood (0(+) group) was collected in citrate-phosphate dextrose solution containing 0.03% adenine (wt/vol), and was used either within 1–2 days after collection, or after 28–36 days of storage at 4°C under sterile conditions. The pH of all solutions was adjusted at room temperature.

Resealed ghosts were prepared in dextran solutions in the presence of an excess of free Ca^{2+} [33]. One vol of packed erythrocytes was lysed for 50 sec at room temperature in 20 vols of a medium containing: ATP · Na_2 2 mM, $MgCl_2$ 2 mM; EGTA-Tris 5 mM; Tris-HCl (pH 7.4) 20 mM 3% dextran (wt/vol) and variable $CaCl_2$ concentrations.

After restoring isotonicity with KCl, ghosts were resealed to monovalent cations by incubating for 40–50 min at 37°C in a medium containing (in mM): KCl 160; adenine 5; inosine 10; KH_2PO_4 4; Tris-HCl (pH 7.4) 20; as reported previously [34]. Ghosts not containing ATP were resealed in a similar medium as above, but in the absence of added substrates. In most experiments with cAMP or its bromide and dibutyryl derivatives, the compounds were present both in the hemolytic and resealing media.

Different Ca^{2+}/EGTA ratios set up experimentally, were used in order to obtain free-Ca^{2+} concentrations between 0.2 μM–2 mM by the end of resealing. The ghosts were then washed three times with a high-Na^+ medium and finally incubated (10% hematocrit) for up to 60 min at 37°C, in a medium containing (in mM) NaCl 160; KCl 2; ouabain 0.1; $LaCl_3$ 0.1; Tris-HCl (pH 7.4) 20, with and without methylxanthines and cAMP or derivatives. Aliquots of the ghost suspension were withdrawn at 20-min time intervals for up to 1 hr and centrifuged through a *n*-dibutyl phthalate cushion (d = 1.042–1.045 g/ml). The sediment was lysed in water and kept for analysis of the ghost contents.

All ghosts from the different experimental conditions employed succeeded in passing through the dibutyl phthalate cushion. Only in a few cases, especially after incubating with theophylline in the absence of ATP, a thin layer of ghosts was found on top of the oil.

Ghosts heterogeneity to Ca^{2+} and K^+ was explored in pilot tests by centrifuging through diethyl (d = 1.117–1.120 g/ml) and dibutyl phthalates mixtures, after a brief exposure to thiocyanate solutions at room temperature [10].

In some experiments, inside-out vesicles (IOV's) were prepared from fresh blood [36] and fragmented by freezing and thawing four times in a solid CO_2-ethanol mixture. The membranes (120 μg protein) were then phosphorylated by endogenous protein kinases by incubating for 15 min at 37°C in a medium containing (in mM): KCl 140; $MgCl_2$ 2; $LaCl_3$ 0.1; ouabain 0.1; EGTA 5; imidazole-HCl (pH 7.4) 20; in the presence of 17.5 μM (γ-^{32}P)ATP (specific activity = 0.25×10^{10} cpm/μmol), 1 μM free Ca^{2+} (Ca^{2+}/EGTA = 0.830) and 0.5 vols of a membrane-free lysate (1 : 8 lytic ratio), with and without cAMP (50 μM), methylxanthines and other additions to be specified later. After washing three times with a large excess of a 160 mM KCl + 1 mM $MgCl_2$ + 10 mM Tris-HCl (pH 7.6) medium, the membranes were dissolved in 3% SDS plus 1% glycerol in 0.7 M mercaptoethanol. Radioactivity was assessed in aliquots of these solutions by liquid scintillation counting.

The K^+ and Ca^{2+} concentrations of ghosts were determined by atomic absorption flame photometry [34]. Ca^{2+}-EGTA buffers were calculated as detailed elsewhere [31] and free-Ca^{2+} concentrations were checked using commercial electrodes (Radiometer Ca^{2+}-Selectrodes).

Protein was assayed according to the Lowry's method [23], using bovine serum albumin as standard.

The K^+ efflux rate constant (^{o}k) was calculated from linear regression analyses of the log (efflux) *versus* time. The determination coefficient (r^2) of the regression lines was above 0.75 in most experiments, thus indicating a relative good fit of the data to single-exponential kinetics.

Results

Ghosts Heterogeneity

Ghosts having about 1 μM free Ca^{2+} and either depleted of or loaded with ATP, were incubated for 3 min at room temperature in an isotonic-Na^+ solution containing 100 mM thiocyanate [10]. Different mixtures of diethyl and *n*-dibutyl phthalates were employed in preliminary tests to study the degree of heterogeneity of the ghost population.

