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European Journal of Pharmacology 489 (2004) 49–54
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# Substance P can stimulate prostaglandin $D_2$ and leukotriene $C_4$ generation without granule exocytosis in murine mast cells

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Received 16 October 2003; received in revised form 26 February 2004; accepted 2 March 2004

**Abstract**

Mast cells play a central role in immediate type hypersensitivity and inflammatory events. Activation of mast cells not only can result in the release of preformed granule-associated mediators generally followed by de novo synthesis of lipid-derived substances. In the present study, we show that mast cell can be activated to release lipid mediators in absence of granule exocytosis. Primary cultured murine mast cells were stimulated with substance P and produced leukotriene $C_4$, and prostaglandin $D_2$ without the release of the granule-associated enzyme β-hexosaminidase. Indomethacin and nordihydroguaiaretic acid caused complete inhibition of arachidonic metabolite generation. Leukotriene $C_4$ and prostaglandin $D_2$ production was blocked by genistein, a specific inhibitor of tyrosine kinases, and bisindolylmaleimide, a protein kinase C inhibitor, indicating a role for both phosphorylation pathways in the substance P-stimulated lipid mediator production. We suggest that the cytokine microenvironment of the mast cell determines whether mast cell stimulation leads to only lipid mediator release or full activation. Analysis of granule-associated mediators only might underestimate the role of mast cell activation under (patho)physiological conditions.


*Keywords:* Mast cell; Substance P; Neurogenic inflammation; Allergy; Exocytosis

## 1. Introduction

Mast cells are often found in close association with blood vessels and nerves, suggesting a role in the control of vascular permeability and in the induction of neurogenic inflammation. The neural network and mast cells form an interactive unit that is able to influence immune and inflammatory responses (Mori et al., 2002). Neuropeptides released from sensory neurons can induce mast cell activation and their mediators can further stimulate the release of neuropeptides from sensory nerves resulting in a bi-directional mast cell–sensory neuron autocatalytic loop (Ansel et al., 1993, 1996; Johnson and Erdos, 1973; Kowalski and Kaliner, 1988).

Activation of mast cells not only causes the release of preformed granule-associated mediators, but also initiates the de novo synthesis of lipid-derived substances (Metcalfe et al., 1997). Large quantities of arachidonic acid are stored in membrane glycerolipids of mast cells. Upon cell activation, arachidonic acid is mobilized from the storage pools by different phospholipases and is converted to prostanoids and leukotrienes by cyclooxygenase and 5-lipoxygenase, respectively (Williams et al., 2000; Marone et al., 1997).

Morphologic and histochemical studies demonstrate the presence of multiple mast cell phenotypes within a single organism. Classically, rodent mast cells are categorized into two subtypes: connective tissue mast cells and mucosal mast cells (Galli, 1997). Both subtypes possibly represent the extreme phenotypes of a wide range of mast cell populations found in vivo and show differences in phenotype, expression of granule proteases, and their response to different stimuli. In contrast to freshly isolated peritoneal mast cells, bone marrow-derived mast cells (Levi-Schaffer and Shalit, 1989), which are generated in interleukin-3 containing medium and resemble immature mucosal mast cells, cannot be activated by substance P to release granule contents (Sakaguchi et al., 1992). On the other hand, primary cultured mast cells respond to cross-linking of the high affinity immunoglobulin (Ig) E receptor (FcεRI) or stimulation by cytokines like stem cell factor leading to degranulation and production of lipid mediators (Haisa et al., 1992; Mencia-Huerta et al., 1983;

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doi:10.1016/j.ejphar.2004.03.003

Murakami et al., 1995a,b; Razin et al., 1982, 1983; Tkaczyk et al., 1996).

