Characterization and regulation of basal calcium influx in human peripheral blood lymphocytes

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Abstract. The basal $^{45}Ca^{2+}$ influx into resting human blood lymphocytes was measured. This process showed biphasic kinetics with first rapid phase followed by the second long-lasting and markedly slower phase. Further, it showed signs of saturability and reaches maximal values at 37°C and extracellular pH 7.2. The basal $^{45}Ca^{2+}$ influx was stimulated by addition of submicromolar concentrations of 4β-phorbol-12-myristate-13-acetate, and this effect was abolished by protein kinase C (PKC) inhibitor Ro-31-8220. In the regulation of basal $^{45}Ca^{2+}$ influx is probably only partially involved adenylate cyclase pathway as show results with intracellular c-AMP elevating agents (dB-c-AMP, 3-isobutyl-1-metylxantine and forskolin). Uncoupler 3,3′,4′,5-tetrachloro-salicylanilide (TCS) in micromolar concentrations stimulated basal $^{45}Ca^{2+}$ influx and its effect was more significant in media with high extracellular concentration of K^+.

Key words: Lymphocytes — Calcium influx — PKC — Amiloride

Introduction

At the present state of knowledge it seems to be generally accepted that there are hardly cells not depending on the production of calcium (Ca^{2+}) signals in the human body, which are responsible for regulation of variety cell functions and triggering of specific responses.

In cells, which act in inflammatory reactions, such a lymphocytes, Ca^{2+} plays a fundamental role as a second messenger in regulating numerous cellular functions, including activation, proliferation, production of superoxide anion and cell death (Berridge 1995; Jambrina et al. 2003; Nagy et al. 2003; Randriamampita and Trautmann 2004). It is well known that these functions are coupled with the increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) that is mediated by Ca^{2+} efflux from intracellular stores and Ca^{2+} influx from extracellular space. Transport of Ca^{2+} into lymphocytes evoked by activation is mediated by voltage-dependent- (Badou et al. 2005) and -non-dependent Ca^{2+} transport systems (Stokes et al. 2004), and is modulated by changes of membrane potential, intracellular pH and cytosolic Na^+ concentration, which affect activity of Na^+/Ca^{2+} antiporter (Balasubramanyam et al. 1994; Lewis and Cahalan 1995; Nofer et al. 1999; Cabado at al. 2000).

The mechanism(s) responsible for the increase in $[Ca^{2+}]_i$ during antigen- or mitogen- induced stimulation of lymphocytes has been widely investigated (Kotturi et al. 2003; Randriamampita and Trautmann 2004; Badou et al. 2005). Early experiments with thapsigargin (Tg), a selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, revealed the existence of so-called capacitative, or store-operated, Ca^{2+} entry pathway in lymphocytes. This consists of two phases: rapid depletion of intracellular stores and consecutive entry of extracellular Ca^{2+} (Putney 1990, 1997; Berridge 1995). Using electrophysiological methods and Ca^{2+}-sensitive fluorescent indicators, essential transport systems responsible for Ca^{2+} influx in antigen-activated (and mitogen-activated) lymphocytes were uncovered, and Ca^{2+} release-activated Ca^{2+} (CRAC) channels, members of the store-operated calcium (SOC) channels family, (for review, see Prakriya and Lewis 2003) were demonstrated. Recently, essential components regulating SOC influx and CRAC channel activity have been characterized (Roos et al. 2005; Zhang et al. 2005).

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In contrast, little is known about the processes underlying $[Ca^{2+}]_i$ homeostasis and Ca^{2+} transport in resting (non-stimulated) cells in spite of the fact that changes in $[Ca^{2+}]_i$ homeostasis are associated with variety of diseases (Aviv 1996; Eckert et al. 1997; Martino et al. 1997; Mooren and Kinne 1998; Ori et al. 1999; Balasubramanyam et al. 2001; Schwarz et al. 2004).

In human neutrophils, it was shown that the Ca^{2+} uptake is in part mediated through Na^+/Ca^{2+} exchanger, and is non-competitively inhibited by benzamil and other amiloride analogues, and it is enhanced by membrane depolarization (Simchowitz and Cragoe 1988). The role of the plasma membrane depolarization of human neutrophils in regulating the Ca^{2+} influx was confirmed also in Ca^{2+}-depleted cells. It was proposed that Ca^{2+} uptake is mediated by voltage-gated calcium channel (Majander and Wikstrom 1989). The role of Na^+/Ca^{2+} exchanger, and involvement of protein kinase C (PKC) in the Ca^{2+} homeostasis regulation through the stimulation of Ca^{2+} pump activity in plasma membrane was demonstrated in Tg-treated human T-lymphocytes (Balasubramanyam et al. 1993, 1994; Balasubramanyam and Gardner 1995; Nofer et al. 1999). However, they are also data referring about no function of Na^+/Ca^{2+} exchanger in calcium homeostasis in lymphocytes (Donnadieu and Trautmann 1993). Using Ca^{2+}-chelator-loaded rat thymic lymphocytes, Mason and co-workers showed that depletion of an internal Tg-sensitive store correlates with and appears to be responsible for the increased permeability of the plasma membrane. Accordingly, the Ca^{2+} fluxes induced by intracellular Ca^{2+} depletion and by Tg were pharmacologically indistinguishable (Mason et al. 1991). Recently, it was proposed that Ca^{2+} influx into resting T-lymphocytes is mediated by CRAC channels as it occur in mitogen-activated cells and that these processes differ only in total number of expressed channels (Fomina et al. 2000). It was also shown that the collapse of mitochondrial proton motive force caused by mitochondrial uncoupler or by other mitochondria-directed agents inhibited a Tg-stimulated Ca^{2+} influx and authors suggested that Ca^{2+} influx into Jurkat cell is under control of mitochondrial energy status (Hoth et al. 1997; Makowska et al. 2000; Zablocki et al. 2003).

The information concerning basal Ca^{2+} influx into resting T-lymphocytes are still only partial and do not provide comprehensive understanding of this process. In this work we characterised the basic parameters of non-stimulated $^{45}Ca^{2+}$ influx into isolated human blood lymphocytes and investigated its possible underlying regulatory pathways.

