In vitro activation of murine DRG neurons by CGRP-mediated mucosal mast cell degranulation

F. De Jonge, A. De Laet, L. Van Nassauw, J. K. Brown, H. R. P. Miller, P.-P. van Bogaert, J.-P. Timmermans, and A. B. A. Kroese. In vitro activation of murine DRG neurons by CGRP-mediated mucosal mast cell degranulation. Am J Physiol Gastrointest Liver Physiol 287: G178–G191, 2004. First published March 11, 2004; 10.1152/ajpgi.00528.2003.—Upregulation of CGRP-immunoreactive (IR) primary afferent nerve fibers accompanied by mastocytosis is characteristic for the Schistosoma mansoni-infected murine ileum. These mucosal mast cells (MMC) and CGRP-IR fibers, which originate from dorsal root (DRG) and nodose ganglia, are found in close apposition. We examined interactions between primary cultured MMC and CGRP-IR DRG neurons in vitro by confocal recording of intracellular calcium concentration ([Ca^{2+}]_i). The degranulatory EC_{50} for the mast cell secretagogue compound 48/80 (C48/80; 10 μg/ml) and the neuropeptides CGRP (2.10^{-8} M) and substance P (SP; 3.10^{-8} M) were determined by measurement of extracellular release of the granule chymase, mouse mast cell protease-1. Application of C48/80 (10 μg/ml) and CGRP and SP (both 10^{-7} M) to Fluo-4-loaded MMC induced a transient increase in [Ca^{2+}]_i, after a lag time, indicative of mast cell degranulation and/or secretion. The CGRP response could be completely blocked by pertussis toxin (2 μg/ml), indicating involvement of G_{i} proteins. Application of MMC juice, obtained by C48/80 degranulation of MMC, to Fluo-4-loaded DRG neurons induced in all neurons a rise in [Ca^{2+}]_i, indicative of activation. Degranulation of MMC by C48/80 in culture dishes containing Fluo-4-loaded DRG neurons also caused activation of the DRG neurons. In conclusion, these results demonstrate a bidirectional cross-talk between cultured MMC and CGRP-IR DRG neurons in vitro. This indicates that such a communication may be the functional relevance for the close apposition between MMC and CGRP-IR nerve fibers in vivo.

De Jonge, F., A. De Laet, L. Van Nassauw, J. K. Brown, H. R. P. Miller, P.-P. van Bogaert, J.-P. Timmermans, and A. B. A. Kroese. In vitro activation of murine DRG neurons by CGRP-mediated mucosal mast cell degranulation. Am J Physiol Gastrointest Liver Physiol 287: G178–G191, 2004. First published March 11, 2004; 10.1152/ajpgi.00528.2003.—Upregulation of CGRP-immunoreactive (IR) primary afferent nerve fibers accompanied by mastocytosis is characteristic for the Schistosoma mansoni-infected murine ileum. These mucosal mast cells (MMC) and CGRP-IR fibers, which originate from dorsal root (DRG) and nodose ganglia, are found in close apposition. We examined interactions between primary cultured MMC and CGRP-IR DRG neurons in vitro by confocal recording of intracellular calcium concentration ([Ca^{2+}]_i). The degranulatory EC_{50} for the mast cell secretagogue compound 48/80 (C48/80; 10 μg/ml) and the neuropeptides CGRP (2.10^{-8} M) and substance P (SP; 3.10^{-8} M) were determined by measurement of extracellular release of the granule chymase, mouse mast cell protease-1. Application of C48/80 (10 μg/ml) and CGRP and SP (both 10^{-7} M) to Fluo-4-loaded MMC induced a transient increase in [Ca^{2+}]_i, after a lag time, indicative of mast cell degranulation and/or secretion. The CGRP response could be completely blocked by pertussis toxin (2 μg/ml), indicating involvement of G_{i} proteins. Application of MMC juice, obtained by C48/80 degranulation of MMC, to Fluo-4-loaded DRG neurons induced in all neurons a rise in [Ca^{2+}]_i, indicative of activation. Degranulation of MMC by C48/80 in culture dishes containing Fluo-4-loaded DRG neurons also caused activation of the DRG neurons. In conclusion, these results demonstrate a bidirectional cross-talk between cultured MMC and CGRP-IR DRG neurons in vitro. This indicates that such a communication may be the functional relevance for the close apposition between MMC and CGRP-IR nerve fibers in vivo.

