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QUARTERLY

Participation of phospholipase A_2 isoforms in the control of calcium influx into electrically non-excitable cells★

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The participation of phospholipase A_2 isoforms in capacitative store-operated Ca^{2+} influx into Jurkat leukemic T and MDCK cells was investigated. Preincubation of Jurkat cells with either bromophenacyl bromide (an inhibitor of secreted phospholipase A_2, $sPLA_2$) or Helss (an inhibitor of calcium independent phospholipase A_2 — $iPLA_2$) resulted in a significant inhibition of the calcium influx. The extent of this inhibition depended on the pH of the extracellular millieu; it increased with alkalisation. The rate of Ca^{2+} influx into MDCK cells was reduced by bromophenacyl bromide. Preincubation of these cells with Helss resulted in the stimulation of the influx. These observations suggest the participation of different PLA_2 isoforms in the regulation of Ca^{2+} influx. They also show that the extent that PLA_2 isoforms control the influx depends on the pH of the medium. Finally, these data indicate that various phospholipase A_2 isoforms may play a role in the control of Ca^{2+} influx in different cell lines.

Calcium cations play a pivotal role in the intracellular signal cascade triggered by many extracellular stimuli, such as hormones and growth factors. An increase in the cytosolic calcium concentration ($[Ca^{2+}]_c$) which occurs after the stimulation of cells, is a result of both the release of Ca^{2+} from intracellular stores localised in the endoplasmic reticulum (ER) and the transport of Ca^{2+} from the extracellular medium into the cell. In electrically non-excitable cells, calcium release from the ER is activated by inositoltrisphosphate (IP_3).

★75th Anniversary of Membrane Lipid Bilayer Concept.

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Abbreviations: AA, arachidonic acid; BPB, bromophenacyl bromide; $[Ca^{2+}]_c$, cytosolic calcium concentration; CIF, calcium influx factor; CHO, chinese hamster ovary cells; $cPLA_2$, cytosolic phospholipase A_2; ER, endoplasmic reticulum; Helss, *E*-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; MDCK, Madin-Darby canine kidney cells; Me_2SO, dimethylsulfoxide; SOC, store-operated channel; $sPLA_2$, secretory phospholipase A_2.

The intracellular concentration of this signal molecule increases due to the stimulation of phospholipase C related to its interaction with protein G or growth factor receptors. IP_3 specifically binds to receptors in the membrane of the ER. These receptors carry the function of Ca^{2+} channels. IP_3 activates these channels and consequently promotes the release of Ca^{2+} from the ER (Berridge, 1993; Pozzan *et al.*, 1993). Calcium release from the ER may also be achieved, at least in some types of cells, by a Ca^{2+}-dependent activation of ryanodine receptors (Wayman *et al.*, 1998; Bennett *et al.*, 1998). The depletion of intracellular calcium stores activates calcium entry from the extracellular fluid into the cytosol *via* so-called stored-operated calcium channels (SOCs) (Putney, 1986; Putney, 1990). The activation of SOCs may also be achieved by treatment of cells with thapsigargin, a selective inhibitor of Ca^{2+}-ATPase in the ER (Thastrup *et al.*, 1994; Parekh & Penner, 1997). The role of SOCs in many physiological processes is well documented. However, the biochemical nature of the coupling between calcium content of the ER and the activity of SOCs is still unclear.

Three main hypotheses have been proposed to explain signaling between the ER and the plasma membrane. These hypotheses assume: 1) a direct conformational interaction between the ER membranes and SOCs, triggered by the activation of IP_3-sensitive receptors in the ER (Irvine, 1990; Berridge, 1995), 2) the participation of secretory vesicles (Somasundaram *et al.*, 1995), 3) the existence of a soluble messenger known as calcium influx factor, CIF. CIF was supposed to be synthesised or released into the cytosol after release of calcium from the ER. It has been postulated that CIF is a small molecule (about 500 kDa) that undergoes phosphorylation/dephosphorylation (Randriamampita & Tsien, 1993). Among other "CIF-related" concepts, it has also been suggested that SOC activation is related to cytochrome P450s (Alvarez *et al.*, 1992), protein G (Petersen & Berridge, 1994), tyrosine kinases (Lee *et al.*, 1993) and small GTP-binding proteins (Fasolato *et al.*, 1993).

