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Activation of Protein Kinase D1 in Mast Cells in Response to Innate, Adaptive, and Growth Factor Signals

Thomas R. Murphy, Henry J. Legere III, and Howard R. Katz

Little is known about the serine/threonine kinase protein kinase D (PKD)1 in mast cells. We sought to define ligands that activate PKD1 in mast cells and to begin to address the contributions of this enzyme to mast cell activation induced by diverse agonists. Mouse bone marrow-derived mast cells (BMMC) contained both PKD1 mRNA and immunoreactive PKD1 protein. Activation of BMMC through TLR2, Kit, or FcεRI with Pam3CSK4 (palmitoyl-3-cysteine-serine-lysine-4), stem cell factor (SCF), and cross-linked IgE, respectively, induced activation of PKD1, as determined by immunohistochemical detection of autophosphorylation. Activation of PKD1 was inhibited by the combined PKD1 and protein kinase C (PKC) inhibitor Gö 6976 but not by broad-spectrum PKC inhibitors, including bisindolylmaleimide (Bim) I. Pam3CSK4 and SCF also induced phosphorylation of heat shock protein 27, a known substrate of PKD1, which was also inhibited by Gö 6976 but not Bim I in BMMC. This pattern also extended to activation-induced increases in mRNA encoding the chemokine CCL2 (MCP-1) and release of the protein. In contrast, both pharmacologic agents inhibited exocytosis of β-hexosaminidase induced by SCF or cross-linked IgE. Our findings establish that stimuli representing innate, adaptive, and growth factor pathways activate PKD1 in mast cells. In contrast with certain other cell types, activation of PKD1 in BMMC is largely independent of PKC activation. Furthermore, our findings also indicate that PKD1 preferentially influences transcription-dependent production of CCL2, whereas PKC predominantly regulates the rapid exocytosis of preformed secretory granule mediators. The Journal of Immunology, 2007, 179: 7876–7882.

Mast cells play key roles in the pathogenesis of allergic diseases but can also provide protection against certain microbial infections (1). Mast cells can be activated by many stimuli (2, 3), leading to rapid exocytosis of preformed granule mediators, synthesis and secretion of eicosanoids (primarily leukotriene C4 and PGD2), and release of a large number of proinflammatory and immunomodulatory cytokines and chemokines (4, 5). However, different activating agents can induce the production of different profiles of mediators from a particular mast cell population, which influences their contributions to immunologic and other processes in vivo. Hence, it is important to understand the signaling events that regulate the responses of mast cells to their activators, and in particular, those that are associated with the release of a particular mediator or class of mediators.

While investigating protein kinase C (PKC)3-dependent phosphorylation induced by several activating agents in mouse bone marrow-derived mast cells (BMMC), we also observed activation of protein kinase D (PKD)1, a member of a related but distinct family of kinases. PKD1 is a serine/threonine kinase consisting of two cysteine-rich zinc finger domains (C1a and C1b) in the N-terminal region, a pleckstrin homology domain, and a C-terminal kinase domain that has homology with calmodulin-dependent kinase (6, 7). The C1b domain of PKD1 to diacylglycerols (8) promotes membrane localization of PKD1 and an increase in enzyme activity (7, 9, 10). PKD1 was originally termed PKCμ because of sequence identity in the diacylglycerol-binding region with PKC and because PKD1 is a serine/threonine kinase (6). However, the preferred consensus amino acids flanking phosphorylated serines and threonines in substrates are distinct for PKD1 and PKC (7, 11). Nevertheless, a biochemical connection exists between PKC and PKD1 because certain PKC can phosphorylate PKD1 in its activation loop, and this event is important for activation of PKD1 in many (12–17) but not all (18, 19) cases. Phosphorylation of PKD1 by PKC is mediated primarily by calcium-independent/diacylglycerol-dependent (“novel”) PKC (20–22), although calcium-dependent/diacylglycerol-dependent (“classic”) PKC may also contribute (23). When activated, PKD1 autophosphorylates at S916 (24).

PKD1 is located in the cytoplasm (9, 25, 26), Golgi (27), and nucleus (28), depending on the cell type. In the Golgi, PKD1 phosphorylates and activates PI4K IIIβ, a key regulator of Golgi structure and function (29). Indeed, PKD1 activity contributes to the reorganization of Golgi membranes, which participates in transport of proteins destined for secretion (29, 30). PKD1 also regulates both fission from the trans-Golgi of transport vesicles carrying proteins to the cell surface (27) and lipid homeostasis in Golgi membranes by phosphorylating a ceramide transfer protein (31).