There is abundant evidence that substance P (SP) released from nerve fibers induces mast cell degranulation (9, 28, 40, 43) and that mast cell-derived mediators, such as histamine and serotonin, can influence neuronal activity (11, 12, 46). The most direct evidence available for such a bidirectional interaction has been obtained in an elegant coculture approach of rat basophilic leukemia cells (RBL) and neurons of the superior cervical ganglion (43, 44). Measurements of intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in both cell types showed that IgE-induced degranulation of RBL cells causes activation of neurites (44) and that bradykinin-induced SP release from neurites causes NK1 receptor-mediated activation of the RBL cells (43). Remarkably, however, most of the information available about the degranulatory properties of neuropeptides has been obtained in studies using SP (9, 28, 40, 43), whereas data on the degranulatory properties of other neuropeptides, such as CGRP, are scarce (21, 49).

So far, most of the direct evidence for mast cell degranulation by neuropeptides (21, 40) has been obtained in studies on connective tissue mast cells (CTMC), which are found in connective and muscle tissues. The other type of mast cells, the mucosal mast cells (MMC), are abundantly present in the inflamed mucosa of the gastrointestinal tract (6, 39). Both mast cell types originate from the same bone marrow precursor cell, but tissue-specific signals drive them toward CTMC or MMC differentiation (26). In the mouse, two mast cell types can be distinguished (47): 1) the atypical, T cell-dependent MMCs, which contain the soluble β-chymase mouse mast cell protease-1 (mMCP-1) and a limited amount of histamine and 2) the T cell-independent CTMC, which contain heparin and large amounts of histamine but no mMCP-I (3, 6). At present, no information is available about the involvement of MMC in bidirectional communication with nerve fibers.

We previously reported (6) that the parasite infection of schistosomiasis in the mouse ileum is accompanied by a mastocytosis, which is characterized by a temporal distribution of distinct mast cell phenotypes. We observed in the mucosal layer a transient increase in MMC, peaking at 8 wk postinfection. Coinciding with this mastocytosis was an increase in the density of CGRP-IR extrinsic primary afferent nerve fibers in the lamina propria of both acute and chronic infected animals (5). Although these CGRP-containing extrinsic primary afferent nerve fibers in the mouse ileum do not contain SP, MMC...
were found in close apposition to the dense CGRP network (5). This suggested a possible role for CGRP in the communication between MMC and the dense CGRP network, to which efferent-like functions have been ascribed (15). We hypothesize that a bidirectional communication between MMC and CGRP-IR nerve fibers is involved in regulating the inflammatory response.

The present study thus aims to determine this functional bidirectional communication between MMC and CGRP-IR extrinsic primary afferent nerve fibers in vitro. To this end, primary cultures of bone marrow-derived MMC and adult dorsal root ganglia (DRG) neurons were characterized morphologically and physiologically. Recordings of \([\text{Ca}^{2+}]_i\) and electrophysiological measurements were used to investigate 1) the degranulation of MMC by the neuropeptide CGRP and 2) the activation of CGRP-containing DRG neurons by mast cell degranulate.

**MATERIALS AND METHODS**

**Animals**

Adult male Swiss mice (Iffa Credo Belgium, Brussels, Belgium) were given food and water ad libitum and were kept in a 12:12-h light-dark cycle. All experimental procedures were approved by the local ethics committee of the University of Antwerp.

**Cell Cultures**

Mast cell cultures. Bone marrow-derived mast cell cultures were made as previously described (51). Briefly, male mice at the age of 10–12 wk were killed by cervical dislocation, and their femurs were removed under sterile conditions. Bone marrow was washed from the femurs using a 23-gauge needle and a 5-ml syringe filled with DMEM (GIBCO Life Technologies, Paisley, UK) containing 10% FCS (Sigma, Poole, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 2 mM L-glutamine, and 1 mM sodium pyruvate (DMEM/FCS). A single cell suspension was obtained by passing the material three times through a 19-gauge needle, followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT). The ganglia were then enzymatically digested with collagenase (5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT). The ganglia were then enzymatically digested with collagenase (5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT). The ganglia were then enzymatically digested with collagenase (5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT). The ganglia were then enzymatically digested with collagenase (5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT). The ganglia were then enzymatically digested with collagenase (5 mg/ml, 45 min, RT; Boehringer-Roche, Mannheim, Germany) followed by incubation with trypsin (GIBCO) and collagenase (each 2.5 mg/ml, 30 min, RT).

