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Direct Neurite-Mast Cell Communication In Vitro Occurs Via the Neuropeptide Substance P

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Communication between nerves and mast cells is a prototypic demonstration of neuroimmune interaction. However, whether mast cell activation occurs as a direct response to neuronal activation or requires an intermediary cell is unclear. Addressing this issue, we used an in vitro coculture approach comprising cultured murine superior cervical ganglia and rat leukemia basophilic cells (RBLs; possesses properties of mucosal-type mast cells). Following loading with the calcium fluorophore, Fluo-3, neurite-RBL units (separated by <50 nm) were examined by confocal laser scanning microscopy. Addition of bradykinin, or scorpion venom, dose-dependently elicited neurite activation (i.e., Ca2+ mobilization) and, after a lag period, RBL Ca2+ mobilization. Neither bradykinin nor scorpion venom had any direct effect on the RBLs in the absence of nerves. Addition of a neutralizing substance P Ab or a neurokinin (NK)-1 receptor antagonist, but not an NK-2 receptor antagonist, dose-dependently prevented the RBL activation that resulted as a consequence of neural activation by either bradykinin or scorpion venom. These data illustrate that nerve-mast cell cross-talk can occur in the absence of an intermediary transducing cell and that the neuropeptide substance P, operating via NK-1 receptors, is an important mediator of this communication. Our findings have implications for the neuroimmunologic signaling cascades that are likely to occur during airways inflammation, intestinal hypersensitivity, and other conditions in which mast cells feature. The Journal of Immunology, 1999, 163: 2410–2415.

During the last decade, there has been an exponential increase in data illustrating that the immune and nervous systems are not disparate entities (1, 2). The mast cell-nerve relationship has served as a prototypic association and has provided substantial evidence for bidirectional communication between nerves and immune cells (3). Early studies elegantly described the nonrandom spatial association of mast cells and nerves in a variety of tissues in which actual membrane-membrane contacts could be observed (4, 5). Many studies have shown that messenger molecules (e.g., neuropeptides) from nerves can elicit mast cell degranulation (6) and that mast cell-derived mediators (e.g., histamine, serotonin, and platelet-activating factor) modulate neuronal transmission (7, 8). For instance, intestine from β-lactoglobulin (β-LG)-sensitized animals respond to in vitro β-LG challenge with an increase in electrogenic chloride secretion that is largely mast cell-mediated (9). The same study showed that the chloride secretory event (as evidence of mast cell activation) was precisely correlated with a concomitant release of acetylcholine and that the release of the neurotransmitter was mimicked by the application of exogenous histamine. Perdue et al. (10) reported that the transient increase in ion transport evoked by electrical field stimulation of nerves in jejunal segments from mast cell-deficient (W/Wv) mice was half that which occurred in congenic mast-cell containing mice. Furthermore, reconstitution of the W/Wv mice with mast cells resulted in a restoration of a normal ion transport response to nerve stimulation. Also, Theoharides and colleagues (11) found that electrical nerve stimulation caused mast cell degranulation in the dura mater.

However, while these tissue studies have shown a functional nerve-mast cell interplay, they do not dismiss the possibility that an intermediate cell transduces or modulates the nerve-mast cell communication. It is this question that is the crux of the present study. Using an in vitro coculture model comprising rat basophilic leukemia cells (RBLs), which display properties of mucosal-type mast cells, and neurite-sprouting murine superior cervical ganglia (12), we have examined direct nerve-mast cell communication. Our findings demonstrate that mast cell activation, as judged by calcium mobilization, can be a direct consequence of contact with a specific activated nerve fiber. Moreover, we provide evidence that this RBL cell activation was mediated, at least in part, by the neuropeptide substance P acting through neurokinin (NK)-1 tachykinin receptors.

