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Properties of the basal calcium influx in human red blood cells

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Abstract

The basal $^{45}Ca^{2+}$ influx in human red blood cells (RBC) into intact RBC was measured. $^{45}Ca^{2+}$ was equilibrated with cells with $t_{1/2}$ = 15–20 s and the influx reached the steady state value in about 90–100 s and the steady state level was 1.5 ± 0.2 μmol/$l_{packed\ cells}$ (n = 6) at 37 °C. The average value of the Ca^{2+} influx rate was 43.2 ± 8.9 μmol/$l_{packed\ cells}$ hour. The rate of the basal influx was pH-dependent with a pH optimum at pH 7.0 and on the temperature with the temperature optimum at 25 °C. The basal Ca^{2+} influx was saturable with Ca^{2+} up to 5 mmol/l but at higher extracellular Ca^{2+} concentrations caused further increase of basal Ca^{2+} influx. The $^{45}Ca^{2+}$ influx was stimulated by addition of submicromolar concentrations of phorbol esters (phorbol 12-myristate-13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu)) and forskolin. Uncoupler (3,3′,4′,5-tetrachloro-salicylanilide (TCS) 10^{-6}–10^{-5} mol/l) inhibited in part the Ca^{2+} influx. The results show that the basal Ca^{2+} influx is mediated by a carrier and is under control of intracellular regulatory circuits. The effect of uncoupler shows that the Ca^{2+} influx is in part driven by the proton-motive force and indicates that the influx and efflux of Ca^{2+} are coupled via the RBC H^+ homeostasis.

Keywords: Red blood cell; Basal Ca^{2+} influx; Uncoupler

1. Introduction

Ca^{2+} ions enter the cytoplasm after the membrane receptors and/or channels are activated by a signalling event [1–3]. On the other hand, Ca^{2+} also enters the cytoplasm without stimulation by external stimuli (including the membrane potential changes) and this could be revealed experimentally, e.g. by labelling of cells with $^{45}Ca^{2+}$. This process is denoted as the basal Ca^{2+} influx. In contrast to ligand- or potential-induced changes in Ca^{2+} fluxes, the basal Ca^{2+} is much less referred to in the literature, usually mentioned as control. The rates of basal Ca^{2+} influx vary in magnitude (expressed in mol/mg protein). The highest influx was observed in synaptosomes [4], but was lower [5,6] in blood platelets. The magnitudes and properties of the basal Ca^{2+} influx may represent a characteristic feature (parameter) of the cell and probably is under genetic control. However, the systematic and exhausting data, which could elucidate the processes underlying the basal Ca^{2+} influx, are still missing.

Human red blood cells (RBC) are a prototype of cells, which exhibit the basal Ca^{2+} transport only, as their membranes do not possess receptor-mediated functions and they apparently do not contain cytoplasmic organelles. There are also numerous reports describing the character of the basal Ca^{2+} transport. Lew et al. [7], McNamara and Wiley [8] and also Pokudin and Orlov [9] have measured the rates of the $^{45}Ca^{2+}$ influx in presence of calcium chelator in cells. These papers showed the saturatability of the transport and its insensitivity to the monovalent cation mediums composition. This approach although enables comfortable measurement, but probably does not yield a true value of the rates due to the (additional) buffering of the cytoplasm, which may distort the original concentration conditions and increases the total Ca^{2+} accumulation in the cytoplasm [10].

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; diSC$_3$-(5), 3,3′-dipropylthiodicarbocyanine iodide; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine-*N*,*N*′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; TCS, 3,3′,4′,5-tetrachloro-salicylanilide

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Desai et al. [11] reported the description of the Ca^{2+} influx observed after eliminating the active Ca^{2+} extrusion and found that it matches the known rate of the Ca^{2+} efflux. Engelmann et al. [12] measured the Ca^{2+} influx without entrapping the Ca^{2+} chelator and showed that the kinetics of the basal Ca^{2+} influx is faster than that in Ca^{2+} chelator-loaded cells and could be stimulated by external ligand, epidermal growth factor. Varečka et al. [13] found that the basal $^{45}Ca^{2+}$ measured without entrapped Ca^{2+} chelator is not sensitive to the changes in external Na^+ and K^+ concentrations. Recently, Andrews et al. [14] using the fura-2 fluorescence measurements published a more detailed description of the basal Ca^{2+} influx in human RBCs and shown that it is stimulated by phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C (PKC). Soldati et al. [15] used the same approach and suggested that the Ca^{2+} influx is mediated by membrane potential-operated channel, and, in another paper [16], studied the mechanism of the parathyroid hormone on the calcium influx. However, the complete transport characteristics of the basal Ca^{2+} influx are still missing.