A mixture consisting of 50% (vol/vol) each of above phthalates (approx. d = 1.080 g/ml) was effective in separating two subpopulations of ghosts, which were more or less of similar size. The Ca^{2+} concentration was identical in both light and heavy fractions and equal to that of the parent mixed population, independently of whether or not ghosts contained ATP. By contrast, the lighter fraction of ATP-loaded ghosts had two to three times more K^+ than the corresponding heavier fraction. This was not the case with ATP-depleted ghosts, both fractions had almost an identical K^+ concentration. Similar results on K^+ distribution were obtained after 1-hr incubation at 37°C in a high-Na^+ medium.

These tests indicate that ghosts are homogeneous to Ca^{2+} after loading. They, however, become heterogeneous to K^+ following incubation in K^+-free media only if loaded with ATP. The experiments to be described below were performed on the entire ghost population and no attempt was made to study subpopulations separately.

Action of Methylxanthines on K^+ Efflux

Depending on internal free Ca^{2+}, theophylline had a dual effect on K^+ loss. At high Ca^{2+} (about 1 mM)

[1] *Abbreviations:* EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether) N,N' tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; DTNB, 5,5'-dithio*bis*(2-nitro)benzoic acid; and SDS, sodium dodecyl sulfate.

Table 1. Effects of theophylline and cAMP on Ca^{2+}-dependent K^+-efflux

Internal Ca^{2+} (μM)	K^+ efflux rate constant ($min^{-1} \times 10^{-4}$) No addition	Theophylline (10 mM)	dibut-cAMP (0.2 mM)	Theophylline plus cAMP
0.15 ± 0.098 (5)	33 ± 13 (5)	16 ± 7 (5) $P < 0.025$		
0.41 ± 0.044 (7)	52 ± 13 (7)	23 ± 13 (5) $P < 0.005$	49 ± 13 (4) $P > 0.1$	20 ± 15 (6) $P < 0.005$
0.52 ± 0.015 (5)	68 ± 16 (5)	34 ± 6 (5) $P < 0.005$		
0.73 ± 0.123 (6)	120 ± 41 (6)	69 ± 49 (4) $P > 0.05$	110 ± 25 (4) $P > 0.1$	43 ± 35 (4) $P < 0.025$
1.43 ± 0.427 (6)	119 ± 48 (6)	90 ± 11 (5) $P > 0.1$	40 ± 6 (5) $P < 0.005$	35 ± 8 (5) $P < 0.005$
998 ± 130 (4)	125 ± 45 (4)		91 ± 15 (4) $P > 0.1$	
1,890 ± 415 (12)	100 ± 30 (12)	110 ± 36 (11) $P > 0.1$		47 ± 3 (7) $P < 0.005$

ATP-loaded ghosts were prepared from fresh cells and contained after resealing, the free-Ca^{2+} concentrations shown above, were as described in the legend to Fig. 1. They were then incubated in a high-Na^+ medium with either theophylline (10 mM), dibutyryl-cAMP (0.2 mM), or theophylline (10 mM) plus dibutyryl-cAMP (0.2 mM). After incubation the K^+ efflux rate constant was determined. Collected results are shown as mean value ± SD with the number of experiments given in parentheses. *P* denotes the probability obtained from a Student's *t* test.

and in the presence of 2 mM ATP, the K^+ efflux was slightly stimulated by 10 mM theophylline (Fig. 1). The effect, however, was not observed at low Ca^{2+} concentrations. Instead, at or below 0.5 μM Ca^{2+}, theophylline reduced $^\circ k$ by almost half (Table 1). This inhibition was nearly maximal as further raising theophylline concentration to 40 mM, only diminished $^\circ k$ slightly (Fig. 2).

Low IBMX concentrations (1 mM or less) were ineffective at any Ca^{2+} concentration tested (Tables 2 and 3). At 10 mM, however, IBMX reproduced results with equimolar theophylline concentrations (*data not shown*). These findings indicate that theophylline inhibits K^+ loss in a way common to other methylxanthines.

On the other hand, the inhibition caused by theophylline was incomplete and the theophylline-resistant K^+ efflux was progressively raised upon increasing free Ca^{2+} (Table 1). In addition, it seemed to increase as the cells were aged in vitro. Thus, in the presence of about 0.4 μM free Ca^{2+}, the residual $^\circ k$ of ghosts from stored cells was nearly three times that obtained with ghosts from fresh cells (*compare* Tables 1 and 4). The actual difference may be even greater as the latter ghosts were incubated with four times more theophylline than that used with the former type.