**TESIS Preparation and Immunocytochemistry**

Cells from the bone marrow-derived mast cell culture were washed three times and resuspended in PBS before being loaded onto Marienfeld Adhesion Slides (Paul Marienfeld, Lauda-Königshofen, Germany) according to the manufacturer’s guidelines. Bound cells were fixed in modified Bouin’s fluid (95% saturated picric acid, 5% concentrated (37%) formaldehyde, and 2.5% glacial acetic acid) for 10 min at RT, permeabilized in absolute methanol for 10 min at RT, and stored in 70% ethanol at 4°C until further processing for immunocytochemistry.

Cells from the primary culture of DRG neurons were fixed with 4% paraformaldehyde for 10 min at RT on the poly-L-lysine-coated glass bottom of the petri dish. Subsequently, they were rinsed three times and kept in PBS until further processing for immunocytochemistry.

All incubations were performed at RT. The primary and secondary antibodies (Table 1) were diluted in PBS containing 10% normal goat serum, 0.01% bovine serum albumin, 0.05% thimerosal, and 0.01% sodium azide (PBS). To block nonspecific immunoglobulin interactions and to enhance permeability, the fixed cells were immersed in the supplemented PBS solution to which 1% Triton X-100 was added.
Next, they were incubated for 3 h with a primary antibody. Subsequently, after being rinsed in PBS, the tissue was incubated with an appropriate secondary antibody for 1 h. For negative controls, primary antisera were omitted in the protocol. The specificity of the commercial antibodies was tested by performing immunoblotting and preabsorption tests.

**Electron Microscopy**

A volume of 10 ml of a 9-day-old culture of both stimulated [compound 48/80 (C48/80); see Drugs] and nonstimulated MMC was centrifuged at 250 g for 7 min. The supernatants were removed, and the pellet was fixed with 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.4). Subsequently, the pellet was broken into small pieces and postfixed in 0.1 M cacodylate buffered 1% OsO₄, followed by dehydration in acetone before embedding in Durcupan. Subsequently, after 2 days, 50-nm-thin sections were cut and contrasted with 2% uranylacetate and Reynolds solution for 10 min each. The sections were then analyzed at a primary magnification of ×6,600 in a transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands).

**Drugs**

C48/80, rat α-CGRP, SP, pertussis toxin (PT), and TTX were purchased from Sigma. Neurobiotin was obtained from Vector Laboratories (Burlingame, CA). Capsaicin was purchased from Fluka (Buchs, Switzerland). Cell culture medium and FCS were supplied by GIBCO-BRL. C48/80, CGRP, SP, and PT were stored as frozen aliquots before use. TTX, neurobiotin, and capsaicin were freshly made before each experiment.

**Preparation of MMC Juice**

Nine-day-old cultured MMC (5 × 10⁵ cells in 100 μl DMEM F-12 buffer; GIBCO) were treated with C48/80 (10 μg/ml) for 1 h at 37°C. After centrifugation at 250 g for 5 min, the supernatants (MMC juice) were carefully removed and stored at RT for immediate experimental use.