The problem intimately connected with the existence of the basal Ca^{2+} influx follows from the charge-compensating properties of the Ca^{2+}-ATPase, which according to several studies [17–19], catalyses the ATP-dependent electroneutral $Ca^{2+}/2H^+$, or partially compensated Ca^{2+}/H^+ exchange [20]. This implies that there is net influx of one to two protons after each cycle of basal Ca^{2+} influx. Such an influx might, if not compensated, dramatically change cytoplasmic pH in the steady state. Another implication of these properties of the Ca^{2+}-ATPase is that there is a net influx of charge during each cycle of the cyclic Ca^{2+} flux. This aspect has been also analysed, in addition to the basic properties of the Ca^{2+} influx, in this paper.

2. Materials and methods

2.1. Red blood cell suspension

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into acid-citrate-dextrose anticoagulant in the local blood transfusion station and was used at a second day after blood withdrawal being stored at 0–4 °C. RBC were isolated after centrifugation of the blood (10 min at $600 \times g$), an aspiration of the supernatant with the buffy coat and three-fold washing and, finally, suspending into a medium containing (in mmol/

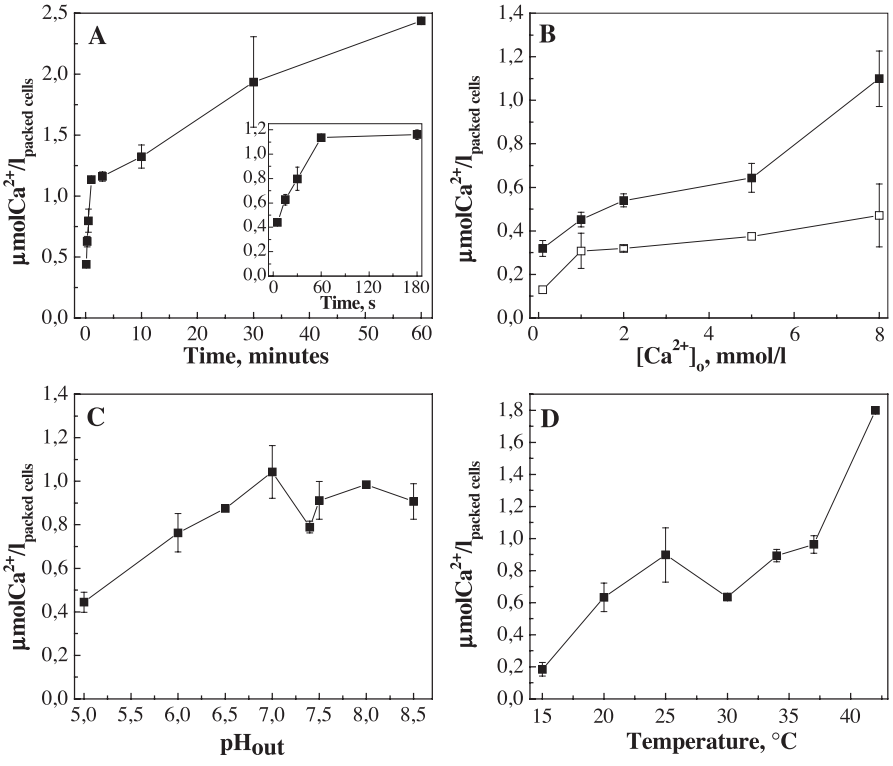

Fig. 1. Basic characteristics of $^{45}Ca^{2+}$ influx into human red blood cells. (A) Kinetics of $^{45}Ca^{2+}$ influx was measured by addition of 2.5 mmol/l Ca^{2+} at 37 °C. Inset shows data of initial phase of the time dependence of basal influx re-plotted in expanded time scale. (B) Concentration dependence of Ca^{2+} influx at 37 °C at zero time (□) and after 3 min (■). (C) Dependence of $^{45}Ca^{2+}$ influx at 37 °C with 2.5 mmol/l Ca^{2+} on external pH. (D) Temperature dependence of $^{45}Ca^{2+}$ influx was measured 10 min after addition of radionuclide. Concentration of Ca^{2+} was 2.5 mmol/l. In panels C and D, the values are corrected for measurement at time zero. All plots are representative from the independent experiments with 30% RBC suspensions. ($n_A = 6$, $n_B = 3$, $n_C = 2$, $n_D = 4$). Points are mean ± S.E. of duplicates.