The Action of cAMP Plus Methylxanthines

The external addition of cAMP and its bromide or dibutyryl derivatives (concentrations up to 1 mM), had no reproducible effects on K^+ loss. A statistically significant inhibition of K^+ efflux was, however, obtained when these compounds where present throughout preparation of the ghosts and during their final incubation. The effect showed a

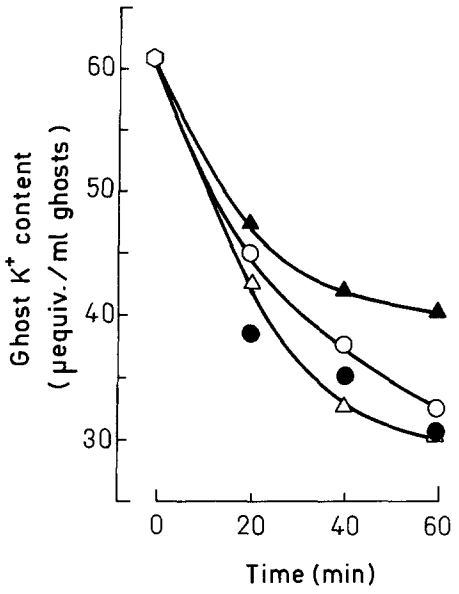

Fig. 1. The inhibition of K^+ loss by theophylline and cAMP. Human erythrocytes from fresh blood, were lysed and resealed to K^+ in dextran solutions containing 2 mM ATP, 2 mM $MgCl_2$ and 5 mM EGTA, with (triangles) and without 0.2 mM dibutyryl-cAMP (circles) and various $CaCl_2$ concentrations in order to obtain by the end of resealing, either 1.15 mM (circles) or 1.65 mM free Ca^{2+} (triangles). The ghosts were then incubated in a medium containing 160 mM NaCl; 2 mM KCl; 0.1 mM ouabain; 0.1 mM $LaCl_3$; 20 mM Tris-HCl (pH 7.4), in the presence (filled symbols) and absence of 10 mM theophylline (open symbols). Those ghosts resealed with cAMP were also incubated with 0.2 mM dibutyryl-cAMP in addition to theophylline. Results from a single experiment are shown as mean values of duplicate determinations

bell-shape dependence on ghost Ca^{2+}, being optimal with 0.2 mM dibutyryl-cAMP at about 1.4 μM Ca^{2+} (Table 1).

Under above conditions, theophylline (10 mM) did potentiate cAMP action and sustained the inhibitory effect at high internal Ca^{2+}. Thus, a highly significant reduction of $^{\circ}k$ by 40% was found at nearly 2 mM free Ca^{2+} (Table 1).

Essentially identical inhibition was also obtained with IBMX (0.5 mM) in ATP-loaded ghosts (Table 2). Unlike the case of theophylline, however, a mixture of IBMX plus cAMP was effective at low Ca^{2+} concentrations (Fig. 3).

Requirements for ATP and Mg^2

The inhibitory action of cAMP plus methylxanthines and that of methylxanthines alone, showed a marked requirement for ATP. In the absence of ATP $^{\circ}k$ remained unaltered, or it was slightly increased, after incubating either with cAMP (0.2 mM) plus IBMX (0.5 mM) or with theophylline (40 mM) or IBMX (10 mM), respectively (*data not shown*). In the presence of ATP $^{\circ}k$ was appreciably reduced.

Pilot experiments with 3-methoxybenzamide (10 mM), a very potent inhibitor of ADP-ribosyl

Table 2. Effects of ATP and cAMP on Ca^{2+}-dependent K^+-efflux

		K^+ efflux rate constant ($min^{-1} \times 10^{-4}$)	
Internal Ca^{2+} (μM)	Additions to hemolytic medium	No cAMP	dibut-cAMP (0.2 mM)
0.32 ± 0.149 (5)	None	206 ± 54(5)	190 ± 55(5) $P > 0.1$
0.38 ± 0.133 (6)	ATP (2 mM)	43 ± 12(7)	40 ± 8(6) $P > 0.1$
0.30 ± 0.150 (6)	ATP (2 mM) + IBMX (0.5 mM)	44 ± 10(6)	23 ± 7(5) $P < 0.01$

Red cells from blood stored for 1–3 days were lysed as described in the legend to Fig. 1, in the presence of 2 mM $MgCl_2$/5 mM EGTA-Ca^{2+} buffer/20 mM Tris-HCl (pH 7.4), with the additions indicated. IBMX and dibutyryl-cAMP were present throughout the preparation and final incubation of ghosts, when added at all. Results are presented as mean value ± 1 SD with the number of experiments given in brackets. *P* denotes the probability obtained from a Student's *t* test.

