Inhibition of protein tyrosine phosphatase 1B by reactive oxygen species leads to maintenance of Ca^{2+} influx following store depletion in HEK 293 cells

Ivan Bogeski [a], Monika Bozem [a], Lutz Sternfeld [a], Hans Werner Hofer [b], Irene Schulz [a],*

[a] *Physiological Institute, University of the Saarland, 66421 Homburg/Saar, Germany*
[b] *Department of Biology, University of Konstanz, 78457 Konstanz, Germany*

Abstract

Depletion of inositol 1,4,5 trisphosphate-sensitive Ca^{2+} stores generates a yet unknown signal, which leads to increase in Ca^{2+} influx in different cell types [J.W. Putney Jr., A model for receptor-regulated calcium entry, Cell Calcium 7 (1986) 1–12]. Here, we describe a mechanism that modulates this store-operated Ca^{2+} entry (SOC). Ca^{2+} influx leads to inhibition of protein tyrosine phosphatase 1B (PTP1B) activity in HEK 293 cells [L. Sternfeld, et al., Tyrosine phosphatase PTP1B interacts with TRPV6 in vivo and plays a role in TRPV6-mediated calcium influx in HEK293 cells, Cell Signal 17 (2005) 951–960]. Since Ca^{2+} does not directly inhibit PTP1B, we assumed an intermediate signal, which links the rise in cytosolic Ca^{2+} concentration and PTP1B inhibition. We now show that Ca^{2+} influx is followed by generation of reactive oxygen species (ROS) and that it is reduced in cells preincubated with catalase. Furthermore, Ca^{2+}-dependent inhibition of PTP1B can be abolished in the presence of catalase. H_2O_2 (100 μM) directly added to cells inhibits PTP1B and leads to increase in Ca^{2+} influx after store depletion. PP1, an inhibitor of the Src family tyrosine kinases, prevents H_2O_2-induced Ca^{2+} influx.

Our results show that ROS act as fine tuning modulators of Ca^{2+} entry. We assume that the Ca^{2+} influx channel or a protein involved in its regulation remains tyrosine phosphorylated as a consequence of PTP1B inhibition by ROS. This leads to maintained Ca^{2+} influx in the manner of a positive feedback loop.
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1. Introduction

Depletion of inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium stores leads to "capacitative" or "store-operated Ca^{2+} influx" (SOC) into pancreatic acinar cells [1] and in other cell types (for review, see Putney et al. [2]). The signal, by which SOC channels are opened, is however still unknown. Previously, we have shown that protein tyrosine phosphatase 1B (PTP1B) modulates store-operated Ca^{2+} influx in cells of the pancreatic acinar cell line AR42J and in HEK 293 cells [3]. In untransfected HEK 293 cells as well as in cells transfected with TRPV6 and/or co-transfected with PTP1B, the constitutive Ca^{2+} entry was not altered in the presence of the tyrosine phosphatase inhibitor DMHV. However, following depletion of intracellular Ca^{2+} stores, endogenous store-operated as well as TRPV6-mediated Ca^{2+} entry were increased in the presence of the tyrosine phosphatase inhibitor DMHV [3,4] and TRPV6 was tyrosine phosphorylated under these conditions [4]. We have demonstrated that Ca^{2+} influx following store depletion and the increase in cytosolic Ca^{2+} concentration led to inhibition of PTP1B activity in both, untransfected cells and cells transfected with TRPV6 [4]. Since PTP1B is not directly inhibited by Ca^{2+} [5] we assumed that Ca^{2+} influx activated a regulatory mechanism that finally led to inhibition

of PTP1B and consequently to increase in Ca^{2+} influx as long as tyrosine phosphorylation of the endogenous Ca^{2+} influx channel or of TRPV6, respectively, was maintained [4].

It was the aim of the present study to examine the regulatory mechanism, which is activated by Ca^{2+} influx and which results in transient inhibition of PTP1B. We have considered the Ca^{2+} signaling pathways, which play a role in the physiological stimulation of cells and which could be related to regulation of PTP1B activity [5–7]. Ca^{2+} is a messenger in different signaling pathways in response to cellular stimulation. Receptor-mediated stimulation of phospholipase C causes production of IP_3 and diacyl glycerol (DAG) in many cells. IP_3 releases Ca^{2+} from intracellular stores and DAG activates protein kinases C in Ca^{2+}-dependent or Ca^{2+}-independent ways, which have been suggested to inhibit PTP1B [8,9]. Furthermore, increase in cytosolic free Ca^{2+} concentrations also leads to stimulation of phospholipase A_2 [10–12], of NAD(P)H oxidases with generation of reactive oxygen species (ROS) and of nitric oxide synthase (NOS) with subsequent nitric oxide (NO^-) generation [13]. Inhibition of PTP1B by ROS due to reversible oxidation of cysteine 215 in the catalytic center of PTP1B has been described [14,15].

Our data provide evidence that Ca^{2+} influx following depletion of Ca^{2+} stores induces generation of ROS and that ROS inhibit PTP1B activity. Ca^{2+}-dependent inhibition of PTP1B activity is abolished and store-operated Ca^{2+} influx is reduced in cells preincubated with catalase. Furthermore, we show that the addition of an external source of ROS (H_2O_2) causes inhibition of PTP1B activity in HEK 293 and pancreatic AR42J cells and an increase in Ca^{2+} influx following store depletion. This increase is abolished in the presence of the Src family kinase inhibitor PP1.

Our data suggest that the cascade of events, which is engendered by depletion of intracellular Ca^{2+} stores by thapsigargin (tg) or acetylcholine (ACh) and followed by Ca^{2+} influx results in ROS generation and subsequent inhibition of PTP1B. We assume that this causes attenuated tyrosine dephosphorylation of target proteins involved in Ca^{2+} influx, which leads to maintenance of store-operated Ca^{2+} entry into the cell.

2. Materials and methods

2.1. Chemicals

Fura-2 AM, BAPTA-AM, $2',7'$-dichlorodihydrofluorescein diacetate (H_2-DCFDA) and thapsigargin were purchased from *Invitrogen* (*Molecular Probes*, Germany), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), diphenyleneiodonium chloride (DPI), 1-oleoyl-2-acetylglycerol (OAG), GF-109203X, bis-(*N,N*-dimethyl-hydroxamido) hydrooxovanadate (DMHV), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-50-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (GOE 6976), 1,2-dioctanoyl-*sn*-glycerol

(DOG), apocynin and acetylcholine were from *Calbiochem* (Germany), 2-amino-ethyldiphenyl borate (2-APB), phorbol 12-myristate 13-acetate (PMA) and arachidonic acid (AA) were purchased from *AXXORA* (Germany), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and penicillin/streptomycin were obtained from *PAA Laboratories* (Germany). Bovine serum albumin (BSA) and all other chemicals (analytical grade) were from *Sigma* (Germany).

