Bi-directional Relationship of in Vitro Mast Cell–Nerve Communication Observed by Confocal Laser Scanning Microscopy

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Communication between nerves and mast cells is a prototypic demonstration of neuro–immune interaction. Recently, we used an in vitro co-culture approach comprising cultured murine superior cervical ganglia (SCG) and rat basophilic leukemia (RBL) cells to study this interaction. Previously, we concentrated mainly on the activation signal from neurites to mast cells (RBL). However, it is proposed that mast cell–nerve communication is not a one-sided relationship but a bi-directional one. In the present work, we studied the communication from mast cells to neurites. We observed that binding of anti-IgE receptor antibodies to mast cells increases calcium ion concentration [Ca$^{2+}$] in SCG neurites. This indicates that mast cell–nerve communication is bi-directional. Confocal fluorescence microscopic images indicated that [Ca$^{2+}$] in neurites increased after an increase of [Ca$^{2+}$] in mast cells. The lag-time of neurite activation was several times longer than that of mast cell activation. The correlation coefficient between the lag-times for mast cell and nerve activation was calculated to be 0.81. In addition, the fluorescence images showed that calcium signals in SCG neurites were able to extend to a long distance (100—200 μm) from the site where mast cells (RBL) attached to neurites.

Key words: neuro–immune interaction; confocal laser scanning microscopy; superior cervical ganglia; RBL cell; calcium signal

MATERIALS AND METHODS

Nerve–Mast Cell Co-culture Following a published protocol, superior cervical ganglia (SCG) were dissected from newborn (0—48 h old) CBA mice (Japan SLC, Shizuoka, Japan) and rinsed in Hank’s’ balanced salt solution 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, U.S.A.). The resultant cell suspension was plated at a density of 0.5—1×10⁶ nerve cells onto matrigel (Becton Dickinson, Bedford, MA, U.S.A.)-coated 35-mm diameter glass dishes. The neurons were grown in F12 culture medium (Life Technologies, Rockville, MD, U.S.A.) supplemented with 0.2 mM l-glutamine, 0.3% glucose, 3% antibiotic/antimycotic (A-7292) (all from Sigma), 10% FBS (BioWhittaker, Walkersville, MD, U.S.A.), and 50 ng/ml murine nerve growth factor (NGF, 2.5 S; Upstate Biotechnol-ogy, Lake placid, NY, U.S.A.). Nonganglionic cells were killed by an initial exposure to cytosine-β-d-arabinofuranoside (Ara-C, 10⁻⁶ M; Sigma) for 24 h. Further details of the experimental procedure are described in the previous papers.6,8,9 For co-culture experiments, RBL-2H3 cells (1×10⁶ cells/dish) were added to 48 h old cultures of SCG neurites and incubated at 37 °C for 72 h.6,8,9

Calcium Mobilization and Differential Interference Images Calcium mobilization was used as an index of cellular activation and was assessed by confocal laser scanning microscopy (CLSM).6,10—12 After 72 h of co-culture, cells were treated with culture medium containing Fluo-3-AM (2.5 μM for 30 min at 37 °C; Molecular Probes, Eugene, OR) and then washed with HEPES buffer. Cells were observed with a CLSM (Zeiss, LSM-410; argon ion laser at 488 nm) and images were captured and analyzed using IBM compatible computer software.6,10—12 Differential interference images of the co-cultured cells were also measured by a Zeiss confocal laser scanning microscope (LSM-410).6 Neurite activation

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 bi-directional communication between nerves and immune cells.³ Early studies elegantly described the non-random spatial association of mast cells and nerves in a variety of tissues in which actual membrane–membrane contacts could be observed.⁴,⁵ To understand these events, we have recently studied direct neurite–mast cell communication using an in vitro co-culture approach and calcium imaging by confocal laser scanning microscopy (CLSM). Our results showed clearly that communication from mast cells to neurites. Our results showed clearly that communication from mast cells to neurites. Our results clearly show that communication from mast cells to neurites exists in the in vitro nerve–mast cell co-culture system.

In the present work, we have studied in more detail the communication from mast cells to neurites. Our results clearly show that communication from mast cells to neurites exists in the in vitro nerve–mast cell co-culture system.

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was evoked by either bradykinin (100 nM; Bachem, Bubendorf, Switzerland) or scorpion venom (*Leiurus quingestriatus herbaeus*, 100 pg/ml; Sigma). Addition of bradykinin, or scorpion venom, dose-dependently elicited neurite activation (i.e., Ca\(^{2+}\) mobilization) and, after a lag period, RBL calcium mobilization. This direct neurite–mast cell communication occurred via the neuropeptide substance P.6

RESULTS AND DISCUSSION

In the neurite–RBL co-culture system, extended neurites attached to RBL cells as shown in Fig. 1. This is a typical example of differential interference images of the neurite–RBL co-culture. After 3 d, co-cultures were examined by differential interference microscopy for evidence of contacts between

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Fig. 1. Differential Interference Image of the Neurite-RBL Co-culture
The extended neurites are attached to an RBL cell. Arrows indicate the portions of the neurite–RBL attachment.