**Optical Recording of Cytosolic [Ca²⁺]**

Calcium mobilization was used as an index of cellular activation and was assessed by confocal laser scanning microscopy (CLSM). Dishes with cells (for details, see below) were incubated with the Ca²⁺ indicator dye Fluo-4 AM (1 μM; Molecular Probes, Eugene, OR) and 0.005% of the detergents Pluronic F-127 (Sigma) in carbogenated (5% O₂-95% CO₂) DMEM F-12 buffer (GIBCO) for 40 min at RT. Approximately 20–30 min after stopping dye loading by refreshing the buffer, the dishes were transferred to an inverted microscope (Axiovert 100M; Zeiss) equipped with a ×25 water immersion objective (Zeiss; Plan-neofluar; numerical aperture 0.8) in a CLSM system (Zeiss; LSM-510). Digital images (size 368.5 × 368.5 μm) were recorded (usually during 240 s) at RT with a spatial resolution of 256 × 256 pixels and a temporal resolution of five images per second. The 488-nm argon laser line (200 mW) was used to excite Fluo-4 fluorescence in the cells, which was measured using a long-pass 505-nm filter. Laser illumination intensity was kept to a minimum (max 1% of laser output) to avoid phototoxicity and photobleaching. Calcium signals of the cultured DRG neurons were recorded in the culture dishes containing 100 μl buffer. For recordings of MMC (day 9 of culture), ~5 × 10⁵ MMC (in 100 μl of buffer) were seeded onto the poly-l-lysine-coated bottom of a well (diameter 10 mm) in a 35-mm-diameter culture dish (MatTek, Ashland, MA) and were then left ~30 min to settle to the bottom. All fluorescence measurements were made from subconfluent areas of the dishes, enabling the ready identification of individual cells. To measure cellular calcium responses to application of CGRP, SP, C48/80, and mast cell juice, these compounds were dissolved in DMEM-F12 buffer and applied directly in the bath as a small drop (10 μl) from a micropipette. The location of the pipette tip was at least 500 μm from the nearest cells to avoid pressure-induced stretching of cell membranes. Because the culture dishes with DRG neurons also contained some fibroblasts, immediately following each experiment, all DRG neurons within the image were identified by visual inspection using differential interference contrast. Criteria used were shape, diameter, and thickness of the soma, presence of neurites, and neurite size.

Image data were analyzed off-line using the Zeiss LSM510 analyzing software V2.53. A selected image in each image set was used as a template for designating each cell as a region of interest. Because Fluo-4 is a single-wavelength indicator, it was not possible to apply the ratiometric method for quantitative determination of [Ca²⁺]. Therefore, data were normalized with respect to the mean fluorescence intensity (F₀) during the first 5–10 s of recording. Temporal fluorescence intensity of the dye was divided by F₀. These relative fluorescence (RF) values represent integrated [Ca²⁺]. The RF values within each region of interest were plotted as a function of time. To determine the lag time of responses to application of compounds, we measured the time between drug application (as recorded by computer) until the first noticeable change in RF in the plots. The amplitude of the transient responses of MMC to application of neuropeptides (see Fig. 6) and of the DRG neurons to mast cell juice (see Fig. 5) was quantified and of the RF reached during the measuring period. Given the individual differences between cell responses, the amplitude of the complex responses of MMC to application of C48/80 (see Fig. 4) was also quantified as the maximum RF reached in the plots.

**Intracellular Electrophysiological Recordings**

Dishes containing a culture of DRG neurons were placed on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan) and were continuously superfused (10 ml/min; RT) with oxygenated Krebs-Ringer solution of the following composition (in mM): 118 NaCl, 4.75 KCl, 2.54 CaCl₂·2H₂O, 1.2 MgSO₄·7H₂O, 1 NaHPO₄·2H₂O, 25 NaHCO₃, and 11.1 glucose. Intracellular recordings were made with borosilicate glass microelectrodes (1-mm outer diameter; Clarc ElectroMedical Instruments, Reading, UK) pulled on a P-97 Brown-Flaming micropipette puller (Sutter Instrument, Novato, CA). The electrodes were backfilled with 1 M KCl containing 2% neurobiotin (resistance 60–100 MΩ). Potentials were recorded with an electrometer (Axoclamp 2A; Axon Instruments, Foster City, CA) through which also rectangular current pulses (generated using pClamp 6.0.2; Axon Instruments) could be injected. After amplification and low-pass filtering (3 kHz), each signal was digitized using a Labmaster TL-1 DMA Interface (Axon Instruments) at a sample rate of 5 kHz and displayed and stored on PC. Electrophysiological measurements were made after allowing the voltage signal to stabilize for a few minutes, without applying intracellular direct current. The input resistance of the neurons was estimated by the responses to hyperpolarizing current pulses of variable amplitude (~0.05 to ~0.3 nA). Durations of action potentials were measured as half widths, i.e., the time interval between the point on the upstroke at which the amplitude is halfway between the membrane resting potential and the maximum potential, and the equivalent point on the downstroke.