2.2. Buffers

Buffer A (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, HEPES 20, glucose 10 (without Ca^{2+}), pH 7.4.
Buffer B (in mM): $CaCl_2$ 1.3, NaCl 135, KCl 5, $MgCl_2$ 1, HEPES 20, glucose 10, BSA 0.1%, pH 7.4.
Buffer C (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, HEPES 20, glucose 10 (without Ca^{2+}), leupeptin 0.2, trypsin inhibitor 20 μg/ml, PMSF 1 μM, Triton X-100 0.2%, pH 7.4.

2.3. Cell culture

HEK 293 and AR42J cells (*ATCC*) were cultured on plastic *Petri* dishes in DMEM, supplemented with 10% fetal calf serum and penicillin/streptomycin, in a humidified atmosphere (8.5% CO_2) at 37 °C. The cells used for experiments were at a culture density of about 80%.

2.4. Determination of protein concentration

Protein concentrations of cell homogenates were determined according to Bradford [16] with bovine serum albumin as standard.

2.5. Measurement of intracellular calcium concentrations

The cells were detached from the *Petri* dish and loaded with fura-2 AM (7 μM) for 30 min at 37 °C. Thereafter cells were washed twice in *buffer B* and once in *buffer A* and transferred to an acryl stirring cuvette. Calcium measurements were performed at 37 °C in a cell suspension in the Ca^{2+} free *buffer A* at a protein concentration of 0.3 ± 0.1 mg/ml using a fluorescence spectrometer (SPEX, DM 3000) 340/380 nm excitation, 505 nm emission and slit-width 0.5 mm. Calcium was added as indicated and concentrations were calculated for each experiment according to Grynkiewicz et al. [17], using a dissociation constant (K_D) for Ca^{2+} of 224 nM.

2.6. Measurement of protein tyrosine phosphatase 1B activity

Attached cells were pretreated within the *Petri* dish according to the respective protocol as indicated in the figure legends. Reactions were stopped precisely at a particular time point by transferring *Petri* dishes to liquid nitrogen until complete freezing. Cells were then collected into ice-cold

buffer C and broken by sonication. PTP1B activity in cell homogenates was assayed as described [3] using reduced, carboxymethylated and maleylated lysozyme (RCML) as substrate. RCML had been phosphorylated with [γ-^{32}P] ATP in the presence of type II protein tyrosine kinase purified from porcine spleen [18] following the procedure described by Tonks [5]. Release of [^{32}P] from cells, taken as a measure for PTP1B activity, was determined by Cerenkov counting for 5 min. Enzyme activities are expressed as specific activity (mean $\pm$ S.D.) in nmol/mg protein $\times$ min.

2.7. Measurement of reactive oxygen species

For determination of ROS generation in intact cells, cells were collected from *Petri* dishes and loaded with 10 μM H$_2$-DCFDA for 20 min at 37 $^\circ$C. After washing twice with *buffer B* and once with *buffer A*, cells were suspended in Ca^{2+} free *buffer A* and placed into an acryl cuvette for measurements of fluorescence. Before starting measurement cells were given 10 min rest to reduce unspecific ROS generation caused by agitation. Measurements were conducted at 37 $^\circ$C, using a fluorescence spectrometer (SPEX, DM 3000) at 498 nm excitation, 522 nm emission and slit-width 0.5 mm. When applying substances and Ca^{2+} to the measuring cuvette, perturbation of the cell suspension was avoided. Fluorescent signals of H$_2$-DCFDA are not reversible. The traces representing ROS generation reflect accumulated values for ROS. ROS generation is expressed as change in the intensity of fluorescence (ΔF; arbitrary units) over time.

2.8. Statistics

For statistical evaluations Student's *t*-test for paired or unpaired values was used. All data are expressed as mean $\pm$ S.D.

3. Results

3.1. Effect of Ca^{2+} influx on generation of reactive oxygen species and on PTP1B activity after Ca^{2+} store depletion

To examine the hypothesis that generation of reactive oxygen species is involved in Ca^{2+} influx-induced inhibition of PTP1B, we measured [Ca^{2+}]$_{cyt}$, ROS generation and PTP1B activity in parallel using the same protocol. Fig. 1 shows that addition of thapsigargin (tg, 200 nM), which causes Ca^{2+} store depletion and a small increase in cytosolic [Ca^{2+}] (Fig. 1A) also induced a small but significant rise in ROS generation in a Ca^{2+} free buffer (Fig. 1B) (see also Table 1). This, however, was not followed by any change of PTP1B activity (Fig. 1C).

After addition of Ca^{2+} (1.3 mM) both, Ca^{2+} influx and ROS generation were significantly increased (Fig. 1A and B and Table 1) and PTP1B activity was decreased (Fig. 1C) by

Table 1
Relative concentrations of reactive oxygen species (ROS) in HEK 293 cells

Experimental condition	ROS (% of control) $\pm$ S.D.	Significance (*p*-value)	*n*
Ca^{2+} free (control)	101 $\pm$ 9		5
tg (Ca^{2+} free)[*]	131 $\pm$ 17	0.01	5
ACh (Ca^{2+} free)[*]	167 $\pm$ 26	0.01	4
Ca^{2+}	130 $\pm$ 41	0.107	6
tg + Ca^{2+}[*]	316 $\pm$ 115	0.00001	9
ACh + Ca^{2+}[*]	344 $\pm$ 81	0.007	6
tg + Ca^{2+} + 2-APB[**]	103 $\pm$ 8	0.01	3
tg + Ca^{2+} + CCCP[**]	127 $\pm$ 38	0.008	5
tg + Ca^{2+} + BAPTA-AM[**]	140 $\pm$ 60	0.03	3
tg + Ca^{2+} + catalase[**]	195 $\pm$ 58	0.047	5
Ba^{2+}[*]	136 $\pm$ 14	0.0002	3
tg + Ba^{2+}[*]	210 $\pm$ 37	0.002	3
tg + Ba^{2+} + CCCP[***]	110 $\pm$ 22	0.004	3

ROS production was determined using the fluorescent dye H$_2$-DCFDA as described in Section 2. The fluorescence traces were recorded similar as shown in Fig. 1B. Values between 0 and 150 s (nominal Ca^{2+} free, control = 100%) and between 850 and 1000 s (experimental condition as indicated in the table), respectively, were averaged for this purpose. When the Ca^{2+} free condition was maintained throughout the experiment, the values between 850 and 1000 s were 101 $\pm$ 9% of the control signal. Concentrations used in the experiments: Ca^{2+} (1.3 mM), Ba^{2+} (2 mM), thapsigargin (tg, 200 nM), acetylcholine (ACh, 10 μM), 2-amino-ethyldiphenyl borate (2-APB, 50 μM), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μM), BAPTA-AM (10 μM) and catalase (1000 U/ml). Where indicated, cells were preincubated with 2-APB, CCCP and BAPTA-AM for 5 min, respectively, and with catalase for 15 min. In addition to *t*-tests using "Ca^{2+} free" as control, "tg + Ca^{2+}" and "ACh + Ca^{2+}" were also significantly different to "Ca^{2+}" (*p* = 0.002 and 0.01, respectively).
[*] *t*-test was performed with "Ca^{2+} free (control)" as reference.
[**] *t*-test was performed with "tg + Ca^{2+}" as reference.
[***] *t*-test was performed with "tg + Ba^{2+}" as reference.

