Altered protein kinase C in a mast cell variant defective in exocytosis

(intracellular calcium serotonin release/phorbol esters/interleukin 3)

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ABSTRACT The murine mast cell line PB-3c is dependent on interleukin 3 (IL-3) with respect to survival and proliferation. These cells also require IL-3 to display antigen-mediated serotonin release, which is coupled to a transient increase of cytosolic free calcium ([Ca²⁺]ₙ). The antigen-mediated exocytosis is inhibited by phorbol 12-tetradecanoate 13-acetate (PTA), an activator of phospholipid/Ca²⁺-sensitive protein kinase. In contrast, the malignant mast cell variant PB-1 is IL-3 independent with respect to proliferation but is unable to undergo antigen-mediated exocytosis. Yet this cell line exhibits basal levels of [Ca²⁺]ₙ, serotonin content, and numbers of IgE receptors comparable to those of PB-3c cells. Subcellular distribution studies revealed that the specific activity of cytosolic protein kinase C of PB-1 cells was only 40% of that found in PB-3c cells. Furthermore, the PB-1 cells showed a significantly higher specific activity of membrane-bound protein kinase C than PB-3c cells. Scatchard plot analysis of [³H]phorbol 12,13-dibutyrate binding to intact PB-1 cells demonstrated the presence of 20% high-affinity (Kd = 6 nM) and 80% low-affinity (Kd = 60 nM) phorbol ester receptors, whereas PB-3c cells displayed only the low-affinity phorbol ester binding. Immunological characterization of protein kinase C from both cell lines revealed the presence of a normal 77-kDa protein kinase C holoenzyme in both cell lines. In addition, a 72-kDa protein kinase C-related protein band was found mainly in the membrane fraction of the PB-1 variant. It is suggested that this altered and membrane-bound form of protein kinase C may be involved in the blockage of the antigen-mediated exocytosis of PB-1 cells.

Cross-linking of receptor-bound IgE antibodies by a multivalent antigen on mast cells causes a transient increase in the intracellular free calcium ([Ca²⁺]ₙ) and results in the exocytotic release of vasoactive amines and other inflammatory mediators (1). This antigen-induced bridging of IgE receptors also induces the formation of diacylglycerol and activation of protein kinase C (2). Whereas translocation of cytosolic protein kinase C to membranes is thought to reflect the intracellular activation of the enzyme (3) and occurs upon stimulation of target cells with a variety of growth factors, hormones, and tumor promoters, the antigen challenge of mast cells stimulated only a membrane-bound protein kinase C activity, not affecting the cytosolic enzyme (2). To further investigate the involvement of protein kinase C during antigen-mediated exocytosis, we studied two murine bone-marrow-derived mast cell lines, PB-1 and PB-3c. The PB-3c cells proliferate and survive only in the presence of interleukin 3 (IL-3) and fail to induce tumors in syngeneic animals, whereas the PB-1 cell line makes tumors in animals and grows without IL-3 in culture (4, 5). The two cell lines exhibit similar mast cell-specific traits (4, 5) and express comparable transcript levels of several cellular protooncogenes (6). In terms of exocytosis, the PB-3c cells were found to display an antigen-mediated increase of [Ca²⁺]ₙ and release of serotonin only in the presence of IL-3. Phorbol 12-tetradecanoate 13-acetate (PTA), a tumor promoter known to activate and bind to protein kinase C (3, 7), blocked the increase in [Ca²⁺]ₙ. In contrast, the PB-1 cells did not respond to antigen with increased [Ca²⁺]ₙ and release of serotonin, and thus they seem to resemble the PTA-treated PB-3c cells. Characterization of protein kinase C from both cell lines demonstrated the presence of an altered protein kinase C in the membranes of PB-1 cells. It is suggested that this altered membrane-bound protein kinase C may be involved in both the malignant phenotype and the lack of response to antigen of this cell line.

MATERIALS AND METHODS

Cell Culture. Origin and culture conditions of PB-1 and PB-3c cell lines have been described earlier (4–6). As source of IL-3, media conditioned by WEHI-3B murine cells (D⁰ subtype) (8) or by pokeweed mitogen-stimulated murine spleen cells (9) were used. Preparation and testing of conditioned medium have been reported earlier (4, 5).

