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Effect of the ionic strength and prostaglandin E_2 on the free Ca^{2+} concentration and the Ca^{2+} influx in human red blood cells

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Abstract

Human red blood cells (RBCs) were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 to investigate the effects of media ionic strength and prostaglandin E_2 (PGE_2) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ of intact RBCs in a Ca^{2+}-containing physiological (high) ionic strength (HIS) solution was 75.1 ± 8.3 nM after 5 min incubation, increasing to 114.9 ± 9.6 nM after 1 h. In Ca^{2+}-containing low ionic strength (LIS) solutions, $[Ca^{2+}]_i$ was significantly lower than in the Ca^{2+}-containing HIS solution ($p = 0.041$ or 0.0385 for LIS solutions containing 200 or 250 mM sucrose, respectively), but, as in HIS solution, an increase of $[Ca^{2+}]_i$ was seen after 1 h. In Ca^{2+}-free (0 Ca^{2+} plus 15 μM EGTA) media, $[Ca^{2+}]_i$ decreased (ranging from 15 to 21 nM), but were not significantly different in HIS or LIS, and did not change following 1 h incubation. The effect of the ionic strength and PGE_2 on passive Ca^{2+} influx was investigated on ATP-depleted RBCs. Ca^{2+} influx was faster during the initial 10 min in comparison with the subsequent time period (10 − 45 min), both in HIS and LIS media, decreasing from 20.3 ± 1.9 to 12.9 ± 1.3 μmol/(l_{cells} × h) in HIS, and from 36.7 ± 5.3 to 8.6 ± 1.2 μmol/(l_{cells} × h) in LIS. Prostaglandin E_2 (PGE_2; 10^{-7}–10^{-11} M), dissolved in deionised water or in ethanol, did not affect $[Ca^{2+}]_i$ in either normal or in ATP-depleted RBCs suspended in Ca^{2+}-containing HIS medium. Finally, the addition of carbachol (100 μM) did not affect $[Ca^{2+}]_i$. The present findings suggest that stimulation of the Ca^{2+}-activated K^+ channel by PGE_2, reported in [J. Biol. Chem. 271 (1996) 18651], cannot be mediated via increased $[Ca^{2+}]_i$.

Keywords: Red blood cells; Ca^{2+} concentration; Ca^{2+} influx; Ionic strength; Prostaglandin

1. Introduction

The total calcium content of red blood cells (RBCs) is about 2 − 5 μmol/l_{cells} [2], but there are large discrepancies in the literature for the value of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), ranging from about 10 to 30 nM (when measured with Ca^{2+} chelators like benz-2, BAPTA, or Quin MF [3–5]) to about 70–90 nM (using Ca^{2+} fluorescent dyes like quin-2 or fura-2 [6–10]). Advantages and disadvantages of these different methods are discussed in detail by Tiffert et al. [11]. Three mechanisms are responsible for the maintenance of the relatively low $[Ca^{2+}]_i$: (i) a powerful Ca^{2+} transport extrusion mechanism realised by the Ca^{2+} pump, (ii) various intracellular Ca^{2+}-binding components such as calmodulin and polar phospholipid head groups on the inner membrane leaflet, and (iii) a low passive Ca^{2+} permeability of the RBC membrane. Whereas the Ca^{2+} pump is relatively well investigated, this does not hold for the passive Ca^{2+} fluxes across the RBC membrane.

It is well known that there is an increase of Na^+ and K^+ influxes as well as effluxes of human red blood cells when the ionic strength of the extracellular solution is reduced [12–16]. One aim of the present study was to establish whether the same effect could be observed also for the Ca^{2+} flux. It is not clear to what extent two recently identified transport pathways, the $K^+(Na^+)/H^+$ exchanger [16,17] and the nonselective voltage-activated cation channel [18–20], participate in the ionic strength effect. It seems unlikely that the $K^+(Na^+)/H^+$ exchanger can transport Ca^{2+}. On the other hand, it was demonstrated by Kaestner et al. [21], using the patch-clamp technique, that the voltage-activated cation