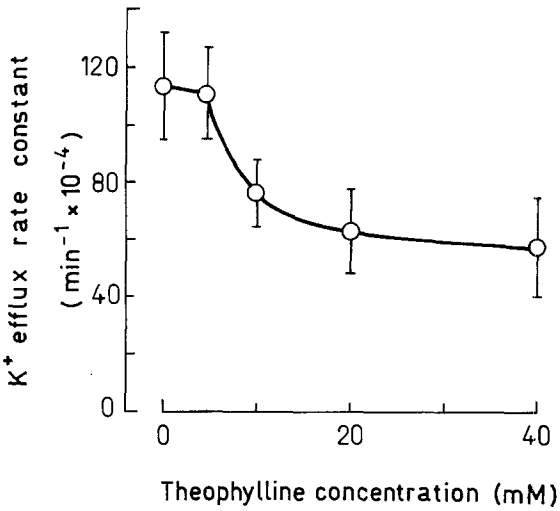

Fig. 2. Incomplete inhibition of K^+ efflux by theophylline. ATP-loaded ghosts from fresh blood, were incubated in the high-Na^+ medium described in the legend to Fig. 1, in the presence of different theophylline concentrations. The results are given as the mean value of four experiments. Vertical bars denote ± 1 SD of mean. Ghost free Ca^{2+} was 0.17 ± 0.088 μM (4) (mean ± 1 SD)

Table 3. Requirement for ATP of methyl-xanthines and cAMP effects

Additions to incubation medium	K^+ efflux rate constant ($min^{-1} \times 10^{-4}$)			
	No ATP		2 mM ATP	
	No cAMP	dibut-cAMP (0.2 mM)	No cAMP	dibut-cAMP (0.2 mM)
None	235 ± 23(4)	—	95 ± 10(5)	—
Theophylline (40 mM)	280 ± 13(4)	263 ± 18(4)	55 ± 8(4)	33 ± 8(4)
IBMX (0.5 mM)	215 ± 11(4)	198 ± 17(4)	93 ± 12(4)	22 ± 4(5)

Erythrocytes from blood stored for 28–30 days at 4°C, were lysed in the presence or absence of 2 mM ATP, with and without dibutyryl-cAMP, in a medium similar to that reported in Table 2. cAMP was present both internally and externally, whereas theophylline and IBMX were only added externally. Results are given as mean values ± 1 SD with the number of experiments given in parentheses. The mean free internal Ca^{2+} (± SD) was 0.23 ± 0.071 μM (5).

transferases [30], showed that it affects K^+ loss in a similar way as methylxanthines alone, i.e., ATP being required for eliciting the inhibitory effect (*data not shown*). On the other hand, the effects of theophylline and methoxybenzamide at 10 mM were additive.

Ghosts from stored blood were also affected by cAMP plus methylxanthines or theophylline alone (Table 3), but unlike those from fresh blood, they were more sensitive to the combined action of cAMP and IBMX. In the presence of 0.2–0.3 μM free Ca^{2+}, IBMX (0.5 mM) plus cAMP (0.2 mM) reduced $^\circ k$ by 42 and 76% in ghosts from fresh and stored blood, respectively (Tables 2 and 3). At about 0.4 μM free Ca^{2+}, cAMP on its own elicited a significant reduction of $^\circ k$ when added to ghosts from stored blood (Table 4), but not with ghosts from fresh blood (Table 1).

The inhibition obtained with theophylline (10 mM) at low free Ca^{2+} (0.2–0.4 μM) in ghosts from fresh cells was about the same whether 0.2 mM cAMP was added, or not (Table 1). It was significantly decreased if cAMP was present in ghosts from stored cells (*compare* Tables 1 and 3).

On the other hand, a marked reduction of K^+ efflux was obtained with ATP alone. Thus, in ghosts from fresh blood and containing about 0.3 μM Ca^{2+}, ATP (2 mM) diminished $^\circ k$ by nearly 80% (Table 2). The nucleotide was also effective in ghosts from stored cells.

Preliminary experiments using nonmetabolizable ATP analogues have demonstrated that the rate of K^+ efflux from ATP-depleted ghosts, loaded with about 1 μM free Ca^{2+}, is practically unaltered by the presence of 0.5 mM β,γ-methylene adenosine 5′-triphosphate (*data not shown*).

Table 4. The Mg^{2+} requirement of ATP, theophylline and cAMP actions

Additions to incubation medium	K^+ efflux rate constant ($min^{-1} \times 10^{-4}$)			
	Additions to hemolytic medium			
	2 mM ATP		2 mM ATP plus 0.2 mM dibut-cAMP	
	No Mg^{2+}	2 mM Mg^{2+}	No Mg^{2+}	2 mM Mg^{2+}
None	128 ± 11 (4)	90 ± 12 (7)	130 ± 15 (4)	60 ± 16 (5)
Theophilline (40 mM)	76 ± 12 (4)	57 ± 8 (5)	71 ± 13 (4)	35 ± 13 (6)

Red cells from blood stored for 28–36 days at 4°C, were lysed as in the legend to Table 2, with the additions shown above. When added, cAMP was present throughout the preparation and final incubation of ghosts. Results are given as mean value ± 1 SD from the different experiments given in parentheses. Ghost free Ca^{2+} was 0.35 ± 0.14 μM (7) and control K^+ efflux rate constant (no ATP, no Mg^{2+}) = 201 ± 53 $min^{-1} \times 10^{-4}$ (7). Without Mg^{2+}, 0.5 mM EDTA was added to the hemolytic medium.