Recent years have brought a growing number of evidence indicating that arachidonic acid (AA) and/or its derivatives are involved in the activation of SOCs. The relevant observations are the following:

- AA as well as 5,8,11,14-eicosatetraynoic acid, its non-metabolizable analogue, produces activation of SOCs in CHO cells (Gailly, 1998).
- Ca^{2+} release from the ER leads to an enhancement of the cellular AA concentration due to the liberation of this fatty acid from membrane glycerophospholipids (Rzigalinski *et al.*, 1996; Nofer *et al.*, 2000).
- Calcium depletion of the ER results in the stimulation of phospholipase A_2 activities (Törnquist *et al.*, 1994).
- Addition of exogenous phosholipase A_2 into the extracellular medium results in the activation of SOCs (Nofer *et al.*, 2000).

It is noteworthy that AA and/or its derivatives are also supposed to activate Ca^{2+} influx that is not coupled to the release of calcium from the ER (so called non-capacitative calcium influx); this was observed after stimulation of the cells with growth factors (Munaron *et al.*, 1997). Therefore, in experiments concerning the effect of AA on SOCs activity, this must also be taken into account (Shuttleworth & Thompson, 1999). Finally, at least in some cell types, it has been observed that fatty acids inhibit SOCs (Gamberucci *et al.*, 1997; Alonso-Torre & Garcia-Sancho, 1997).

The phospholipase A_2 family consists of a growing number of enzymes that catalyse the hydrolysis of the ester bound at the sn-2 position of glycerophospholipids. The products of this reaction (free fatty acids and lysophospholipids) are important for cell signalling and the biosynthesis of several biologically active lipids (including eicosanoids and platelet-activating factors). Numerous intracellular and secreted PLA_2s have been de-

scribed to date. The PLA_2 family is divided into at least three main classes according to their structures and enzymatic properties.

Class 1. Secretory PLA_2s ($sPLA_2$s) are small enzymes (about 14 kDa), requiring millimolar [Ca^{2+}] for full activity. They seem to exhibit only little selectivity towards fatty acids (Tischfield, 1997). Until now, five $sPLA_2$s have been described in mammalian tissues (Murakami *et al.*, 1998). They differ in tissue localization and participation in various physiological processes. Some of them are known to be involved in the signalling pathway of renal messangial cells activated by proinflammatory cytokines (Huwiler *et al.*, 1997). Apart from their enzymatic activities, $sPLA_2$s are supposed to interact with some soluble and membrane-bound receptors. The exact role of these receptors has not yet been identified but to date two main types of them, M-type (muscle-type, detected originally in muscle) and N-type (neuronal-type, detected originally in brain), have been described (Lambeau & Lazdunski, 1999).

Class 2. Cytosolic PLA_2s. This class is divided into two sub-classes: the first one containing $cPLA_2$s, commonly known as a "cytosolic" PLA_2s, and the second one, $iPLA_2$s or Ca^{2+}-independent PLA_2. $cPLA_2$ (an 85-kDa enzyme) is usually found in many mammalian tissues. It plays a role in signal transduction after receptor-mediated excitation of cells (Leslie, 1997). $cPLA_2$ requires 1 μM [Ca^{2+}] for maximal activity and exhibits a strong preference for AA containing glycerophospholipids (Clark *et al.*, 1991; Sharp *et al.*, 1991). Upon stimulation of the cell, $cPLA_2$ is translocated in a Ca^{2+}-dependent manner from the cytosol to the ER or perinuclear membranes (Glover *et al.*, 1995; Schievella *et al.*, 1995). This enzyme has a few phosphorylation sites, among which Ser^{505} seems to be the most important amino-acid residue phosphorylated by mitogen-activated protein kinase (MAPK) (Lin *et al.*, 1993). $cPLA_2$ is believed to release arachidonic acid that is subsequently converted into prostanoids and leukotrienes.