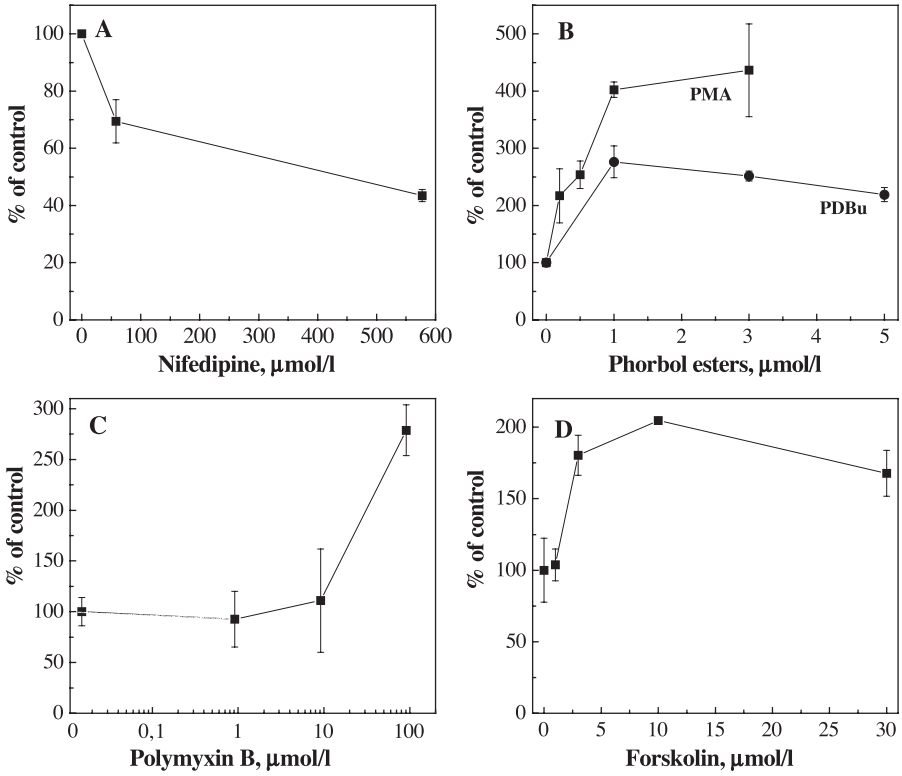

Fig. 2. Effect of compounds modulating signalling pathways on the basal $^{45}Ca^{2+}$ influx into human red blood cells. (A) Effect of nifedipine on basal Ca^{2+} influx. (B) Effect of phorbol esters (PMA (■) and PDBu (●) on basal Ca^{2+} influx. (C) Effect of polymyxin B on basal Ca^{2+} influx. (D) Effect of forskolin on basal Ca^{2+} influx. All experiments were measured under identical conditions: radionuclide final concentration 2.5 mmol/l, 30% cell suspension, 37 °C, concentrations of additions are indicated in the figures. Plots are representative from independent experiments (n>2). Points are mean ± S.E. of duplicates and corrected for values obtained at time zero.

l): 20 Tris−Cl (pH 7.4); 130 NaCl; 5 KCl; 1.2 NaH_2PO_4, 1 $MgCl_2$ and 10 glucose (further referred to as the suspension medium), to the final haematocrit of 30%, and immediately used for experiment.

2.2. Measurement of the basal $^{45}Ca^{2+}$ influx

The procedure described by Pokudin and Orlov [9] was modified as follows: red cells (0.5 ml, 30% haematocrit)