about 20–30% of the control to a similar extent as described previously [4]. During the following decrease in cytosolic [Ca^{2+}] and ROS generation, the PTP1B activity recovered (see Fig. 1). In the absence of tg, addition of Ca^{2+} (1.3 mM) caused increase in [Ca^{2+}]$_{cyt}$ by only $\sim$10–20% as compared to that after store depletion, i.e. in the presence of tg. Under the same conditions, i.e. addition of Ca^{2+} in absence of tg, ROS generation was $\sim$14% of that following store depletion with tg (see Table 1). PTP1B activity remained unchanged without tg [4].

A similar inhibition of PTP1B as observed after endogenous ROS generation subsequent to Ca^{2+} influx (see Fig. 1 and Table 1) was also seen following addition of 10 μM H$_2$O$_2$ to HEK 293 cells. One hundred micromolar H$_2$O$_2$, a concentration at which inhibition of PTP1B is still reversible [15], had a slightly higher effect (Fig. 2). H$_2$O$_2$ at these relative low concentrations had no effect on base [Ca^{2+}]$_{cyt}$ both in the presence and absence of Ca^{2+} and in the absence of thapsigargin (data not shown).

In the presence of catalase, an enzyme that breaks down H$_2$O$_2$, the inhibitory effect of Ca^{2+} influx on PTP1B activity was abolished (Fig. 3A and B). Calcium influx following store depletion was reduced when the cells had been preincubated for 15 min and incubated with catalase (Fig. 3C).
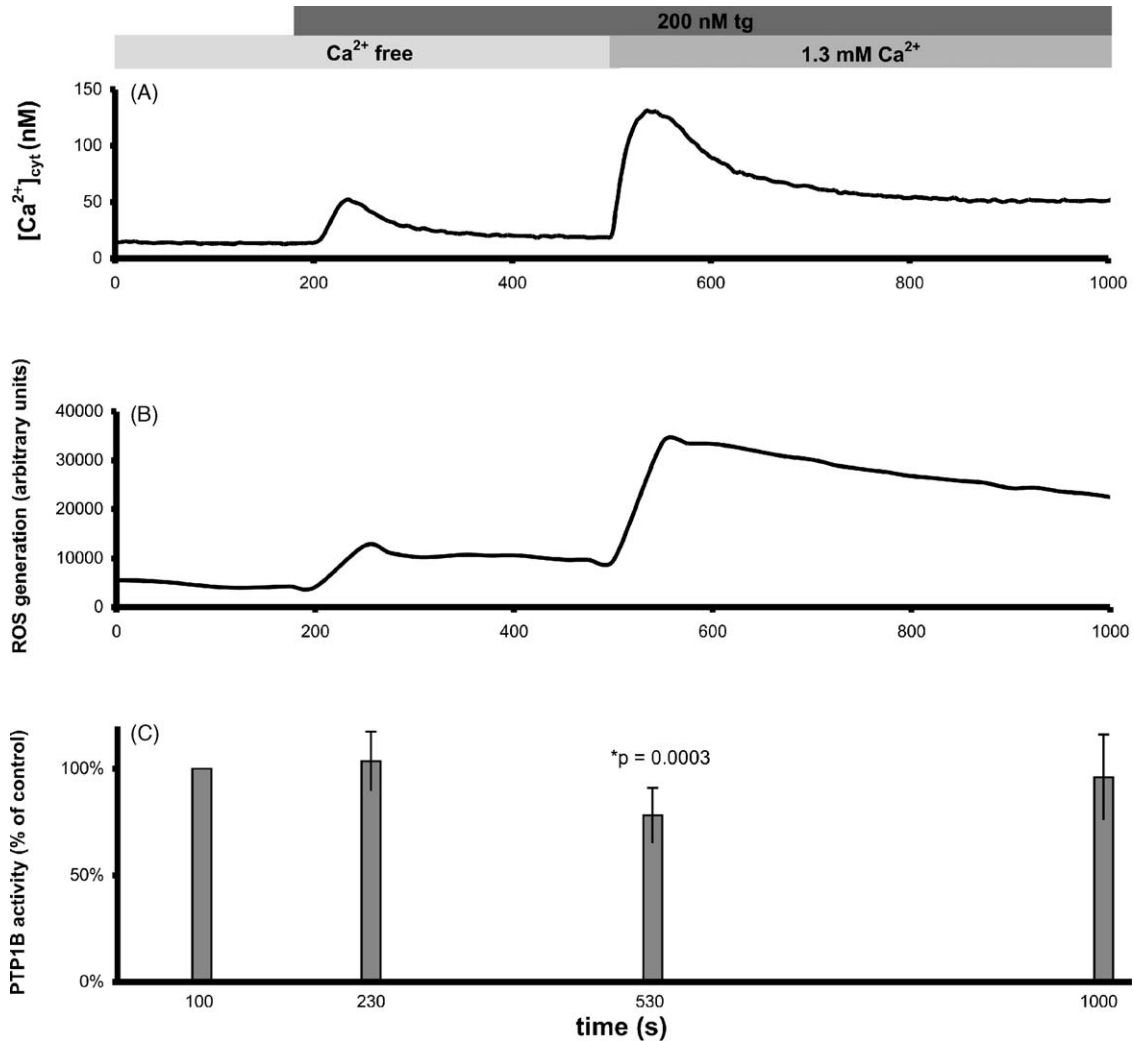
Fig. 1. Effects of Ca^{2+} store depletion by thapsigargin (tg) and addition of Ca^{2+} to nominal Ca^{2+} free buffer in HEK 293 cells on: (A) cytosolic $[Ca^{2+}]$, (B) generation of reactive oxygen species (ROS) and (C) PTP1B activity. $[Ca^{2+}]_{cyt}$ and ROS generation were continuously measured using the fura-2 fluorescence method and the fluorescent indicator H_2-DCFDA, respectively, PTP1B activity was determined at indicated time points using phosphorylated RCML as substrate (see Section 2). Each trace in (A) and (B) is representative for nine similar experiments. PTP1B activity (C) is expressed as mean values $\pm$ S.D. of six experiments.