The effect of TTX (2 μM) on the action potential was determined by adding TTX to the superfusion solution for 5–10 min. Capsaicin (10⁻⁶ M in pipette) was applied in the vicinity of the impaled neuron by pressure-pulse ejection (50 ms) using a Picospritzer II (General Valve, Fairfield, NJ). Mast cell juice (see Preparation of Mast Cell Juice) was applied by consecutive (5-s intervals) pressure-pulse ejections with increasing duration (50–550 ms). Data analysis was performed using the Clampfit for Macintosh, version 6.0.2 (Molecular Devices, Sunnyvale, CA).

During electrophysiological experiments, impaled neurons were iontophoretically filled with neurobiotin by passing depolarizing cur-
rent pulses (0.5–1 nA, 100- to 500-ms duration) through the recording electrode. To reveal the presence of neurobiotin in the impaled neurons, the cultures were incubated with streptavidin coupled to Cy3 (1:4,000; Jackson ImmunoResearch Laboratories) after fixation (see Tissue Preparation and Immunocytochemistry).

Statistics

For quantification of mMCP-I levels in culture (n = 4) supernatants, median mMCP-I concentrations were compared using the non-parametric Mann-Whitney U-test.

The responses to stimulation of MMC with C48/80, CGRP, and SP were analyzed via an ANOVA test followed by a Bonferroni test to check for differences between groups when comparing the number of responding MMC and amplitudes (RF) of the responses. For all experiments, these statistical tests revealed no differences among dishes within one culture (usually 3 dishes per culture). Subsequently, results obtained on the dishes within one culture were pooled, and statistical analysis was performed among the different cultures (usually 3 cultures per experiment). Because no differences were observed, the results of all cultures were pooled, resulting in mean values for the percentage of MMC responding and the response amplitude. Stimulation of DRG neurons with MMC juice was analyzed similarly: no significant differences were found, and therefore, results of all cultures were pooled, resulting in a mean value for response amplitude. The same statistical test was performed for neurons activated by C48/80-induced MMC degranulation (n = 2). Finally, an unpaired t-test with Welch correction was used to compare the mean lag time and mean amplitude of directly (by MMC juice) and indirectly activated (by C48/80-induced MMC degranulation) DRG neurons.

All values are expressed as means ± SD. Data were considered statistically significant when P < 0.05.

RESULTS

Validation and Characterization of the Cell Cultures

MMC cultures. The bone marrow-derived MMC cultures (n = 20) were maintained for 9 days. Cell viability was decreased by days 2 and 4 (90 ± 0.8%; n = 3 cultures) but restored at day 9 (96 ± 0.3%; P < 0.05). The concentration of mMCP-I (a marker for MMC) in culture supernatants was 176 ± 31 ng/ml (n = 3 cultures) on day 4 and increased to 1,170 ± 50 ng/ml on day 9 (P < 0.05). Our results are in accordance with previous observations showing that addition of SCF, TGF-β, IL-3, and IL-9 to the culture medium results in differentiating mast cell-like phenotypes and that this quartet of cytokines has good results on MMC viability (93%) and mMCP-I concentration (1,250 ng/ml) in differentiated MMC (51).

Leishman’s staining (Fig. 1A) clearly showed the presence of granules in the cytoplasm of MMC. Immunocytochemical staining for mMCP-I (Fig. 1B) demonstrated that this protease is present in the granules, and electron microscopic (EM) analysis further revealed densely packed granules in the cytoplasm of MMC (Fig. 1C).

The degranulation properties of MMC were measured using the detection of extracellular mMCP-I by ELISA in response to application of C48/80, a general mast cell secretagogue (20, 52). The dose-response relationship (Fig. 2A) indicated that the EC_50 of C48/80 was 10 μg/ml (20). After stimulation with 10 μg/ml C48/80, EM analysis showed predominantly a noncytolytic anaphylactic-type degranulation (Fig. 1D), although a variability in the type and degree of mast cell degranulation was observed. With the use of the same mMCP-I ELISA, dose-response relationships (Fig. 2B) were determined for the degranulation of MMC by CGRP and SP. Responses to CGRP and SP were expressed as percentages of the C48/80 (10 μg/ml) response. On the basis of the EC_50 (CGR 1.7 × 10^-8 M; SP 2.7 × 10^-8 M), working concentrations for C48/80 (10 μg/ml; 100% degranulation), CGRP (10^-7 M; 39 ± 4%), and SP (10^-7 M; 57 ± 5%) were chosen for further experiments. Finally, the concentration of two main mast cell mediators in the supernatants of C48/80-degranulated MMC was determined by ELISA. Histamine and serotonin were both present in a concentration of 638 ± 52 and 602 ± 18 nM, respectively.

