Phosphoinositide-dependent Kinase PDK1 in the Regulation of Ca^{2+} Entry into Mast Cells

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Key Words

Calcium • Ca^{2+} channels • K^{+} channels • Mast cells • Mouse

Abstract

The function of mast cells is modified by the phosphoinositide-3 (PI3)-kinase pathway. The kinase signals partially through the phosphoinositide-dependent kinase PDK1, which on the one hand activates the serum- and glucocorticoid-inducible kinase SGK1 and on the other hand activates protein kinase PKC δ . SGK1 participates in the stimulation of Ca^{2+} entry and degranulation, PKC δ inhibits degranulation. The present experiments explored the role of PDK1 in mast cell function. As mice completely lacking PDK1 are not viable, experiments have been performed in mast cells isolated from bone marrow (BMMCs) of PDK1 hypomorphic mice (*pdk1^{hm}*) and their wild-type littermates (*pdk1^{wt}*). Antigen stimulation via the Fc ϵ RI receptor was followed by Ca^{2+} entry leading to increase of cytosolic Ca^{2+} activity in *pdk1^{wt}* BMMCs, an effect significantly blunted in *pdk1^{hm}* BMMCs. In contrast, Ca^{2+} release from intracellular stores was not different between BMMCs of the two genotypes. The currents through Ca^{2+} -activated K^{+} channels follow-

ing antigen exposure were again significantly larger in *pdk1^{wt}* than in *pdk1^{hm}* cells. The Ca^{2+} ionophore ionomycin (1 μM) increased the K^{+} channel conductance to similar values in both genotypes. β -hexosaminidase and histamine release were similar in *pdk1^{wt}* BMMCs and *pdk1^{hm}* BMMCs. PKC δ inhibitor rottlerin increased β -hexosaminidase release in *pdk1^{wt}* BMMCs but not in *pdk1^{hm}* BMMCs. Phosphorylation of PKC δ and of the SGK1 target NDRG1, was stimulated by the antigen in *pdk1^{wt}* but not in *pdk1^{hm}* cells. The observations reveal a role for PDK1 in the regulation of Ca^{2+} entry into and degranulation of murine mast cells.

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Introduction

Mast cells underly IgE-dependent allergic reactions [1, 2] including allergic rhinitis [3], asthma [4], anaphylaxis and delayed type hypersensitivity reactions [5-8]. They release cytokines and regulate the function of neutrophils and T cells [7, 9]. Mast cell function is regu-

lated by the activity of ion channels such as Ca^{2+} channels [10–14], K^{+} channels [10, 15–17], and Cl^{-} channels [12, 13, 18]. The Ca^{2+} channels mediate the entry of Ca^{2+} which is critically important for degranulation and thus release of cytokines [19]. The K^{+} channels maintain the cell membrane potential, which provides the electrical driving force for Ca^{2+} entry [15, 17]. The effect of Cl^{-} channels on cell membrane potential depends on cytosolic Cl^{-} concentration. In theory, Cl^{-} channels could polarize the cell and thus facilitate Ca^{2+} entry or depolarize the cell membrane thus acting as a brake during mast cell activation [13].

Mast cell function is regulated by signaling pathways including the phosphatidylinositol (PI) 3 kinase-pathway [20–23]. The PI3 kinase pathway is partially effective through alteration of ion channel activity [24].

PI3 kinase-dependent signaling includes activation of the phosphoinositide-dependent kinase PDK1 [25, 26], which phosphorylates and thus activates the protein kinase B/Akt isoforms [27, 28], the serum- and glucocorticoid-inducible kinase (SGK) isoforms [29, 30], and the protein kinase PKC δ [31, 32].

SGK1 stimulates a variety of ion channels [30] including Ca^{2+} channels in mast cells [33]. Accordingly, the antigen induced Ca^{2+} entry and degranulation is blunted in mast cells lacking SGK1 [33]. Degranulation is further regulated by PKC δ [34, 35].

The present study has been performed to define the role of PDK1 in the regulation of mast cell function. The PDK1 knockout mouse is not viable [36]. Thus, mast cells have been isolated from PDK1 hypomorphic mice expressing some 10 to 25% PDK1 (*pdk1^{hm}*) and from age and sex matched wild type mice (*pdk1^{wt}*) [36].

Materials and Methods

Animals

Generation and basic properties of PDK1 hypomorphic mice were described previously [36]. Genotyping was made by PCR on tail DNA using PDK1 and neo-R-specific primers as described [36]. Mice had free access to standard mouse diet (Altromin, Lage, Germany) and tap water. All animal experiments were conducted according to the German law for the welfare of animals and were approved by the respective governmental authorities.