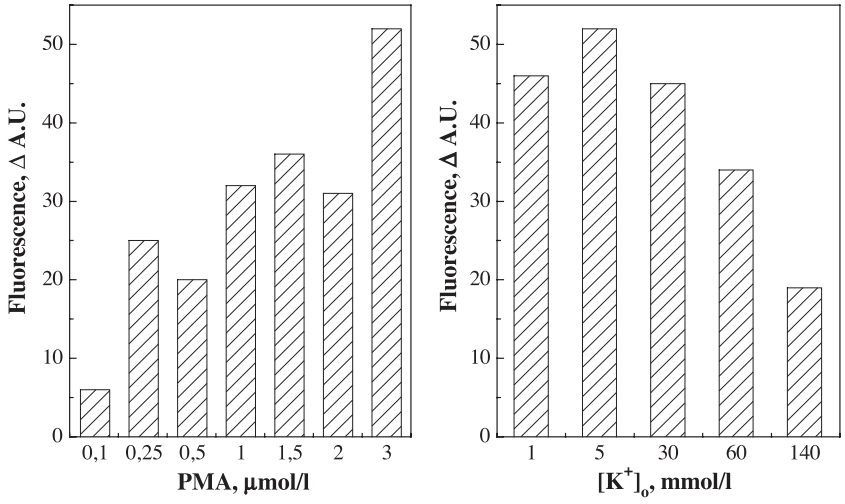

Fig. 3. Effects of PMA on fluorescence of $diSC_3$-(5) in the suspension of human erythrocytes. Changes in the fluorescence $F_{651/675}$ were measured using 3.6 µmol/l $diSC_3$-(5) and 20% suspension of red cells in the suspension medium. Left panel: fluorescence change depended on the PMA concentration in presence of 5 mmol/l K^+ in medium. Right panel: fluorescence change depended on concentration of extracellular potassium after addition of the maximal efficient concentration of PMA (3 µmol/l). In both panels, changes in $[K^+]_{ex}$ were compensated with Na^+ to isotonicity.

were pre-incubated at 37 °C, $^{45}Ca^{2+}$ (2.5 mmol/l; spec. act. approx. 4000–8000 cpm/nmol) was added, and incubation was continued at 37 °C. At times indicated (or after 20 min when not indicated specifically) 0.5 ml aliquots of the suspension were withdrawn and added to 5 ml of medium containing (in mmol/l): 20 Tris–Cl (pH 7.4); 65 NaCl; 70 KCl; 5 EGTA and 10 glucose cooled in an ice–water mixture, and immediately spun down on a microcentrifuge at 2–4 °C (the experiments were carried out in a cold room). Two more washing steps followed, and the radioactivity of pellets was determined after precipitation of cells with 0.5 ml of 10% trichloroacetic acid containing 10 mmol/l $LaCl_3$ and centrifugation of precipitates (5 min, 12,000 × *g*). The substances to be tested were added 5 min prior to the addition of radionuclide.

2.3. The measurement of the membrane potential changes

These changes were measured by means of the fluorescence probe 3,3′-dipropylthiodicarbocyanine iodide (diSC_3-