These results validate the cultured MMC and demonstrate their degranulatory properties in response to activation by C48/80 and the neuropeptides CGRP and SP.

DRG neuron cultures. After 2 days in culture, the DRG neurons exhibited outgrowths of neurites that form contacts with each other (Fig. 1E) and with other neurons over relatively long distances. Immunocytochemical double staining for protein gene product (PGP) 9.5 (labels all neurons) (48) and CGRP (specifically labels primary afferent, ileum-projecting DRG neurons in mice) (5) revealed that ~40% of the cultured DRG neurons were CGRP immunoreactive (IR; Fig. 1, F–H). Electrophysiological experiments confirmed that two populations of DRG neurons, each having specific electrophysiological properties, can be distinguished. One population of neurons had short-duration action potentials (1.1 ± 0.7 ms; n = 11) with no shoulder on the falling phase (Fig. 3Aa). These cells displayed TTX-sensitive action potentials (2 μM; n = 9; Fig. 3Ab) and did not respond to pressure-pulse application of capsaicin (10^-6 M; n = 5; Fig. 3Ac). The other population of neurons exhibited long-duration action potentials (2.8 ± 1.1 ms; n = 27) with a shoulder on the falling phase (Fig. 3Ba). In the latter neurons, the action potentials were resistant to TTX (2 μM; n = 8; Fig. 3Bb) and the cells depolarized (34 ± 11 mV) and reached firing threshold in response to pressure-pulse application of capsaicin (10^-6 M; n = 5; Fig. 3Bc). On the basis of these electrophysiological properties, this population could be identified as nociceptive afferent neurons (27). Moreover, immunocytochemical analysis of the impaled neurons revealed that the neurons belonging to this population were immunoreactive for CGRP (Fig. 3C). This finding is in accordance with earlier morphological observations that capsaicin treatment results in loss of CGRP-IR (5). These results validate the cultured neurons and identify part of the cultured neurons used in this study as ileum-projecting, extrinsic primary afferent, CGRP-containing neurons.

C48/80, CGRP, and SP increase [Ca^{2+}]_i of MMC

On MMC loaded with Fluo-4, we measured the [Ca^{2+}]_i response to bath application of C48/80 as a reference and of the neuropeptides CGRP and SP. Calcium images of MMC (day 9 of culture) were measured (5 images/s) during a period of 3–4 min. Application of the secretagogue C48/80 (10 μg/ml) (20, 52) induced a complex increase in [Ca^{2+}]_i in the MMC. A typical example of sequential Fluo-4 fluorescence images depicting the response of hundreds of MMC to C48/80 in a single microscopic field is shown in Fig. 4A. The regions of interest defined for each individual MMC in the field were used to calculate the RF intensity vs. time for all MMC. The calcium
Fig. 1. Morphological characterization of the mucosal mast cell (MMC) primary culture (A–D) and dorsal root ganglia (DRG) neurons (E–H). A: Leishmann staining for the visualization of mast cell granules. B: immunocytochemical staining for mouse mast cell protease-1 (mMCP-I; chymase, Cy-3 epifluorescence), located in the granules. C: electron microscopical image of the ultrastructure of an MMC, showing conspicuous electron-dense granules. D: after stimulation with compound 48/80 (C48/80; 10 μg/ml), the granules in MMC lost their content, which is indicative of degranulation. E: phase-contrast photomicrographs of DRG neurons after 2 days in culture. DRG neurons exhibit outgrowths of neurites (arrows) that form contacts with each other and with neurites over longer distances. F–H: immunocytochemical staining for PGP 9.5 (neuronal marker, Cy-3 epifluorescence (F)) and CGRP (marker for mouse extrinsic primary afferent neurons, FITC epifluorescence (G)) showed that the primary culture consisted of a heterogeneous population of neurons. Double labeling (H) revealed that not all PGP 9.5-IR neurons (*) were CGRP-IR (arrows).
The differences in amplitude and pattern of the Ca oscillations and the maximum \([\text{Ca}^{2+}]\) level reached (RF) were quantified. The degranulatory responses were determined from the soma responses. The calcium signals of three representative MMC are depicted in Fig. 4B. These signals show that the application of C48/80 induced, after a considerable lag period, an abrupt oscillatory increase in \([\text{Ca}^{2+}]\). After a few oscillatory cycles, the calcium signal settled to a temporarily sustained elevated level and then usually started to decrease slowly. These properties are considered characteristic of a “rapid-release” degranulatory response of mast cells (32, 52).