Measurement of Serotonin Release. About 10⁵ cells were incubated for 24 hr at 37°C in 10 ml of Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) supplemented with 10% calf serum (GIBCO) containing 1.2–2.5 μmol of [³H]serotonin (10–20 Ci/mmol, Amersham; 1 Ci = 37 GBq). Cells were washed free of [³H]serotonin, passively sensitized by incubation with ascites fluid containing monoclonal IgE antibodies specific for 2,4-dinitrophenyl (DNP) hapten (10) for 1 hr at 4°C in 96-well flat-bottomed microtiter plates (Nunclon, GIBCO) (10² cells in 100 µl in each well). After removal of unbound IgE, cells were challenged with 150 µl of IMDM containing 1.0 ng of antigen (DNP coupled to bovine serum albumin (DNPₙ-albumin)) and 10 µg of L-α-phosphatidylyl-L-serine at 37°C. After centrifugation at 800 x g for 5 min, released [³H]serotonin was determined in the supernatant. Total [³H]serotonin was estimated by dissolving cells in 1 M NaOH. Serotonin release was expressed as the percentage of the total [³H]serotonin incorporated into cells.

Measurement of [Ca²⁺]ₙ. Exponentially growing cells (0.5–1.0 x 10⁶ cells per ml) were loaded with Quin-2-AM (CIBA-Geigy) for 15 min at 37°C in 137 mM NaCl/2.7 mM KCl/0.4 mM NaH₂PO₄/10 mM Hepes/5 mM glucose/1.8 mM CaCl₂/1.0 mM MgCl₂/0.1% bovine serum albumin, pH 7.4. The intracellular free calcium concentration was monitored with a fluorescence spectrophotometer (Molecular Dynamics). The fluorescence of the digital image was digitized (Concanavalin A (Con A); 20 µg/ml) and analyzed by computer software (BioRad). The percentage of cells exhibiting fluorescence was determined for each time point.

Abbreviations: [Ca²⁺]ₙ, intracellular free calcium concentration; DNP, 2,4-dinitrophenyl; IL-3, interleukin 3; PTA, phorbol 12-tetradecanoate 13-acetate; PTA, phorbol 12-tetradecanoate 13-acetate.

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from [83-32P]ATP phosphatidyl-L-serine, and 7.4, as described previously (11). Changes in [Ca2+]i were monitored in an Amicon-Bowman fluorimeter as described earlier (11).

**Quantitation of Protein Kinase C Activity.** Protein kinase C activity was assayed by analytical polyacrylamide gel electrophoresis (PAGE) measuring the 32P incorporation from [β-32P]ATP (10 Ci/mmol; New England Nuclear) into lysine-rich histone (type V-S, Sigma) as described previously (12). The assay was carried out in a final volume of 50 μl containing 50 μg of histone, 20 mM Tris-HCl at pH 7.4, 10 mM Mg(NO3)2, 100 μg of l-α-phosphatidyl-l-serine, and 50 nM [β-32P]ATP (12.4 Ci/mmol, New England Nuclear) in the presence and absence of 2 μM PTA. After an incubation of 3 hr on ice the bound [32P]ATP was separated from unbound [32P]ATP by filtration through GF/C glass fiber filters (Whatman).

[32P]ATP binding to intact cells was measured as described earlier (13). Intact cells (4×8×106) were incubated in 1 ml of IMDM containing IL-3 and bovine serum albumin at 1 mg/ml with increasing concentrations of [32P]ATP (1-150 nM) in the presence or absence of a 200-fold excess of unlabeled PTA. After an incubation period of 30 min at 37°C, the tubes were chilled on ice and the contents were collected on GF/C fiber filters. Binding data were analyzed by a computer program developed by Bürgisser (14).

