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Intracellularly regulated Ca^{2+} influx or remnants of extracellularly activated signalling pathway in human red blood cells?

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Abstract

Phorbol-14-myristate-13-acetate (PMA) (10^{-7}-10^{-6} mol/l) inhibited the Ca^{2+}-dependent K^+ efflux (the Gárdos effect - GE) induced by Ca^{2+}, the hyperpolarisation accompanying the GE, the vanadate-induced $^{45}Ca^{2+}$ influx, and depolarised the membrane, in vanadate-treated human red blood cells (RBC). The GE induced by propranolol (PLL) was not inhibited by PMA. Both PMA and PLL stimulated the basal $^{45}Ca^{2+}$ influx. These results suggest that a) protein kinase C activity prevents the activation of GE by vanadate but PLL bypasses this mechanism, b) the stimulation of the Ca^{2+} influx by PMA and the GE inhibition are caused by the membrane depolarisation, c) the basal Ca^{2+} influx in human RBC is regulated in a complex manner, and d) the effect of vanadate resembles to the activation of agonist-stimulated signalling pathway in non-excitable cells.

Key words: Human red blood cells; Ca^{2+} influx; Gárdos effect; Ca^{2+}-activated K^+ channel, propranolol; vanadate; PMA

Introduction

In non-excitable cells, receptor agonists often induce the Ca^{2+} influx followed by a membrane hyperpolarisation which is a consequence of the $[Ca^{2+}]_{cyt}$ increase and subsequent opening of the Ca^{2+}-activated K^+ channel (K_{Ca}). Such a sequence of events could be inferred from experiments with blood platelets activated by thrombin (Sage 1986), rat basophilic leukaemia cells activated by antigen of the IgE receptor (Mohr 1987, Labrecque 1991), human neutrophils (DiVirgilio 1987) or promyelocytic HL-60 cell line (Pittet 1990) activated by fMet-Leu-Phe (fMLP) chemotactic peptide and with in mast cells activated by their secretagogues (6Penner 1988, Cabado 1999). Also the acetylcholine-induced Ca^{2+} influx into

hamster ovary cells (Carroll 1998) or EDRF (endothelium-derived relaxing factor)-induced Ca^{2+} influx into endothelial cells (Luckhoff 1990) were inhibited by depolarisation. These properties of agonist-induced Ca^{2+} influx mirror the coupling between the action of agonists on their receptors, Ca^{2+} influx, activation of K_{Ca} and the membrane hyperpolarisation. One of the characteristical properties of the Ca^{2+} influx observed under these circumstances is its inhibition by increased $[K^+]_o$, or by the membrane depolarisation achieved by other instruments (e.g., ionophores, or K^+ channel blockers).

The Ca^{2+} influx could be elicited by vanadate in A431 epidermal carcinoma cells was also inhibited by depolarisation (Macara 1987). This observation reminisces vanadate - induced changes in human red blood cells (RBC) described earlier (Varečka 1982, Varečka 1987, Varečka 1997a, Varečka 1997b) which demonstrated the stimulation of the Ca^{2+} influx by this compound and the role of the K_{Ca} in generating the membrane hyperpolarisation which provided additional Ca^{2+}-motive force for the Ca^{2+} influx. Although vanadate-induced Ca^{2+} influx in RBC is an artificial phenomenon, it has a value as a model. Human RBCs have no intracellular organelles and the interference of the machinery of capacitative Ca^{2+} influx (involved in agonists-induced events in other cells) could be avoided.

Phorbol esters, tumor promoters, are known to exert multiple changes on partial reactions of cellular Ca^{2+} homeostasis and are activators of protein kinase C (PKC) (Govekar 2001). Their effects on the basal Ca^{2+} influx is stimulatory in human blood platelets (Rosado 2000), in human RBC (Andrews 2002, Murphy 1994), in rat artery endothelial cells (Hudec 2004), α-T3-1 pituitary gonadotroph cells (Anderson 1992), pituitary adenoma cells (Prevarskaya 1994). In other cells (Miller 1994, Chao 1992) no effect was observed. On the other hand, the agonist-induced Ca^{2+} influx and/or $[Ca^{2+}]_{cyt}$ increase seems to be regularly inhibited by the pre-incubation with PMA in a number of non-excitable cells and structurally non-related agonists (Anderson 1992, Aoyama 1995, Barbar 2003, Chau 1993, Chen 2000, Cooper 1985, Merritt 1993, Racke 1993, Staddon 1986, Sugita 1999, Tornquist 1995, Zoukhri 2000). However, PMA had no effect on fMLP-stimulated $^{45}Ca^{2+}$ influx into murine macrophage cell line (Kong 1993) and human neutrophils (Chen 2000). Thus, PMA is an important tool for studying the molecular events involved in establishing the rate of both basal and agonist-induced Ca^{2+} influx.