Culture of bone marrow derived mast cells (BMMCs)

Femoral bone marrow cells from 6–8 week-old *pdk1^{+/+}* and *pdk1^{hm}* naive mice were cultured for 4 weeks in RPMI 1640 (GIBCO, Carlsbad) containing 10% fetal calf serum, 1% penicillin/streptomycin, 20 ng/ml interleukin-3 (RD systems,

Wiesbaden-Nordenstadt, Germany), and 100 ng/ml of the c-kit ligand stem cell factor (SCF, Peprotech, Tebu-bio, Rocky Hill, NJ, USA). BMMC maturation was confirmed by flow cytometry (FACS Calibur, Becton Dickinson) using the following specific fluorescent-labelled antibodies: Phycoerythrin-labelled anti-Fc ϵ RI (eBioscience GmbH, Frankfurt, Germany), allophycocyanin-labelled anti-CD117 (BD Pharmingen, Heidelberg, Germany) and fluorescein-isothiocyanate-labelled anti-CD34 (BD Pharmingen). For experiments BMMCs were sensitized for 1 h with monoclonal mouse anti-dinitrophenyl mouse IgE (anti-DNP IgE, 10 $\mu\text{g}/\text{ml}$ per 1×10^6 cells, clone SPE-7, Sigma-Aldrich, Munich, Germany) in culture medium and then challenged acutely with dinitrophenyl-human serum albumin (DNP-HSA, 50 ng/ml, Sigma-Aldrich).

Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} measurements were performed as described [37] using Fura-2AM. Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 nm and 380 nm and the light was deflected by a dichroic mirror into the objective (Fluar 40 $\times$ /1.30 oil, Zeiss). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was performed using specialized computer software (Metafluor, Universal Imaging, Downingtown, PA). As a measure of the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the 340 nm/380 nm ratio were calculated for each experiment.

BMMCs were sensitized with IgE (10 $\mu\text{g}/\text{ml}$) for 1 h at 37°C and subsequently loaded with Fura-2/AM (2 μM , Molecular Probes, Goettingen, Germany) for 20 min at 37°C. Intracellular Ca^{2+} was measured prior to and following addition of DNP-HSA to IgE-sensitized BMMCs.

For intracellular calibration purposes, ionomycin (10 μM) was applied at the end of each experiment. Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+} -free conditions, experiments were performed using Ca^{2+} -free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 Na_2HPO_4 , 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4.

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode [38]. The cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (2–4 M Ω m tip resistance; GC 150 TF-10, Harvard Apparatus, March-Hugstetten, Germany) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Whole-cell currents were determined as 10 successive 400-ms square pulses from the -35 mV holding potential to potentials between -115 mV and +65 mV. The currents

were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered.

The pipette solution contained (in mM): 140 K-gluconate, 5 KCl, 1.2 MgCl₂, 2 EGTA, 1.26 CaCl₂ (pCa 7), 2 Na₂ATP and 10 HEPES/KOH (pH 7.2), and was used in combination with NaCl Ringer bath solution: 145 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 10 glucose, 10 HEPES/NaOH (pH 7.4). Where indicated the antigen DNP-HSA (50 ng/ml, Sigma-Aldrich) or the Ca²⁺ ionophore ionomycin (1 μ M, Sigma-Aldrich) were added to the bath solution.

The offset potentials between both electrodes were zeroed before sealing, and the potentials were corrected for liquid junction potentials as estimated according to Barry & Lynch [39]. The original whole-cell current traces are depicted without further filtering and currents of the individual voltage square pulses are superimposed. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

Degranulation assay (β -hexosaminidase release) and histamine release

Mature BMMCs (0.3 $\times$ 10⁶ per well) were seeded in a 96-well plate in fresh medium with 10 μ g/ml anti-DNP IgE antibody for 1 h. Afterwards cells were washed in Tyrode's salts (Sigma-Aldrich) and challenged with DNP-HSA (50 ng/ml) for 15 min at 37°C. 20 μ l supernatant and 20 μ l of 2 mM p-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma-Aldrich, Munich) diluted in 0.2 M citrate buffer, pH 4.5, were mixed and incubated for 2 hours at 37°C. The reaction was terminated by the addition of 60 μ l 1M TRIS buffer, pH 9.0, and the absorbance was measured at 405 nm in an ELISA microplate reader.