**Preparation of Cytosol and Membrane Extracts.** Cells were disrupted by sonication at 4°C in HB buffer (20 mM Tris-HCl, pH 7.4/2 mM EGTA/2 mM EDTA/6 mM 2-mercaptoethanol/20 μg of leupeptin per ml/2 μg of aprotinin per ml) as described earlier (15). The homogenate was centrifuged at 100,000 × g for 1 hr, yielding the cytosol. The 100,000 × g pellet was resuspended in HB buffer containing 1% (wt/vol) Nonidet P-40 at a protein concentration of 2 mg/ml, sonicated, and recentrifuged as described before. The resulting supernatant was referred to as "membrane fraction." For immunoblotting, total extracts were prepared by homogenization of approximately 107 cells at 95°C in 0.5 ml of 2% NaDodSO4/10 mM EDTA/10 mM Tris-HCl, pH 7.4.

**Purification of Protein Kinase C Antigen and Production of Anti-Protein Kinase C Antiserum.** A detailed description of the purification methods and characterization of the anti-protein kinase C antiserum will be published elsewhere. In brief, the protein kinase C holoenzyme (77 kDa) was purified from pig brain according to Uchida and Filburn (16). The enzyme was lyophilized and subjected to NaDodSO4/PAGE as described by Rudolf and Krueger (17). Protein kinase C was identified by comigration of purified autophosphorylated 77-kDa holoenzyme. Gel pieces containing the 77-kDa protein kinase C protein band were electroeluted and injected into rabbits in complete Freund's adjuvant. Antiserum was collected after the fourth boost. Titers were determined by immunoblotting.

**Immunoblot Analysis of Protein Kinase C.** Subcellular fractions and total cell extracts were subjected to NaDodSO4/PAGE and transferred electrophoretically to nitrocellulose blotting membranes (Bio-Rad) as described by Towbin et al. (18). To block nonspecific binding sites the nitrocellulose sheets were incubated for 1 hr at 25°C in 20 mM Tris-HCl, pH 7.4/200 mM NaCl containing 3% bovine serum albumin and 10% horse serum (GIBCO). After a 90-min incubation at 25°C with the anti-protein kinase C antiserum (1:500), the immunoreactive proteins were enzymatically visualized by goat anti-rabbit antibody coupled to horseradish peroxidase (Behringwerke).

**Other Analytical Methods.** Protein was determined by the method of Bradford (19), using Bio-Rad reagents with bovine serum albumin as standard. Statistical significance was analyzed by the Wilcoxon rank-sum test.

**RESULTS**

**Modulation of Antigen-Mediated Exocytosis by IL-3.** Changes in both [Ca2+]i, and serotonin release were monitored after the addition of antigen (DNP8-albumin) to PB-1 and PB-3c cells sensitized with the corresponding IgE antibody. As shown in Table 1, only PB-3c cells could be induced to release serotonin by challenge with antigen, whereas PB-1 cells did not respond. The discrepancy between this finding and an earlier report that the PB-3c cells were unable to release histamine (4) is explained by the fact that the radiometric technique employed is far more sensitive than the fluorimetric assay used previously (4). In contrast to PB-1, PB-3c cells exhibited exocytosis accompanied by a rapid increase of [Ca2+]i (Fig. 1, trace 1), although both cell lines displayed similar basal [Ca2+]i (Table 1). After antigen stimulation of sensitized PB-3c cells the [Ca2+]i peaked at 350 nM after 30 sec, and it returned to basal values within 10 min. The antigen-induced increase of [Ca2+]i and serotonin release of sensitized PB-3c cells was dependent on IL-3 (Table 1, Fig. 1A). Removal of IL-3 for 2 hr resulted in the loss of the antigen-mediated calcium signal and serotonin release. Re-addition of IL-3 restored the capacity to respond to antigen

| Table 1. Serotonin release and [Ca2+]i in challenged PB-1 and PB-3c cells |

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Serotonin release, %</th>
<th>Net after addition of antigen</th>
<th>[Ca2+]i, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>30 sec after addition of antigen</td>
<td>Basal</td>
</tr>
<tr>
<td>PB-1</td>
<td>-IL-3</td>
<td>1.5</td>
<td>1</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>+IL-3</td>
<td>2.0</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>+IL-3A</td>
<td>1.5</td>
<td>1.2</td>
<td>90</td>
</tr>
<tr>
<td>PB-3c</td>
<td>+IL-3</td>
<td>3.0</td>
<td>40</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>+IL-3, +PTA</td>
<td>3.2</td>
<td>3.5</td>
<td>95</td>
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<tr>
<td></td>
<td>-IL-3 5 hr</td>
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</tr>
<tr>
<td></td>
<td>+IL-3 7 hr</td>
<td>4.0</td>
<td>40</td>
<td>95</td>
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</table>