In this work we used vanadate-treated RBC as a model and show that PMA acts on Ca^{2+} fluxes and on the Ca^{2+}-activated K^+ efflux in way similar to its effect on agonist-induced events. In addition, we show that propranolol, K_{Ca} activator and Ca^{2+} influx inducer in RBC acts by PMA-independent mechanism.

Materials and Methods

Red blood cell suspension

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into chelatonate or 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 ^{o}C. No differences in experiments were found when our own (R.H., B.L.) blood was collected into the same anticoagulant mixture and used at the same day. RBC were isolated as described previously (Varečka 1997a) and were finally suspended into the suspension medium containing (in mmol/l): 20 Tris-Cl (pH 7.4); 140 NaCl; 5 KCl; 1.2 NaH_2PO_4; 1 $MgCl_2$ and 10 glucose to the haematocrit of 30%, and immediately used for experiments.

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

The modified procedure by Pokudin and Orlov (Pokudin 1986), which was described in (Varečka 1997b) was used. RBCs (0.5 ml, 30% haematocrit) were incubated at 25 or 37 °C and $^{45}Ca^{2+}$ (2.5 mmol/l; spec. act. approx. 4 000-8 000 cpm/nmol) was added. At times indicated (or after 20 min when not indicated otherwise) the transport was stopped by 5 ml of ice-cold stopping medium (in mmol/l): 20 Tris-Cl (pH 7.4); 65 NaCl; 70 KCl; 5 EGTA and 10 glucose, and immediately spun down on a centrifuge (for 2 min at 7,000 x g) at 2-4 ^{o}C. Two more washing steps followed, and the radioactivity retained in cells was determined by liquid scintillation counting after precipitation of cells with 0.5 ml of 10% (w/v) trichloroacetic acid (TCA) contained 10 mmol/l $LaCl_3$ and centrifugation of precipitates (5 min, 12,000 x g). Radioactivity was measured in supernatants.

$^{45}Ca^{2+}$ uptake induced by vanadate

The procedure described previously (Varečka 1997b) was used. Aliquots of 30% suspension were preincubated with 1 mmol/l $NaVO_3$ (further referred to as vanadate) for 15 min at 25 ^{o}C, following by the addition of $^{45}Ca^{2+}$ (2.5 mmol/l) and the incubation for 60 min at the same temperature. The incubation was stopped by the addition of the same volume of the stopping medium (described above) followed by the rapid centrifugation on the microcentrifuge (30 s, 12,000 x g). The supernatant was sucked off and pellet was washed with the same medium three times more and, finally, processed as indicated in the previous paragraph. Control cells without vanadate were treated in parallel.

Measurement of the Gárdos effect

The Gárdos effect (Ca^{2+}_i-dependent K^+ efflux) was monitored by the measurement of K^+ concentration in the medium by flame photometry (770 nm). The Gárdos effect was elicited by RBC treatment with 1 mmol/l $NaVO_3$ or 1 mmol/l propranolol. At time zero, $CaCl_2$ (2.5 mmol/l) was added to the 30% suspension of RBC, and the aliquots were withdrawn after 45 min incubation at 25 ^oC. After spinning down RBC suspension through a silicone oil layer the supernatant was used for flame photometry measurement. Controls (2.5 mmol/l EGTA was used instead of $CaCl_2$) were treated in parallel. Experiments were done in duplicates. Concentrations of K^+ in the media are shown in figures which were corrected for the the value of K^+ concentration added to the medium (5 mmol/l).

The measurement of the membrane potential changes

These changes were measured by means of the fluorescence probe 3,3'-dipropylthiodicarbocyanine iodide ($diSC_3$-(5)) as described by Sims (Sims 1974) 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.