Activation stimuli induce translocation of PKD1 to the plasma membrane (16, 25, 26, 32), and migration to membranes is a key step in PKC-mediated phosphorylation of PKD1 (16). Cell activation can also induce bidirectional movement of PKD1 between the cytoplasm and nucleus (9, 25, 28, 32). Particularly notable are the findings that phosphorylation of histone deacetylases 5 and 7 by...
PKD1 regulates their export from the nucleus, leading to derepression of the transcription of certain genes (33–37). In addition, PKD1 phosphorylates members of the cAMP-response element-binding protein family of transcription factors, leading to increased transcriptional activity (38). PKD1 belongs to a family of three homologous enzymes (6, 39, 40) that can differ in subcellular localization (41–43) and function (29, 37, 44).

We now report that mouse BMMC express PKD1, and that cell activation induced by certain TLR ligands, SCF, or cross-linked IgE induce activation of PKD1. Activation in all cases was inhibited by the indolocarbazol Gö 6976 that inhibits PKD1 and calcium-dependent PKC (45), but not by Bim I that inhibits classic and novel PKC but not PKD1 (14, 46). Release of the chemokine CCL2 measured 2 h after stimulation with each activating agent was also inhibited by Gö 6976, but not by Bim I. In contrast, both Gö 6976 and Bim I inhibited the rapid exocytosis of the preformed secretory granule mediator β-hexosaminidase induced within 15 min by SCF or cross-linked IgE. Our findings establish that stimuli representing innate, adaptive, and growth factor pathways activate PKD1 in mast cells in a largely PKC-independent manner, and reveal distinct influences of PKD1 and PKC on rapid and delayed release of mediators from activated mast cells.

Materials and Methods

BMMC

BMMC were obtained from the femurs and tibias of male BALB/c mice (obtained originally from The Jackson Laboratory and generated from breeding pairs in-house) or from Myd88−/− and Myd88+/+ mice (N11 on the C57BL/6 background) provided by Dr. S. Akira (Osaka University, Osaka, Japan). Cells were cultured for 3–6 wk in 50% enriched medium (RPMI 1640 medium containing 2 mM t-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mM amphotericin B, and 10% FBS) and 50% WEHI-3 (American Type Culture Collection) conditioned medium (WCM). Nonadherent cells were passed weekly. After 3 wk, the cells were >95% mast cells as indicated by metachromatic staining with toluidine blue. The use of mice for these studies was reviewed and approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA).

Detection of PKD1 mRNA by RT-PCR and quantification of CCL2 mRNA by quantitative RT-PCR

For detection, total RNA was isolated from BMMC with TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. cDNA was generated by reverse transcription with an oligo(dT) primer using the Advantage RT-PCR system (Clontech Laboratories). To generate a PKD1-specific product, the cDNA from BMMC and (forward) 5′-TGGCATGGGATTTAGGAGA-3′ and (reverse) 5′-AGATGGGAGACTTTTGCTAAAGC-3′ PKD1-specific primers (47) were subjected to 50 cycles of PCR using REDExtract-N-Amp PCR mix (Sigma-Aldrich) in an Apollo ATC401 thermal cycler as follows: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. After amplification, the products were separated in a 1% agarose minigel. Primers for mouse GAPDH (Clontech Laboratories) were used as a positive control.

For quantification, total RNA was isolated and purified from BMMC with the RNeasy Mini kit with RNeasy-free DNase (Qiagen), according to the manufacturer’s instructions. cDNA was generated by reverse transcription with the iScript cDNA Synthesis kit (Bio-Rad). The cDNA from BMMC and the CCL2- and GAPDH-specific primers (SuperArray Biosciences) were subjected to incubation at 95°C for 15 min and then 40 cycles of PCR (15 s at 95°C, 1 min at 60°C, and 30 s at 72°C) using the Quantitect SYBR Green PCR kit (Qiagen) in a Stratagene Mx3000P thermal cycler. Amplification plots, dissociations curves, and threshold cycle (Ct) values were generated by the Mx3000P software after data collection. Changes in threshold cycle values (∆Ct) were calculated by subtracting the threshold cycle values for GAPDH from those for CCL2; ∆∆Ct values were calculated by subtracting the ∆Ct value for cells incubated in medium from those for treated cells, and 2−∆∆Ct was calculated.