Class 3. $iPLA_2$s are an extraordinary group of PLA_2s; they do not need Ca^{2+} for enzymatic activity. $iPLA_2$s are subdivided into three types (Ackermann & Dennis, 1995) for a review): (1) lysosomal $iPLA_2$; (2) intracellular $iPLA_2$ types I and II and platelet-activating factor acetylhydrolases; their substrate specificities are limited toward platelet-activating factor and oxidised phospholipids; (3) 85-kDa $iPLA_2$-VI – forms oligomers of about 300 kDa. This latter enzyme does not exhibit any selectivity toward fatty acid and is postulated to be mostly involved in the remodelling of phospholipids (Balboa *et al.*, 1997; Balsinde & Dennis, 1997). Moreover, some experimental data indicate the role of $iPLA_2$-VI in the stimulus-dependent liberation of arachidonate. $iPLA_2$-VI was found to be activated in myocardial cells after depletion of intracellular calcium stores. The activity of this enzyme was lowered at high calcium concentration and increased in the presence of calmodulin inhibitors (Wolf & Gross, 1996). These findings suggest that $iPLA_2$ is inhibited by Ca^{2+}-calmodulin complex and the decrease in the calcium level in the ER results in the activation of $iPLA_2$ and formation of signalling molecules (Wolf *et al.*, 1997; Gross, 1998).

The purpose of this study was to test the hypothesis that different PLA_2s participate in the regulation of store-operated calcium entry into electrically non-excitable cells. We studied how two inhibitors of PLA_2s, one specific towards $sPLA_2$s and the other towards $iPLA_2$, influence store-operated calcium entry. In these experiments, we used two different electrically non-excitable cell lines, Jurkat lymphoidal T-cells and kidney epithelial cells (MDCK). We found that PLA_2s play a role in the control of SOCs and, that in various cell lines, different types of PLA_2 can modulate SOCs activity.

MATERIAL AND METHODS

Materials. *E*-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (Helss) and thapsigargin were from Calbiochem (La Jolla, CA, U.S.A.). Fura-2 AM and *p*-bromophenacyl bromide (BPB) were purchased from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin was from Sigma Chemicals, Co (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. The standard assay medium contained 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 25 mM Hepes, 1 mM pyruvate and 5 mM glucose; pH, as indicated.

Cell cultures. Jurkat lymphoidal T-cells were cultured in RPMI-1640 medium (Wrocław, Poland) supplemented with 10% foetal bovine serum (GIBCO BRL), 2 mM glutamine (GIBCO BRL), penicillin (100 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C, as described previously (Makowska *et al.*, 2000). Madin-Darby canine kidney cells, MDCK (passage 63–70) were grown on glass coverslips under the same conditions with the exception that the RPMI medium was replaced by a DMEM medium (GIBCO BRL).

Intracellular calcium measurements. Cytosolic free Ca^{2+} concentration was measured flurometrically using the fluorescent dye Fura-2 (Grynkiewicz *et al.*, 1985). Jurkat cells (10^6 cells/ml) suspended in the culture medium were loaded with 1 μM Fura-2 for 15 min at 37°C. Then, the cells were washed by centrifugation with the standard assay medium supplemented with 0.1 mM $CaCl_2$ and suspended in a nominally calcium-free assay medium (containing 1 mM EGTA) at the appropriate pH. MDCK cells growing on glass coverslip were loaded with Fura-2 by the same procedure, then rinsed with the standard assay medium containing 0.1 mM $CaCl_2$. The coverslips with cell monolayers were inserted into flurometric cuvettes containing the nominally calcium-free assay medium, pH 7.4. Fluorescence was measured at 30°C in a Shimadzu RF5000 spectrofluorometer set in the ratio mode using 340/380 nm wavelengths for excitation and 510 nm as the emission wavelength. The time resolution of the measurements was 1 s. In order to calculate $[Ca^{2+}]_c$, the system was calibrated in each run in the presence of 3 mM externally added $CaCl_2$ and 3 μM ionomycin plus 0.002% digitonin.

RESULTS AND DISCUSSION

We tested the action of two compounds on PLA_2s: BPB which inhibits $sPLA_2$s (Hernandez *et al.*, 1998) and Helss which specifically inhibits $iPLA_2$ (Hazen *et al.*, 1991). SOCs activities and corresponding rates of calcium influx were evaluated from the rate of changes in $[Ca^{2+}]_c$ triggered by the addition of 3 mM $CaCl_2$ to cells initially suspended in the nominally calcium-free standard medium and pretreated with thapsigargin. As expected, 10 μM Helss decreased the rate of calcium influx into Jurkat cells (Zabłocki & Duszyński, 1999). A similar action was also found in the case of 10 μM BPB (Fig. 1). The degree of BPB inhibition was time- and concentration-dependent. However, at higher concentration (> 20 μM) BCB additionally induced the calcium depletion of the ER.