Chemicals

$^{45}CaCl_2$ - ICN, U.S.A.; valinomycin - Calbiochem, Luzern, Switzerland; propranolol, phorbol-12-myristate-13-acetate (PMA) - Sigma; Tris base and dimethyl sulfoxide (DMSO) - AppliChem; Darmstadt, Germany, $NaVO_3$ - Reachim, Moscow, Russia; 3,3'-dipropylthiodicarbocyanine iodide ($diSC_3$-(5)) - Fluka, Buchs, Switzerland. Other used chemicals (all of analytical grade) were purchased from Lachema, Brno, Czech Republic.

Results

The Ca^{2+}-dependent K^+ efflux (Gárdos effect-GE) elicited by the pre-incubation with 1 mmol/l $NaVO_3$ followed by addition of 2.5 mmol/l Ca^{2+} was inhibited by increasing concentrations of PMA from 0.1 to 20 μmol/l. The degree of inhibition approached 60% (58.2 ± 3.1, n=5) at the highest PMA concentration used (Fig. 1). On the other hand, the GE induced by 1 mmol/l propranolol (PLL) and Ca^{2+} (2.5 mmol/l) was rather stimulated than inhibited by comparable concentrations of PMA. The effect of PLL was biphasic, the degree of stimulation decreased upon increasing the PMA concentrations over 10 μmol/l (Fig. 1). No

effect of PMA was observed in control cells without Ca^{2+} in the presence of either vanadate or PLL (Fig. 1).

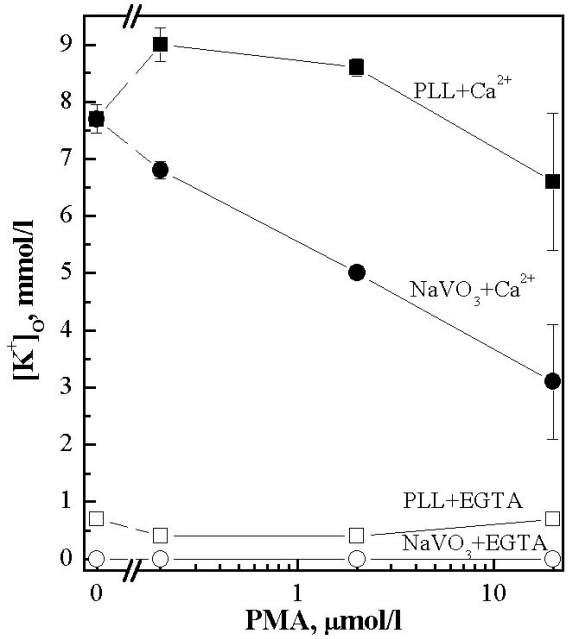

Fig. 1: The effect of PMA on the Ca^{2+}-induced K^+ efflux (GE) in vanadate- or propranolol-treated red blood cells. GE was monitored in presence of indicated concentration of PMA in 1 mmol/l vanadate (●)- or 1 mmol/l propranolol (■)-treated RBC suspension by measuring the K^+ concentration in the medium after 45 min incubation with 2.5 mmol/l Ca^{2+} at 25 °C by flame photometry. EGTA (2.5 mmol/l) was added to the controls (○,□). All values were diminished by 5 mmol/l (the nominal K^+ concentration in the medium). Plot is representative from the 5 independent experiments with 30% RBC suspensions. All data are expressed as mean ± standard error of duplicates.

In order to find whether the effect of PMA is indeed caused by its effect on the Ca^{2+}-activated K^+ channel (K_{Ca}) and not on the anion channel (which would also cause the inhibition of the GE), the membrane potential changes accompanying the GE were measured by the potential probe - cyanine dye, 3, 3-dipropylthiodicarbocyanine iodide (diS-C_3-(5)). The presence of PMA itself caused the increase of fluorescence which was dependent on both PMA and $[K^+]_o$ (Fig. 2A, B). The hyperpolarisation induced by the addition of Ca^{2+}in vanadate-treated RBC due to the opening of the K_{Ca} was inhibited by the pre-incubation of these cells with PMA but not with the same volume of solvent (Fig. 2C).