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channel, which opens at positive transmembrane potentials (i.e. in LIS solutions), is permeable to both monovalent cations and also divalent cations. Before this direct demonstration of a channel permeable to Ca^{2+}, several reports have already suggested the existence of facilitated diffusion mechanism for Ca^{2+} entry (i.e. a channel) in the human RBC membrane [22–24,32]. They are based on the observation that the majority of passive Ca^{2+} influx under physiological conditions occurs via a saturable transport mechanism (with a K_m of approximately 1 mM), which can be trans-stimulated by Ca^{2+}. However, it is still unclear whether other Ca^{2+} transport pathways are present in the human RBC membrane. The Na^+/Ca^{2+} antiport system was not observed in these cells [6]. In addition, reports in the literature on the Ca^{2+} influx in human RBCs in LIS media are controversial. Lew et al. [3] showed an increase of the Ca^{2+} influx in low (Na^+ and K^+), choline, or sucrose media, whereas Ellory et al. [25] found a slight decrease of this flux in sucrose-containing LIS solutions.

A further aim of the present paper was to investigate the effect of PGE_2 on $[Ca^{2+}]_i$ in human RBCs. Li et al. [1] have reported that the addition of PGE_2 to RBC suspensions leads to an increase of K^+ efflux mediated by the Ca^{2+}-activated K^+ (Gardos) channel. The maximal effect was observed at 10^{-10} M PGE_2 resulting in a loss of intracellular K^+ of about 36 mM/h over the first 5 min of stimulation. However, in these investigations $[Ca^{2+}]_i$ was not measured. In contrast, Rasmussen et al. [26] observed a decrease in size of the intracellular Ca^{2+} pool upon addition of 10^{-11} M PGE_2 to RBCs. To determine whether activation of the Ca^{2+}-activated K^+ channel by PGE_2 is caused by an increase of $[Ca^{2+}]_i$ or due to a direct activation of the channel, it is important to measure $[Ca^{2+}]_i$ after PGE_2 addition. Higher activity of the Ca^{2+}-activated K^+ channel, caused by an increase of $[Ca^{2+}]_i$, could result from activation by PGE_2 of the nonselective voltage-activated cation channel, as shown by Kaestner and Bernhardt [27]. As these authors showed, addition of PGE_2 leads to a channel opening at negative transmembrane potentials, i.e. in HIS solutions. As stated above, Ca^{2+} can pass through the channel, which might result in a rise in intracellular Ca^{2+}, in turn activating the Ca^{2+}-activated K^+ channel.

In the present paper, therefore, we used the Ca^{2+}-sensitive fluorescence dye fura-2 to measure $[Ca^{2+}]_i$ in normal human RBCs as well as the Ca^{2+} influx in ATP-depleted RBCs, both in HIS and LIS solutions. In addition, we investigated the effect of PGE_2 on $[Ca^{2+}]_i$ in normal and ATP-depleted cells in HIS media.

2. Experimental

2.1. Blood and solutions

Fresh human RBCs were collected from healthy donors and washed three times (2000 × *g*, 8 min) at room temperature in the HIS solution containing (mM): NaCl 145, glucose 10, morpholinoethanesulphonic acid/tris-(hydroxymethyl)aminomethane (MES/Tris) 10, pH 7.4. Supernatant and buffy coat were removed by aspiration. The cells were then washed once in the appropriate medium used for the experiment. Experiments were carried out in the HIS solution or in solutions where 145 mM NaCl was replaced by 250 or 200 mM sucrose (LIS). For the measurements in Ca^{2+}-containing or Ca^{2+}-free HIS and LIS solutions, the cells were added directly to the solutions immediately before the experiment (without washing them in these media). These solutions had the same composition as the HIS and LIS solutions, with the addition of 2 mM Ca^{2+} (Ca^{2+}-containing solutions) or 15 μM EGTA (Ca^{2+}-free solutions). The pH of all solutions was 7.4 at 37 °C.

2.2. Measurement of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

The cell-permeable acetoxymethyl ester of fura-2 (fura-2/AM; Molecular Probes, Leiden, Netherlands) was used to monitor $[Ca^{2+}]_i$ in RBCs. 10 – 20 μl of packed RBCs were resuspended at ~ 1% haematocrit in the Ca^{2+}-containing or Ca^{2+}-free HIS solution at 37 °C. Erythrocytes were loaded with 0.5 μM fura-2 AM (taken from a 5 mM stock solution, in DMSO) in 2-ml Eppendorf tubes under vigorous stirring. The RBC suspension was then incubated for 45 min at 37 °C with intermittent mixing as described in [9,10]. Finally, the cells were washed three times in the corresponding HIS solution (15 s, 12,000 × *g*) to remove any extracellular fura-2. The RBCs were stored at ~ 40% haematocrit at 37 °C for 15 – 30 min. Aliquots of the RBC suspension were diluted to ~ 0.067% haematocrit in Ca^{2+}-containing or Ca^{2+}-free HIS solution and transferred into a quartz cuvette for fluorescence measurements (using a spectrophotometer, FluoroMax-2, Jobin Yvon, France). When investigations were carried out in Ca^{2+}-containing or Ca^{2+}-free LIS solution, the RBCs were transferred to these solutions from the corresponding HIS solutions immediately before the measurements.