The signal transduction pathways leading to granule exocytosis and lipid mediator production has been suggested to operate through protein kinase C/$Ca^{2+}$ and mitogen-activated protein kinase pathway, respectively (Kawakami and Galli, 2002). Cross-linking of FcεRI results in activation of both pathways, but stimulation of mast cells by cationic compounds such as substance P and compound 48/80 does not always result in mast cell activation and degranulation (Ferry et al., 2002). The cytokine environment or chemical treatment of mast cells can alter this sensitivity to cationic compounds. For instance, quercetin-treated RBL-2H3 mast cells can be stimulated by compound 48/80 and substance P (Senyshyn et al., 1998; Swieter et al., 1993). In recent studies, it has been shown that bone marrow-derived mast cells cocultured with fibroblasts or with stem cell factor and interleukin-4 (Karimi et al., 1999; Ogasawara et al., 1997) gained responsiveness to substance P.

In this study, we provide evidence that bone marrow-derived mast cells do not show significant degranulation after stimulation with substance P, but do release lipid mediators prostaglandin $D_2$ and leukotriene $C_4$. These results illustrate that the involvement of mast cells in neurogenic inflammation cannot be studied by monitoring the release of mast cell granule constituents only.

## 2. Materials and methods

The following were purchased: culture medium RPMI 1640, Tyrode's buffer powder, fetal calf serum, nonessential amino acids (GibcoBRL Life Technologies, Paisley, Scotland), penicillin–streptomycin, L-glutamine, sodium pyruvate, 2-mercaptoethanol, dinitrophenyl-conjugated human serum albumin (DNP–HSA), herbimycin, pertussis toxin, indomethacin, nordihydroguaiaretic acid (Sigma, St. Louis, MO, USA), nonidet P40 (BDH Laboratory Supplies, Poole, England), dinitrophenol (DNP)-specific IgE (H1DNPe26.82 hybridoma, kindly provided by J. Rivera, NIH, USA), albumin, fraction V and bisindolylmaleimide (Boehringer Mannheim, Germany), tissue-culture flasks (Costar, Cambridge, MA, USA), toluidine blue, HEPES (E. Merck, Darmstadt, Germany), genistein (Biomol. Res. Lab.), substance P (Novabiochem. Laufelfingen, Switzerland), leukotriene $C_4$, prostaglandin $D_2$ assay kits (Amersham Life Science).

### 2.1. Mouse bone marrow cultures

Bone marrow cells from BALB/c mice ($2 \times 10^5$ cells/ml) were cultured for three weeks in complete RPMI (RPMI 1640 medium containing 4 mmol/l L-glutamine, $5 \times 10^{-5}$ mol/l 2-mercaptoethanol, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mmol/l nonessential amino acids) containing 10% vol/vol fetal calf serum in presence 1 ng/ml of IL-3. After 3 weeks, the cells in the culture were identified as mast cells, as assessed by histochemical staining with toluidine blue.

### 2.2. Activation of bone marrow-derived mast cells with substance P

Bone marrow cells were cultured for three weeks to develop into mast cells. Bone marrow-derived mast cells were washed twice with Tyrode's buffer supplemented with 0.1% bovine serum albumin plus 10 mM HEPES and resuspended in this modified Tyrode's buffer at density of $0.6 \times 10^6$ cells/ml. A total of $2–3 \times 10^4$ cells were aliquoted in 96-well plates and activated with different concentrations of substance P (0–100 μM) for 30 min. Total release was established by adding nonidet P40 to get complete lysis of cells. After 1 h of incubation of aliquots with assay solution containing 4-methylumbelliferyl glucosaminide in 0.1 M citrate buffer (pH = 4.5), the reaction was stopped by adding 0.2 M glycine buffer (pH = 10.7). Fluorescence was measured using a multi-well plate reader at an emission wavelength $\lambda = 360$ nm and excitation wavelength $\lambda = 450$ nm. The percentage of degranulation was calculated as: $\{(a-b)/(t-b)\} \times 100$, where $a$ is the amount of β-hexosaminidase released from stimulated cells, $b$ is that released from unstimulated cells and $t$ is total cellular content.