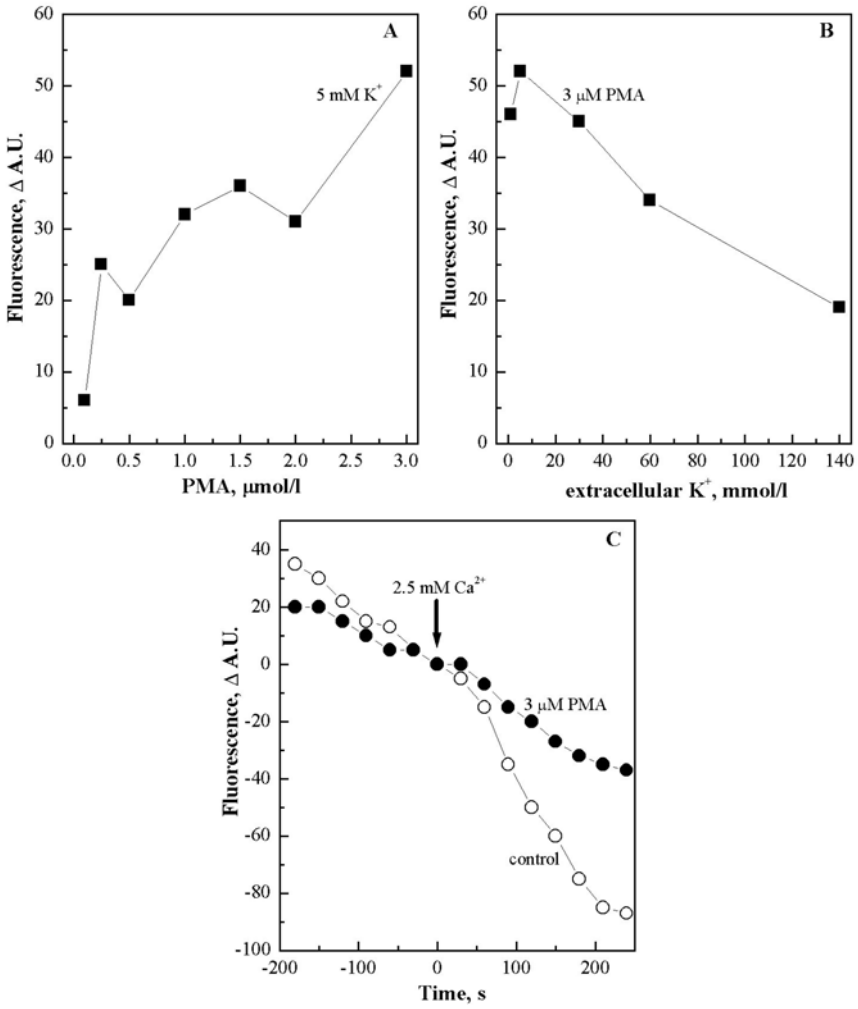

Fig. 2: Changes in fluorescence of 3,3´-dipropylthiodicarbocyanine iodide (diS-C_3-(5)) induced by PMA in non-treated RBC (A, B) and its effect on the hyperpolarisation induced by Ca^{2+} in vanadate-treated RBC (C). A. Fluorescence measurements were performed as described in Materials and Methods with non-treated RBC at 5 mmol/l K^+ in external medium using indicated PMA concentrations. B. Changes of fluorescence elicited by 3 μmol/l PMA in media with $[K^+]_o$ indicated in the figure. Equilibrium fluorescence was plotted. C. Fluorescence change induced by the addition of 2.5 mmol/l Ca^{2+} (arrow) to vanadate-treated RBC was measured in absence (○) or presence of 3 μmol/l PMA (●). Data in A - C are representative from 2 independent experiments and represent continuous fluorescence tracking manually converted to discontinuous form.

The inhibition of the K_{Ca} was found to inhibit the vanadate-induced Ca^{2+} influx in human RBC (Varečka 1982, Varečka 1997a). Therefore, the effect of PMA on the vanadate-induced $^{45}Ca^{2+}$ influx was investigated. These experiments revealed that the $^{45}Ca^{2+}$ influx induced by $NaVO_3$ was inhibited from the 0.1 to 3 μmol/l (Fig. 3A, B). The $^{45}Ca^{2+}$ influx into control (i.e., non-treated) cells was stimulated at the same conditions and 3 μmol/l PMA caused approximately the 100% increase of the calcium accumulation in comparison to the addition of the same volume of solvent (DMSO) (Fig. 3C). The inhibition of the vanadate-

induced $^{45}Ca^{2+}$ influx and the stimulation of the basal $^{45}Ca^{2+}$ influx could be increased by the prolonged pre-incubation with PMA (Fig. 3A).