These results suggest the participation of both $iPLA_2$ and $sPLA_2$s in the activation of the SOCs in Jurkat cells. Moreover, we established that these inhibitory effects were dependent on the acidity of the extracellular medium. As shown in Fig. 1, the rate of Ca^{2+} influx in control samples (without inhibitors) was lowest when the cells were incubated in the medium of pH 7.2 and it gradually increased in media of higher pH. Such a dependency of the rate of SOCs activity on the extracellular pH was described earlier for primary cultures of rat hepatocytes (Zhang *et al.*, 1991). On the other hand, inhibitory effects of both inhibitors were most expressed in cells

incubated in the medium of pH 7.8. It suggests that the PLA_2s-dependent step in the SOCs activating mechanism has a greater control power at pH 7.8 than at pH 7.2. At lower pH, the participation of PLA_2s in the activation of SOCs seems to be less crucial. We also cannot exclude the possibility that the relatively stronger effects of PLA_2 inhibitors at pH 7.8 results from the significantly enhanced activities of these enzymes under these conditions. Thus, the higher SOCs activity observed at pH 7.8 may be, at least partially, due to "hyperactivation" of PLA_2s. So, the inhibition of these enzymes has a greater impact on SOCs activity. This seems to be especially true in the case of $sPLA_2$-IIA. The activity of this enzyme is markedly elevated at alkaline pH (Kudo *et al.*, 1993). As the addition of $sPLA_2$ into the cell suspension stimulates the activity of SOCs (Nofer *et al.*, 2000) and, moreover, the activation of cells by the tumor necrosis factor (TNF) results in secretion of $sPLA_2$ (Kudo *et al.*, 1993), it may be suggested that, after cell stimulation and the release of calcium from the ER, endogenous $sPLA_2$ is secreted and acts as a paracrinic or autocrinic ligand affecting SOCs activity. In such a scenario, $sPLA_2$ would be transported to the extracellular medium and its activity could be directly modulated by extracellular pH. However, this hypothesis needs further confirmation. The inhibitory effect of Helss on the calcium influx into Jurkat cells is in accordance with data showing that $iPLA_2$ participates in AA release triggered by the depletion of intracellular calcium stores (Gross, 1998).

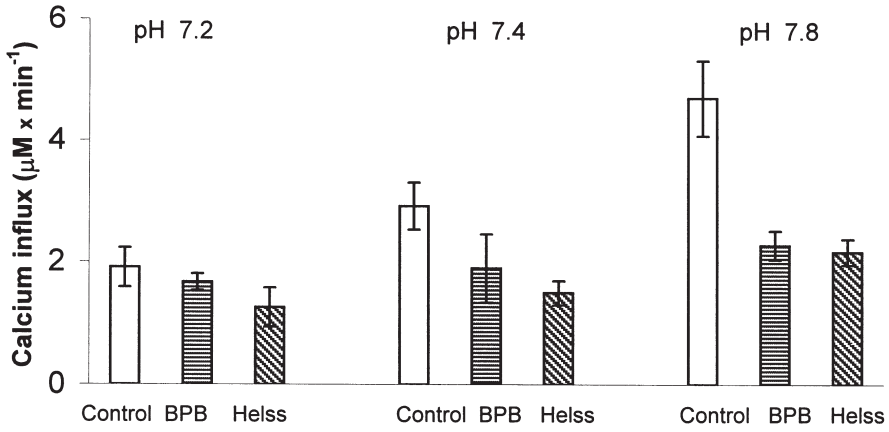

Figure 1. Effects of BPB and Helss on the rate of calcium influx into Jurkat cells incubated at various pH.

Fura-2 loaded Jurkat cells suspended in nominally calcium-free standard media supplemented with 1 mM EGTA, pH as indicated, were preincubated for 5 min with 1 μM thapsigargin. Then, the PLA_2 inhibitors (10 μM BPB or 10 μM Helss or Me_2SO in the control sample) were added. After 2 min preincubations with the inhibitors, the medium was supplemented with $CaCl_2$ (final concentration 3 mM). The rate of calcium influx was calculated from the changes in the Fura-2 fluorescence. The data are shown as means ± S.D. from 3–5 experiments.

As shown in Fig. 2, the activation of store-operated calcium influx into MDCK cells is also impaired by 50 μM BPB. This suggests the involvement of $sPLA_2$s in SOCs activation in this cell line. MDCK cells grow as a monolayer and hydrophobic agents may have restricted access to these cells. It is our experience that they are effective at higher concentration than in freely floating Jurkat cells. Thus, 50 μM BPB does not cause the ER depletion of calcium in the case of MDCK cells. This side effect is observed with BPB concentrations higher than 100 μM.