Intracellular free Ca^{2+} levels were determined using a two-wavelength method as described in Ref. [28]. The emission wavelength was set at 510 nm, with excitation wavelengths of 340 and 380 nm. All experiments were performed at 37 °C under magnetic stirring. Data were analysed by the software package (DataMax, version 2.20) that allows a fluorescence record every 1.3 s. $[Ca^{2+}]_i$ was determined from the standard equation: $[Ca^{2+}]_i = K_D \times Q \times (R - R_{min})/(R_{max} - R)$, where K_D is the Ca^{2+} dissociation constant of fura-2. R represents the ratio of the fluorescence intensities measured at 340 and 380 nm; R_{max} and R_{min} were found when fura-2 was saturated with Ca^{2+} and when completely free of Ca^{2+}, respectively. Q is the ratio of the minimum/maximum fluorescence intensity at 380 nm, i.e. the fluorescence intensity measured when fura-2 is free of Ca^{2+}, and saturated with Ca^{2+}, respectively. R_{min} and R_{max} were estimated under all experimental conditions separately (but

see below). For the determination of R_{max}, 0.01% Triton was added to the cuvette with the fura-2-loaded RBCs. At the end of the experiment, 10 – 20 mM EGTA was added to estimate R_{min}.

K_D values were determined after fura-2-loaded RBCs were lysed with Triton X-100 in HIS solution. Part of the lysate was transferred into a HIS medium of a known free Ca^{2+} concentration (0 – 11.98 μM). A series of solutions with different known free Ca^{2+} concentrations were obtained from the mixing of two main stock solutions (denoted A and B), in various proportions [29]. Both solutions were the same as the HIS solution. In addition, solution A contained 10 mM EGTA, whilst solution B contained 10 mM nominally equimolar Ca-EGTA. The dissociation constant of Ca^{2+} for fura-2 measured under our experimental condition was 155 nM. This value is in good agreement with the $K_D = 140$ nM estimated by Molecular Probes in MOPS buffer, pH 7.2 at 22 °C in the absence of Mg^{2+}.

To evaluate the effect of PGE_2 on $[Ca^{2+}]_i$ of RBCs, experiments were carried out on normal and ATP-depleted RBCs. PGE_2 dissolved in ethanol or deionised water was added to the cuvette immediately before the beginning of the fluorescence recordings. Precautions were taken to minimise a possible prostaglandin oxidation, with PGE_2 stock solutions being prepared and stored under nitrogen.

2.3. Analysis of $[Ca^{2+}]_i$ in LIS media

The use of the equation given above for the calculation of $[Ca^{2+}]_i$ requires an accurate determination of R_{max} and R_{min} at each individual fluorescence ratio measurement. Correct measurement of these calibration parameters requires lysis of the cells and the titration of the dye released. Although methods have been proposed to obtain R_{max} and R_{min} without lysis, e.g. using ionophores (A23187 or ionomycin) to saturate the fluorescent dye with Ca^{2+} and then to quench it with Mn^{2+}, there is no evidence that such determinations give the true end points (minimal and maximal fluorescence intensity), which should be identical to those obtained after cell lysis. Since it has been reported that the solution viscosity, pH and ionic strength affect the fluorescence properties of fura-2, the influence of the pH and the ionic strength of the solution on the ratio of the fluorescence intensities at 340 and 380 nm was investigated in LIS solutions. No significant change of the fluorescence ratio (340 nm/380 nm) in the pH interval 6.8 – 7.8 could be determined (not shown). However, quenching of the fura-2 fluorescence ratio in LIS medium was observed after lysis of the RBCs (Fig. 1). Therefore, the values for R_{max} and R_{min} obtained in the experiments with RBCs in HIS solution were used for the experiments in LIS solutions.