Table 1 shows that the effect of Ca^{2+} influx after store depletion with either thapsigargin or with the neurotransmitter acetylcholine (10 μM) on ROS generation was abolished in the presence of 2-APB (50 μM), an inhibitor of store-
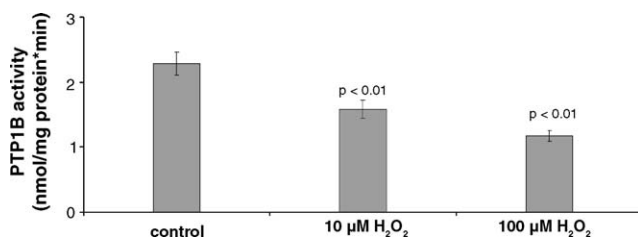

Fig. 2. Inhibition of PTP1B activity by H_2O_2 in HEK 293 cells. Cells were treated with 10 or 100 μM H_2O_2 in nominal Ca^{2+} free buffer for 10 s, then frozen in liquid nitrogen and homogenized. PTP1B activity was measured with RCML as substrate following the method described in Section 2. Data are mean values $\pm$ S.D. for three experiments.

operated Ca^{2+} channels, and by the Ca^{2+} chelator BAPTA-AM (10 μM). We have already shown previously that the prevention of a rise in cytosolic $[Ca^{2+}]$ by 2-APB or BAPTA-AM also prevented the inhibition of PTP1B [4].

ROS generation in the presence of thapsigargin and Ca^{2+} was also reduced by catalase, although not completely (see Table 1). This might be due to the fact that catalase does not enter the cell and only mops up any H_2O_2 that crosses the membrane, secondarily reducing the overall concentration inside the cell.

3.2. The source of ROS

We examined the contribution to the generation of ROS from three sources which are known to rely on Ca^{2+}-dependent mechanisms: (1) NAD(P)H oxidases [13], (2) the lipooxygenase- or cyclooxygenase-catalyzed reac-
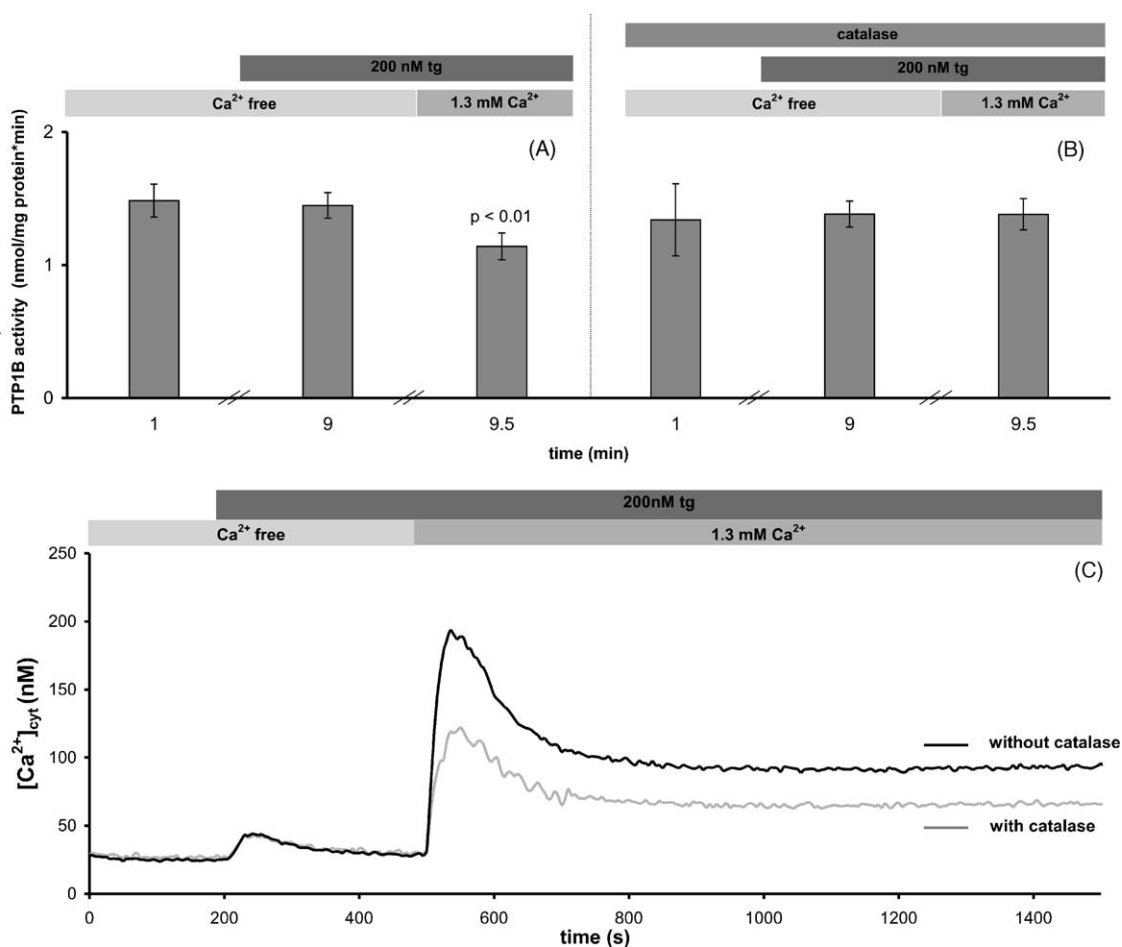
Fig. 3. Effect of catalase (1000 U/ml, 15 min preincubation) on PTP1B activity (A and B) and $[Ca^{2+}]_{cyt}$ (C) following store depletion with thapsigargin (tg, 200 nM) and addition of Ca^{2+} (1.3 mM) in HEK 293 cells. PTP1B activity was measured at the indicated times as described in Fig. 1 and Section 2. $[Ca^{2+}]_{cyt}$ was measured as described in the legend to Fig. 1 and Section 2. The experiments were performed either in the absence or presence of catalase as indicated. Data are mean values ± S.D. from three experiments (A and B) and show one representative out of five similar experiments (C).

tions that convert arachidonic acid to prostaglandins and leukotrienes and generate oxygen radicals as a by-product [19] and (3) the mitochondrial electron transport chain [19,20].

3.2.1. NAD(P)H oxidases

Cellular stimuli, such as hormones, usually lead to ROS generation via NAD(P)H oxidases [13,21,22]. As shown in Table 1, addition of acetylcholine (10 μM) in nominal Ca^{2+} free buffer induced significant ROS generation of 167 ± 26% of the control ($p = 0.01$). However, under these conditions no change in PTP1B activity could be observed (data not shown) [4]. Following Ca^{2+} influx after store depletion by ACh or tg we observed ROS generation by 344 and 316%, respectively, and a subsequent transient inhibition of PTP1B (see Figs. 1 and 3 for tg). Inhibitors of plasma membrane located NAD(P)H oxidases such as DPI (10 μM) or apocynin (500 μM) had no effect on PTP1B activity and on ROS generation following Ca^{2+} influx after store depletion ($n = 5$, data not shown).

3.2.2. Arachidonic acid pathway

A pathway involving hormonal and Ca^{2+}-induced stimulation of phospholipase A_2 with subsequent formation and oxidative metabolism of arachidonic acid also leads to ROS generation [19].