Suprisingly, the preincubation of MDCK cells with Helss resulted in a significant stimulation of the capacitative calcium influx. We cannot provide a satisfactory explanation for this phenomenon. MDCK are epithelial cells with a highly heterogenous topography of plasma membrane proteins. It has been postu-

lated that SOCs are localised mostly in the basolateral membranes that are not freely exposed to the extracellular medium (Gordjani *et al.*, 1997). Because $iPLA_2$ is involved in membrane remodeling, it seems possible that the inhibition of this enzyme by Helss results in an impaired rearrangement of membrane

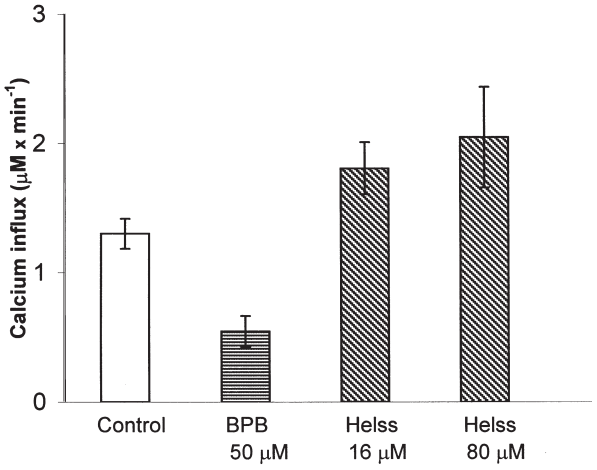

Figure 2. The effect of Helss and BPB on the rate of calcium influx into MDCK cells.

MDCK cell monolayers grown on glass coverslips were preincubated for 15 min at 37°C in growth medium containing 1 μM Fura-2 dye and the concentration of BPB as indicated or Helss or Me_2SO in the control sample. Subsequently, the monolayers were rinsed with the standard medium supplemented with 0.1 mM $CaCl_2$, pH 7.4, and transferred into the fluorometric cuvette containing nominally calcium-free standard medium. Then, the cells were preincubated for 7 min with 1 μM thapsigargin. After this period the medium was supplemented with $CaCl_2$ (final concentration 3 mM). The rate of calcium influx was calculated from changes in the Fura-2 fluorescence. The data are shown as means ± S.D. from 3 experiments.

phospholipids. In turn, this might lead to changes in the shape of cells and increased availability of calcium channels for Ca^{2+} added into the medium. However, this speculative explanation needs further investigation.

Recently, it has been shown that BPB and Helss inhibit Na^+ influx into thapsigargin treated human lymphocytes. It has also been found that this effect of thapsigargin on Na^+ entry could be mimicked either by exogenously added PLA_2 or AA. These data indicate a similarity between the regulation of Ca^{2+} and Na^+ influxes activated by the release of calcium from the ER. It was also shown (Nofer *et al.*, 2000) that Na^+ entry was associated with a tyrosine kinase-dependent activation of PLA_2; the inhibition of the kinase by genistein resulted in a decrease in both thapsigargin-triggered effects: release of AA and Na^+ influx into the cells. These observations are relevant to our findings since it is known that $iPLA_2$ activity is regulated by the tyrosine kinase pathway and inhibited by genistein (Olivero & Ganey, 2000). The participation of tyrosine kinase in the regulation of SOCs entry was suggested by Lee *et al.* (1993). So, it may not be precluded that this hypothesis will be employed again to explain the mechanism involving $iPLA_2$ in the regulation of SOCs activity. The results presented in this paper clearly show that $sPLA_2$s, as well as $iPLA_2$, are involved in the regulation of calcium influx into electrically non-excitable cells. According to other authors, in some cell lines this function is ascribed to $cPLA_2$ isoenzymes (Gailly, 1998; Leslie, 1997; Shuttleworth & Thompson, 1999). Summarizing, one can conclude that PLA_2 isoforms which belong to three distinguished classes – $sPLA_2$, $cPLA_2$ and $iPLA_2$, may play a role in the regulation of calcium homeostasis. However, the data presented, as well as the results obtained for different cell lines in other laboratories, indicate a strong variability in the mechanism of regulation of SOCs activity in various cell types.

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