The data are evaluated as the percent of the total release (0.1 % Triton X-100) corrected for spontaneous release.

Histamine release from mast cells was measured by ELISA according to the instructions of the manufacturer (IBL-Hamburg GmbH, Hamburg, Germany).

Immunoblotting

Mast cells were seeded in a 6-well plate in fresh medium with 10 μ g/ml anti-DNP IgE antibody for 1 h. Afterwards cells were washed in PBS and challenged with DNP-HSA (50 ng/ml) for 5 min at 37°C. Then the cells were washed twice in PBS, then solubilized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X, 0.5% Nadeoxycholat) containing protease inhibitor cocktail (Roche), 0.4% β -mercaptoethanol and phenyl-methanesulfonyl fluoride solution (PMSF, 10 μ g/ml, Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at -80°C until use for western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline (TBS) and 0.1% Tween-20. Then the blots were probed overnight with one of the following rabbit polyclonal antibodies: phospho (p) NDRG1 (Thr³⁴⁶), p-PKC δ (Thr⁵⁰⁵), PKC δ or rabbit monoclonal GAPDH (Cell Signaling, Germany). p-PKC δ was diluted 1:500, all other antibodies were diluted

Fig. 1. Antigen induced Ca²⁺ entry into BMMCs from *pdk1^{wt}* and *pdk1^{hm}* mice. **A.** Representative tracings showing the ratio of 340/380 nm Fura-2 fluorescence in Fura-2/AM loaded BMMCs prior to and following acute addition of antigen (Ag, 50 ng/ml). At the end of each experiment, ionomycin (10 μ M) was added for calibration. For quantification of the Ca²⁺ entry into the BMMCs, the slope (delta ratio/s) and peak (delta ratio) were calculated following addition of Ag. **B.** Mean ($\pm$ SEM) of the peak (upper panel) and slope (lower panel) of the fluorescence ratio change for *pdk1^{wt}* (n = 25, open bars) and *pdk1^{hm}* (n = 15, closed bars) BMMCs following stimulation with antigen (Ag, 50 ng/ml). ** (p < 0.01) and *** (p < 0.001) indicate significant difference between both groups (two-tailed unpaired *t*-test). **C.** Representative traces showing the Fura-2 fluorescence ratio before and after addition of Ag (50 ng/ml) in the absence of extracellular Ca²⁺ in Fura-2AM loaded BMMCs. To create a Ca²⁺-free environment, EGTA (0.5 mM) was added to the Ca²⁺-free bath solution. Means ($\pm$ SEM) of the peak value (left side) and slope (right side) of the fluorescence ratio change for *pdk1^{wt}* (n = 4, open bars) and *pdk1^{hm}* (n = 5, closed bars) BMMCs upon stimulation with Ag in Ca²⁺-free solution.

Fig. 2. Ca^{2+} -activated K^{+} current in *pdk1*^{wt} and *pdk1*^{hm} BMMCs upon antigen-IgE-induced stimulation. A. Mean I-V relationships ($\pm$ SEM, $n = 6$) in *pdk1*^{wt} BMMCs (left panel) or *pdk1*^{hm} BMMCs (right panel) prior to (control, squares) or 3 min after stimulation with antigen added to 1 mM Ca^{2+} -containing standard NaCl bath solution (Ag, triangles). B. Mean whole-cell conductance in paired experiments ($\pm$ SEM, $n = 6-14$) as recorded in (A) prior to or 3 min after stimulation with antigen or ionomycin (1 μM). Data were calculated by linear regression between -95 and +45 mV. * indicates significant difference between *pdk1*^{wt} and *pdk1*^{hm} cells ($p < 0.05$; ANOVA).

1:1000 in TBS with 5% BSA 0.1% Tween-20, washed 3 times for 15 min/wash, probed with secondary antibodies (anti-rabbit, Cell Signaling) diluted 1:5000 for 1 h at room temperature, and washed 3 times for 15 min/wash. Antibody binding was detected with the Western blotting detection reagents (Amersham ECL Western Blotting Detection Reagents, GE Healthcare, UK). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany).

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed t -test or ANOVA (Dunnets test), where applicable. Results with $p < 0.05$ were considered statistically significant.

Fig. 3. Histamine release from antigen-stimulated *pdk1*^{wt} and *pdk1*^{hm} BMMCs. Mean histamine release ($\pm$ SEM, $n = 4$ individual experiments) from cultured *pdk1*^{hm} BMMCs (closed bars) and their wild-type littermates *pdk1*^{wt} (open bars) stimulated for 15 min with 50 ng/ml antigen. The stimulated histamine release in each experiment was corrected for the spontaneous release.