Materials and Methods

Nerve-RBL cell coculture

Following a published protocol (12, 13), superior cervical ganglia (SCG) were dissected from newborn (0–48 h old) CBA mice (Japan SLC, Shizuoka, Japan) and rinsed in HBSS containing 10 mM HEPES (pH 7.4). Each ganglion was divided into two to four pieces and incubated for 60 min at 37°C in 2 ml of HEPES containing 0.125% trypsin (grade II, Sigma, St. Louis, MO). The resultant cell suspension was plated at a density of 0.5–1 × 105 nerve cells onto matrigel (Becton Dickinson, Bedford, MA)-coated 35-mm diameter glass dishes. The neurons were grown in F12 culture...
medium (Life Technologies, Rockville, MD) supplemented with 0.2 mM l-glutamine, 0.3% glucose, 3% antibiotic/antimycotic (A-7292) (all from Sigma), 10% PBS (BioWhittaker, Walkersville, MD), and 50 ng/ml murine nerve growth factor (NGF, 2.5S; Upstate Biotechnology, Lake Placid, NY). Nonganglionic cells were killed by an initial exposure to cytosine-β-D-arabinofuranoside (Ara-C, 10-6 M; Sigma) for 24 h.

RBLs (RBL-2H3, these cells display some of the properties of mucosal mast cells and are generally accepted as a model of mucosal-type mast cells) were originally a gift from Dr. R. P. Siragianan (National Institutes of Health, Bethesda, MD) and were maintained in F12 culture media containing 50 ng/ml NGF (RBLs cultured in NGF-free medium displayed similar cell growth and morphology to those maintained in NGF-containing medium). The pattern of β-hexosaminidase secretion from the RBLs used in this study was similar to previous studies with this cell clone (14). For coculture experiments, RBLs (104/dish) were added to 48-h-old cultures of SCG-neurites and incubated at 37°C for 72 h.

Cellular activation

Calcium mobilization and activation of fluorophores were used as an index of cellular activation and were assessed using confocal fluorescence microscopy (15, 16). After 72 h of coculture, cells were treated with cell membrane-permeant fura-2-AM (2.5 μM for 30 min at 37°C; Molecular Probes, Eugene, OR) and then washed three times with HEPES buffer. Cells were observed with a confocal laser scanning microscope (Zeiss, Oberkochen, Germany; LSM-410; argon ion laser at 488 nm), and images were captured and analyzed using IBM compatible computer software. In coculture studies, neurites and RBLs in contact with neurites (defined as cell membranes within <50 nm of each other) were examined. In some studies, RBL size was measured (cell circumference determined by computer image analysis) and membrane morphology examined as other indices of RBL activity.

Neurite activation was evoked by either bradykinin (BK; 1–100 nM; Bachem, Bubendorf, Switzerland) or SV (SV; Leituris quinquiesstratus herbarus, 1–100 pg/ml; Sigma). Examining RBL-to-neurite communication, the RBLs were specifically stimulated by adding anti-IgE receptor Abs (BC4, an ascites solution used at a dilution of 1/100,000; a gift from Dr. R. P. Siragianan) to the cocultures. This Ab causes histamine release from RBLs (18).

In addition studies, following 72 h of coculture, RBLs were retrieved, loaded with Fluo-3, and then treated with BK (10 or 100 nM), SV (10 or 100 pg/ml), or substance P (10-6 or 10-8 M). Calcium mobilization in RBLs derived from coculture studies in response to these agents was compared with naive RBLs (i.e., those not cocultured with neurites).

Mechanism of neurite-to-RBL communication

The neuropeptide substance P was selected as the possible neurite-derived mediator responsible for the RBL activation.

Immunohistochemistry.

Seventy-two-hour neurite cultures were fixed for 10 min in 0.05% gluteraldehyde, rinsed, and then preincubated in 5% (w/v) BSA for 30 min. Cells were then incubated for 24 h at 4°C with primary rabbit anti-substance P Ab (1 μg/ml diluted in PBS/1% BSA/5% normal mouse serum). Substance P immunoreactivity was detected by incubation with goat-anti-rabbit Ab-HRP conjugates for 45 min at room temperature and visualized with diaminobenzidine. The degree of positivity was assessed by a single investigator in a blinded fashion. All of the appropriate controls were performed, including omission of the primary antiserum, substitution of rabbit non-specific IgG (Dako, Carpinteria, CA) for the primary Ab, and inclusion of mouse gut tissue as a positive control for substance P immunostaining.