2.4. Measurement of the Ca^{2+} influx of ATP-depleted RBCs

Ca^{2+} uptake was measured in ATP-depleted RBCs that had been pretreated by the method described by others [9,10,30]. RBCs at 1% haematocrit were incubated in a medium containing (mM) NaCl 140, KCl 5, iodoacetate 6, inosine 6, pH 7.4 (medium C). After at least 2 h from the beginning of the incubation 0.5 μM fura-2 was added to the medium (45 min loading procedure). The cells were then washed three times with the medium C to eliminate the excess of extracellular fura-2, diluted (also in medium C) to an haematocrit ~ 40% and stored for 15 – 30 min. Aliquots of the RBC suspension were diluted to ~ 0.067% haematocrit in Ca^{2+}-containing HIS or LIS solution and transferred into a quartz cuvette to measure the fluorescence. Ca^{2+} influx was calculated from the changes of $[Ca^{2+}]_i$ and expressed in μmol per l cells per hour.

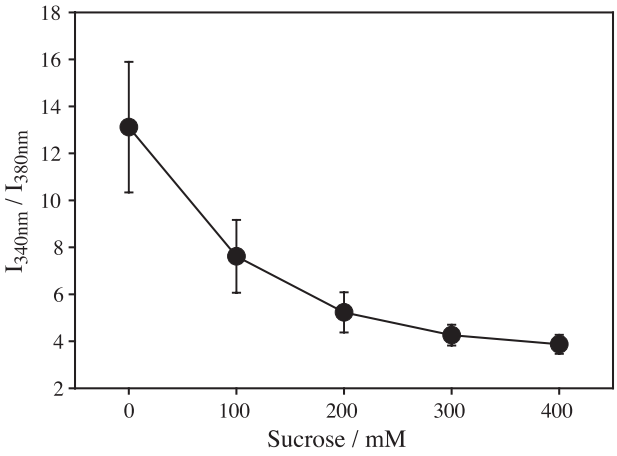

Fig. 1. Quenching of fura-2 fluorescence in LIS media of various sucrose concentrations in the presence of 2 mM $CaCl_2$ in comparison with HIS solution (zero sucrose concentration).

2.5. Reagents

Inorganic salts, sucrose and glucose were of analytical grade. Inosine, iodoacetate, and PGE_2 were obtained from Sigma (St. Louis, MO, USA), Tris and EGTA from Fluka (Buchs, Switzerland), MES from SERVA (Heidelberg, Germany), and fura-2/AM from Molecular Probes.

2.6. Statistical treatment of the results

Values are given as means of at least three independent experiments carried out on blood of different donors ± S.D. Where errors are not shown they were smaller than the symbols. Where necessary, a paired *t*-test was used to determine the significance of the difference between values. The values were taken as significantly different when $p < 0.05$.

3. Results

$[Ca^{2+}]_i$ of intact RBCs in Ca^{2+}-containing HIS solutions was 75.1 ± 8.3 nM. This value is similar to those reported

Table 1
Intracellular free calcium concentration ($[Ca^{2+}]_i$) in low ionic strength (LIS) solution in the presence (2 mM) or absence (15 μM EGTA added) of extracellular Ca^{2+} determined after 5 min and 1 h of incubation in the corresponding solutions

	$[Ca^{2+}]_i$ (nM) after 5 min	$[Ca^{2+}]_i$ (nM) after 1 h
LIS solution containing 2 mM Ca^{2+}		
200 mM sucrose	50.0 ± 6.5	67.1 ± 8.6
250 mM sucrose	35.7 ± 6.5	59.7 ± 8.7
LIS solution free of Ca^{2+} (15 μM EGTA added)		
200 mM sucrose	19.2 ± 4.8	17.7 ± 1.6
250 mM sucrose	21.4 ± 3.7	14.7 ± 3.0

The values represent means of at least four independent experiments ± S.D.

by others [6,9,10,22,30]. After 1 h of incubation in the Ca^{2+}-containing HIS medium, $[Ca^{2+}]_i$ increased to 114.9 ± 9.6 nM. $[Ca^{2+}]_i$ in a Ca^{2+}-free HIS medium (15 μM EGTA added to the medium) was 17.3 ± 4.3 nM. No significant change was observed after 1 h of incubation in this medium (17.0 ± 4.5 nM). These results in Ca^{2+}-free HIS solutions are in good agreement with those reported by Lew et al. [3] and Foder and Scharff [31].