For comparison to IgE-dependent activation, mast cells were resuspended in culture medium (complete RPMI) and incubated with dinitrophenol-specific IgE for 1 h in 5% $CO_2$ at 37 °C (approximately $0.8 \times 10^6$ cells/ml). After sensitization with IgE, cells were washed twice with Tyrode's buffer supplemented with 0.1% bovine serum albumin and resuspended in the modified Tyrode's buffer at a density of $0.6 \times 10^6$ cells/ml. A total of $2–3 \times 10^4$ of cells were aliquoted in 96-well plates and activated with different concentrations of DNP–HSA (0–25 ng/ml) for 30 min.

In indicated experiments, the effect of indomethacin, nordihydroguaiaretic acid, genistein, and bisindolylmaleimide on prostaglandin $D_2$ and leukotriene $C_4$ production was assessed. Mast cells were incubated with indicated concentrations of the inhibitors or solvent only for 5–15 min and were activated as mentioned above with 100 μM of substance P.

### 2.3. Determination of prostaglandin $D_2$ and leukotriene $C_4$ production

Leukotriene $C_4$ and prostaglandin $D_2$ production was determined using a competition radioimmunoassay kit according to manufacturer's instruction (Amersham Life Science). Bone marrow-derived mast cells were primed with IgE supernatant following by activation with different concentration of dinitrophenyl-conjugated human serum albumin or stimulated with different concentrations of substance P as stated above. Supernatants were stored at −20 °C until further assay.

### 2.4. Presentation of data

All culture experiments were repeated two to three times with different bone marrow preparations pooled from three animals.

## 3. Results

### 3.1. Bone marrow-derived mast cell degranulate in response to cross-linking of IgE but not to substance P

After sensitization with dinitrophenol-specific IgE and challenge with multivalent Ag (DNP–HSA), interleukin-3 grown bone marrow-derived mast cells released β-hexosaminidase. Cells released 40.3 ± 1.5% of their β-hexosaminidase content at 2.5 ng/ml DNP–HSA, while no substantial granule exocytosis was observed when the mast cells were treated with 100 μM of substance P (4.4 ± 0.5%).

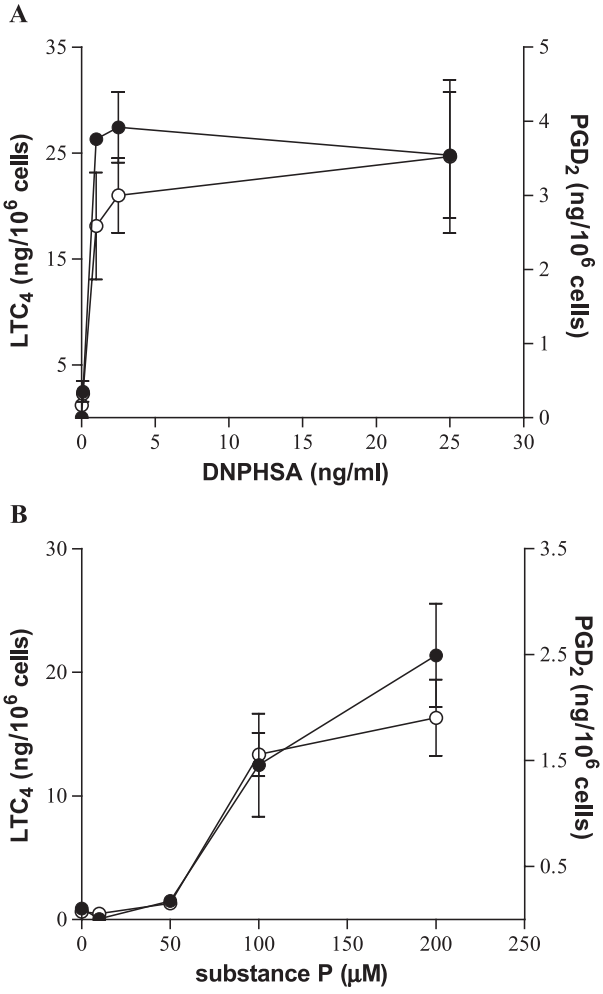


Fig. 1. IgE-mediated and substance P-stimulated leukotriene $C_4$ and prostaglandin $D_2$ production from mouse bone marrow-derived mast cells. Mast cells labeled with IgE followed by activation with different concentrations of DNP–HSA (A) or stimulation with different concentrations of substance P (B) for 30 min. Leukotriene $C_4$ (closed symbol) and prostaglandin $D_2$ (open symbol) production was determined using a radioimmunoassay. The figure indicates representative experiment from four independent isolations (mean ± S.E.M.).