Materials and Methods

Isolation of human lymphocytes

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. Lymphocytes were prepared by centrifugation of blood on Ficoll-Paque Plus (30 min, 400 × *g*, 15°C) followed by three washing steps and finally suspended in medium containing (in mmol/l): Tris 20, NaCl 135, KCl 5, glucose 10, $MgCl_2$ 1, NaH_2PO_4 1; pH = 7.4 (further referred to as the suspension medium). Cell viability was estimated by using Trypan Blue Dye exclusion test and usually was about 90–95%. Such a suspension was immediately used for experiments.

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

One volume of cell suspension was added to nineteen volumes of suspension medium (or medium with desired composition) and pre-incubated for 5 min at 37°C. After this step, substances to be tested were added to the cell suspension and pre-incubation continued for desired time. $^{45}Ca^{2+}$ was added (2.5 mmol/l; spec. activity approx. 4000 cpm/nmol, if not indicated otherwise). At times indicated (or after 30 min when not indicated otherwise) aliquots of the suspension (usually 100–200 µl) were withdrawn and added to five volumes of ice-cold medium containing (in mmol/l): Tris 20, NaCl 65, KCl 75, EDTA 10 and glucose 10 layered on top of 150 µl of dibuthylphtalate (DBP) and dioctylphtalate (DOP) mixed in ratio 3 : 2 and immediately spun down on micro-centrifuge (45 s; 14,000 × *g*). Inorganic phase of supernatant was sucked out and space over organic phase was rinsed three times with 1 ml of distilled water. Organic phase was sucked out after last washing step and the cell pellet was precipitated with 200 µl of 10% trichloroacetic acid containing 10 mmol/l $LaCl_3$. Radioactivity of supernatants was measured after centrifugation of precipitates (2 min; 14,000 × *g*).

Measurement of $^{45}Ca^{2+}$ efflux

Suspension of freshly isolated cells was incubated 1 h at 37°C with $^{45}Ca^{2+}$ (100 µmol/l; spec. activity 26 kcpm/nmol). After centrifugation (5 min, 1000 × *g*) suspension was washed twice in suspension medium supplemented with 0.4 mmol/l EDTA, and, finally, re-suspended in suspension medium without EDTA. Cells were then incubated at 37°C with different substances and at times indicated samples (200 µl) were withdrawn and layered on top of 150 µl of mixture DBP : DOP. Samples were immediately spun down on micro-centrifuge (45 s; 14,000 × *g*) and supernatants with small portion of organic layer were transferred into scintillation liquid for radioactivity measurements.

Chemicals

$^{45}CaCl_2$, forskolin, dB-c-AMP (N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate), IBMX (3-isobutyl-1-methylxan-

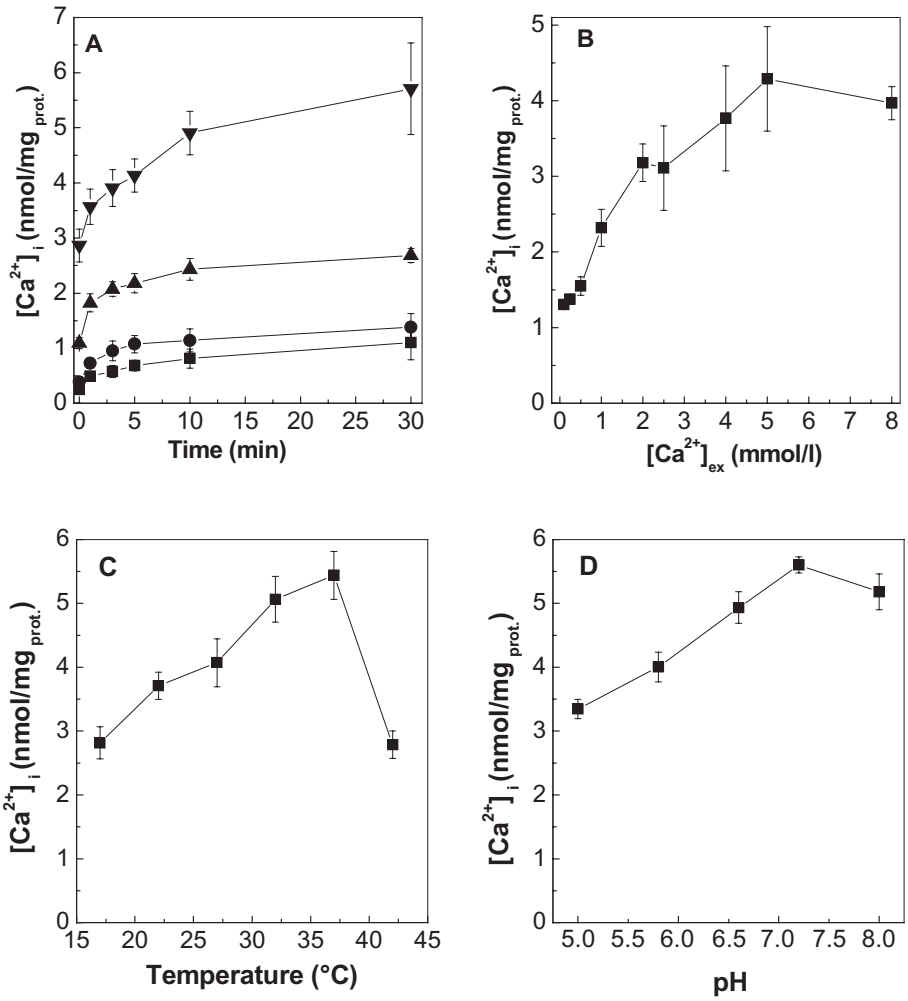

Figure 1. Basic parameters of basal $^{45}Ca^{2+}$ influx into resting human lymphocytes. A. Time course of Ca^{2+} influx measured at 0.5 (■), 1.0 (●), 2.5 (▲) and 5.0 (▼) mmol/l extracellular calcium ($[Ca^{2+}]_{ex}$) and 37°C. Concentration (B), temperature (C) and pH (D) dependence of Ca^{2+} influx into the human lymphocytes. Data represent values of Ca^{2+} influx 30 min after addition of $^{45}Ca^{2+}$: $^{40}Ca^{2+}$ mixture (final concentration 2.5 mmol/l – panel C and D) and are corrected for values obtained at zero time.

thine) – MP Biomedicals (Irvine, USA); TCS (3,3',4',5-tetrachlorosalicylanilide) – Eastman-Kodak (Rochester, USA); DMSO (dimethyl-sulfoxide) – Applichem (Darmstadt, Germany); PMA (4β-phorbol 12-myristate-13-acetate), Ro 31-8220, amiloride – Sigma; dibutylphtalate, dioctylphtalate – Fluka; Ficoll-Paque Plus – Amersham Bioscences (Uppsala, Sweden).