Immunochromatographic detection of proteins

Cells (1 × 10⁶) were pelleted by centrifugation and resuspended in Tris-Glycine SDS sample buffer (Invitrogen Life Technologies) containing 5% 2-ME, boiled for 5 min, and electrophoresed in 6 or 10% Tris-Glycine polyacrylamide gels (Invitrogen Life Technologies). The resolved proteins were transferred to Immobilon P membranes (Millipore) and immunoblotted with rabbit anti-mouse total PKD1, rabbit anti-mouse PKD1 phosphorylated S916, rabbit anti-mouse PKD1 phosphorylated S744/748, antitubulin (Cell Signaling Technology), or anti–heat shock protein 27 (hsp27) phosphor S85 (Affinity BioReagents). Immunoreactive proteins were detected with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) and visualized by chemiluminescence with SuperSignal West Pico (Ferrie) on Kodak BioMax MR film.

Activation of BMMC and measurements of release of mediators

Stock solutions of Bim I and Gö 6976 (Calbiochem) were prepared at 60 nM in DMSO and stored at −80°C and 4°C, respectively. For activation with Pam3CSK4 (InvivoGen) or mouse recombinant SCF (R&D Systems), BMMC (1 × 10⁶ cells/ml) were preincubated with Bim I, Gö 6976, diluted DMSO (vehicle) or WCM for 30 min at 4°C (vehicle controls were indistinguishable from WCM controls in all studies). Pam3CSK4, or SCF was added, and the cells were incubated at 37°C. For IgE-mediated activation, BMMC (1 × 10⁶ cells/ml) were incubated in WCM alone or containing rat monoclonal IgE anti-DNP (clone LO-DNP-30, 3.1 μg/ml; Zymed Laboratories) at 4°C for 1 h. During the last 10 min of this sensitization period, Bim I, Gö 6976, vehicle, or WCM was added. Cells were washed by centrifugation, and the cell pellets were resuspended at 4°C in their original volume with WCM alone or containing 25 μg/ml F(ab)2 mouse anti-rat IgG (MAR) (H chain and L chain reactive; Jackson ImmunoResearch Laboratories). MAR binds the L chain of IgE and cross-links FcεR1 leading to cell mast activation (48). Samples that received MAR were incubated in MAR alone or containing fresh inhibitors, vehicle, or WCM. Cells were then incubated at 37°C, and for all routes of activation, cells were centrifuged at 4°C to stop the reactions. The net activation-induced amount of CCL2 in supernatant samples was quantified by ELISA (R&D Systems). For measurement of exocytosis, supernatants were decanted and held on ice while the pellets were resuspended in their original volume with cold WCM. Samples were exposed to three cycles of freezing on dry ice and thawing at 37°C and were centrifuged to remove residual cell debris. β-Hexosaminidase activity in the samples was measured with a spectrophotometric assay (49). The percentage release of β-hexosaminidase was calculated according to the formula S/(S + P) × 100, where S and P are the mediator contents of the samples of each supernatant and cell pellet, respectively.

Results

Identification of PKD1 in BMMC

Total RNA was isolated from BMMC, cDNA was generated using an oligo(dT) primer, and the samples underwent PCR with primers specific for PKD1. Primers for GAPDH were used as a positive control. Products with the predicted sizes of 411 and 150 bp were obtained with GAPDH and PKD1 primers, respectively (Fig. 1a), demonstrating the presence of PKD1 mRNA. To ascertain that BMMC translate PKD1 mRNA, cell extracts were immunoblotted with an anti-mouse PKD1 directed to C-terminal amino acids. An
immunoreactive band that migrated near the predicted molecular mass of 102 kDa (Fig. 1b) indicated that BMMC constitutively express PKD1 protein.

**Pam3CSK4-induced activation of PKD1 in BMMC**

BMMC were incubated with the TLR2 ligand Pam3CSK4, and the activation of PKD1 was assessed by immunoblotting for phosphorylated S916, the autophosphorylation site of PKD1 (24). Pam3CSK4 induced an increase in the phosphorylation of S916 that was evident by 15 min, reached a plateau level by 45 min, and decreased slightly at 60 min (Fig. 2a). The phosphorylation at 45 min was inhibited in a dose-responsive manner by Go 6976, an inhibitor of PKD1 and classic PKC (45) (Fig. 2b). The phosphorylation at 45 min was also not inhibited by Go 6983 or Ro 31-8220 (data not shown), two other Bims that inhibit a broad array of PKC (45, 50), including atypical PKC that can be inhibited by Go 6983 (45). Hence, activation of PKD1 in response to Pam3CSK4, and PKD1 in BMMC proceeded when most, if not all, PKCs were inhibited.