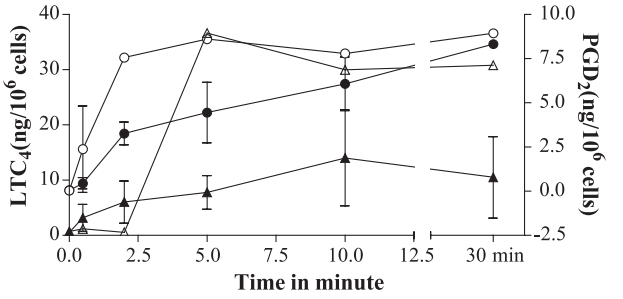


Fig. 2. Determination of time course substance P and IgE/antigen-mediated leukotriene $C_4$ and prostaglandin $D_2$ production from mouse bone marrow-derived mast cells. Three weeks IL-3-dependent primary cultured mast cells from BALB/c mice were incubated with 100 μM of substance P or 2.5 ng/ml antigen (DNP–HSA) (after sensitization with IgE) for 30 s to 30 min and subsequently leukotriene $C_4$ and prostaglandin $D_2$ production was assessed using a competition radioimmunoassay. Open circles represent Ag/IgE-induced $PGD_2$, closed circles SP-induced $PGD_2$, open triangles Ag/IgE-induced $LTC_4$, and closed triangles SP-induced $LTC_4$. Data are expressed as mean ± S.D. of duplicate samples.

### 3.2. Leukotriene $C_4$ and prostaglandin $D_2$ generation in response to substance P

We measured the release of lipid mediators leukotriene $C_4$ and prostaglandin $D_2$ after cross-linking of IgE or stimulation with substance P. IgE/antigen-activated bone marrow-derived mast cells produced 27.4 ± 3.3 ng/$10^6$ cells leukotriene $C_4$ and 3.0 ± 0.5 ng/$10^6$ cells prostaglandin $D_2$ at 2.5 ng/ml DNP–HSA (Fig. 1A). Surprisingly, substance P (100 μM) stimulated the cells to generate 13.4 ± 1.8 ng/$10^6$ cells leukotriene $C_4$ and 1.5 ± 0.5 ng/$10^6$ cells prostaglandin $D_2$ (Fig. 1B). Eicosanoid generation by substance P-stimulated mast cells was dose dependent. The cells produced 0.5 ± 0.1, 1.3 ± 0.1, 13.4 ± 1.7 and 16.3 ± 3.0 ng/ml of leukotriene $C_4$ in response to 10, 50, 100 and 200 μM of substance P, respectively. The amounts of prostaglandin $D_2$ generation by substance P-stimulated mast cells were 0.2 ± 0.0 ng/ml at 50 μM, 1.5 ± 0.5 ng/ml at 100 μM and 2.5 ± 0.5 ng/ml at 200 μM (Fig. 1B).

### 3.3. Time course of lipid generation by mast cells after substance P-initiated and IgE-mediated activation

Leukotriene $C_4$ and prostaglandin $D_2$ production was rapidly detected after activation with IgE/Ag (Fig. 2). It reached a maximum of 14.0 ± 8.7 ng leukotriene $C_4$ or 6.1 ± 1.5 ng prostaglandin $D_2$/$10^6$ cells at 10 min. The release of leukotriene $C_4$ and prostaglandin $D_2$ from mast cells stimulated by 100 μM of substance P was measurable within 30 s (Fig. 2). The generation of leukotriene $C_4$ reached near maximal at 10 min post activation (15 ng/

$10^6$ cells), while prostaglandin $D_2$ production was more gradual and maximum was reached at 30 min post activation (8.4 ng/$10^6$ cells).