Other chemicals used (all of analytical grade) were purchased from Lachema (Brno, Czech Republic).

Statistical analysis

Data are expressed as means ± SE of three (if not indicated otherwise) independent experiments measured in duplicates. Significance was assessed by the Student's *t*-test for paired samples; *p* values less than 0.05 were considered as significant.

Results

Basic parameters of basal (non-stimulated) Ca^{2+} influx

At first, the basic parameters of basal Ca^{2+} influx using $^{45}Ca^{2+}$ were measured in human lymphocytes prepared by Ficoll-Paque Plus centrifugation method. Obtained data shows that this transport is rapid in the first 5 min with initial rate strongly dependent on extracellular Ca^{2+} concentration. Its value for 2.5 mmol/l extracellular Ca^{2+} is 5.0 ± 1.0 $pmol \cdot mg^{-1}_{prot} \cdot s^{-1}$. After initial rapid phase basal Ca^{2+} influx slows down but did not stop during the measuring time (Fig 1A). Under conditions used in our experiments basal Ca^{2+} influx seems to be saturable up to the 5 mmol/l concentration of extracellular Ca^{2+} (Fig. 1B). The basal Ca^{2+}

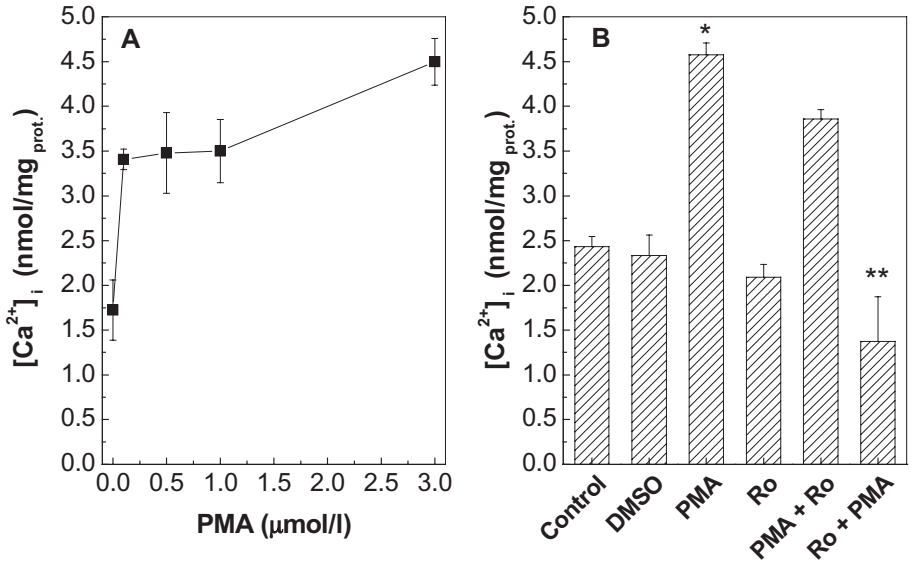

Figure 2. Effect of PMA on $^{45}Ca^{2+}$ influx in resting human lymphocytes. **A.** Stimulation of Ca^{2+} influx dependent on PMA concentration. Cells were preincubated 30 min with indicated concentrations of PMA or vehicle (DMSO – final concentration less then 0.5% v/v). **B.** Inhibition of PMA-stimulated influx of Ca^{2+} by PKC inhibitor Ro 31-8220. Concentration of PMA and Ro 31-8220 used in experiment was 3 μmol/l. Last two columns differ in the sequence of addition of compounds: PMA + Ro – phorbol ester was added 15 min before PKC inhibitor and then cells were incubated another 15 min; Ro + PMA – inhibitor was added 15 min before PMA. Data represent mean ± SE of four independent experiments measured in duplicates. * significantly different from control; ** significantly different from PMA.

uptake was stimulated by the increase in temperature (Fig. 1C) with the optimum at 37°C. Over this temperature the Ca^{2+} influx rapidly decreased. From the measurements of the Ca^{2+} influx temperature dependencies ($n = 3$) plotted in an Arrhenius plot, the activation energy of 24.4 kJ/mol and temperature quotient Q_{10} of 1.24 were calculated (from the ascending part of the curve in the Fig. 1C). The basal $^{45}Ca^{2+}$ influx was maximal at pH 7.2 and was inhibited by lowering (to pH 5) or increasing (to pH 8) the pH of the medium (Fig. 1D).

Regulatory systems involved in basal Ca^{2+} influx

With the aim to find which signalling pathways and transport systems are involved in regulation of basal Ca^{2+} influx, the effect of some effectors was studied. The uptake of $^{45}Ca^{2+}$ in resting cells was markedly increased by PKC activator PMA. After 30 min pre-incubation of cells with 100 nmol/l concentration, PMA increased Ca^{2+} influx two-fold and at maximal concentration used in experiments (3 μmol/l) PMA evoked increase 260% of control (Fig. 2A). An inactive analogue of PMA (4α-PMA) at the same concentrations did not exert any effect (not shown). Moreover, a potent inhibitor of PKC Ro-31-8220, which itself was without effect on Ca^{2+} influx fully abolished the effect of PMA if added before activator (Fig. 2B).