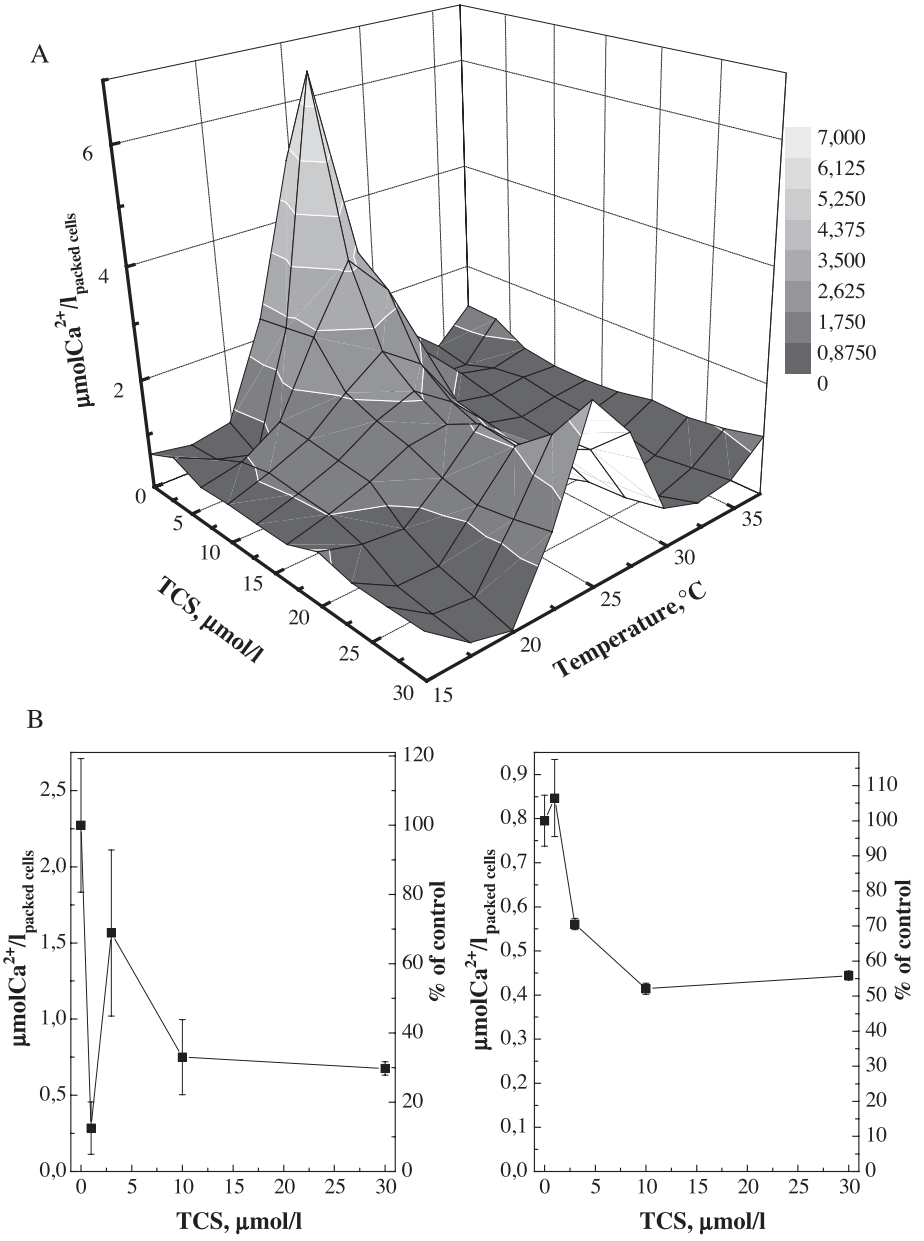

Fig. 4. The effect of TCS on basal $^{45}Ca^{2+}$ influx into human red blood cells. (A) Three-dimensional plot describes the effect of TCS in the concentration range 0–30 μmol/l on the basal $^{45}Ca^{2+}$ influx at temperatures between 15 and 37 °C (1% methanol was present in control samples treated in parallel). Other conditions as described in Materials and methods. Gray scale on the right site is in μmol $Ca^{2+}/l_{packed\ cells}$. Area was corrected for measurements at time zero. The pilot experiment. (B) The dependence of the basal $^{45}Ca^{2+}$ influx on the concentration of TCS at 30 °C (left panel) and 37 °C (right panel). Experimental conditions as above. Representative from independent experiments ($n = 6$). Data in panels A and B are from independent experiments. Points are mean ± S.E. of duplicates and corrected for measurement at time zero.

(5)) as described by Sims et al. [21] using 651 and 675 nm as excitation and emission wavelengths, respectively. The measurements were performed in the continuously stirred thermostatted cuvette in a Carl Zeiss (Opton) PMQ fluorometer.

2.4. Materials

$^{45}CaCl_2$ from ICN, USA; valinomycin from Calbiochem, Luzern, Switzerland; forskolin, phorbol-12-myristate-13-acetate (PMA), phorbol-12,13-dibutyrate (PDBu) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Sigma; Tris base and polymyxin B from Serva; 3,3′,4′,5-tetrachloro-salicylanilide (TCS) from Eastman-Kodak; $diSC_3$-(5) from Fluka, Buchs, Switzerland and dimethyl sulfoxide (DMSO) from Applichem, Darmstadt, Germany. Other chemicals used (all of analytical grade) were purchased from Lachema, Brno, Czech Republic.

3. Results

The addition of $^{45}Ca^{2+}$ into the RBC suspension kept at 37 °C led to its rapid equilibration with cells, which was completed after about 90–100 s (Fig. 1A). A prolonged incubation induced the gradual and slow increase of cell radioactivity. After 1 h of incubation the cell Ca^{2+} content attained the value up to 2.5 μmol/$l_{packed\ cells}$. The uptake was saturatable by Ca^{2+} concentrations smaller then 5 mmol/l (Fig. 1B) but the basal $^{45}Ca^{2+}$ influx was further stimulated upon exceeding this Ca^{2+} concentration (Fig. 1B). The basal $^{45}Ca^{2+}$ influx was maximal at pH 7.0 and was moderately inhibited by lowering (to pH 5) or increasing (to pH 8.5) the pH of the medium (Fig. 1C). The basal Ca^{2+} influx was stimulated by the increase of temperature (Fig. 1D) with the optimum at 25 °C. Over this temperature the Ca^{2+} influx decreased, although at 42 °C, another phase of stimulation appeared (Fig. 1D). In some experiments, only an ascending phase of the Ca^{2+} influx was observed (not shown). From the measurements of the Ca^{2+} influx temperature dependencies ($n=4$) was calculated the activation energy of 62 kJ/mol a temperature quotient Q_{10} of 1.3 by an Arrhenius plot of the ascending part of the curves.