Because the $^{45}Ca^{2+}$ influx induced by vanadate is inhibited by high $[K^+]_o$, a phenomenon found to be directly caused by the opening of the K_{Ca} (Varečka 1997a), the experiment was done which should explore whether this feature of vanadate accelerated $^{45}Ca^{2+}$ influx has been preserved in the presence of PMA. It is shown in the Fig. 3B that the differences between Na^+ - rich and K^+ - rich media were preserved also in the presence of high PMA concentrations and the $^{45}Ca^{2+}$ influx was inhibited by PMA also in K^+ - rich medium (Fig. 3B). On the other hand, in control RBC, the stimulatory effect of PMA on basal Ca^{2+} influx was not observed in K^+- rich medium (Fig. 3C).

Valinomycin, a K^+- ionophore, is expected to impose a membrane potential dependent on both magnitude and orientation of the K^+ gradient across the RBC membrane even under conditions of the K_{Ca} inhibition. Therefore, it should abolish any effect of PMA caused by the inhibition of membrane hyperpolarisation in vanadate-treated RBC. It was found, however, no effect of valinomycin on the $^{45}Ca^{2+}$ influx in the presence of PMA in either Na^+- rich or in K^+- rich medium (Fig. 3B). In control cells, an inhibition of the $^{45}Ca^{2+}$ influx by valinomycin was observed in both media, with the degree of inhibition by 3 μmol/l PMA of about 55 % and 33 %, respectively (Fig. 3C).

PLL (1 mmol/l), under identical conditions stimulated the $^{45}Ca^{2+}$ influx similarly as did PMA (3 μmol/l). The simultaneous addition of both compounds also stimulated the $^{45}Ca^{2+}$ influx and their effects were not additive (Fig. 3D).