Fig. 4. Hexosaminidase release from antigen-stimulated *pdk1*^{wt} and *pdk1*^{hm} BMMCs. Mean β -hexosaminidase release ($\pm$ SEM, $n = 4$ individual experiments) from cultured *pdk1*^{hm} BMMCs (closed bars) and their wild-type littermates *pdk1*^{wt} (open bars) stimulated for 15 min with 50 ng/ml antigen. The experiments have been performed in the absence (left bars, control) and presence (right bars) of PKC δ -inhibitor rottlerin (10 μM). The stimulated β -hexosaminidase release in each experiment was corrected for the spontaneous release. * indicates significant difference ($p < 0.05$; ANOVA).

Results

To stimulate the Fc ϵ RI receptor of bone marrow derived mast cells (BMMCs), the BMMCs were incu-

Fig. 5. Phosphorylation status of PKC δ and the SGK1 target NDRG1 in *pdk1^{wt}* and *pdk1^{hm}* BMMCs. A. Original Western blot of mast cells from *pdk1^{wt}* and *pdk1^{hm}* mice untreated (control) or stimulated for 5 min with 50 ng/ml antigen (Ag). Protein extracts were analyzed by direct Western blotting using antibodies directed against phospho (p)-NDRG1 (Thr³⁴⁶) and p- PKC δ (Thr⁵⁰⁵). Equal protein loading was confirmed with GAPDH or PKC δ antibody. B. Arithmetic mean $\pm$ SEM (n = 4) of the abundance of p-NDRG1 (Thr³⁴⁶) and p- PKC δ (Thr⁵⁰⁵) as the ratio of p-NDRG1/GAPDH and p-PKC δ /PKC δ normalized to *pdk1^{wt}* (control). * (p<0.05) indicates significant difference from *pdk1^{wt}* (control), ANOVA.

bated with IgE (10 μ g/ml, 1h) directed against the hapten dinitrophenyl (DNP), washed and then stimulated with DNP coupled to the carrier protein human serum albumin (DNP-HSA, 50 ng/ml).

According to Fura-2 fluorescence, the activation of the Fc ϵ RI receptor resulted in a rapid increase of cytosolic Ca²⁺ concentration. The increase was significantly blunted in *pdk1^{hm}* BMMCs (Fig. 1A, B), i.e. the maximal fluorescence Δ ratio (peak) and the slope of the ratio (Δ ratio/time) upon stimulation with antigen were significantly smaller in *pdk1^{hm}* BMMCs than in *pdk1^{wt}* BMMCs (Fig. 1B). Stimulation of the BMMCs with antigen in Ca²⁺-free solution allowed an estimate of Ca²⁺ release from intracellular stores (Fig. 1C). This release was not significantly different between the genotypes (Fig. 1D).

According to whole cell patch clamp experiments the stimulation of BMMCs with antigen was followed by a rapid increase of the K⁺-selective conductance (Fig. 2). The same current could be induced by Ca²⁺ ionophore ionomycin (1 μ M). The current was thus carried by Ca²⁺-activated K⁺ channels, which have recently been identified as K_{Ca}3.1 [37] and are known to be activated following Fc ϵ RI cross-linking in human mast cells [12, 16]. The antigen induced current was significantly smaller in *pdk1^{hm}* BMMCs than in *pdk1^{wt}* BMMCs (Fig. 2). Treatment of the cells with ionomycin led to an increase of the K⁺ current to similar values in *pdk1^{wt}* and *pdk1^{hm}*

BMMCs (Fig. 2B). The observations suggested that the Ca²⁺-activated K⁺ channels were similarly expressed and similarly activated by increase of cytosolic Ca²⁺ in *pdk1^{wt}* and *pdk1^{hm}* BMMCs. The blunted activation of the K⁺ channels by antigen in *pdk1^{hm}* BMMCs thus resulted from impaired Ca²⁺ entry.

Additional experiments were performed to explore the effect of antigen exposure on degranulation of *pdk1^{wt}* and *pdk1^{hm}* BMMCs. Activation via the Fc ϵ RI receptor was followed by release of histamine and of β -hexosaminidase, an enzyme stored in mast cell granules [40]. The release of both histamine (Fig. 3) and β -hexosaminidase (Fig. 4) was similar in *pdk1^{hm}* BMMCs and *pdk1^{wt}* BMMCs.