The cell-permeable analogue of c-AMP, dB-c-AMP, stimulated the basal Ca^{2+} influx, although the maximal stimulation was only about 40% (Fig. 3A). Forskolin, a cell permeable activator of adenylate cyclase, did not induce significant and reproducible changes in the Ca^{2+} influx, when used in concentrations 10 μmol/l. Higher forskolin concentrations (up to 20 μmol/l) caused the inhibition rather than stimulation of Ca^{2+} influx (Fig. 3B). IBMX, an inhibitor of phosphodiesterase which is expected to increase intracellular concentration of c-AMP, displayed similar stimulation effect as dB-c-AMP (Fig. 3C). When dB-c-AMP and IBMX were added simultaneously in concentrations lower than maximal used in previous experiments, a slight synergic effect was observed (Fig. 3D). These changes were not significantly different from controls in all measurements.

Role of H^+ gradient and the monovalent cations in regulation of basal Ca^{2+} fluxes. Effect of TCS

An uncoupler of oxidative phosphorylation and protonophore TCS (in micromolar concentrations) stimulated the basal Ca^{2+} influx into resting leukocytes. This effect was concentration-dependent and the maximal stimulation at maximal TCS concentration (50 μmol/l) was about 250% (Fig. 4A). In further experiments we used lower

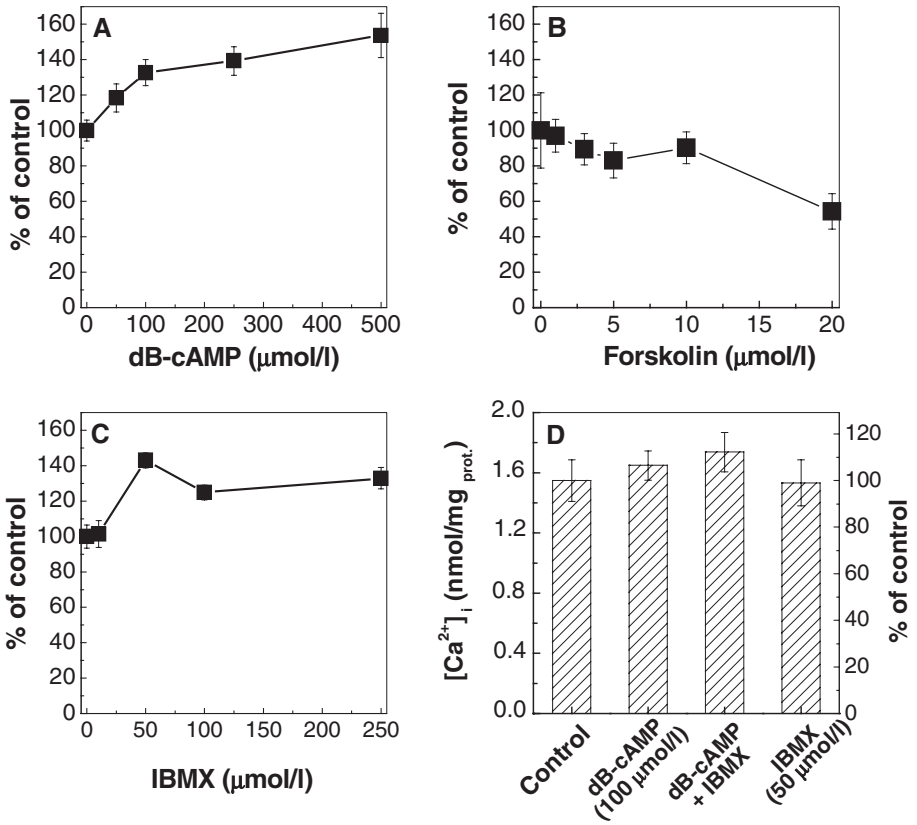

Figure 3. Involvement of c-AMP signalling pathway in regulation of basal Ca^{2+} influx. Cells were preincubated 15 min with indicated concentrations of effectors: dB-c-AMP (A), forskolin (B), IBMX (C) and influx was measured at the time zero and 30 min after addition of calcium. Concentration of vehicle (DMSO, for B and C did not exceed 0.5% v/v). Results are expressed as % of control (vehicle) and represent values of Ca^{2+} influx after 30 min corrected for values obtained at time zero. D. Effect of simultaneous addition of dB-c-AMP and IBMX on Ca^{2+} influx. Cells were preincubated 15 min with indicated concentrations of effectors added separately or in combination and influx was measured as mentioned before.

concentration of TCS (30 μmol/l), which had satisfactory stimulatory effect without having side effects on plasma membrane. Stimulation of Ca^{2+} influx by TCS was boosted by increasing extracellular concentration of K^+ ($[K^+]_{ex}$) (Fig. 4B) and decreasing extracellular concentration of Na^+ ($[Na^+]_{ex}$) (when Na^+ was replaced by choline) (Fig. 4C). Using another uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), or tetrachlorotrifluoromethyl benzimidazol (TTFB) we were unable to observe similar effect on Ca^{2+} influx even at concentrations exceeding those commonly used for uncoupling of oxidative phosphorylation in this type of cells (not shown).

As the stimulation of Ca^{2+} influx by TCS was probably due to its effect on H^+ gradient of plasma membrane we interested if such a change affects also efflux of Ca^{2+}. $^{45}Ca^{2+}$ preloaded cells were treated with TCS in the presence or absence of extracellular Ca^{2+}. As shown in Fig. 4D, TCS significantly stimulated efflux of Ca^{2+} from cells in the presence and absence of extracellular Ca^{2+}.

Effect of extracellular medium composition on Ca^{2+} influx. Role of Na^+ and K^+

Basal Ca^{2+} influx was markedly dependent on extracellular concentration of monovalent cations. With increasing $[K^+]_{ex}$ we observed inhibition of Ca^{2+} uptake (Fig. 4B). On the other hand, when extracellular Na^+ was replaced by choline and $[K^+]_{ex}$ was maintained stable, influx of Ca^{2+} increased with decreasing $[Na^+]_{ex}$ (Fig. 4C). In media with physiological concentration of Na^+, Ca^{2+} influx was markedly inhibited in the presence of amiloride. This inhibitor did not exert significant effect in the media with high $[K^+]_{ex}$ (Fig. 5A). However, the addition of 1 mmol/l amiloride to cells pre-incubated with 30 μmol/l TCS in the medium with high $[K^+]_{ex}$ caused strong inhibition of influx stimulated by TCS (Fig. 5B).