The basal $^{45}Ca^{2+}$ influx was inhibited by nifedipine (up to 0.58 mmol/l) (Fig. 2A). The degree of inhibition (max. 55%) was substantially lower than that observed in the vanadate-induced $^{45}Ca^{2+}$ influx [22]. Furthermore, the effect of other compounds with possible effect on calcium influx was studied. Phorbol derivatives, PMA and PDBu exerted a stimulatory effect in the submicromolar concentration range, PMA with the half-maximal stimulation at 0.5 μmol/l (Fig. 2B). The extent of the stimulation was from 2.3 to 5 times in various experiments. Polymyxin B, PKC inhibitor, activated the basal $^{45}Ca^{2+}$ influx at concentrations higher than 10 μmol/l (Fig. 2C). Forskolin, activator of adenylate cyclase, also exerted a stimulatory effect on the basal $^{45}Ca^{2+}$ influx (Fig. 2D) with the half-maximal stimulation at about 2 μmol/l. Several other compounds were tested for their effect on the basal $^{45}Ca^{2+}$ influx. Secalonic acid D, a teratogen and PKC inhibitor, rugulosin and scopathricin (mycotoxins) exerted only a minor stimulatory effect (about 15%) in the concentration range 0–50 μmol/l). Gliotoxin (apoptosis inductor in liver cells) [23] had a minor inhibitory effect (about 10%) on the basal Ca^{2+} influx (not shown).

PMA, if added to the suspension of RBCs equilibrated with the membrane potential fluorescence probe $diSC_3$-(5), induced a concentration-dependent increase of the fluorescence. This increase was found to be dependent on the concentration of K^+ with a biphasic response (Fig. 3) being maximal at 5 mmol/l K^+ and inhibited at higher K^+ concentrations (completed with Na^+ to isotonicity) suggesting that the effect of PMA could be ascribed to the depolarisation the membrane (Fig. 3).

The fact that Ca^{2+}-ATPase catalyses the ATP-driven $Ca^{2+}/1-2H^+$ exchange suggests that the basal $^{45}Ca^{2+}$ influx should be influenced by the permeability to H^+ of the human RBC membrane. Therefore, the next experiments were carried out to test the effect of uncoupler on the basal $^{45}Ca^{2+}$ influx. The last was sensitive to uncoupler, TCS, which inhibited the basal $^{45}Ca^{2+}$ influx up to 60% in various experiments (Fig. 4A). The maximal degree of inhibition was obtained at 10 μmol/l TCS and apparently occurred at various temperatures. At lower concentrations (e.g. 2–5 μmol/l) irregular phenomena appeared, which were different from one experiment to another and which were characteristic of the scattering of experimental points of the basal Ca^{2+} influx (Fig. 4B) indicating that the

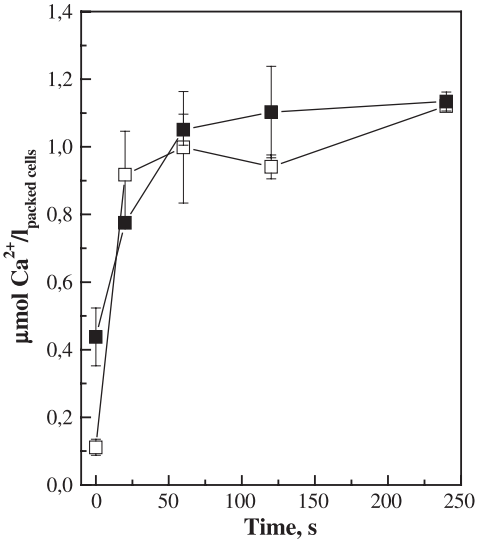

Fig. 5. The effect of DIDS on basal $^{45}Ca^{2+}$ influx into human red blood cells. Kinetics of the basal $^{45}Ca^{2+}$ influx was measured as described in Materials and methods in the presence (■) or absence (control, 0.1% DMSO (□)) of 25 μmol/l DIDS in 30% human RBC suspension at 37 °C. Plot is representative from three independent experiments. Points are mean ± S.E. of duplicates.