The measurements of $[Ca^{2+}]_i$ in LIS media were carried out in the presence or absence of extracellular Ca^{2+} in solutions containing 200 or 250 mM sucrose. The solution that is commonly used to replace 145 mM NaCl contains 250 mM sucrose to maintain osmolarity. However, to avoid an initial shrinkage of the cells, that happens in the solution containing 250 mM sucrose, a solution was used that containing only 200 mM sucrose. Results are presented in Table 1. After 5 min or 1 h of incubation in both Ca^{2+}-containing LIS solutions, $[Ca^{2+}]_i$ was significantly lower than in the Ca^{2+}-containing HIS solution ($p = 0.041$ and 0.0385, in 200 and 250 mM of sucrose, respectively), but, as for HIS solution, an increase of $[Ca^{2+}]_i$ was also seen after 1 h in both Ca^{2+}-containing LIS solutions. In addition, a significant fall of $[Ca^{2+}]_i$ in both LIS media was observed when the extracellular solution lacked Ca^{2+} ($p = 0.034$ and

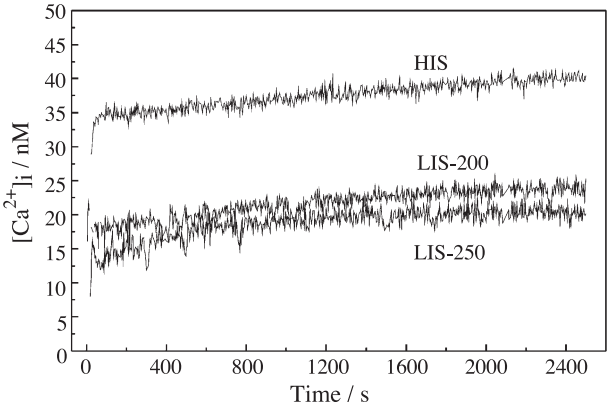

Fig. 2. Intracellular free calcium concentration ($[Ca^{2+}]_i$) in human RBCs in physiological (HIS) and low ionic strength (LIS) solutions. $[Ca^{2+}]_i$ was determined using fura-2. Traces are taken from a single experiment, representative of at least three others.

Table 2
Ca^{2+} influx into ATP-depleted cells in Ca^{2+}-containing physiological (HIS) and low ionic strength (LIS) solutions estimated over the time intervals $t = 0 - 10$ min and $t = 10 - 45$ min

	Ca^{2+} influx, μmol/(l_{cells} × h)	
	$t = 0 - 10$ min	$t = 10 - 45$ min
HIS	20.3 ± 1.9	12.9 ± 1.3
LIS (200 mM sucrose)	33.5 ± 4.6	9.7 ± 1.7
LIS (250 mM sucrose)	36.7 ± 5.3	8.6 ± 1.2

The values represent means of four independent experiments ± S.D.

0.0025 for 200 and 250 mM of sucrose, respectively). Again, as for HIS solution, there was no significant change of $[Ca^{2+}]_i$ after 1 h of incubation in the absence of extracellular Ca^{2+}. Furthermore, all values for $[Ca^{2+}]_i$ obtained in the Ca^{2+}-free HIS and LIS solutions are not significantly different.

Representative measurements of the Ca^{2+} uptake of ATP-depleted RBCs in the Ca^{2+}-containing HIS, LIS-200 and LIS-250 solutions are shown in Fig. 2. The average value of the Ca^{2+} influx obtained from four independent experiments is given in Table 2. Ca^{2+} influx was faster during an initial period of 10 min in comparison to the subsequent time period (10 − 45 min). However, the values for Ca^{2+} influx obtained in the Ca^{2+}-containing HIS solution are in good agreement with previous estimates using different methods [6,22,30,32].

As for HIS solutions, in LIS solutions, there was also a decrease in the flux with time. During the first time interval (0 − 10 min), there was a slight increase of the Ca^{2+} influx in both LIS solutions (compared to HIS); in the second time interval (10 − 45 min), these fluxes were slightly smaller than those in HIS solution. Over the 45 min, fluxes in HIS and LIS media were not significantly different.