Cells were stimulated by DNP8-albumin after passive sensitization with IgE, and serotonin release and [Ca2+]i were analyzed. Amounts of IL-3 added corresponded to those needed for optimal proliferation. PTA was added at 50 nM. Each value represents the mean of 10 independent determinations (standard deviations were less than 10%).
a single peak of phospholipid and Ca\(^{2+}\)-sensitive protein kinase activity coeluting with specific \(^{3}H\)PBt2 binding (Fig. 3). Similar results were obtained when cytosols were analyzed by DEAE-cellulose chromatography (data not shown). Examination of subcellular fractions revealed that membrane extracts of PB-1 cells exhibited a significantly higher \((P < 0.01)\) protein kinase C specific activity and phorbol ester-binding activities than membrane extracts of PB-3c cells, whereas the corresponding value for the cytosol of PB-1 cells was only about 30–40% of the enzyme activity of PB-3c cells (Table 2).

**Phorbol Ester Binding to Intact Cells.** Estimation of protein kinase C by \(^{3}H\)PBt2 binding to intact cells showed that both cell lines bound similar amounts of the phorbol ester. Saturation analysis of \(^{3}H\)PBt2 binding to PB-3c cells resulted in linear Scatchard plots (Fig. 4A) and could be fitted by a one-site binding model (14) with a \(K_d\) of 60 nM. However, PB-1 cells revealed two classes of \(^{3}H\)PBt2 receptors (Fig. 4B), which fitted to a two-site binding model (14). The apparent high-affinity class consisted of 2.5 \(\times\) 10\(^5\) receptors per cell and had an estimated \(K_d\) of 6 nM, whereas the apparent low-affinity class was represented by 12 \(\times\) 10\(^5\) binding sites per cell with a \(K_d\) of 60 nM. The two receptor classes of PB-1 cells were also detected at 4°C (data not shown). Since in both cell lines the phorbol ester binding reached equilibrium after 30 min at 37°C, different down-regulation rates of \(^{3}H\)PBt2 receptor can be excluded.

**Immunological Characterization of Protein Kinase C.** The two phorbol ester receptor populations as well as the subcellular distribution of protein kinase C activity of PB-1 cells indicated the presence of different pools of protein kinase C. It remained to be clarified, however, whether the lower specific protein kinase C activity in PB-1 cells reflected merely differences in the amounts of membrane-bound enzyme or could be related to modifications of the enzyme protein. Therefore, both cell lines were extracted with hot NaDodSO\(_4\) and subjected to immunoblot analysis. As shown in Fig. 5 (lanes a and b), the rabbit polyclonal anti-protein

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**Fig. 1.** Effects of removal and addition of IL-3-containing conditioned medium on the antigen-induced changes in [Ca\(^{2+}\)]. Fluorescence measurements were performed after loading cells with Quin-2. Antigen (DNP\(_6\)-albumin) was added at 0.5 \(\mu\)g/ml after passive sensitization with IgE. Arrows indicate the addition of antigen. (A) PB-3c cells. Trace 1, PB-3c cells with IL-3; trace 2, PB-3c cells first deprived of IL-3 for 1.5 hr and then exposed to IL-3 for 3 hr; trace 3, PB-3c cells deprived of IL-3 for 1.5 hr. (B) PB-1 cells. Trace 1, PB-1 cells with IL-3; trace 2, PB-1 cells deprived of IL-3.

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**Fig. 2.** Effects of PTA (50 nM) on the antigen-induced changes in [Ca\(^{2+}\)]. (A) Effects of 50 nM PTA added before the antigen (upward-pointing arrow). (B) Effects of 50 nM PTA added 1 min after addition of antigen. The antigen was DNP\(_6\)-albumin at 0.5 \(\mu\)g/ml.