presence of uncoupler induces an instability in the extent of the $^{45}Ca^{2+}$ influx. The degree of inhibition varied among experiments between 40% and 80% ($n = 10$). In order to find out whether the anion channel, the site of Cl^-/OH^- exchange [24], plays a role in the basal Ca^{2+} influx, the effect of anion channel inhibitor DIDS was tested. DIDS did not cause a significant effect on the basal $^{45}Ca^{2+}$ influx at the concentration of 25 μmol/l (Fig. 5). In a pilot experiment, valinomycin (1 μg/ml), stimulated the basal Ca^{2+} influx by 15%, however, its stimulatory effect was observed also in K^+-containing medium (not shown).

4. Discussion

The results presented above demonstrate that the basal Ca^{2+} influx occurs in the membrane of human RBC, which has all characteristics of the carrier-mediated transport. As the Ca^{2+} efflux is mediated solely by the Ca^{2+}-ATPase (there is no evidence for the presence of a Na^+/Ca^{2+} antiporter in human red cells), then it is possible to ascribe the Ca^{2+} influx to a cyclic flow of Ca^{2+}, which occurs without added stimuli and which consists of these transport systems. This statement could be supported not only by the transport characteristics shown in Fig. 1 but also by its magnitude which was rather high and varied between 20.8 and 47.6 μmol/$l_{packed\ cells}$ h at 37 °C and falls into the range observed under similar conditions by other authors. From the paper of Engelmann et al. [12] the value between 30 and 40 μmol/$l_{packed\ cells}$ h could be derived from data shown in Fig. 2B at 37 °C. On the other hand, other papers where the $^{45}Ca^{2+}$ influx into RBC containing entrapped Ca^{2+} chelators was measured presented different but essentially similar results: 2–20, 14–20, 59 μmol/$l_{packed\ cells}$ h [7–9], respectively, less than 50 μmol/$l_{packed\ cells}$ h [25]. Here we do not discuss the values of the Ca^{2+} influx in ATP-depleted and vanadate-treated cells, where the net Ca^{2+} accumulation occurs and the qualitative changes in properties of the Ca^{2+} transport were observed [22,26,27]. Under conditions used in this work, no inhibition of Ca^{2+}-ATPase is expected. Concentration of ATP was found to be 0.6 mmol ATP/$l_{packed\ cells}$ (unpublished results). In this case, the net Ca^{2+} accumulation does not take place (Fig. 1A) and only the turnover of calcium cyclic flow is measured.

The kinetics of the $^{45}Ca^{2+}$ influx show the clear biphasicity, which was not observed before (Fig. 1A). The rapid phase has similar time-course as that observed by Engelmann et al. [12]. The second phase may indicate that the longer pre-incubation of RBC with $^{45}Ca^{2+}$ in artificial medium leads to the slow accumulation, and possibly the precipitation of $^{45}Ca^{2+}$ in the cytoplasm. The temperature optimum of the basal $^{45}Ca^{2+}$ influx was similar to that induced by vanadate [28] and could be explained similarly, i.e. by the differences in the temperature sensitivities of both transports systems participating in the cyclic Ca^{2+} flow.

Unlike our [13,22] and other [26] findings, in presented experiments we observed an inhibition of the basal $^{45}Ca^{2+}$ by nifedipine (Fig. 2A) up to 55% as compared with about 10–20% inhibition in the quoted papers. This difference is probably to explain by the fact that in this work, we used cells which were stored 1 day since the blood withdrawal.

The question of the physiological role of the basal Ca^{2+} influx remains strictly speaking essentially unanswered. However, some experimental results provide useful hints in this respect. First, the effects of phorbol esters [14], Fig. 2B in this work, and the effects of polymyxin B and forskolin (Fig. 2C and D) indicate that changes in intracellular variables of signalling pathways mediated by PKC, or adenylate cyclase, respectively, can modify the rate of the basal influx. These changes occur without involvement of a specific receptor, therefore, these signalling pathways may represent remnants of those present in the preceding developmental stages of erythropoiesis, or they reflect interventions from a functional intracellular signalling pathway(s). These explanations have to be tested in the future.