Inhibitor studies.

An affinity column-purified anti-substance P rabbit IgG polyclonal Ab at 0.2–20 μg/ml was added to neurite-RBL cocultures 20 min before BK or SV stimulation, and cellular responses were measured microscopically. This Ab has no cross-reactivity with neurokinin A, or other tachykinins, and has been used as a neutralizing Ab in in vivo studies (A. Stanisz, unpublished observation). Controls included the use of an irrelevant rabbit IgG (20 μg/ml) and addition of the anti-substance P Ab to single cultures of neurites or RBLs only. A similar approach was used to investigate the tachykinin receptor subtype involved in this communication. Before neurite stimulation, cocultures were pretreated with the non-peptide NK-1 receptor antagonist, CP-99,994-1 (1–100 ng/ml; Pfizer, Groton, CT) (this is an upgrade on the NK-1 antagonist, CP-96,345, which has been shown to block the effects of substance P (19, 20)) or an NK-1 receptor antagonist, SR 49685 (1 μg/ml; Sonofi Roche, Basel, Switzerland). Both agents were gifts to Dr. A. Stanisz (McMaster University, Hamilton, Canada), and the concentrations used were based on unpublished in vivo and in vitro studies in Dr. Stanisz’s laboratory. Both NK-receptor antagonists were tested in single neurite and RBL cultures stimulated with BK or anti-IgE receptor Abs, respectively.

Data presentation

Data are presented as arbitrary fluorescence units or as the percentage of RBLs responding to simulation (this is an all or nothing measure, based on Fluo-3 activity), where n values represent the number of cells or the number of RBL-neurite cultures examined.

Results

Effect of nerve stimulation on RBL Ca2+ mobilization

Bradykinin. Addition of BK to SCG cultures resulted in a dose-dependent neurite activation: 1 nM BK = 25.6 ± 5.8, 10 nM BK = 34.2 ± 4.9, and 100 nM BK = 38.9 ± 7.6 units of fluorescence intensity/neurite (n = 7; Fig. 1, A and B), but did not evoke an increase in fluorescence when added directly to Fluo-3-loaded RBLs in the absence of SCG neurites (n > 100 RBLs examined). Addition of BK (10 nM) to SCG cultures only, resulted in an increase in neurite fluorescence within 5 s that peaked within 15 s. In coculture studies, BK-induced neurite activation was invariably followed by RBL activation, as indicated by increased fluorescence (Fig. 1B). The number of RBLs responding increased in accordance with increasing concentrations of BK (Fig. 1C). The interval between neurite activation and RBL activation was 5.0 ± 1.0, 4.4 ± 2.5, and 3.8 ± 1.2 s for 1, 10, and 100 nM BK, respectively (n = 9–20). Additionally, RBLs in contact with BK (10 nM)-stimulated neurites displayed cell membrane ruffling, and cell size was increased by 19.4 ± 2.7% (n = 7). In the absence of neurites, BK had no direct effect on RBL membrane morphology or size. In addition, RBLs (n > 50) retrieved after 72 h of coculture and examined in isolation were, like naive RBLs, unresponsive to direct BK (10 or 100 nM) stimulation, as gauged by Ca2+ signaling (data not shown).

Scorpion venom. SV also caused an almost instantaneous and dose-dependent increase in neurite fluorescence, which was maximal within 5 s of addition of the venom (Fig. 2). Thus, 100 pg/ml or 10 pg/ml SV caused an average increase in neurite fluorescence of 30.0 ± 5.5 and 25.9 ± 3.5 units of fluorescence, respectively (n = 7); at 1 pg/ml, SV had negligible effects on neurite activity. SV (100 pg/ml) added directly to Fluo-3-loaded RBLs only did not evoke any Ca2+ response (n > 100 RBLs examined). When SV was added to SCG-RBL cocultures, the expected increase in neurite activation (i.e., fluorescence) was followed by an increase in RBL fluorescence (Fig. 2A). The increase in RBL fluorescence remained elevated for at least 60 s (end of experiment) after addition of SV to the coculture. In all cocultures examined, SV-evoked neurite activation preceded RBL activation, with a dose-dependent average lag phase of 8.3 ± 5.3 and 3.5 ± 1.9 s at concentrations of 10 and 100 pg/ml, respectively (n = 8). Concentrations of 10 and 100 pg/ml SV resulted in activation in 67 and 73% of the RBLs, respectively (n = 15; Fig. 2B). Similar to the BK experiments, RBLs (n ≥ 50 cells) isolated after 72 h of coculture did not respond directly to 10 or 100 pg of SV (data not shown).