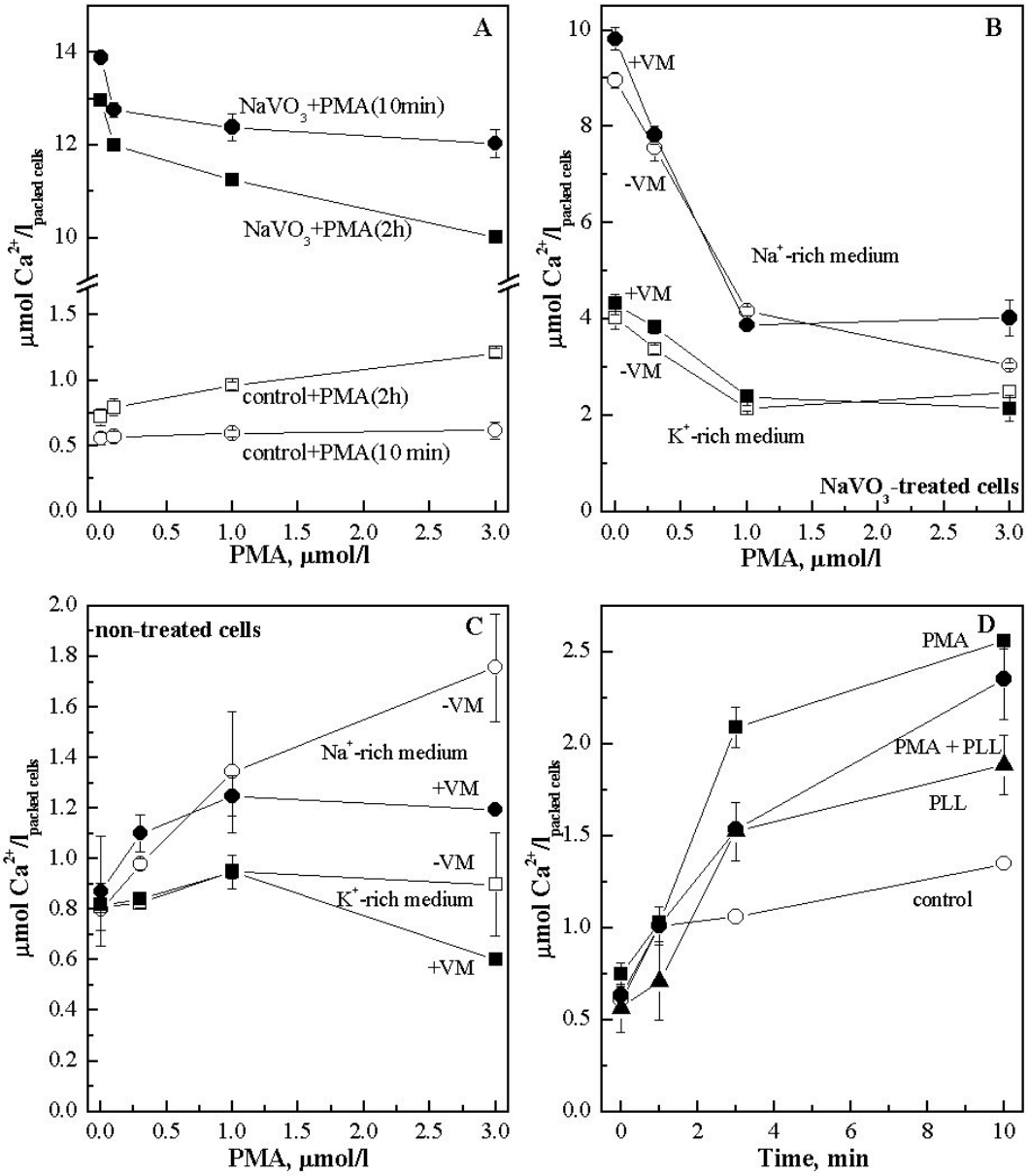

Fig. 3: $^{45}Ca^{2+}$ fluxes induced by PMA, PLL and vanadate.

A. Vanadate-induced $^{45}Ca^{2+}$ influx (●, ■) was measured according to Materials and Methods in the presence of indicated concentrations of PMA. PMA was added 10 min (●, ○) or 120 min (■,□) before addition of $^{45}Ca^{2+}$. The effect on basal $^{45}Ca^{2+}$ influx in non-treated cells (o,□) was measured in parallel. **B**. The effect of PMA in vanadate-treated RBC suspended in Na^+-rich (●,○) and K^+-rich medium (■, □) in the presence (●,■) or absence (○, □) of 1 µg/ml valinomycin (VM). **C**. The effect of PMA on the basal $^{45}Ca^{2+}$ influx under the same condition described in paragraph B. **D**. The time course of the $^{45}Ca^{2+}$ influx was measured as described in Material and Methods in the presence of 3 µmol/l PMA (■), 1 mmol/l PLL (●) or both compounds together (▲). Control (○) contains the same volume of solvents (max. 0.5% v/v). Data in A - D are representative from 3, 5, 5 and 3 independent experiments and are expressed as mean ± standard error of duplicates.

Discussion

Data presented above resulted from the screening of series of compounds intended to find a specific inhibitor of K_{Ca} (i. e. the Gárdos effect-GE) different from classical inhibitors of this transport system. They show that the known PKC activator inhibits the K_{Ca} activated by Ca^{2+} in vanadate-treated cells (Fig. 1). Its effect on the GE differs from classical K_{Ca} inhibitors (quinine, oligomycin) because it is dependent on the method of activation the K_{Ca} (Fig. 1). This observation reveals the complexity of events controlling the $^{45}Ca^{2+}$ influx and the K_{Ca} activation in human RBC.

The $^{45}Ca^{2+}$ accumulation in vanadate-treated cells was found to be partially inhibited by prototypal inhibitors of K_{Ca}, such as quinine, oligomycin, or 4-aminopyridine, (Varečka 1997b) in accordance with previous effects of these inhibitors on ATP-depleted cells (Armando-Hardy 1975, Blum 1972), and the inhibition was partially reversed by valinomycin (Varečka 1997a). The effect of PMA on the $^{45}Ca^{2+}$ uptake in $NaVO_3$-treated RBC is similar but cannot be reversed by valinomycin (Fig. 3B). It is difficult to interpret this finding as a direct inhibition of the Ca^{2+} transport system because the same compound stimulates the basal Ca^{2+} influx (Fig. 3C), unless adopting the suggestions by other authors (Engelmann 1989, Romero 2003) that vanadate opens a different Ca^{2+} transport pathway, or by us (Varečka 1997b), that the Ca^{2+} transport pathway was modified by the presence of vanadate. The latter possibility conforms the observation of Andrews (Andrews 2002) who found only one band reacting to the antibody against the α_{1a}-subunit of L-type Ca^{2+} channel. It seems, however, feasible that the inhibitory action of PMA on the Ca^{2+} influx may be caused by its depolarizing action (Fig. 2) as the membrane potential was found to be a component of driving force in vanadate-treated cells (Varečka 1997b). It is also feasible that the PMA-induced membrane depolarisation could contribute to the inhibition of the K_{Ca} and, subsequently, of the GE.