The action of cAMP on K^+ loss showed a strict requirement for both ATP and Mg^{2+} (Table 4). By contrast, the inhibition caused by theophylline, or ATP alone, could be obtained in the absence of added Mg^{2+} (0.5 mM EDTA being present within

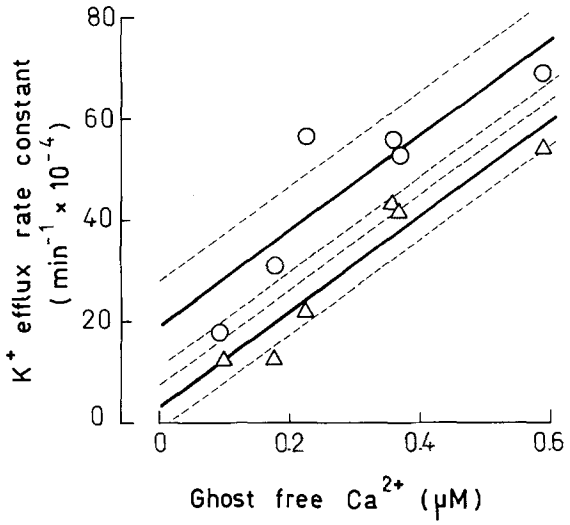

Fig. 3. The inhibitory action of IBMX. ATP-loaded ghosts from fresh blood, were prepared in the presence of 0.2 mM dibutyryl-cAMP and an excess of Ca^{2+} over EGTA, to give after resealing the free-Ca^{2+} concentrations shown. Ghosts were incubated in a high-Na^+ medium similar to that described in the legend to Fig. 1, but containing 0.2 mM dibutyryl-cAMP, in the presence (triangles) and absence of 0.5 mM IBMX (circles). Collected results from at least six experiments are presented. The curves drawn correspond to the regression lines $Y_1 = 9.76X + 2.37$ and $Y_2 = 96.5X + 18.9$ for ghosts incubated with and without IBMX, respectively. Dotted lines above and below the solid lines represent ± 1 SD of the whole regression lines

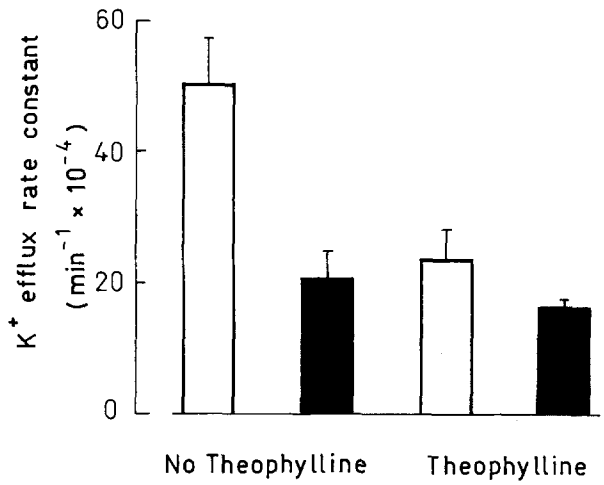

Fig. 4. Inhibitory action of GTP on K^+ channel. Ghosts from 1-day-old stored cells, were prepared containing both 2 mM ATP and 2 mM $MgCl_2$, with (black bars) and without addition of 0.5 mM GTP (open bars). The K^+-efflux rate constant ($min^{-1} \times 10^{-4}$) was determined after incubation in the presence and in the absence of 40 mM theophylline. Bars show the mean value from four experiments. Vertical lines indicate ± 1 SD. Ghost free Ca^{2+} was 0.35 ± 0.019 μM (4) (mean value ± 1 SD)

ghosts) but was potentiated upon adding 2 mM $MgCl_2$ (Table 4).

Inhibitory Action of GTP on K^+ Channel

As GTP and nonhydrolysable analogues have been shown to modulate Ca^{2+} and K^+ channels in excitable cells [18, 28], it was of interest to test the effect of this nucleotide on Ca^{2+}-dependent K^+ loss.

The incorporation of GTP (0.5 mM) into ATP-loaded ghosts containing about 0.4 μM Ca^{2+}, decreased $^\circ k$ to the same level achieved in ATP-, or ATP plus GTP-loaded ghosts both of which had been incubated with theophylline (40 mM) (Fig. 4).