Mechanism of neurite-RBL communication

Using standard indirect immunohistochemical techniques, 72-h neurite cultures were consistently positive for substance P-like immunoreactivity (data not shown). Since substance P can cause mast cell degranulation and at pM doses can prime RBLs for subsequent activation at a lower threshold (6, 13), we proceeded to assess substance P as a putative mediator responsible for the direct neurite-RBL communication demonstrated here. Experiments in
which SCG-RBL cocultures were stimulated with BK in the presence of neutralizing anti-substance P Abs revealed that the inclusion of the Abs dose-dependently prevented the RBL activation (Fig. 3, A and B). Treatment with a control isotype-matched irrelevant Ab (n = 7) did not affect the RBL changes evoked in response to neurite activation by BK (Fig. 3C). As an additional control, we tested the ability of the anti-substance P Abs to abrogate the increase in RBL fluorescence evoked by anti-IgE receptor Abs. Inclusion of the anti-substance P Abs (20 μg/ml) (or an NK-1 receptor antagonist) did not affect the increase in RBL fluorescence elicited directly by anti-IgE receptor Abs: 127.0 ± 7.1, 124 ± 7.2, 122.3 ± 7.2 fluorescence units for anti-IgE receptor Ab-treated RBLs, and time-matched cultures, including an anti-substance P Ab or the NK-1 receptor antagonist, respectively (n = 10). These data confirm the postulate that the effect of the anti-substance P Abs in the coculture model was due to their substance P-neutralizing effects and not a nonspecific effect on the RBL. Also, the anti-substance P Ab had negligible effects on BK-induced neurite activation (data not shown).

Next, we examined the ability of tachykinin receptor antagonists to abrogate the neurite-RBL communication. Inclusion of the NK-1 receptor antagonist, CP99,994-01, in the SCG-RBL cocultures did not affect the neurite activation evoked by BK, but significantly and dose-dependently inhibited the subsequent increase in RBL activation (Fig. 4, A and B). In contrast, the NK-2 receptor antagonist, SR48,968 (1 μg/ml), did not affect either BK-stimulated neurite activity or the subsequent Ca^{2+} mobilization in RBLs in coculture studies (Fig. 4C). The receptor antagonists did not directly affect neurite stimulation or the RBLs when incubated in the absence of coculture. Also, coculture with neurites did not enhance the RBL responsiveness to exogenous substance P when the RBLs were examined in isolation after coculture. Thus, 51 and 21% of RBLs retrieved from cocultures responded with an increase in Ca^{2+} mobilization in response to 10^{-7} M and 10^{-8} M substance P, respectively, as compared with 50 and 18% of naive RBLs. The response of both naive and cocultured RBLs to exogenous substance P was blocked (i.e., <5% of cells responded) by a 20-min pretreatment with 100 ng/ml of the NK-1 receptor antagonist.

The ability of the anti-substance P Abs and the NK-1 receptor antagonist to inhibit neurite activation of RBLs was also observed.
in cocultures treated with SV. For example, the number of RBLs in coculture responding to 10 pg/ml of SV was reduced from 67% to 33% and 31% in the presence of anti-substance P Abs (20 μg/ml) or the NK-1 receptor antagonist (100 ng/ml), respectively.