However, ebselen (10 μM) an inhibitor of both, lipo- and cyclooxygenases, did not show any effect on ROS generation upon addition of Ca^{2+} to the cells, with or without store depletion ($n = 3$, data not shown). Furthermore, direct addition of AA (20 μM) to cells with or without store depletion and in the presence or absence of Ca^{2+} had no effect on ROS generation ($n = 9$, data not shown).

3.2.3. Mitochondrial electron transport chain

ROS generation by mitochondria in response to Ca^{2+} has been described [19,20,23]. Ca^{2+} enhances ROS generation in mitochondria by stimulating the tricarbonic acid (TCA)-cycle and oxidative phosphorylation [19]. In the presence of uncouplers of oxidative phosphorylation, Ca^{2+}-dependent ROS generation is diminished [19].
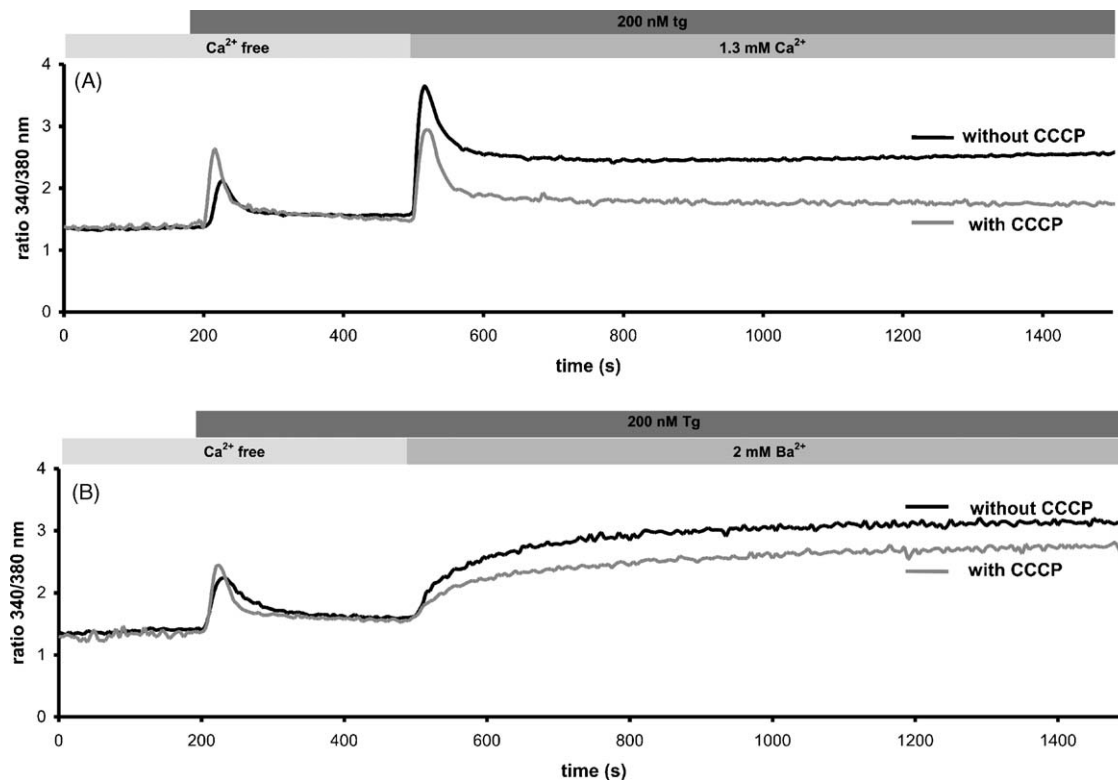
Fig. 4. Effects of CCCP (10 µM, 5 min preincubation), an uncoupler of oxidative phosphorylation on $[Ca^{2+}]_{cyt}$ (A) and $[Ba^{2+}]_{cyt}$ (B) in HEK 293 cells following store depletion with thapsigargin (tg). The particular incubation conditions are indicated on the top of the figures. Both, the initial Ca^{2+} influx peak and the Ca^{2+} influx plateau are reduced in the presence of CCCP (A). Ba^{2+} influx is slower without an initial peak and also diminished in the presence of CCCP (B). The figures show representative traces of four similar experiments each. Values for $[Ca^{2+}]_{cyt}$ and $[Ba^{2+}]_{cyt}$ are expressed as ratios using fura-2 as described in Section 2.

As shown in Table 1, mitochondrial inhibition with CCCP (10 µM), an uncoupler of oxidative phosphorylation, reduced ROS generation. Consequently, inhibition of mitochondrial ROS generation by CCCP should also decrease Ca^{2+} influx. As shown in Fig. 4A the peak showing the initial Ca^{2+} influx as well as the sustained Ca^{2+} influx were severely diminished in the presence of CCCP. This effect could be due to two regulatory mechanisms. One of them is the mitochondrial Ca^{2+} buffering which prevents Ca^{2+}-induced feedback inhibition of CRAC [24]. The other one is the generation of ROS from mitochondria, which subsequently leads to an increase in Ca^{2+} influx as described in this study. It was important therefore to find out, to what extent inhibition of ROS generation by CCCP contributes to this inhibitory effect shown in Fig. 4A. We therefore replaced Ca^{2+} in the buffer by Ba^{2+} and conducted the experiment presented in Fig. 4B. Ba^{2+} has no inhibitory effect on CRAC or SOC [25]. As can be seen from Fig. 4B, inhibition by CCCP can be observed, although Ba^{2+} influx is much slower as compared to Ca^{2+} influx. Generation of ROS during Ba^{2+} influx following store depletion was 210% of control (see Table 1). Under the same conditions we could show inhibition of PTP1B activity by 13% with 2 mM of Ba^{2+} as compared to 20% with 1.3 mM Ca^{2+} ($n = 2$). With 1.5 mM of Ba^{2+} inhibition was $5.4 \pm 0.6\%$ as compared to $25 \pm 1.8\%$ with Ca^{2+} ($n = 5$). With 1.3 mM Ba^{2+} no significant inhibition of PTP1B activity could be observed.

We therefore conclude that the inhibition of Ba^{2+} influx in the presence of CCCP (Fig. 4B) is mainly due to inhibition of ROS generation and consequent prevention of PTP1B inhibition.