The effect of PGE_2 on $[Ca^{2+}]_i$ of RBCs was studied after 5 and 45 min of incubation in the Ca^{2+}-containg HIS medium. Experiments were carried out with PGE_2 at concentrations ranging from 10^{-7} to 10^{-11} M. For one set of experiments PGE_2 was dissolved in ethanol, for the other it was dissolved in deionised water. In all cases, there was no significant effect on $[Ca^{2+}]_i$ (data not shown). These experiments were repeated with ATP-depleted cells, with the same result (i.e. no significant effect of PGE_2, 10^{-7}–10^{-11} M, on $[Ca^{2+}]_i$). Finally, the effect of PGE_2 was investigated in the presence of carbachol (100 μM), which simulates the opening of the nonselective voltage-activated cation channel [21,33]. Again, no significant change of $[Ca^{2+}]_i$ was observed.

4. Discussion

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated in human RBCs incubated in solutions of physiological and low ionic strength using the Ca^{2+}-sensitive fluorescent dye fura-2. Values of $[Ca^{2+}]_i$ obtained in

controls (i.e. Ca^{2+}-containing HIS medium) were similar to those already reported in literature [8–10]. The slight increase of $[Ca^{2+}]_i$ after 1 h of incubation in the Ca^{2+}-containing HIS solution could result from reduction in the activity of the Ca^{2+} pump. The incubation of RBCs in Ca^{2+}-free HIS solution containing EGTA (resulting in a level of ionised Ca $<10^{-13}$ M [24]) lead to a reduction of $[Ca^{2+}]_i$ within the first 5 min of incubation. Such an effect has already been reported [24,31]. It has been proposed that this decrease in $[Ca^{2+}]_i$ results from Ca^{2+} extrusion by the Ca^{2+} pump [24], subsequent to removal of Ca^{2+} bound to the outer membrane surface by EGTA and also Ca^{2+} release from intracellular binding sites.

RBCs transferred from the Ca^{2+}-containing into the Ca^{2+}-free LIS solution showed the same response to the reduction of extracellular Ca^{2+} as those in HIS media. Interestingly, the measured $[Ca^{2+}]_i$ in Ca^{2+}-containing LIS solution is lower than in the corresponding HIS solution, although the passive Ca^{2+} influx is higher in the LIS solution in the first 10 min of incubation (estimated with ATP-depleted cells). To explain this decrease in $[Ca^{2+}]_i$, one has to assume (i) an activation of the Ca^{2+} pump immediately after the beginning of a higher Ca^{2+} uptake in LIS solution, and/or (ii) a higher binding capacity of $[Ca^{2+}]_i$ at intracellular binding sites of the RBCs in LIS solutions. Taking into account alkalinisation of the cytoplasm after transfer to the LIS solution (reaching a $pH_i \sim 7.7$, which is reduced to the normal level of ~ 7.2 over the next 30 min (see below and Ref. [34]), an increase in calcium binding capacity of low-affinity intracellular binding sites of phospholipids can be expected. Moreover, there are no available data suggesting that alkalinisation, unlike acidification, affects calcium binding properties of another important cytosolic calcium buffer – calmodulin (CaM), which is present in human erythrocytes at micromolar (3 – 5 μM) concentration [35]. It was shown that 5% of the CaM in erythrocytes is involved in the interaction with Ca^{2+}-ATPase and that 95% of CaM is available for the interaction with other membrane proteins and modulation of their interactions [36]. There is an absolute Ca^{2+} requirement for CaM-induced regulation of all protein interactions. Small increases in Ca^{2+} concentrations would result in rapid Ca^{2+} binding to the amino-terminal domain of CaM, which has the potential to ensure fast activation of Ca^{2+}-ATPase necessary for rapid calcium removal with Ca^{2+} pump [37]. It is also worth noting that subsequent activation of the Ca^{2+}-ATPase requires an association with CaM bound to two Ca^{2+}. Taking into account the dissociation constants of the $(Ca^{2+})_2$–CaM complex (23 nM [35]) and the Ca^{2+}–fura-2 complex (155 nM, estimated in the present paper), CaM appears to compete with fura-2 for Ca^{2+}, since it has a slightly lower Ca^{2+} binding energy (– 10.82 kcal/mol) as fura-2 (– 9.5 kcal/mol). In Ca^{2+}-free HIS and LIS solution, however, $[Ca^{2+}]_i$ was nearly the same and was not altered by incubation over 1 h, suggesting that this free intracellular Ca^{2+} concentration represents the nonextractable pool of $[Ca^{2+}]_i$, which cannot be further decreased. The estimated low level of $[Ca^{2+}]_i$ is in agreement with the corresponding values reported by Lew et al. [3] and Foder and Scharff [31].