It has been shown earlier that nordihydroguaiaretic acid completely blocks the generation of leukotrienes and partially inhibits prostaglandin production from RBL-2H3 mast cells after IgE-or calcium ionophore-mediated activation. Also, indomethacin was reported to inhibit prostanoid generation via inhibition of the cyclooxygenase pathway (Andersson et al., 1983; Chakravarty, 1984; Obata et al., 1996). Indeed, we found that the substance P-induced production of the arachidonic acid metabolites could be inhibited with nordihydroguaiaretic acid and indomethacin. Substance P-induced $PGD_2$ production in presence of indomethacin was completely blocked ($0.0 \pm 0.0\%$ of control, $n = 4$), while $LTC_4$ generation in presence nordihydroguaiaretic acid was reduced to $7.7 \pm 0.8\%$ of control ($n = 4$).

### *3.4. Signal transduction pathways in substance P-initiated lipid generation*

To investigate the role of protein tyrosine kinases in arachidonic metabolite production induced by substance P, bone marrow-derived mast cells were preincubated with genistein, a well-known tyrosine kinase inhibitor. Genistein (1 μM to inhibit prostaglandin $D_2$ and 10 μM for leukotriene $C_4$ production) effectively inhibited substance P-induced leukotriene $C_4$ and prostaglandin $D_2$ production (Fig. 3), indicating that activity of protein tyrosine kinases was required for arachidonic metabolite generation. In addition also a protein kinase C inhibitor, bisindolylmaleimide (2.5 μM), greatly reduced the lipid generation by mast cells in response to substance P, as shown in Fig. 3.

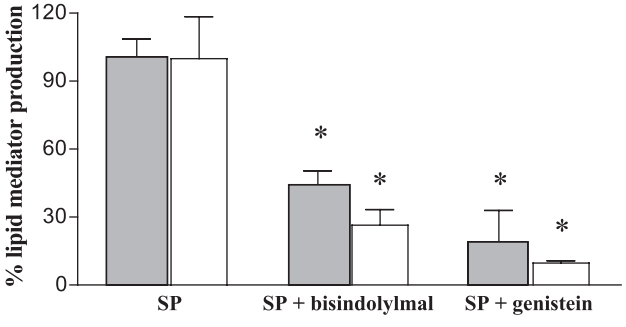


Fig. 3. Inhibitory effect of inhibitors for protein kinase C (bisindolylmaleimide) and protein tyrosine kinase (genistein) on substance P-mediated leukotriene $C_4$ (open bar) and prostaglandin $D_2$ (shaded bar) production by mouse bone marrow-derived mast cells. Three-week-old IL-3-dependent primary cultured mast cells from BALB/c mice were incubated with vehicle, 2.5 μM bisindolylmaleimide or 10 μM genistein and subsequently stimulated with 100 μM substance P (SP). Leukotriene $C_4$ and prostaglandin $D_2$ production was assessed using a competition radioimmunoassay. Data are expressed as mean ± S.D. of duplicate samples; *significantly different from control (no inhibitor added), $P < 0.05$.

## 4. Discussion

Release of mast cell mediators can be induced by a variety of stimuli including antigens cross-linking cell-surface-bound IgE or $IgG_1$, complement products, neuropeptides and many physical agents (Mecheri and David, 1997). The response of mast cells to the neuropeptide substance P is dependent on the subtype of mast cell. It has been reported that mucosal mast cells including bone marrow-derived mast cells are not responsive to substance P (Lawrence et al., 1987), while substance P activates rat brain, peritoneal and human skin mast cells, leading to rapid histamine release by granule exocytosis (Church et al., 1991; Jozaki et al., 1990; Mousli et al., 1989). However, in contrast to IgE/antigen-induced prostaglandin $D_2$ and leukotriene $C_4$ production, in these human mast cells, substance P caused minimal eicosanoid generation. This suggests a fundamental difference in the biochemical mechanisms of cell activation via the IgE-receptor and substance P. In previous studies, we demonstrated that interleukin-3-dependent bone marrow-derived mast cells treated with recombinant stem cell factor plus interleukin-4 gain functional responsiveness to substance P and can be activated by substance P to release β-hexosaminidase and to produce arachidonic acid metabolites (Karimi et al., 1999). In subsequent studies, we showed that stem cell factor and interleukin-4 induced an up-regulation of the neurokinin-1 receptor expression (Van der Kleij et al., 2003) and activation at low concentrations of substance P was neurokinin-1 receptor-dependent. At higher neuropeptide concentrations mast cells are activated through a receptor-independent mechanism (Van der Kleij et al., 2003). In our present study, we show that mouse mast cells derived from a culture of bone marrow cells with interleukin-3 do not respond to substance P by degranulation, but surprisingly generate prostaglandin $D_2$ and leukotriene $C_4$ (Fig. 1).