The lack of difference between histamine and β -hexosaminidase release in *pdk1^{wt}* and *pdk1^{hm}* BMMCs could be due to the opposite effects of PDK1-dependent activation of serum- and glucocorticoid- inducible kinase 1 (SGK1), that is known to positively regulate mast cell degranulation [33] and PDK1 dependent activation of PKC δ , which is a negative regulator of degranulation [35]. Further experiments have been made in the presence of PKC δ -inhibitor rottlerin. As illustrated in Fig. 4, β -hexosaminidase release was significantly enhanced by rottlerin in *pdk1^{wt}* BMMCs but not in *pdk1^{hm}* BMMCs. The observation points to PDK1 sensitive inhibition of mast cell degranulation by PKC δ .

The activity of the PDK1 sensitive kinases SGK1 and PKC δ was assessed by western blotting of phosphorylated (activated) PKC δ and phosphorylated NDRG1 (N-myc downstream-regulated gene 1), a target of SGK1 [41]. Phosphorylation of PKC δ (Thr⁵⁰⁵) and NDRG (Thr³⁴⁶), a site of SGK1-mediated phosphorylation, was induced by antigen stimulation in *pdk1^{wt}* but not in *pdk1^{hm}* BMMCs (Fig. 5), confirming that both downstream targets of PDK1, SGK1 and PKC δ , are affected upon antigen stimulation in mast cells.

Discussion

The present study demonstrates that the stimulation of Ca²⁺ entry, but not Ca²⁺ release from intracellular stores, following exposure to antigen is significantly less pronounced in mast cells from PDK1 hypomorphic mice (*pdk1^{hm}*) than in mast cells from their wild type littermates (*pdk1^{wt}*). PDK1 deficiency thus blunts Ca²⁺ entry and the currents through Ca²⁺-activated K⁺ channels. The stimulation of the release of inflammatory mediators, as well as the depolymerisation of the cortical F-actin barrier require increase in intracellular Ca²⁺ [10-14, 42, 43]. Ca²⁺ further activates Ca²⁺-sensitive K⁺ channels [10, 15-17, 37], which maintain the membrane potential and thus the electrical driving force for Ca²⁺ entry [44].

The PI3 kinase has previously been shown to regulate mast cell proliferation, adhesion and migration, as well as antigen-IgE-induced degranulation and cytokine release [20]. Moreover, the PI3 kinase was suggested to regulate the mast cell TRPV2 Ca²⁺ channel [45]. PDK1 activates the serum- and glucocorticoid-inducible kinase SGK1 [25, 26, 46-50]. SGK1 is genomically upregulated by glucocorticoids [29], mineralocorticoids [51-53], 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) [54], cell shrinkage [55], gonadotropins [56-59], and TGF β [60, 61].

Surprisingly, partial PDK1 deficiency did not abrogate degranulation despite the reduced Ca²⁺ entry.

Accordingly, both β -hexosaminidase release and histamine release were not significantly different between *pdk1^{wt}* and *pdk1^{hm}* BMMCs. It should be kept in mind that the *pdk1^{hm}* still express some 10-25% of regular PDK1. The determination of cytosolic Ca²⁺ concentration and the K⁺ channel activity could be more sensitive functions than degranulation. Moreover, degranulation depends on localized increases of cytosolic Ca²⁺ activity, which may not be apparent in Fura2 fluorescence of the whole cell [62, 63]. Alternatively, PDK1 may, in addition to stimulating Ca²⁺ entry, inhibit degranulation by some other mechanism. PDK1 activates protein kinase PKC δ [31, 32] which has in turn been shown to negatively regulate mast cell degranulation [35]. Reduced activity of PDK1 may on the one hand compromise the stimulation of degranulation by decreasing Ca²⁺ entry and on the other hand disinhibit degranulation by decreasing activation of PKC δ . The net result could be seemingly unchanged degranulation. The observation that the PKC δ -inhibitor rottlerin enhances β -hexosaminidase release in *pdk1^{wt}* BMMCs but not in *pdk1^{hm}* BMMCs is in support of a dual role of PDK1 in the regulation of mast cell degranulation.

In conclusion, PDK1 participates in the regulation of Ca²⁺ entry and Ca²⁺-activated K⁺ channels of mast cells. At the same time, PDK1 may influence degranulation through activation of PKC δ . The present observations thus identify a further element in the signaling linking PI3 kinase activation and mast cell function.

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