3.3. Calcium effects on PTP1B activity are not mediated by protein kinase C

Since Ca^{2+} can activate Ca^{2+}-dependent protein kinase C (PKC) we examined the possibility that PTP1B is inhibited by PKC [8,26] via an increase in cytosolic $[Ca^{2+}]$. We therefore measured PTP1B activity in cells, which had been treated with activators of PKC such as PMA (200 nM), DOG (100 µM) or OAG (100 µM), or with inhibitors of PKC such as GOE 6976 (2 µM) and GF-109203X (10 µM). The phorbolester PMA added to cells in a nominal Ca^{2+} free buffer before ($n = 5$) or after addition of tg ($n = 5$) or in a Ca^{2+} containing buffer ($n = 4$) had no effect on PTP1B activity. DAG analogues of PKC activators such as DOG and OAG had inhibitory effects of 32 ± 14 and $24 \pm 6\%$, respectively. However, this effect was also observed without previous depletion of Ca^{2+} stores and was not abolished in the presence of the Ca^{2+} influx inhibitor 2-APB. Furthermore, PTP1B was also inhibited, when DOG or OAG was added to cell homogenates (data not shown). This suggests that the inhibitory effect of DOG and OAG on PTP1B activity was independent of Ca^{2+}
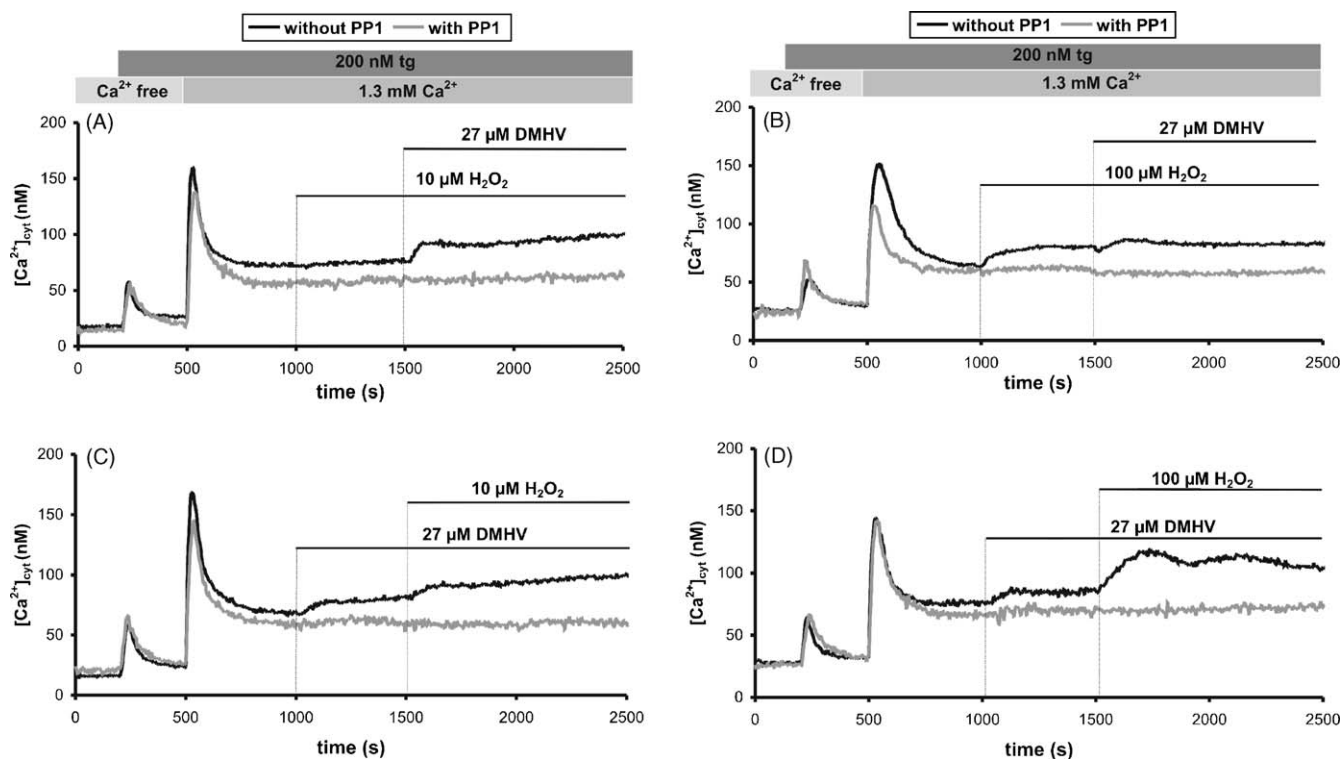
Fig. 5. (A–D) Effects of H_2O_2 (10 and 100 μM) and DMHV (27 μM) on Ca^{2+} influx following depletion of Ca^{2+} stores with thapsigargin (tg) in HEK 293 cells. The incubation conditions are indicated on the top of the figure. $[Ca^{2+}]_{cyt}$ of cells in suspension were continuously recorded with the fura-2 method in the absence (dark traces) or in the presence of the Src family kinases inhibitor PP1 (light traces, 30 min preincubation). DMHV and H_2O_2 were given to cell suspensions in the absence and in the presence of PP1 at similar time points.

influx following store depletion and may be a direct effect of these lipids on PTP1B.

Inhibitors of PKC such as GOE 6976 or GF-109203X did not change PTP1B activity and did neither prevent the inhibitory effect of Ca^{2+} influx nor of DOG and OAG (data not shown). These data therefore suggest that PKC is not involved in Ca^{2+} influx-induced PTP1B inhibition.

3.4. Store depletion-induced Ca^{2+} influx in the presence of the PTP1B inhibitors DMHV and H_2O_2

We have previously shown that inhibition of protein tyrosine dephosphorylation by PTP1B with DMHV increases store depletion-dependent Ca^{2+} influx into HEK 293 and AR42J cells [3,4]. At concentrations between 4.5 and 27 μM, DMHV had no effect on base $[Ca^{2+}]_{cyt}$ in the absence of thapsigargin [3]. Inhibition of PTP1B by DMHV also leads to protein tyrosine phosphorylation of TRPV6, a model Ca^{2+} channel [4]. Since H_2O_2 inhibits PTP1B [5,27] we anticipated that H_2O_2 would also increase Ca^{2+} influx following store depletion similar as DMHV [3,4]. As can be seen from Fig. 5, this was indeed the case. At 10 μM H_2O_2 had no or a small effect on Ca^{2+} influx (Fig. 5A). At 100 μM, H_2O_2 caused a clear increase in Ca^{2+} influx in all experiments (Fig. 5B). Both concentrations of H_2O_2 are known to reversibly inhibit PTP1B [15]. Addition of 27 μM DMHV, a maximal inhibitory concentration for PTP1B [3] caused fur-

ther Ca^{2+} influx, which was smaller following Ca^{2+} influx induced by 100 μM H_2O_2 as compared to 10 μM H_2O_2 (Fig. 5). The effects of both inhibitors seem not to be additive. Subsequent addition of 10 μM H_2O_2 and 27 μM DMHV or vice versa (Fig. 5A and C) caused an increase in cytosolic $[Ca^{2+}]$ by 49 or 57%, respectively, as compared to the value before application of the inhibitors. Subsequent additions of 100 μM H_2O_2 and 27 μM DMHV or vice versa (see Fig. 5B and D) caused an increase by 48 and 59%, respectively. More importantly, the effect of both inhibitors was completely abolished in the presence of the Src family kinases inhibitor PP1 (see Fig. 5, grey trace) and the Ca^{2+} plateau was lowered. This again indicates that tyrosine phosphorylation of target proteins is responsible for Ca^{2+} influx. Similar as with 2-APB [3,4] effects of DMHV and H_2O_2 on store depletion-dependent Ca^{2+} influx was completely prevented in the presence of 5 μM Gd^{3+}, which blocks store-operated Ca^{2+} channels [28] (data not shown). Similar data as with HEK 293 cells were also obtained with cells of the pancreatic cell line AR42J (data not shown).