Protein Kinases and K^+ Channel Activity

The human erythrocyte possesses a number of protein kinases, both cytosolic and membrane bound [16, 22, 29, 38]. With the interest of establishing if channel activity is related to protein phosphorylation mediated by these kinases, two different approaches were employed.

Initially, the action of the following chemicals on K^+ loss was studied. First, DTNB, which has been reported as being effective against the catalytic subunit of cAMP-dependent kinase [27]; second, fluoride ions, known inhibitors of cAMP-independent protein kinases [38]; and third, acridine orange, a potent inhibitor against protein kinase C [17].

At the highest concentration tested in the hemolytic medium, with free Ca^{2+} between 0.2–0.7 μM, DTNB (10 mM) or acridine orange (100 μM) showed no effect on K^+ loss from ATP-loaded ghosts. If these ghosts were incubated with external fluoride (10 mM) plus equimolar amounts of Mg^{2+}, however, a twofold increase of $^\circ k$ was elicited. A further twofold rise in $^\circ k$ resulted from the simultaneous presence of theophylline (40 mM) plus F_2Mg (10 mM) (*data not shown*).

In the second approach, fragmented membranes from fresh cells were incubated with radioactive ATP under various conditions altering K^+ channel activity.

The membranes were effectively phosphorylated and the extent of phosphorylation was affected by above conditions. Thus, phosphorylation was increased fivefold in the presence of 1 μM free Ca^{2+} while it was reduced by about 30% upon addition of cAMP or cAMP plus IBMX (0.5 mM) (Table 5). Moreover, it was decreased either to a half after adding theophylline (15 mM) or by nearly 80% when cAMP plus theophylline (10 mM), GTP (0.5 mM) or methoxybenzamide (20 mM) were added. By con-

Table 5. Membrane phosphorylation by protein kinases

Incubation conditions	Phosphate incorporated (nmol ^{32}P/mg protein)	Net phospho-rylation (b-a)	Inhibition (%)
Inactivated by 10 min at 98°C (a)	0.35	—	—
Not inactivated (b):			
Without additions	1.75	1.40	0
AMPc (50 μM)	1.36	1.01	27.9
AMPc (50 μM) + theophylline (10 mM)	0.66	0.31	77.9
AMPc (50 μM) + IBMX (0.5 mM)	1.17	0.82	41.4
MgF_2 (5 mM)	4.74	4.39	−213.4
MgF_2 (5 mM) + theophylline (5 mM)	5.38	5.03	−259.3
GTP (0.5 mM)	0.77	0.42	70.8
Theophylline (15 mM)	1.00	0.65	53.6
Methoxybenzamide (20 mM)	0.42	0.07	95.0

Fragmented membranes from IOV's were phosphorylated in the presence of 17.5 μM (^{32}P)ATP and the different additions shown above, as detailed in the text. Results are presented as mean values from two different experiments.

trast, phosphorylation was raised threefold above control levels after incubating with 5 mM fluoride and further increased when fluoride and theophylline were added together (Table 5).

Very preliminary electrophoretic runs of the above phosphorylated membranes, using continuous acrylamide gradients between 5 and 15% in the presence of SDS, showed that radioactivity associated to polypeptides followed in general the same distribution pattern as that found in the whole membranes. Furthermore, in all cases radioactivity was confined to two high molecular weight polypeptides, about 240 and 320 kDa, respectively (*results not shown*).

Discussion

The present work has examined some effects of metabolism on the net K^+ efflux from Ca^{2+}-loaded, human red cell ghosts. Although there may be various routes for K^+ exit, most of the efflux detected in ATP-loaded ghosts should occur through Ca^{2+}-dependent K^+ channels, as K^+ loss is largely increased upon raising internal free Ca^{2+} (*see* Table 1). A reduction of efflux under these conditions can thus be safely interpreted as an effect on the K^+ channel. This is not necessarily true, however, for the K^+ efflux from ATP-depleted ghosts or for a potentiation of K^+ loss.

Ghosts Heterogeneity

The use of a method analogous to that employed on human erythrocytes to demonstrate heterogeneity of Ca^{2+} distribution [10], has revealed the existence of subpopulations among dextran-resealed ghosts after being incubated in thiocyanate media. Unlike intact cells, ghosts loaded with Ca^{2+} in the presence or absence of ATP, show an homogeneous Ca^{2+} distribution. By contrast, K^+ appears to be distributed dissimilarly only in ATP-loaded ghosts.

Comparable findings in intact cells have been interpreted on the basis of an all-or-none behavior of the K^+ channel [10]. This behavior, however, is associated to an heterogeneous Ca^{2+} distribution, due to a differential pumping activity from cells containing an equally dissimilar ATP concentration [10–12, 42]. In spite of such intrinsic heterogeneity, the channel shows no apparent difference in affinity to Ca^{2+} among the distinct subpopulations when it is analyzed in inside-out vesicles in the absence of ATP [2].