**MyD88 dependence of PKD1 activation induced by TLR ligands in BMMC**

MyD88 is a proximal signal transducing element for TLR2 (51). To determine whether MyD88 was required for Pam3CSK4-induced activation of PKD1, BMMC were generated from Myd88−/− and Myd88+/+ mice, incubated with 3 μM Pam3CSK4 for 45 min, and the amounts of phosphorylated S916 were compared. The absence of MyD88 prevented Pam3CSK4-induced phosphorylation of S916 (Fig. 2e), demonstrating an essential role for MyD88 in transducing the signal of the TLR2 ligand to activation of PKD1.

**SCF-induced activation of PKD1 in BMMC**

Mast cells are also activated by SCF binding to the receptor tyrosine kinase Kit (55). To determine whether this interaction activates PKD1, BMMC were incubated with SCF, and the activation of PKD1 was assessed by phosphorylation of S916. SCF induced a rapid increase in the phosphorylation that was maximal within 5 min after addition and maintained through 60 min (Fig. 3a). Thus, maximal activation of PKD1 occurred more rapidly in response to SCF than to Pam3CSK4. However, in common with the activation of PKD1 by Pam3CSK4, preincubation of BMMC for 30 min with 5 μM Go 6976 before addition of SCF inhibited the phosphorylation of S916 measured 45 min later, whereas 5 μM Go 6976 inhibited phosphorylation of S916 was not reduced in Myd88−/− BMMC (data not shown), indicating that phosphorylation induced

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**FIGURE 2. Activation of PKD1 by Pam3CSK4 in BMMC.** Cells were incubated for the indicated times at 37°C with medium (Med.) or 100 ng/ml of SCF (a) or for 45 min after preincubation of the cells for 30 min with vehicle (Veh.), 5 μM Bim I, or 5 μM Go 6976 (b). Comparable results were obtained in two additional experiments. For activation with cross-linked IgE, cells were incubated in medium alone or containing rat monoclonal IgE anti-DNP at 4°C for 1 h (c and d). Bim I (5 μM), Go 6976 (5 μM), or medium was added during the last 10 min of this sensitization period (d). Cells were washed by centrifugation, and the cell pellets were resuspended at 4°C in their original volume with either medium, MAR (e), or MAR in medium alone or with fresh inhibitors added (f). Cells were then incubated at 37°C for 45 min. Phosphorylation of S916 was determined by immunoblotting as described in Fig. 2. Comparable results were obtained in one additional experiment.

**FIGURE 3. Activation of PKD1 by SCF and cross-linked IgE in BMMC.** Cells were incubated for the indicated times at 37°C with medium (Med.) or 50 ng/ml of SCF (a) or for 45 min after preincubation of the cells for 30 min with vehicle (Veh.), 5 μM Bim I, or 5 μM Go 6976 (b). Comparable results were obtained in two additional experiments. For activation with cross-linked IgE, cells were incubated in medium alone or containing rat monoclonal IgE anti-DNP at 4°C for 1 h (c and d). Bim I (5 μM), Go 6976 (5 μM), or medium was added during the last 10 min of this sensitization period (d). Cells were washed by centrifugation, and the cell pellets were resuspended at 4°C in their original volume with either medium, MAR (e), or MAR in medium alone or with fresh inhibitors added (f). Cells were then incubated at 37°C for 45 min. Phosphorylation of S916 was determined by immunoblotting as described in Fig. 2. Comparable results were obtained in one additional experiment.
by SCF was not due to contamination with a TLR ligand, and consistent with the absence of any known dependence of SCF-induced signaling on MyD88.

**Activation of PKD1 by cross-linked IgE in BMMC**

Cross-linked IgE bound to FceRI induces activation of PKD1 in continuous mast cell lines (56, 57). To determine whether cross-linked IgE induces activation of PKD1 in nontransformed, cytokine-dependent mast cells and how the time course of activation compared with Pam3CSK4 and SCF, BMMC were sensitized for 1 h with rat IgE alone or with the addition of Bim I or Gö 6976 for the last 10 min. The cells were washed by centrifugation and incubated with MAR alone (to cross-link the IgE) or with the inhibitors that had been added during the sensitization step. Cross-linked IgE induced phosphorylation of S916 that was evident after 5 min, maximal at 15 min, and decreased but maintained through 60 min (Fig. 3c). As with Pam3CSK4 and SCF, Gö 6976, but not Bim I, inhibited the phosphorylation of S916 (Fig. 3d). The activation-induced increases in phosphorylated S916 levels and their inhibition by Gö 6976 but not Bim I were not due to gel loading and/or blot transfer variations as determined by stripping anti-phospho-S916 blots and reprobing them with antitubulin (Fig. 4).