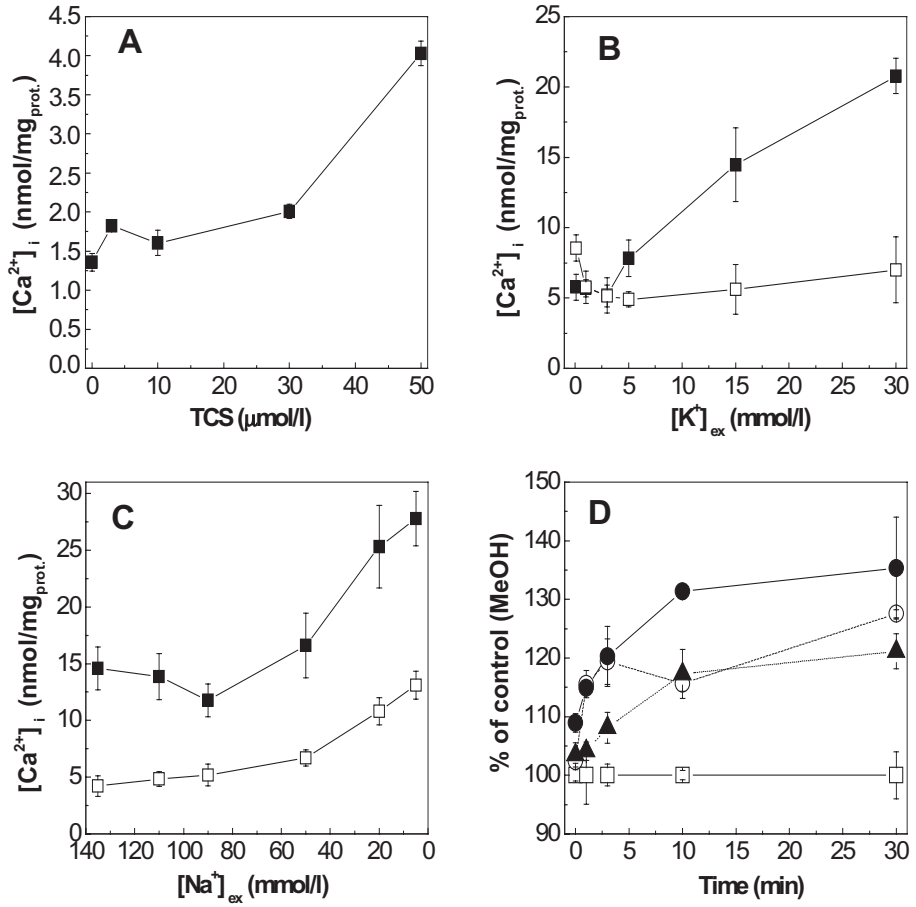

Figure 4. Effect of TCS on basal influx and efflux of $^{45}Ca^{2+}$. **A.** Stimulation of basal Ca^{2+} influx into human lymphocytes by TCS measured in suspension medium (see Materials and Methods). Cells were pre-treated 15 min with indicated concentrations of TCS. Data represent values of Ca^{2+} influx 30 min after addition of $^{45}Ca^{2+}$ and are corrected for values obtained at zero time. Points are means ± SE of at least four independent experiments measured in duplicates. **B.** Effect of different $[K^+]_{ex}$ on Ca^{2+} influx in the presence (■) or absence (□) of 30 μmol/l TCS. **C.** Effect of substitution of Na^+ by choline$^+$ in extracellular medium with stable concentration of K^+ (5 mmol/l) on Ca^{2+} influx in presence (■) or absence (□) of 30 μmol/l TCS. Data in graphs B and C were obtained as mentioned above for graph A. **D.** Stimulation of Ca^{2+} efflux from the Ca^{2+} loaded cells in the absence (○) or presence of 2.5 mmol/l extracellular Ca^{2+} (□), 30 μmol/l TCS (▲) or TCS + extracellular Ca^{2+} (●). Final concentration of vehicle (MeOH) in all experiments did not exceed 0.5% v/v. Data in graphs B and C represent values of Ca^{2+} influx 30 min after addition of $^{45}Ca^{2+}$ and are corrected for values obtained at zero time. Points are means ± SE of at least four independent experiments measured in duplicates.

Effect of potassium channel inhibitors

The effect of $[K^+]_{ex}$ changes on the Ca^{2+} influx indicates the involvement of changes in membrane potential. In order to confirm or disprove this possibility, we employed inhibitors of the voltage-gated K^+ channel 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP), and quinine. Both 4-AP and 3,4-DAP stimulated basal Ca^{2+} influx, although 4-AP only in concentrations above 1 mmol/l (Fig. 6). Effect of quinine was also stimulatory but with lower efficiency (not shown).

Discussion

Results presented above demonstrate that the basal Ca^{2+} influx occurs in the membrane of human blood lymphocytes, and has all characteristics of the carrier-mediated transport. Addition of 2.5 mmol/l extracellular Ca^{2+} to isolated cells at 37°C evoked a quite rapid rise in $[Ca^{2+}]_i$ (Fig. 1A) without lag time, which was found by other authors in cells treated by BAPTA (1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid) (Fomina et al. 2000). The kinetics of $[Ca^{2+}]_i$ shows two phases: first – rapid phase observed by