RBL-to-neurite communication

SCG-RBL cell cocultures were established, and, following Fluo-3 loading, the RBLs were stimulated with anti-IgE receptor Abs (anti-IgE receptor Ab had no direct effect in pure SCG cultures). This treatment resulted in the expected increase in RBL Ca$^{2+}$ and was followed by an increase in neurite fluorescence (22.0 ± 5.8 units of fluorescence) (Fig. 5) with a lag time of 36.9 ± 16.6 s (n = 8). As opposed to coculture experiments involving neurite stimulation with SV or BK, where RBL activation was dependent on proximity to a neurite, RBL activation of neurites was observed at much greater distances, up to 160 μm.

Discussion

Considerable evidence exists for a consistent anatomical association between mast cells and nerves in tissues throughout the body (see Ref. 4). The morphological juxtaposition of mast cells and nerves would, by itself, be of little interest if it were not for evidence of physiological connectedness. Ag activation of mast cells results in the release of a variety of effector molecules (e.g., arachidonic acid metabolites, 5-hydroxytryptamine, histamine, cytokines) that can modulate neuronal activity. In the reciprocal communication pathway, in vitro studies have revealed that neuronal-derived messengers, such as neuropeptides, can evoke or regulate...
mast cell activation and/or degranulation in a tissue- and species-dependent manner. Thus, while it is widely accepted that mast cell-nerve bidirectional communication occurs on a regular basis, it has been unclear if an intermediary cell is required to facilitate this functional cross-talk. Using an in vitro model, we examined neurite-mast cell (i.e., RBL) units and have demonstrated that activation of a nerve fiber elicits an activation event (as indicated by Ca\(^{2+}\) mobilization) in an RBL in contact with the activated neurite.

Electrical stimulation of nerves has been shown in vivo to cause mast cell activation, but this is dependent upon the intensity and type of current used, and usually occurs only with prolonged stimulation (21). Direct stimulation of the vagus nerve can result in activation of mast cells in the dura mater (11), and Gottwald et al. (22) have presented morphological and histochemical evidence of mast cell activation, or even degranulation in the gut following electrical vagal stimulation in vivo. Yet, electrical stimulation of nerves is not always associated with mast cell activation, and vagal stimulation in vivo was found to inhibit mast cell degranulation in a feline model of asthma (23). In the present investigation, we show that both BK and SV evoked neuronal activation and that neither agent had any direct effect on RBL Ca\(^{2+}\) flux. However, addition of either agent to SCG-RBL cocultures resulted in RBL activation that was always preceded by neurite activation, as gauged by Ca\(^{2+}\) imaging. Increased RBL Ca\(^{2+}\) mobilization was sustained throughout the duration of the 60-s observation period and, in the case of BK stimulation, was invariably accompanied by membrane ruffling and an increase in cell diameter, morphological features associated with activation and/or subsequent degranulation. An intriguing possibility was that neurite contact influenced the RBL’s ability to respond directly to BK or SV; however, RBLs retrieved from cocultures were still unresponsive to direct BK or SV application.

Following detection of substance P-like immunoreactivity in the cultured neurites, we postulated that this tachykinin could be responsible for the RBL Ca\(^{2+}\) flux observed after neurite activation since: 1) substance P at high doses can cause mast cell degranulation (6); 2) lower concentrations of substance P prime mast cells for activation at subthreshold concentrations (13); and 3) immunocytochemical characterization of nerves juxtaposed to mast cells in situ has found many of these to be substance P-positive (24). Addition of purified neutralizing anti-substance P Abs to the coculture milieu, dose-dependently prevented the RBL activation elicited by BK or SV stimulation of neurites, but did not affect the neuronal activation. This procedure did not have any direct effect on RBLs in single culture (i.e., neurites not present), indicating that, following activation, neuronal-derived substance P was effecting the RBLs in contact with the activated neurite.