Murakami et al. showed that in contrast to the IgE-mediated production of arachidonic acid metabolites, culturing of murine bone marrow-derived mast cells with stem cell factor induced a progressive loss of their capacity to undergo IgE-mediated exocytosis. In addition, cytokine generation by human mast cells induced by cysteinyl leukotrienes and uridine diphosphate was shown without histamine release or prostaglandin $D_2$ generation (Mellor et al., 2002). In the RBL-2H3 mast cell line, at least two cascades are activated via the high affinity IgE receptor and the G protein-coupled muscarinic m1 receptor. Phospholipase C/protein kinase C are important for the regulation of granule secretion, while the mitogen activated protein kinase/phosholipase $A_2$ pathway accounts for arachidonic metabolite generation (Hirasawa et al., 1995a,b). In addition, it has been suggested that the basic secretagogue compound 48/80 stimulates rat peritoneal mast cells via two distinct signaling pathways, the first leading to exocytosis and release of preformed mediators such as histamine and the second pathway mediates the metabolism of arachidonic acid (Shefler et al., 1998). It was later demonstrated

that phospholipase-β and -γ are in control of mast cell stimulation by basic secretagogues leading to granule exocytosis and arachidonate release, respectively (Ferry et al., 2001). Taken together, these findings support a differential regulation of the respective signal transduction pathways leading to exocytosis and lipid mediator production in mast cells. Monitoring of degranulation (measured as release of granule mediators) may therefore not suffice as an adequate marker for mast cell activation.

The mechanism behind substance P-induced lipid mediator production remains to be further elucidated. In a recent study, we showed that stimulation of mast cells grown interleukin-3 with substance P is independent of the neurokinin-1 receptor (Van der Kleij et al., 2003) and likely through direct interaction with G proteins (Shefler et al., 1998; Ferry et al., 2001). Our study shows that substance P-induced lipid mediator production in mouse bone marrow-derived mast cells is completely blocked with a specific tyrosine kinase inhibitor, implicating activation of protein tyrosine phosphorylation in arachidonic acid metabolite production. Also, protein kinase C was shown to be important because pretreatment with bisindolylmaleimide greatly reduced the substance P-induced lipid generation (Fig. 3). These findings point to an involvement of protein phosphorylation by protein tyrosine kinase(s) and protein kinase C in the substance P-stimulated production of lipid mediators by mast cells. A role for these enzymes has been suggested earlier for stimulation of mast cells by basic secretagogues (Shefler and Sagi-Eisenberg, 2001; Ferry et al., 2001).

In neurogenic inflammation, actions of mast cells can be triggered by the release of transmitters from sensory nerves, mainly neuropeptides (Foreman and Jordan, 1983). Products of mast cells, including leukotrienes, have also been shown to evoke the release of substance P in the airways (Martins et al., 1991; Saria, 1988). Our study shows that mast cells do not necessarily release granule contents upon stimulation by neuropeptides, but can synthesize and release arachidonic acid metabolites. Indeed, these products can stimulate c-fibers and thereafter propagate and worsen a neurogenic inflammation. Our results further suggest that monitoring mast cell granule release in vivo only may underestimate the role of mast cells in these situations.

## Acknowledgements

The authors thanks GlaxoWellcome, The Netherlands, and the Ferdowsi University, Iran, for their financial support for this study.

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