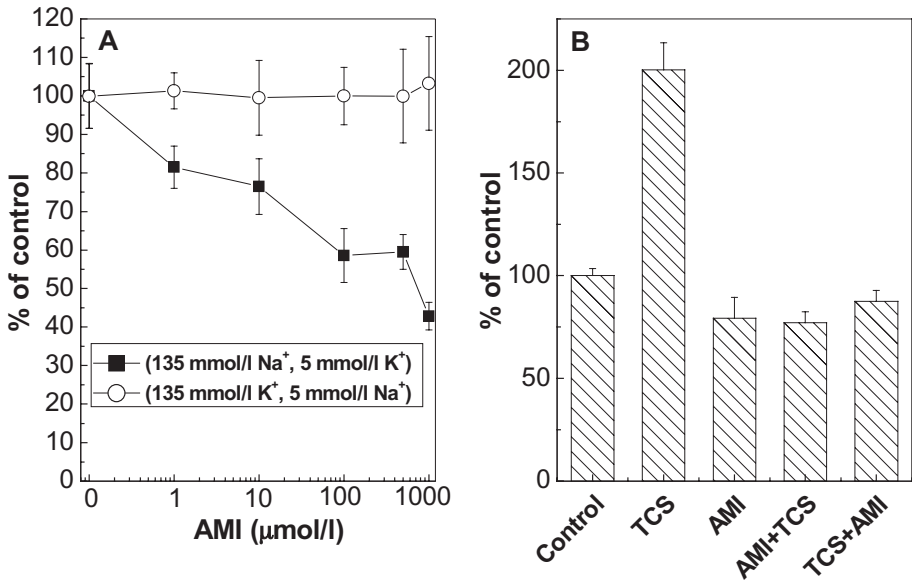

Figure 5. Effect of amiloride (AMI) on Ca^{2+} influx in suspension media with different composition. **A.** Cells were 15 min pre-treated with indicated concentrations of AMI in media with physiological monovalent cation composition (■) or in media with high concentration of K^+ and low concentration of Na^+ (○). **B.** Cells were 5 min preincubated in suspension medium with 135 mmol/l $[K^+]_{ex}$ then incubation continued 15 min in the presence of 30 μmol/l TCS, 1 mmol/l AMI or combination of compounds. When we used combination of effectors cells were 15 min incubated with first indicated compound and another 15 min with the second. Data represent values of calcium influx 30 min after addition of $^{45}Ca^{2+}$ and are corrected for values obtained at zero time. Points are means ± SE of three (A) or two (B) independent experiments measured in duplicates.

other authors in activated or Tg-treated cells (Negulescu et al. 1994; Choudry et al. 1996; Makowska et al. 2000), followed by second slower phase. This phase could indicate that the longer pre-incubation of lymphocytes with $^{45}Ca^{2+}$ in artificial medium leads to slow accumulation of $^{45}Ca^{2+}$ in calcium stores. Other explanation is also possible – storage of blood in acid-citrate-dextrose anticoagulant and isolation steps without calcium in medium provoke emptying of intracellular Ca^{2+} pools what triggers re-filling of stores by Ca^{2+}. Biphasicity of this uptake strongly resembles to that found in resting human erythrocytes (Hudec et al. 2004) what could suggest presence of the similar transport system responsible for basal Ca^{2+} influx. Elevation of $[Ca^{2+}]_i$ was found dependent on extracellular Ca^{2+} concentration and seems to be saturable (Fig. 1B) what support the idea of carrier-mediated transport system. Basal $^{45}Ca^{2+}$ influx rose with increase in temperature with an optimum at 37°C. Additional increase in temperature caused sharp decrease in $[Ca^{2+}]_i$ (Fig. 1C) what could be related to increased activity of Ca^{2+} releasing transport systems. Basal Ca^{2+} influx was sensitive to changes of extracellular pH and reached an optimum at pH 7.2 (Fig. 1D).

PKC is involved in regulation of many cellular events including Ca^{2+} transport. It was shown in activated Jurkat T-cells that stimulation of PKC reduced inflow of Ca^{2+} by

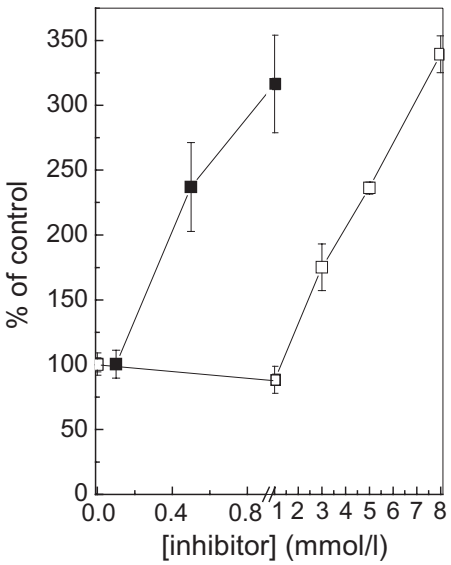

Figure 6. Effect of voltage-gated potassium channel inhibitors on basal $^{45}Ca^{2+}$ influx. Cells were preincubated 15 min with indicated concentrations of inhibitors 4-AP (□), 3,4-DAP (■), and influx was measured at the time zero and 30 min after addition of calcium. Results are expressed as % of control (vehicle) and represent values of Ca^{2+} influx after 30 min corrected for values obtained at time zero. Points are means ± SE of two independent experiments measured in duplicates.

Ca^{2+}-ATPase activity stimulation (Balasubramanyam and Gardner 1995) or direct inhibition of Ca^{2+} influx (Haverstick et al. 1997). Here we report an opposite effect of PKC. Preincubation with PMA evoked significant elevation of $[Ca^{2+}]_i$ (Fig. 2A) but inactive analogue 4α-PMA had no effect on Ca^{2+} influx. Moreover, the effect of PMA was fully abolished by a potent PKC inhibitor Ro-31-8220 (Fig. 2B) suggesting not only involvement of PKC in the regulation of Ca^{2+} influx in resting cells but also presence and activity of another Ca^{2+} channel distinct of CRAC in membrane of human blood lymphocytes. Such a transport system (sensitive to activation by PKC) was found in human erythrocytes (Hudec et al. 2004) or in human platelets (Rosado and Sage 2000). The stimulatory effect of PMA could be explained by a direct stimulation by phosphorylation of putative Ca^{2+}-carrier or a hypothetical regulatory protein(s) as it was proposed for human erythrocytes (Hudec et al. 2004). Another possible explanation is that PKC activate Na^+/H^+ exchanger whose activity affects intracellular concentration of monovalent cations, which could lead to opening of Ca^{2+}-selective or non-selective channels. These hypotheses remain to be proved in the future studies.