The above observation is at variance with this work. Heterogeneity of ghost K^+ distribution as presently found may be due to a variable channel activity arising from a differential metabolic control of ghost subpopulations.

The results reported here demonstrate that the K^+ channel of human red cells is under metabolic regulation. Depending on internal Ca^{2+}, various kinds of control appear to be involved: a cAMP-mediated effect; another directly related to ATP; and a third one associated with theophylline. To our knowledge, this is the first time that modulation of the Ca^{2+}-dependent K^+ channel by cAMP and theophylline is described in human red cells.

On the other hand, the presence of a theophylline-resistant K^+ efflux, activated by Ca^{2+}, supports the idea of a second type of K^+ channel or an altered form of the original channel, which may be no longer under metabolic control. As this efflux increases with aging in vitro, such a channel behavior may predominate in older cells.

Our findings do not allow us to distinguish between multiple modulation of a single channel type, or the presence of various sets of independently regulated channels. The existence of subpopulations of ghosts which may behave differently is a further complication to consider. We shall simply refer to the different types of metabolic control.

The Control of K^+ Channel by cAMP

Human red cells are permeable to cAMP and they possess an extrusion mechanism for this nucleotide [9, 41]. The present results showed that K^+ efflux from high-K^+ ghosts was reduced significantly by cAMP, when it was present throughout the preparation and final incubation of ghosts. It seems possible that the effectiveness of such a procedure arises from a combination of both high permeability and the pumping capacity of these ghosts. Additionally, phosphodiesterase activity could reduce cAMP to such low levels during resealing to monovalent cations (nearly 45 min at 37°C), that a further supply of the nucleotide is needed in order to show an effect.

The latter view is compatible with the finding that cAMP was more effective when added together with phosphodiesterase inhibitors such as theophylline and IBMX, indicating the presence of active esterases within ghosts. As the action of cAMP showed a strict requirement for both ATP and Mg^{2+}, and not for ATP alone, it can be inferred that ATP hydrolysis is essential for modulating the K^+ channel via cAMP.

Early work has shown that cAMP catalyzes phosphorylation of membrane proteins in human erythrocytes [6, 41]. On the other hand, a number of channels are regulated by cAMP-mediated protein phosphorylation, employing ATP as a phosphoryl donor [20]. The Ca^{2+}-dependent K^+ channel of heart sarcolemma remains in the open state after phosphorylation with a cAMP-dependent protein kinase [43]. A similar effect is obtained with the Ca^{2+}-dependent K^+ channel from molluscan neurons, reconstituted in planar bilayers, after adding both ATP and the catalytic subunit of a cAMP-dependent protein kinase [5]. This effect was not observed if a subunit, previously inactivated with DTNB, was employed.

In view of above findings, it is possible that the red cell K^+ channel can be regulated by cAMP-mediated phosphorylation catalyzed by protein kinases. Such a control may occur either directly, through which channel phosphorylation should lead to its closure, or in an indirect way. The results to be discussed below are consistent with the second alternative.

The possibility of inhibiting cAMP-dependent protein kinases was explored by adding DTNB to the hemolytic medium, with the result that DTNB was both internal and external to the cell membrane. No effect on K^+ loss from ATP-loaded ghosts was found with 10 mM DTNB. This finding, however, should be taken cautiously as SH-reacting compounds, such as glutathione, are abundant in the red cell cytosol. This situation may have restricted reacting of DTNB with cAMP-dependent kinases.

The Modulation by ATP

A dramatic effect on K^+ loss was observed upon ATP loading; $^{\circ}k$ was decreased by almost one order of magnitude. The extent of this inhibition, when compared to that attained with cAMP, would suggest major importance of ATP in K^+ channel modulation. The effect was greater in stored cells due to their larger K^+ loss.

The requirement for Mg^{2+} by the underlying mechanism is low. Although $^{\circ}k$ was decreased by ATP in EDTA-loaded ghosts, it was further diminished after adding 2 mM $MgCl_2$. As ghost Ca^{2+} was buffered with 5 mM EGTA, it is unlikely that 0.5 mM EDTA could have altered the free-Ca^{2+} content significantly, so as to reduce K^+ loss.

The above findings do not allow us to determine if the metabolism of ATP is a requirement for its inhibitory activity. The influence that Mg^{2+} omission might have on the overall ATP effect is not necessarily valid as a criterion for demonstrating ATP metabolism. Ca^{2+} ions can also be cofactors in kinase reactions involving ATP [24]. However, the results with the nonmetabolizable ATP analogue in pilot tests, albeit preliminary, lend strong support for the idea that the metabolism of ATP is essential for its inhibitory action.