The absence of the valinomycin caused effect in vanadate-treated cells (Fig. 3B,C) is intriguing and contradicts the possibility that the Ca^{2+} influx is mediated by a hyperpolarization-driven uniporter. This suggests that the Ca^{2+} influx is mediated by an electroneutral mechanism in the presence of both vanadate and PMA. In the absence of vanadate, the depolarising action of PMA may be the cause of stimulation of $^{45}Ca^{2+}$ influx in agreement with the observation of Soldati et al. (Soldati 1997).

PLL stimulates the $^{45}Ca^{2+}$ influx as was observed earlier (Varečka 1987, Szász 1977) and the degree of stimulation of the $^{45}Ca^{2+}$ transport by PMA and PLL were comparable (Fig.

3D). Thus, the lack of inhibition by PMA of the GE induced by PLL is not caused by the surplus of the Ca^{2+} availability for K_{Ca}, and suggests that vanadate and PLL activate K_{Ca} by different mechanisms. This shows that the differences in the activation of K_{Ca} between vanadate-and PLL-treated RBC must involve other step(s) that precede(s) the direct activation of the K_{Ca} by Ca^{2+}. Such a conclusion is in contrast to the numerous observations made on patches of RBC membranes reviewed by Maher and Kuchel (Maher 2003) which showed that the presence of Ca^{2+} is the only requirement for the K_{Ca} activation. On the other hand, it is in agreement with earlier observations with sarcolemmal preparations (Wen 1984) or intact human RBC (Varečka 1990) which could be reconciled with the extrinsic regulation of K_{Ca} activation, possibly by protein phosphorylation and/or dephosphorylation.

The difference in the effect of PMA observed in Na^+ -rich and K^+-rich media may reveal an important clue for understanding the effect of PMA on both the activity of K_{Ca}, and the Ca^{2+} influx observed earlier (Andrews 2002). If the stimulatory effect of PMA on the $^{45}Ca^{2+}$ influx requires $[Na^+]_o$ (Fig. 3B,C), and if PMA inhibits the GE via the inhibition of the K_{Ca} (Fig. 1,2), then it is feasible that the Ca^{2+} influx is regulated in a complex manner, both by the activity of K_{Ca}, and Na^+ gradient. Another aspect, which should be emphasized, is that these changes occur without intervention of intracellular Ca^{2+} stores, which are not present in human RBC. Therefore, underlying molecular mechanisms observed in complete cells activated with agonists should be present in the cytoplasmic membranes only.

Our previous study (Varečka 1987) demonstrated the inhibitory effect of PKC inhibitor polymyxin B (PXB) on the K_{Ca} activation and the GE elicited by all ATP-depletion, vanadate, and PLL which were accompanied with the increase of the $^{45}Ca^{2+}$ influx. Thus, both PKC activator (PMA) and inhibitor (PXB) suppress the activity of K_{Ca} with opposite effect on the Ca^{2+} influx demonstrating the flexibility of its coupling to the membrane potential changes imposed by the K_{Ca} activity. This is also compatible with the possibility that under some conditions Ca^{2+} can permeate the membrane by electrically silent mechanism.

The molecular mechanism of PMA action could not be resolved in this paper. As PXB exerts a multiple inhibitory action on the phosphorylation of RBC membrane proteins (Kaiserová 2002) it is not possible to infer whether the dephosphorylation of one or more specific protein(s) is responsible for its effects. Experiments designed to compare the effects of PMA and PLL on the protein phosphorylation showed marked differences between the effects of these agents (stimulation and inhibition of phosphorylation by PMA, inhibition only by PLL) on the phosphorylation of RBC membrane proteins (R. Hudec, unpublished) but did not enable to unequivocally assign these effects to a formulatable biochemical mechanism.

PMA-elicited changes in intact RBC may reflects the role of PKC in determining the rate of basal Ca^{2+} influx or modulating it as a response to (yet not determined) intracellular stimuli. Changes induced by vanadate may fall to the same category. In addition, effects of vanadate (including effects of PMA, or PLL) could be regarded also as activation of a remnant of an agonist-receptor-activated signalling pathway originally present in RBC predecessor cells which lost its receptor part during RBC differentiation but preserved intact transduction and effector mechanisms (sensitive to vanadate, PMA or PLL).

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