The nonlinear kinetics of the Ca^{2+} influx seen in Ca^{2+}-containing HIS and LIS solutions is characterised by two phases: (i) a fast increase of $[Ca^{2+}]_i$ over the first 10 min followed by (ii) a slower increase over the next 35 min. As can be seen from Table 2, the Ca^{2+} uptake is faster in LIS media compared to HIS solution over the first 10 min of the experiment. Such an effect might be expected from the change in transmembrane potential from about – 10 mV in the HIS solution to about +40 mV in the LIS solutions. However, such a relative high positive transmembrane potential occurs only immediately after the cells are transferred into the LIS solutions. During the next 30 min, the cells will lose a significant amount of KCl. In addition, the intracellular pH, which increases to about 7.7 after transferring the RBCs into the LIS solutions [17], is reduced below its original value in HIS solution of 7.2. Therefore, it is evident that the transmembrane potential of the RBCs in the LIS solutions will return to its original physiological value over a 30-min time period in these media (for detailed discussion of this process, see Ref. [34]). At conditions where the RBCs have a high (positive) transmembrane potential, i.e. immediately after the cells are transferred into the LIS solutions, there will be an activation of the nonselective voltage-activated cation channel resulting in an increase of the Ca^{2+} influx in comparison to the HIS solution [21]. This effect is small in the absence of carbachol, which simulates the channel opening [33]. Such an explanation also fits with the findings of Donlon and Rothstein [13] who found that the stimulation of the K^+ efflux in LIS solutions was more pronounced when the transmembrane potential was higher than +40 mV. This effect could be interpreted as an activation of the $K^+(Na^+)/H^+$ exchanger at reduced ionic strength of the solutions and an additional activation of the nonselective voltage-activated cation channel at transmembrane potentials higher than +40 mV. The presence of a nonlinear kinetics in the HIS solutions can be explained by various mechanisms: (i) the existence of more than one unknown passive Ca^{2+} pathway present in the RBC membrane, and/or (ii) the activation of the Ca^{2+} pump. The second possibility cannot be ruled out although the intracellular ATP concentration is low (but not zero). Therefore, an immediate increase of $[Ca^{2+}]_i$ can result in an activation of the Ca^{2+} pump [11,38]. As it was already shown by Wüthrich et al. [39], a large Ca^{2+} influx can generate intracellular ATP, restoring pump activity.

To evaluate the mechanism of PGE_2 activation of the Ca^{2+}-activated K^+ channel postulated by Li et al. [1], we studied the effect of PGE_2 on $[Ca^{2+}]_i$ in fura-2-loaded cells. Our results show that the addition of PGE_2, dissolved either in deionised water or in ethanol, over a wide range of concentrations ($10^{-7} - 10^{-11}$ M), had no significant effect on $[Ca^{2+}]_i$. In this respect, it seems of importance to note that Rasmussen et al. [26] described

a decrease of the intracellular Ca^{2+} pool of RBCs after the addition of 10^{-11} M PGE_2. How can activation of the Ca^{2+}-activated K^+ channel be explained in the absence of an increase of $[Ca^{2+}]_i$? Two possibilities should be considered: (i) a direct activation and/or an increase of the sensitivity of the Ca^{2+}-activated K^+ channel to Ca^{2+} induced by PGE_2 or (ii) a Ca^{2+} uptake by the RBCs that is immediately buffered, increasing the overall Ca^{2+} pool (although this would be in contrast to the findings of Rasmussen et al. [26]) and/or transported out of the cells by an activated Ca^{2+} pump (in this case, an activation of the Ca^{2+} pump in ATP-depleted cells has to be assumed, see above). The first possibility is supported by the fact that PGE_2 also directly affects the nonselective voltage-activated cation channel [27]. However, it is still surprising that no significant change of $[Ca^{2+}]_i$ after the addition of PGE_2 to the RBC suspension could be observed since under these conditions Ca^{2+} should enter the cells via the nonselective voltage-activated cation channel [27]. In this respect, the second possibility cannot be ruled out. However, the underlying mechanism is unclear at the moment and deserves further investigations.

Acknowledgements

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