In addition, a direct effect of ATP on the K^+ channel can be discarded. Nonmetabolizable analogues of ATP, like ATP in the absence of Mg^{2+}, have been reported to block K^+ channels in heart, pancreatic β-cells and skeletal muscle [4, 19, 40].

The work of Simkowski and Tao [38] may be of significance in understanding the mechanism of action of ATP, since they showed inhibition by fluoride of cAMP-independent protein kinases from human red cells. A large stimulation of K^+ loss by fluoride was obtained in our work. The effect was not due to an altered free Ca^{2+} or Mg^{2+} content, since internal Ca^{2+} was buffered with EGTA, and fluoride and magnesium ions were added stoichiometrically. This suggests the possibility that ATP could be substrate for a cAMP-independent protein kinase, whose activity should lead to channel closure.

Modulation by ATP of Ca-independent K^+ channels has been already reported in pancreatic β-cells, where channel phosphorylation results in its closure [7, 26]. It is possible that a similar situation occurs in ghosts, and that a large fraction of the K^+ efflux found in the absence of ATP may not be due to activity of Ca^{2+}-dependent channels.

Influence of Theophylline

Theophylline alone, like IBMX, was also found to be inhibitory. This effect was clearly distinct from that involving cAMP, as it was observed in its absence and was overcome by raising free Ca^{2+}. Theophylline required the presence of ATP in order to be effective, thus ruling out the possibility of a direct blocking effect.

Theophylline is a rather nonspecific compound which, depending on the concentration used, may bind to adenosine receptors [8], block cAMP phosphodiesterases [3] and may, finally, inhibit ADP-ribosyl transferases (*cf.* ref. [30]).

It is perhaps in the latter context that theophylline action might be seen. As shown in pilot experiments, 3-methoxybenzamide, a very potent inhibitor of ADP-ribosyl transferases [30], affects K^+ loss in a similar way as theophylline. It seems therefore probable that theophylline action might occur via ADP-ribosyl transferases.

In addition, our findings have demonstrated that GTP reduces $^{\circ}k$ in ATP-loaded ghosts and that MgF_2 enhances the K^+ loss. Since G proteins are greatly stimulated by fluoride ions in the presence of Mg^{2+} [14], the results could suggest an involvement of G proteins in channel regulation. Evidence is now accumulating in support of channel modulation by ADP-ribosylating G proteins [18, 28]. It is tempting therefore, to suggest additionally the possibility of such control on the red cell K^+ channel.

Membrane Phosphorylation

An important finding was that in the presence of Ca^{2+}, membranes become phosphorylated from radioactive ATP when incubated under conditions affecting the K^+ channel. Furthermore, the degree of phosphorylation achieved is in direct correspondence with the effect of such conditions on channel activity. Thus, the amount of tracer phosphate incorporated is increased when the K^+ channel is in a conducting state, as occurs in the presence of an increased free-Ca^{2+} concentration and when fluoride is also added. By contrast, phosphorylation is diminished by a wide variety of conditions decreasing channel activity, such as presence of either cAMP plus theophylline, theophylline alone, GTP or methoxybenzamide.

These results indicate that membrane phosphorylation is closely related to channel activity. Although many different protein kinases may be contributing to phosphorylation, having complete opposite effects on channel activity, the above findings demonstrate that the net phosphorylation level in the presence of Ca^{2+} determines the state of activity of the K^+ channel. This suggests that phosphorylation of the channel by a Ca^{2+}-dependent protein kinase leads to its open state.

As the level of membrane phosphorylation most probably reflects an steady state, perhaps the simplest explanation for the effect of cAMP plus methylxanthines described above, could be through activation of a phosphatase by a cAMP-dependent kinase. This enzyme in turn would decrease the level of channel phosphorylation, resulting in its closure. According to this idea, cAMP-dependent kinase inhibitors should not decrease K^+ efflux, in agreement with the lack of effect DTNB reported previously.

In the above line of thought, early work has shown that low concentrations of fluoride ions inhibit a phosphoprotein phosphatase present in the human red cell cytosol [15].

Aging and the Metabolic Control of K^+ Channel

An involvement of the Ca^{2+}-dependent K^+ channel in the trapping and destruction of senescent cells has been suggested previously [32]. The presence of various mechanisms for modulating K^+ channel activity, far from being redundant, would seem to maintain the red cell life span within physiological limits.

It is suggestive that human erythrocytes do not possess adenylate cyclase activity [44] while having all the enzymatic battery to metabolize cAMP, in addition to the presence of a wide variety of protein kinases. The present work has shown an increased susceptibility of the K^+ channel in stored cells to the action of ATP, or cAMP plus methylxanthines. The possibility exists that the progressive loss of metabolic control over the K^+ channel, arising from a protein kinase-mediated membrane signalling during aging, might trigger the physiological removal of senescent cells.

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