The stimulatory effect of PMA on the basal could be explained by a direct stimulation by phosphorylation of the putative Ca^{2+} carrier or a hypothetical regulatory protein(s). It seems to be a feasible explanation as we found several proteins phosphorylated by PMA at conditions similar to those used in this study (unpublished results). However, it was found that PMA caused the depolarisation of the membrane (Fig. 3, left panel). Thus, alternative explanation is possible that the depolarisation-induced Ca^{2+} influx is activated with PMA, in a Na^+-dependent manner (Fig. 3, right panel). Such an explanation is in agreement with data of Soldati et al. [15], but there are no data which could discriminate between these possibilities.

The observation of Niggli et al. [17], that the Ca^{2+}-ATPase catalyses the electrically silent $Ca^{2+}/2H^+$ exchange, reveals an important problem intimately connected with the existence of RBC basal Ca^{2+} transport. It could be inferred that the cyclic flow of Ca^{2+} (of the magnitude indicated above) should lower the cytoplasmic pH by several orders of magnitude within 1 h (down to pH 3.3). Such a change apparently does not happen in RBC under normal conditions. The inhibitory effect of the uncoupler on the basal Ca^{2+} influx (Fig. 4) indicates that the H^+ electrochemical potential contributes to the driving force of the basal Ca^{2+} influx. This effect of uncoupler cannot be expected if the basal Ca^{2+} influx pathway is mediated by an uniport (i.e. channel-like) transport system as the inflow of H^+ (due to the Ca^{2+}-ATPase activity) should be compensated by a mechanism which is located outside of both transport proteins involved in the basal Ca^{2+} cyclic flow excluding, however, the anion channel (Fig. 5).

The most plausible interpretation of the effect of uncoupler is that it releases the constraint of Ca^{2+}-ATPase due to the influx of H^+, and accelerates efflux of Ca^{2+}. This should mask a part of the Ca^{2+} influx and decrease the radioactivity in cells. This explanation, however, also has some weak-

nesses. First, the capacity of the Ca^{2+}-ATPase exceeds the capacity of the Ca^{2+} influx by order of magnitude (see Ref. [25] for review). Second, this interpretation does not explain the net flux of two charges (carried by entering Ca^{2+}) per transport cycle.

There are two other ways to explain the effect of uncoupler; though it must be said that none of them is fully supported by experimental data. The first explanation is based on the inhibition of the vanadate-induced $^{45}Ca^{2+}$ uptake by uncoupler [10,29], which was shown to be due to the elimination of the driving force imposed by the opening of the Ca^{2+}-activated K^+ channel. In this case, the compensation of Ca^{2+} charge could be accomplished by the efflux of K^+. Such an explanation would contradict the established opinion that the RBC membrane does not discriminate between Na^+ and K^+ ions [30] and would suggest that the Ca^{2+}-activated K^+ channel is also active under the conditions used in this study. The second explanation is based on the assumption that H^+ influx is compensated within the pertinent Ca^{2+} inward-transporting system. In other words, the transport system conveying Ca^{2+} into the cell cytoplasm may be a Ca^{2+}/nH^+ antiporter. Such a suggestion may give rise to several questions. First, there is only one protein in the human RBC membrane, which binds the antibody against α_1-subunit of the Ca^{2+} channel has (approximately) the molecular mass corresponding to this channel (i.e. Ca^{2+} uniport) [14]. This fact, however, does not necessarily contradict our results if one takes into account that the slippage phenomenon occurs among ion transport systems (see Ref. [31] for review). Second, the variability of the uncoupler inhibition we observed (Fig. 4B) indicates that other mechanisms of H^+ gradient compensation are also involved. Such mechanism(s) exist in the RBC membranes. A feasible candidate, the anion channel, which is able to catalyses the Cl^-/OH^- antiport seems to be excluded by the effect of DIDS (Fig. 5) and by the fact that the compensation of the H^+ influx (due to the activity of the Ca^{2+}-ATPase) by the Cl^-/OH^- antiport would cause osmotic effect that could increase the water activity in the cytoplasm and could drive the chloride electrochemical gradient out of equilibrium. Thus, the available data are not sufficient to explain exhaustively the effect of uncoupler on the Ca^{2+} influx. However, irrespective of the validity of both explanations of the effect of uncoupler, its effect demonstrates that the influx and efflux of Ca^{2+} (in human RBC) are coupled by the RBC H^+ homeostasis and brings the independent line of evidence that the Ca^{2+}-ATPase operates as Ca^{2+}/nH^+ antiporter.

Acknowledgements

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