Elevation of intracellular concentration of c-AMP was shown to inhibit mitogen-stimulated increase in $[Ca^{2+}]_i$ in human lymphocytes (van Tits et al. 1991). To assess the role of c-AMP in the regulation of basal Ca^{2+} influx we used several agents elevating intracellular concentration of c-AMP. Plasma membrane-permeable c-AMP analogue, dB-c-AMP, and the phosphodiesterase inhibitor IBMX slightly increased $[Ca^{2+}]_i$ but forskolin failed to increase $[Ca^{2+}]_i$ and, at higher concentration, had an inhibitory action. We believe that the effect of c-AMP elevating agents on $[Ca^{2+}]_i$ is not due to their direct impact on inward-directed Ca^{2+} transporter but on endoplasmic reticulum Ca^{2+}-ATPase as was proposed by Tintinger and co-workers (2002) in activated human neutrophils.

Contribution of mitochondria to the regulation of Ca^{2+} influx into activated or Tg-treated lymphocytes was analysed by several authors (Makowska et al. 2000; Bautista et al. 2002; Zablocki et al. 2003, 2005). For evaluation of possible role of mitochondria in the control of basal Ca^{2+} influx in human blood lymphocytes we used uncouplers FCCP and TCS. Unlike published results, using of FCCP even at 10 μmol/l concentration did not change markedly the basal Ca^{2+} influx (data not shown). On the other hand, TCS stimulated Ca^{2+} influx in a concentration-dependent manner, and at 50 μmol/l TCS a significant effect was obtained (Fig. 4A). Detailed study of TCS effect showed that it is strongly affected by $[K^+]_{ex}$ and $[Na^+]_{ex}$. Increase in $[K^+]_{ex}$ markedly elevated Ca^{2+} influx in TCS-treated cells unlike the non-treated ones where increase in $[K^+]_{ex}$ induced an opposite effect (Fig. 4B). When extracellular Na^+ was isotonically replaced by $choline^+$, similar stimulation of Ca^{2+} influx was observed in TCS-treated cells. In this case, control cells exerted also stimulation of Ca^{2+} influx with decreasing $[Na^+]_{ex}$ (Fig. 4C). In the present state of knowledge we can only speculate about the effect of TCS on Ca^{2+} efflux. It seems that the increase in Ca^{2+} efflux from lymphocytes after TCS treatment is likely due to the release of Ca^{2+} from (an unknown) intracellular store triggered by changes in intracellular H^+ concentration. Simultaneous addition of TCS and Ca^{2+} into extracellular medium evoked larger increase in Ca^{2+} efflux.

These results together with our observations on human erythrocytes (Hudec et al. 2004; Hudec, unpublished data) led us to speculate that TCS affects rather H^+ gradient of plasma membrane than the mitochondrial one. Coupling of the basal Ca^{2+} influx with homeostasis of monovalent cations could be explained by changes in Na^+/H^+ exchanger activity. This is supported by different effect of amiloride on Ca^{2+} influx in the high $[Na^+]_{ex}$ and high $[K^+]_{ex}$ (Fig. 5A). Inhibitory effect of amiloride on TCS-stimulated Ca^{2+} influx in high $[K^+]_{ex}$ medium could be accounted for by formation of non-active bipolar compound or to direct blockade of Ca^{2+} carrier in plasma membrane as shown by other authors (Tang et al. 1988).

In conclusion, the present data provide a basic characteristic of basal Ca^{2+} influx into intact human blood lymphocytes and suggest pathways possibly involved in the regulation of this process.

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References

Aviv A. (1996): Recent advances in cellular Ca^{2+} homeostasis: implications to altered regulations of cellular Ca^{2+} and Na^+-H^+ exchange in essential hypertension. Curr. Opin. Cardiol. **11**, 477–482

Badou A., Basavappa S., Desai R., Peng Y. Q., Matza D., Mehal W. Z., Kaczmarek L. K., Boulpaep E. L., Flavell R. A. (2005): Requirement of voltage-gated calcium channel β4 subunit for T lymphocyte functions. Science **307**, 117–121

Balasubramanyam M., Kimura M., Aviv A., Gardner J. P. (1993): Kinetics of calcium transport across the lymphocyte plasma membrane. Am. J. Physiol. **265**, C321–327

Balasubramanyam M., Rohowsky-Kochan C., Reeves J. P., Gardner J. P. (1994): Na^+/Ca^{2+} exchange-mediated calcium entry in human lymphocytes. J. Clin. Invest. **94**, 2002–2008

Balasubramanyam M., Gardner J. P. (1995): Protein kinase C modulates cytosolic free calcium by stimulating calcium pump activity in Jurkat T cells. Cell Calcium **18**, 526–541

Balasubramanyam M., Balaji R. A., Subashini B., Mohan V. (2001): Evidence for mechanistic alterations of Ca^{2+} homeostasis in Type 2 diabetes mellitus. Int. J. Exp. Diabetes Res. **1**, 275–287

Bautista D. M., Hoth M., Lewis R. S. (2002): Enhancement of calcium signalling dynamics and stability by delayed modulation of the plasma-membrane calcium-ATPase in human T cells. J. Physiol. **541**, 877–894

Berridge M. J. (1995): Capacitative calcium entry. Biochem. J. **312**, 1–11

Cabado A. G., Alfonso A., Vieytes M. R., Gonzalez M., Botana M. A., Botana L. M. (2000): Crosstalk between cytosolic pH and intracellular calcium in human lymphocytes: effect of 4-aminopyridin, ammoniun chloride and ionomycin. Cell Signal. **12**, 573–581

Donnadieu E., Trautmann A. (1993): Is there a Na^+/Ca^{2+} exchanger in macrophages and in lymphocytes? Pflügers Arch. **424**, 448–455

Eckert A., Forstl H., Zerfass R., Hennerici M., Muller W. E. (1997): Free intracellular calcium in peripheral cells in Alzheimer's disease. Neurobiol. Aging **18**, 281–284

Fomina A. F., Fanger Ch. M., Kozak J. A., Cahalan M. D. (2000): Single channel properties and regulated expression of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in human T cells. J. Cell Biol. **150**, 1435–1444

Haverstick D. M., Dicus M., Resnick M. S., Sando J. J., Gray L. S. (1997): A role for protein kinase CbetaI in the regulation of Ca^{2+} entry in Jurkat T cells. J. Biol. Chem. **272**, 15426–15433

Hoth M., Fanger C. M., Lewis R. S. (1997): Mitochondrial regulation of store operated calcium signaling in T lymphocytes J. Cell Biol. **137**, 633–648