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**Fig. 3.** PAGE analysis of protein kinase C activity and \(^{3}H\)PBt2 binding of PB-1 and PB-3c cytosols. PAGE of cytosols (300 \(\mu\)g) was performed in a 10% gel. Protein kinase C activity was determined in the presence of Ca\(^{2+}\), phosphatidylserine, and diolein (●) or in their absence but in the presence of 0.5 mM EGTA (○).
kinase C antiserum raised against the porcine brain 77-kDa protein kinase C holoenzyme (lanes g and h) recognized on immunoblots of both cell lines with a protein with a similar molecular mass (77 kDa). Furthermore, in the NaDodSO4 extract of PB-1 cells the antiserum reacted specifically with an additional polypeptide of 72 kDa (Fig. 5, lane b). Interestingly, the 72-kDa protein kinase C-related band was present mainly in the membrane fraction of PB-1 cells (Fig. 5, lanes c–f). The possibility that this membrane-bound 72-kDa band was generated by proteolysis from the protein kinase C holoenzyme (77 kDa) during cell homogenization can be ruled out because it was found also in hot NaDodSO4 extracts of whole PB-1 cells (Fig. 5, lane b).

DISCUSSION

The physiological similarity between cultured and mucosal mast cells has been reviewed recently (20). The antigen-mediated serotonin release and increase of [Ca2+]i in PB-3c cells was therefore not surprising. However, this physiological response could be demonstrated only in the presence of IL-3, which is also required for the proliferation of these cultured mast cells (21). The molecular mechanism for this IL-3 dependency is at present unclear, but it is known that IL-3 enhances glucose uptake and maintains normal cellular levels of ATP (22, 23). In contrast, the PB-1 variant grows in the absence of IL-3 and does not show antigen-induced exocytosis, even in the presence of IL-3, although histamine content and IgE receptor numbers are comparable to those of PB-3c cells (4). Yet the PB-1 cell line appears to have functional IL-3 receptors since it responds to IL-3 with increased growth rates and cloning efficiencies (4). Under optimal growth conditions both cell lines also exhibited similar levels of normal c-myc and c-fos transcripts, but their expression strictly depended on IL-3 in PB-3c cells (6). The finding that both protooncogenes appear to be constitutively expressed in PB-1 cells makes it more likely that the lesion leading to IL-3 independence may reside upstream of the regulation of c-myc and c-fos expression.

PTA inhibited the transient calcium increase and serotonin release of PB-3c cells, confirming earlier reports on normal mast cells (24) and suggesting the involvement of protein kinase C in the exocytotic process. As antigen-induced exocytosis is constitutively blocked in the PB-1 variant, as in PTA-treated PB-3c cells, it can be argued that the PB-1 cells may have an altered phorbol ester receptor (i.e., protein kinase C). In support of this hypothesis, these cells showed a reduced specific cytosolic protein kinase C and higher amounts of membrane-bound enzyme as compared with PB-3c cells. Furthermore, the PB-1 variant displayed about 20% high-affinity phorbol ester receptor. Finally the anti-protein kinase C antiserum clearly recognized in the membrane fraction of PB-1 cells a protein kinase C-related 72-kDa protein, whereas the 77-kDa protein kinase C holoenzyme was predominantly localized in the cytosol of both cell lines. At present it is not clear how such a “truncated” membrane-bound protein kinase C could be involved in the blockage of the antigen-mediated exocytosis. Activation of protein kinase C by IL-3 is believed to result in the transient translocation of cytosolic protein kinase C to the plasma membrane (3, 25), whereas antigen-mediated exocytosis of mast cells is accompanied by the direct activation of a membrane-bound protein kinase C rather than by translocation of cytosolic enzyme to membranes (2). The finding that the calcium-dependent recruitment of protein kinase C to membranes generates a “primed protein kinase C” with high-affinity phorbol ester binding (26) raises the possibility...
that the membrane-associated 72-kDa protein kinase C of PB-1 cells is able to bind to membranes in the absence of IL-3. This altered form of membrane-bound protein kinase C may be involved in the IL-3-independent expression of c-myc and c-fos, since both protooncogenes are known to play a role in the growth factor-promoted nuclear signal transduction. The next step is to examine the properties of this membrane-associated 72-kDa protein kinase C and to investigate its involvement in the regulation of mast cell functions.

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