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Fig. 2. A) Representative Fluorescence Images Showing the Effect of Anti-IgE Receptor Antibodies on the Neurite–RBL Co-culture System at 37 °C
Each image was measured at (a) 6, (b) 26, (c) 28 and (d) 48 s after addition of antibodies. The white bar is 50 μm.

B) Time-courses of Fluo-3 Fluorescence Intensity Changes in a Mast Cell and in a Neurite
Data are the same as shown in Fig. 1A. In this example the lag-time of mast cell activation was rather shorter. Closed arrow heads show the points where we measured the lag-time of the mast cell and the neurite.

C) Example of Time-courses in Fluo-3 Fluorescence Intensity Changes in a Mast Cell and in a Neurite
Here, the lag-time of mast cell activation was much longer. Closed arrow heads show the points where we measured the lag-times.
RBL cells and neurites. The characteristic spherical-to-bipolar shape of the RBL cells distinguished them from the occasional non-neuronal accessory cells that were carried over from the dissociation of the SCG.

After loading both RBL cells and neurites with fluo-3, we activated RBL cells in the co-culture system with anti-IgE receptor antibodies (a diluted ascites with 1/100000 solution, BC4)\(^1\)\(^3\) and measured Ca\(^{2+}\) mobilization in both cells to study the communication from mast cells to SCG neurites. Calcium images of fluo-3 loaded mast cells and neurites in the co-cultured system were measured every 2 s by CLSM. Typical examples of CLSM images of mast cells (RBL) contacted with neurites are shown in Fig. 2A. Sequential fluo-3 fluorescence images of mast cells and neurites are shown in Figs. 2A(a) to 2A(d). Fluo-3 fluorescence intensities increased abruptly in mast cells after a short lag time (see Figs. 2A(b) and 2B (upper curve)). Thereafter, fluo-3 fluorescence intensities increased in neurites attached to the mast cell (see Figs. 2A(c) and 2B (lower curve)). There was a much longer lag-time for neurite activation than for mast cells, as shown in Fig. 2B. Fluorescence pseudo-images of fluo-3 loaded neurites showed that calcium ion concentration apparently increased in thin neurites, as shown in Fig. 2A(c). All of these images showed clearly that calcium ion concentration increased first in mast cells with a short lag-time and that it increased in neurites with a much longer lag-time after stimulation with anti-IgE receptor antibodies, as shown in Fig. 2B.

In the absence of mast cells, neurites themselves were never activated by anti-IgE receptor antibodies, however, they were activated by bradykinin, as shown in Fig. 3.\(^6\)\(^7\) Thus, this result showed that neurites were activated in response to mast cell activation caused by binding of anti-IgE receptor antibodies. This showed that mast cell–nerve interaction is not a one-sided relationship but a bi-directional one.\(^3\)\(^6\)

We have done similar experiments many times using fluo-3 loaded co-culture cells. Another example of the time-course of fluorescence intensity changes of mast cells and neurites is shown in Fig. 2C. In Fig. 2C, calcium ion concentration increased after a 50 s lag-time in the mast cell and increased in the neurite with a much longer lag-time (about 200 s). We found that these lag-times for the calcium signal varied from 10 to 50 s for mast cells (RBL) and from 30 to 300 s for neurites. Variation of the observed lag-times for mast cells and neurites is shown in Fig. 4. In Fig. 4, the correlation coefficient was calculated to be 0.81. These results indicated that the lag-time of neurite activation was several times that of mast cell activation and it took many seconds to communicate from mast cells to neurites. Although we do not know yet what soluble factors from mast cells induced calcium signals in SCG neurites, we assume that pre-formed mediators in secretory granules such as histamine, serotonin, proteoglycan, neutral proteases and ATP would be the most plausible candidates to communicate with neurites. Since there is a large variation in the lag-times (30 to 300 s) of calcium signals in neurites (Fig. 4), it may indicate great variations in lag-time for degranulation in RBL cells.\(^10\)\(^17\)

The lag-time of mast cell activation with anti-IgE receptor antibodies varied from 10 to 50 s in the present experimental conditions. This variation was not due to the diffusion velocity of the antibody solution in the observation chamber for CLSM. Similar variation of lag-time was observed in mast cells (RBL) when they were stimulated with antigen. Thus, the variation itself was maybe due to cell cycles of mast cells and/or population of IgE receptors on the cell surfaces.

The present results showed clearly that mast cells were able to communicate to neurites and nerve-mast cell communication is bi-directional. The activation of mast cells affected the calcium signals of neurites at relatively large distances (100—200 \(\mu\)m) from the mast cell attached to them.

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