4. Discussion

Reactive oxygen species have been implicated as mediators of cell signaling responses, such as growth, proliferation, differentiation, metabolism and migration, particu-

larly in pathways involving tyrosine phosphorylation [29]. Reversible oxidation and inactivation of the tyrosine phosphatase PTP1B by ROS is an important step in different stimulatory pathways controlling protein tyrosine phosphorylation [30].

Intracellular ROS, including H_2O_2 are produced in response to a variety of stimuli such as growth factors [31], insulin [32] or secretagogues [33]. There are at least three known sources of ROS within cells: (1) the mitochondrial electron transport chain, (2) NAD(P)H oxidases and (3) the lipoxygenase- or cyclooxygenase-catalyzed reactions that convert arachidonic acid to prostaglandins and leukotrienes [34]. Calcium can act at different sites in the generation of ROS. We have previously reported that Ca^{2+} influx into HEK 293 and pancreatic AR42J cells following depletion of intracellular Ca^{2+} stores results in inhibition of PTP1B activity [4]. Furthermore, inhibition of PTP1B with DMHV increased Ca^{2+} influx following store depletion [3,4] and led to tyrosine phosphorylation of the Ca^{2+} influx channel TRPV6 [4]. Our interpretation of the data implicated a model, in which inhibition of PTP1B enhances protein tyrosine phosphorylation, that in turn keeps the Ca^{2+} influx channel open as long as inhibition of PTP1B is maintained [3,4]. Since PTP1B is not directly inhibited by Ca^{2+} [5] we tested different possibilities to find the link between Ca^{2+} influx and inhibition of PTP1B. One of those could involve stimulation of a Ca^{2+}-dependent protein kinase C, which in turn could inhibit PTP1B [8,26]. However, neither an activator (PMA) nor inhibitors of PKC (GF-109203X, GOE 9676) had any effect on the activity of PTP1B in our system. Fatty acids such as DOG and OAG led to a small inhibition of PTP1B. Since this effect was also seen in the presence of the PKC inhibitors GF-109203X and GOE 6976 and in cell homogenates, this cannot be explained by PKC-mediated inhibition of PTP1B.

4.1. Inhibition of PTP1B by reactive oxygen species

Reactive oxygen species, generated in response to a physiological stimulus, had been reported to inhibit PTP1B by reversible oxidation of cysteine 215 in the catalytic center of PTP1B forming a sulphenic acid intermediate [5,27]. This leads to a reversible inhibition of PTP1B, and transient suppression of protein dephosphorylation. At high concentrations of H_2O_2 (>100 μM), however, PTP1B is irreversibly oxidized by the formation of sulphinic and sulphonic acid derivatives [15]. Similarly, DMHV like other vanadate derivatives, inhibits PTP1B irreversibly by oxidation of the active-site cysteine 215 to sulphonic acid. H_2O_2 added to cells at concentrations supposed to induce reversible inhibition of PTP1B (10 and 100 μM; see Fig. 2) [15] decreased PTP1B activity by 30 and 50%, respectively.

When Ca^{2+} influx was induced by Ca^{2+} store depletion, Ca^{2+} influx and subsequent rise in cytosolic $[Ca^{2+}]$ was followed by increase in ROS generation (see Fig. 1 and Table 1). At the same time PTP1B activity was inhibited by about the same degree as by addition of 10 μM H_2O_2

(see Figs. 1 and 2) and this inhibitory effect of Ca^{2+} influx on PTP1B activity was abolished by catalase that eliminates H_2O_2 (see Fig. 3A). In addition, catalase-induced attenuation of Ca^{2+} influx (Fig. 3C) also favors our model that Ca^{2+} influx itself stimulates Ca^{2+} channel activity via ROS generation and subsequent PTP1B inhibition, which leads to tyrosine phosphorylation. The inhibitory effect of catalase does not imply that all initially generated ROS are H_2O_2. Other more reactive oxygen species (O_2^-, OH^-, NO^-, etc.) are quickly converted intracellularly to H_2O_2 by enzymes of the superoxide dismutase family (SOD) [35].

4.2. Ca^{2+} influx following store depletion is increased by ROS and decreased by tyrosine kinase inhibition

We have previously shown that inhibition of PTP1B leads to increase in Ca^{2+} influx following store depletion. We also provided evidence that protein tyrosine phosphorylation is involved in this process [3]. As shown in Fig. 5 both, reversible inhibition of PTP1B by H_2O_2 as well as irreversible inhibition by DMHV led to increase in Ca^{2+} influx after store depletion. The effects of both were not additive and maximal at ~60% over the Ca^{2+} influx plateau before addition of these inhibitors.