Hudec R., Lakatoš B., Kaiserová K., Orlický J., Varečka Ľ. (2004): Properties of the basal calcium influx in human red blood cells. Biochim. Biophys. Acta. **1661**, 204–211

Jambrina E., Alonso R., Alcalde M., del Carmen Rodríguez M., Serrano A., Martínez-A. C., García-Sancho J., Izquierdo M. (2003): Calcium influx through receptor-operated channel induces mitochondria-triggered paraptotic cell death. J. Biol. Chem. **278**, 14134–14145

Kotturi M. F., Carlow D. A., Lee J. C., Ziltener H. J., Jefferies W. A. (2003): Identification and functional characterization of voltage-dependent calcium channels in T lymphocytes. J. Biol. Chem. **278**, 46949–46960

Lewis R. S., Cahalan M. D. (1995): Potassium and calcium channels in lymphocytes. Annu. Rev. Immunol. **13**, 623–653

Majander A., Wikstrom M. (1989): The plasma membrane potential of human neutrophils. Role of ion channels and the sodium/potassium pump. Biochim. Biophys. Acta. **980**, 139–145

Makowska A., Zablocki K., Duszynski J. (2000): The role of mitochondria in the regulation of calcium influx into Jurkat cells. Eur. J. Biochem. **267**, 877–884

Martino G., Filippi M., Martinelli V., Brambilla E., Gobbi C., Comi G., Grimaldi L. M. (1997): Interferon-gamma induced increases in intracellular calcium in T lymphocytes from patients with multiple sclerosis precede clinical exacerbations and detection of active lesions on MRI. J. Neurol. Neurosurg. Psychiatry **63**, 339–345

Mason M. J., Mahaut-Smith M. P., Grinstein S. (1991): The role of intracellular Ca^{2+} in the regulation of the plasma membrane Ca^{2+} permeability of unstimulated rat lymphocytes. J. Biol. Chem. **266**, 10872–10879

Mooren F. C., Kinner R. K. H. (1998): Cellular calcium in health and disease Biochim. Biophys. Acta **1406**, 127–151

Nagy G., Koncz A., Perl A. (2003): T cell activation-induced mitochondrial hyperpolarization is mediated by Ca^{2+}- and redox-dependent production of nitric oxide. J. Immunol. **171**, 5188–5197

Negulescu P. A., Shastri N., Cahalan M. D. (1994): Intracellular calcium dependence of gene expression in single T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. **91,** 2873–2877

Nofer J. R., Pulawski E., Junker R., Seedorf U., Assmann G., Zidek W., Tepel M. (1999): $Na^{+/}Ca^{2+}$ exchange inhibitors modulate thapsigargin-induced Ca^{2+} and Na^+ influx in human lymphocytes. Int. J. Clin. Lab. Res. **29**, 89–92

Ori Y., Korzets A., Malachi T., Gafter U., Breitbart H. (1999): Impaired lymphocyte calcium metabolism in end-stage renal disease: enhanced influx, decreased efflux, and reduced response to mitogen. J. Lab. Clin. Med. **133**, 391–400

Prakriya M., Lewis R. S. (2003): CRAC channels: activation, permeation and the search for a molecular identity. Cell Calcium **33**, 311–321

Putney J. W. Jr. (1990): Capacitative calcium entry revisited. Cell Calcium **11**, 611–624

Putney J. W. Jr. (1997): Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. Cell Calcium **21**, 257–261

Randriamampita C., Trautmann A. (2004): Ca^{2+} signals and T lymphocytes: "New mechanisms and functions in Ca^{2+} signalling". Biol. Cell. **96**, 69–78

Roos J., DiGregorio P. J., Yeromin A. V., Ohlsen K., Lioudyno M., Zhang S., Safrina O., Kozak J. A., Wagner S. L., Cahalan M. D., Velicelebi G., Stauderman K. A. (2005): STIM1, an essential and conversed component of store-operated Ca^{2+} channel function. J. Cell Biol. **169**, 435–445

Rosado J. A., Sage S. O. (2000): Protein kinase C activates non-capacitative calcium entry in human platelets. J. Physiol. **529**, 159–169

Schwarz A., Tutsch E., Ludwig B., Schwarz E. C., Stallmach A., Hoth M. (2004): Ca^{2+} signaling in identified T-lymphocytes from human intestinal mucosa: relation to hyporeactivity, proliferation and inflammatory bowel disease. J. Biol. Chem. **279**, 5641–5647

Simchowitz L., Cragoe E. J. Jr. (1988): Na^+-Ca^{2+} exchange in human neutrophils. Am. J. Physiol. **254**, C150–164

Stokes L., Gordon J., Grafton G. (2004): Non-voltage-gated L-type Ca^{2+} channels in human T cells: pharmacology and molecular characterization of the major alpha pore-forming and auxiliary beta-subunits. J. Biol. Chem. **279**, 19566–19573

Tang C. M., Presser F., Morad M. (1988): Amiloride selectively blocks the low threshold (T) calcium channel. Science **240**, 213–215

Tintinger G. R., Anderson R., Ker J. A. (2002): Regulation of calcium homeostasis in activated human neutrophils – potential targets for anti-inflammatory therapeutic strategies. S. Afr. Med. J. **92**, 990–996

van Tits L. J., Michel M. C., Motulsky H. J., Maisel A. S., Brodde O. E. (1991): Cyclic AMP counteracts mitogen-induced inositol phosphate generation and increases in intracellular Ca^{2+} concentrations in human lymphocytes. Br. J. Pharmacol. **103**, 1288–1294

Zablocki K., Makowska A., Duszynski J. (2003): pH-dependent effect of mitochondria on calcium influx into Jurkat cells; a novel mechanism of cell protection against calcium entry during energy stress. Cell Calcium **33**, 91–99

Zablocki K., Szczepanowska J., Duszynski J. (2005): Extracellular pH modifies mitochondrial control of capacitative calcium entry in Jurkat cells. J. Biol. Chem. **280**, 3516–3521

Zhang S. L., Yu Y., Roos J., Kozak J. A., Deerinck T. J., Ellisman M. H., Stauderman K. A., Cahalan M. D. (2005): STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature **437**, 902–905

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