The putative mechanism of action of substance P on mast cells in vivo is currently a controversial issue; consequently, we tested the ability of NK-1 and NK-2 tachykinin receptor antagonists to modulate the RBL activation evoked in response to neurite activation. In short, and somewhat unexpectedly, only the NK-1 receptor antagonist inhibited the RBL Ca\(^{2+}\) mobilization, while having no effect on SV- or BK-induced increases in neurite Ca\(^{2+}\). The effectiveness of the NK-1 receptor antagonist in this model system was initially surprising in view of the lack of documented evidence that NK-1 receptors are expressed on mast cells. It was feasible that the coculture milieu and neurite contact increased expression of an NK-1-type receptor on the RBLs. We present no data in support of this postulate, since assessment of RBLs after coculture showed them to be no more responsive to direct substance P application than naïve RBLs. However, recent radiolabel and RT-PCR studies by Cooke et al. (25) have shown that RBL-2H3 cells do indeed express ~2225 high affinity NK-1 receptors, corroborating the findings in this study that used the same RBL cell clone. Substance P effects upon mucosal mast cells have been thought to occur more often as a result of interaction between the N terminus of the neuropeptide and G proteins in the mast cell membrane, rather than via interaction with specific tachykinin receptors (i.e., NK-1–3). In the light of our findings and those of Cooke et al. (25), it is clear that the issue of receptor-mediated effects of tachykinins, or at least substance P, on mast cells with a mucosal-type phenotype should be revisited.

In vitro studies have shown that high concentrations of substance P are necessary to cause mast cell degranulation (6), which might suggest a limited physiological role for this interaction in vivo. For instance, at \(\leq 1 \mu M\), substance P has been found to be incapable of directly eliciting histamine or hexosaminidase release from RBLs (27, 28). However, it is becoming increasingly apparent that cellular activation and selective mediator release are not synonymous with mast cell degranulation. As only one example of this, it has been shown that peritoneal mast cells in culture will synthesize and release IL-6 in response to cholera toxin in the absence of degranulation measured by histamine release (26). Moreover, we have shown that pM doses of substance P cause changes in ion conductance of the plasma membrane of mast cells, as detected by electrophysiological patch clamp analysis. Furthermore, when exposed to repeated pM doses of substance P, even after a prolonged interval, most peritoneal mast cells displayed an increased Cl\(^{-}\) membrane conductance, increases in cell diameter, and ~60% of the cells actually degranulated (13). Similarly, substance P has been shown to induce whole cell current in RBLs, although higher doses of the tachykinin were used in that study (29). These data, in conjunction with the present findings suggest that low dose substance P, perhaps via intracellular Ca\(^{2+}\) signaling and in the absence of major degranulation, can prime or sensitize mast cells to other stimuli. The physiological consequence of lowering the threshold to subsequent stimuli has clear implications for neuronal-mast cell modulation of physiological events, such as the “sensory perception” of Ag (30).

Finally, we considered mast cell-to-neurite communication. Our data illustrate that neurites were activated in response to RBL activation caused by anti-IgE receptor Abs. Even neurites at considerable distance (\(\leq 160 \mu m\)) from the mast cells were affected, and we surmise that this is consistent with the increased amounts, and variety of mediators, that are released upon mast cell activation/degranulation, as compared with the lower concentrations of mediator that would be released at neuronal synapses or varicosities. Thus, in terms of bidirectional communication, our data precisely
show that neurite or mast cell activation can result in activation of the reciprocal cell type in the absence of any modulating or transducing effects of an intermediary cell. Clearly, afferent communication (i.e., mast cell-to-neuron) and efferent communication (neur-on-to-mast cell), of which less is currently known, and the consequent effects of such potentially reverberating circuits on local physiology may have great significance in the initiation or perpetuation of disease states, such as bronchial hyperreactivity and asthma, idiopathic functional bowel disorders, food allergy, and eczema.

Thus, while it has been intuitive to accept direct functional bidirectional communication within the structural confines of the neurite-mast cell unit, in no instance before this report has actual direct communication been demonstrated or proven between these two cell types. Our data clearly illustrate that nerves can communicate directly with RBLs, an accepted model of a mucosal-type mast cell (25) without the participation of an intervening cell or cells. As a caveat, we would add that our data do not dismiss the likelihood of other cell types in vivo modulating nerve-mast cell communication. Nevertheless, we have shown that an activation event in a specific neurite can result in activation in the mast cell-like RBL cell in contact with the specific neurite, where Ca\(^{2+}\) mobilization was used as an indicator of RBL activity.

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References