**Phosphorylation of a PKD1 substrate in BMMC**

Hsp27 is an established substrate of PKD1 (36, 58) that protects against cell death in response to various agents (59). S82 of hsp27 is a stream substrate of PKD1 in response to Pam3CSK4 and SCF. In the absence and presence of 5 μM Bim I or Gö 6976, an increase in hsp27 phosphorylation was observed when measured after 2 h (Fig. 6a). The addition of Bim I at 30 min before exposure of the cells to 3 μM Pam3CSK4 failed to inhibit the release of CCL2 at doses of Bim I up to 20 μM (Fig. 6b). In contrast, Gö 6976 inhibited the release of CCL2 with an IC50 value of ~0.4 μM (Fig. 6c). Similarly, Bim I at concentrations up to 5 μM did not inhibit the release of CCL2 measured 2 h after the addition of SCF, whereas Gö 6976 induced a dose-related inhibition of CCL2 release with an IC50 value of ~1 μM (Fig. 6d). The addition of 5 μM Bim I also failed to inhibit CCL2 release induced by cross-linked IgE, whereas Gö 6976 at the same concentration completely inhibited release (Fig. 6e). Hence, the patterns of resistance and sensitivity to Bim and Gö 6076, respectively, were analogous for phosphorylation of S916 and release of CCL2 induced by each of the three activating agents.

**Effects of inhibitors on exocytosis by BMMC**

Activation of BMMC induced by SCF and cross-linked IgE was not due to contamination with a TLR ligand, and consistent with the absence of any known dependence of SCF-induced signaling on MyD88.

**PKD1 activation and CCL2 mRNA levels in BMMC**

The increase in PKD1 activation during mast cell activation initiated by Pam3CSK4 and SCF and its inhibition by Gö 6976 but not Bim I in parallel with phosphorylation of hsp27 S85 suggested that the enzyme might play a role in transmitting signals that culminate in the release of proinflammatory mediators from mast cells. To investigate this potential relationship, we measured with quantitative RT-PCR the mRNA levels for the chemokine CCL2 in BMMC 90 min after activation with Pam3CSK4 or SCF in the absence and presence of 5 μM Bim I or Gö 6976. Both agonists induced ~2-fold increases in CCL2 mRNA levels compared with cells incubated in medium alone (Fig. 5). The addition of Gö 6976 completed prevented the agonist-induced increases in CCL2 mRNA and inhibited the basal levels of mRNA by 50%. In contrast, the addition of Bim I did not inhibit the increases in CCL2 mRNA induced by Pam3CSK4 and SCF, but rather increased them, reminiscent of the increases in phosphorylation of S916 of PKD1 (Figs. 2d and 3b, respectively). Hence, activation of PKD1 induced by Pam3CSK4 and SCF in BMMC is associated with an increase in the transcription and/or stabilization of CCL2 mRNA.
of S916 and release of CCL2 induced by SCF and FcεRI cross-linking extended to exocytosis. BMMC were incubated as described for the CCL2 measurements, except that samples of supernatants and cell pellets were taken 15 after the addition of the activators for quantification of the percentage release of β-hexosaminidase. In contrast with CCL2, both Bim I and Gö 6976 inhibited exocytosis measured 15 min after addition of SCF (IC\textsubscript{50} values of ∼2 and ∼0.5 μM, respectively) (Fig. 7a). Similarly, the release of β-hexosaminidase induced by cross-linked IgE was inhibited by both Bim I and Gö 6976 with IC\textsubscript{50} values of ∼4 and ∼1 μM, respectively (Fig. 7b). Hence, Bim I suppressed exocytosis without inhibiting PKD1 activation, whereas Gö 6976 inhibited both PKD1 activation and CCL2 release, neither of which was suppressed by Bim I that lacks the ability to inhibit PKD1 activation.

Discussion
We establish in this study that the serine/threonine kinase PKD1 is expressed in BMMC and is activated by Pam3CSK4, SCF, and cross-linked IgE that reflect innate, growth factor, and adaptive pathways of mast cell activation, respectively. Hence, the diverse activation signaling pathways that are induced by these agents converge on PKD1. Furthermore, we found that activation of PKD1 is preferentially associated with the accumulation of mRNA for, and secretion of, the chemokine CCL2 rather than the rapid exocytosis of a secretory granule mediator, thereby demonstrating a functional distinction not previously appreciated.