One hundred micromolar of H_2O_2 was more effective than 10 μM on Ca^{2+} influx and PTP1B activity was only inhibited by maximal 50% under these conditions (see Fig. 2). The irreversible inhibitor of PTP1B, DMHV, at a maximal inhibitory concentration [3] caused further Ca^{2+} influx (see Fig. 5). The maximal effect of both inhibitors together, assuming complete inhibition of PTP1B never exceeded 60% increase in the Ca^{2+} plateau value. Interestingly, our assumption that protein tyrosine phosphorylation is involved in sustained Ca^{2+} influx was further supported by the observation that PP1, an inhibitor of Src family kinases, not only abolished both, H_2O_2 and DMHV-induced increase, but also reduced the Ca^{2+} plateau following readmission of Ca^{2+} (see Fig. 5). In a previous paper [4], we could show that inhibition of tyrosine dephosphorylation by inhibition of PTP1B led to tyrosine phosphorylation of the Ca^{2+} channel TRPV6 in TRPV6- and src-co-transfected HEK 293 cells. Since the molecular identity of the store-operated Ca^{2+} channel or "Ca^{2+} release-activated Ca^{2+} channel" (CRAC) is not yet known, we cannot prove, if the Ca^{2+} influx channel or a regulatory protein of it, is indeed tyrosine phosphorylated in our experiments. However, given that inhibitors of the tyrosine phosphatase PTP1B increase Ca^{2+} influx and those of Src family kinases inhibit this increase, it appears very likely that the Ca^{2+} influx channel or proteins involved in its regulation are involved in a mechanism that keeps Ca^{2+} influx increased as long as tyrosine phosphorylation is maintained. Other mechanisms, which could also lead to increase in Ca^{2+} influx such as activation of K^+ channels or inhibition of plasma membrane located Ca^{2+} pumps by tyrosine phosphorylation are possible. They seem, however, to be less likely, since we see the effects of DMHV and H_2O_2 on increase in Ca^{2+} influx only after store
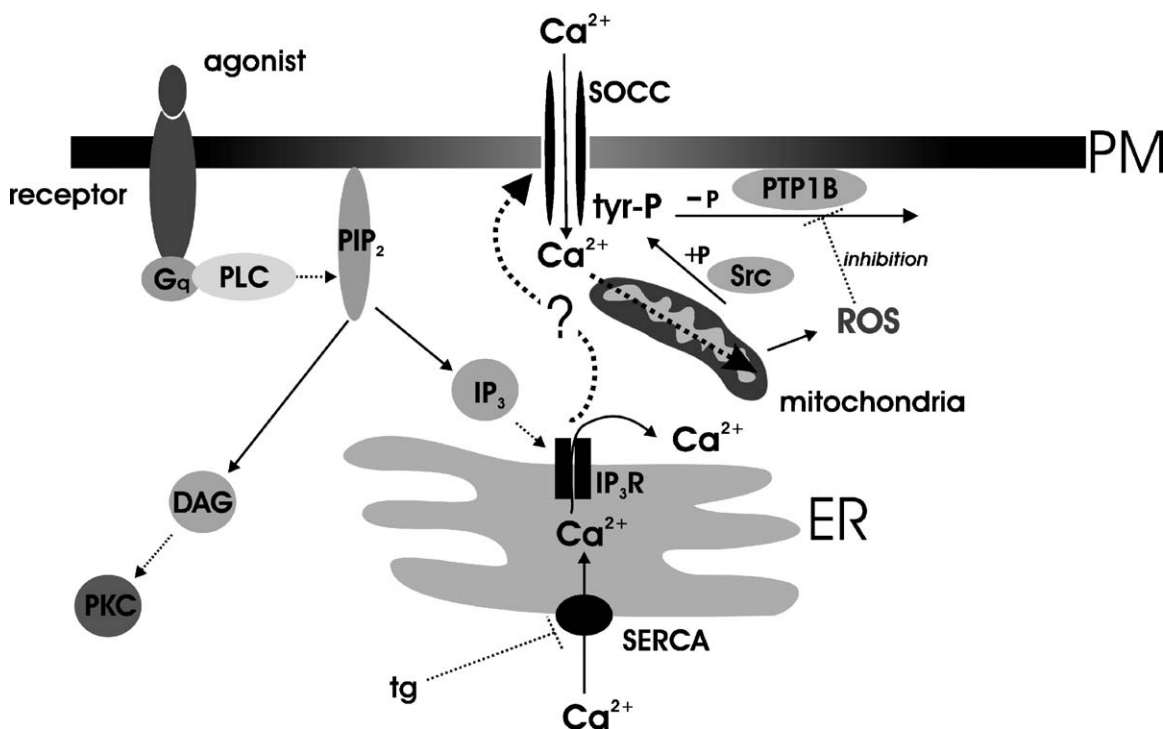
Fig. 6. Model for modulation of store-operated Ca^{2+} influx by protein tyrosine phosphorylation in HEK 293 cells. The link that leads to initial opening of store-operated Ca^{2+} influx is still not known. However, our data give evidence that Ca^{2+} influx following store depletion induces generation of reactive oxygen species (ROS) from mitochondria. ROS causes inhibition of protein tyrosine phosphatase 1B (PTP1B) that in turn by prevention of tyrosine dephosphorylation leads to prolonged tyrosine phosphorylation of a protein regulating Ca^{2+} influx and consequently to maintained Ca^{2+} influx. For details see text.

depletion. They are still present in depolarized cells in the presence of 10 mM K^+ in the bath (data not shown) and Ca^{2+} extrusion was not affected by these PTP1B inhibitors [3].

Our experiments support the conclusion from previous publications [3,4]. They add an important link to the signal cascade, which follows depletion of Ca^{2+} stores and leads to opening of a Ca^{2+} channel, by a yet unknown mechanism. The initial Ca^{2+} influx peak seems to be unaffected by tyrosine phosphorylation but it leads to production of ROS presumably from mitochondria, which reversibly inhibit PTP1B. Consequently, tyrosine dephosphorylation is transiently suppressed, channel opening is increased and Ca^{2+} influx is maintained as long as the protein responsible for this further increase is tyrosine phosphorylated. A second mechanism shown by Hoth et al. involving closure of the channel by increased $[Ca^{2+}]$ near to the cytosolic part of the channel could terminate Ca^{2+} influx [24]. Mitochondria close to the Ca^{2+} influx channel take up inflowing Ca^{2+} and prevent generation of a high Ca^{2+} concentration near to the channel and thereby its closure [24]. Although this role of mitochondria to buffer inflowing Ca^{2+} and thereby preventing closure of the Ca^{2+} channel plays a significant role [24], this role does not seem to be the only one to keep the Ca^{2+} channel open. When Ca^{2+} was replaced by Ba^{2+}, which has no inhibitory effect on store-operated Ca^{2+} channels or CRAC [25], generation of ROS from mitochondria was still present (see Table 1). Inhibition of Ba^{2+} influx in the presence of CCCP (Fig. 4B)

and inhibition of PTP1B was also observed. We therefore come up with a model (see Fig. 6), in which Ca^{2+} influx and uptake of Ca^{2+} by mitochondria generates ROS (see Fig. 1 and Table 1) [23]. This leads to inhibition of PTP1B (see Fig. 2) [5,27], and maintenance of tyrosine phosphorylation and of Ca^{2+} influx. We cannot exclude that other sources but mitochondria are also involved in ROS generation. We could not show any inhibition of ROS generation by NAD(P)H oxidase inhibitors. However, it is possible that Ca^{2+}-dependent hormonal stimulation involves activation of NAD(P)H oxidases as had been described for cerulein-activated NAD(P)H oxidases in pancreatic AR42J cells [21]. Further studies are necessary to clarify regulatory mechanisms that lead to hormonal activation of store-operated Ca^{2+} channels.

5. Conclusions

We have shown that Ca^{2+} influx following store depletion induces generation of reactive oxygen species and inhibition of protein tyrosine phosphatase 1B. Similar as the vanadate derivative, DMHV, H_2O_2 added to HEK 293 cells also leads to inhibition of PTP1B and to increase in store-operated Ca^{2+} influx, whereas catalase prevents Ca^{2+} influx-induced inhibition of PTP1B and reduces both ROS generation and Ca^{2+} influx. In the presence of PP1, an inhibitor of Src family kinases, H_2O_2-induced Ca^{2+} influx is abolished. We con-

clude that protein tyrosine phosphorylation is involved in the regulation of store-operated Ca^{2+} influx.

Acknowledgements

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