BMMC cultured in IL-3-containing medium constitutively expressed PKD1 mRNA and protein (Fig. 1). PKD1 is also expressed in mouse and rat transformed mast cell lines (9, 15, 56). Hence, our data establish that cytokine-dependent mast cells also express this enzyme. The activation of PKD1 induced by Pam3CSK4 (Fig. 2), SCF, and cross-linked IgE (Fig. 3) in BMMC was evident from phosphorylation of S916, an autophosphorylation site (24). This reaction was suppressed by Gö 6976 that inhibits the activity of PKD1 and classic PKC (45), but not by Bim I that inhibits classic and novel PKC, but not PKD1 (46) (Figs. 2 and 3). The inability of Bim I to inhibit phosphorylation of S916 was not due to a general failure of this agent to act in BMMC because it inhibited the release of CCL2 and β-hexosaminidase induced by the activating agents (Figs. 6 and 7) at concentrations that did not prevent phosphorylation of S916. In addition, the phosphorylation of S916 was not inhibited by Gö 6983 and Ro 31-8220 (data not shown), two other broad spectrum PKC inhibitors (45, 50). Although pharmacologic inhibitors can have off-target effects, the fact that three PKC inhibitors did not inhibit phosphorylation of S916, whereas an additional PKC inhibitor that also inhibits PKD1 did, suggests

FIGURE 6. Effects of Bim I and Gö 6976 on release of CCL2 from BMMC. Cells were incubated for 2 h at 37°C with the indicated concentrations of Pam3CSK4 alone (a) or with 3 μg/ml Pam3CSK4 (b and c) or 100 ng/ml SCF (d) after a 30-min preincubation with the indicated concentrations of Bim I (b and d) or Gö 6976 (c and d). e, Cells were also incubated with IgE at 4°C for 1 h, Bim I (5 μM), Gö 6976 (5 μM), or medium were added during the last 10 min of this sensitization period, cells were washed by centrifugation, and the cell pellets were resuspended at 4°C in their original volume with MAR alone or with fresh inhibitors added; cells were then incubated at 37°C. The amounts of CCL2 in the supernatants were measured by ELISA. Data are expressed as mean for n = 2–4 (a), n = 3 (b), n = 2–5 (c), n = 2–4 (d), and n = 4 (e) experiments performed, and error bars denote the range for n = 2 and SEM for n = 3–5 data.

FIGURE 7. Effects of Bim I and Gö 6976 on exocytosis of β-hexosaminidase from BMMC. Cells were activated with SCF (a) or by cross-linking FcεRI (b) with or without inhibitors as described in Fig. 3 except that samples of supernatants and cell pellets were taken 15 after the addition of SCF or cross-linking of FcεRI. The percentage of release of β-hexosaminidase was quantified with a spectrophotometric assay of enzyme activity. Data are expressed as mean for n = 2–4 (a) and n = 2–7 (b) experiments, and error bars denote the range for n = 2 and SEM for n = 3–7 data.
that the inhibition of phosphorylation by Gö 6976 is the result of direct inhibition of PKD1 activation rather than an upstream effect on PKC. Furthermore, the addition of Pam3CSK4 and SCF to BMMC induced phosphorylation of hsp27, an established substrate of PKD1 (36), and this event was inhibited by Gö 6976 but not Bim I (Fig. 4), demonstrating that the PKC-independent activation of PKD1 in BMMC extended to one of its substrates.

In contrast with our findings with BMMC, Bim I, Gö 6983, and Ro 31–8220 inhibit PKD1 activation induced by a variety of activating agents in several other cell types, including lymphocytes, as assessed by reductions in phosphorylation of S916 detected immunocytochemically (20, 57) and immunoprecipitated PKD1 auto-phosphorylation activity measured by incorporation of phospho-

mokine CCL2 in BMMC, and the more prominent involvement of PKD1 in production of CCL2 likely reflects its effect on mRNA accumulation, an event that occurs well after exocytosis is complete in BMMC. Overall, our finding that PKD1 is activated by TLR ligands, SCF, and cross-linked IgE reveal a shared component of mast cell activation pathways induced by innate, growth factor, and adaptive signals that regulate the diverse biologic functions of mast cells.

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Disclosures
The authors have no financial conflict of interest.

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