The percentage of responding MMC was determined from the RF traces of all MMC. Furthermore, the lag time between the application of C48/80 and the first noticeable rise in \([\text{Ca}^{2+}]\) and the maximum \([\text{Ca}^{2+}]\) level reached (RF) were quantified. The differences in amplitude and pattern of the Ca oscillations were not analyzed. Of all MMC to which C48/80 was applied (see MATERIALS AND METHODS for details on pooled experiments), \(\sim 56 \pm 1\%\) (134/260 cells; 3 cultures) responded to C48/80 with an increase in \([\text{Ca}^{2+}]\). The mean lag time of those MMC was found to be 47 ± 21 s. The distribution of the lag times is depicted in Fig. 5. The wide range of lag times (10–100 s) is in good accordance with earlier reports on mast cell degranulation (31, 44). The maximum \([\text{Ca}^{2+}]\) level reached was 6.5 ± 2.

The calcium response of MMC to the neuropeptides SP and CGRP (both \(10^{-7}\) M) consisted of a transient increase in \([\text{Ca}^{2+}]\). A typical example of sequential Fluo-4 fluorescence images depicting the response to CGRP is shown in Fig. 6A, and the calcium signals of three representative MMC are depicted in Fig. 6B. These signals show that CGRP, similar to SP (Fig. 6C), did not evoke any oscillations in \([\text{Ca}^{2+}]\), but induced a rapid increase in \([\text{Ca}^{2+}]\), followed by a slow exponential-like decrease toward the baseline. Compared with C48/80 (56 ± 1%), smaller percentages \((P < 0.01)\) of MMC were activated by CGRP (15 ± 3%; 66/599 cells; 3 cultures) and SP (12 ± 3%; 76/547 cells; 2 cultures). Furthermore, the amplitude (RF) of the responses of MMC to stimulation by CGRP (2.7 ± 0.3) and SP (2.6 ± 0.1) was smaller \((P < 0.001)\) than that of the responses to C48/80 (6.5 ± 2). Remarkably, the lag times of the MMC responses to SP (21 ± 16 s) and CGRP (33 ± 26 s) were much shorter \((P < 0.001)\) than those observed with C48/80 (47 ± 20 s). Figure 5 depicts the lag time distributions for C48/80, CGRP, and SP. The lag time of C48/80-activated MMC was larger than 40 s in 57% of the cases, whereas those for CGRP and SP were shorter than 40 s \((P < 0.0001)\) in 65 and 89% of MMC, respectively.

To determine whether the effect of CGRP was mediated by \(G_i\) proteins, MMC were pretreated with PT (2 \(\mu\)g/ml) for 1, 2, and 3 h before determining their response to CGRP stimulation. When compared with control conditions (15 ± 6%; 66/599 cells), a marked decrease in the percentage of MMC responding to CGRP was observed after treatment with PT for 2 (5 ± 2%; 16/345 cells; \(P < 0.05\)) and 3 h (1 ± 2%; 4/1,297 cells; \(P < 0.001\)). With respect to the amplitude (RF; control: 2.7 ± 0.3; PT 3 h: 2.9 ± 0.7) and the lag period of the responses, no effect of PT treatment was observed. These results clearly indicate that the effect of CGRP was mediated by PT-sensitive \(G_i\) proteins, which is in accordance with literature on connective tissue mast cells (8, 29).

**MMC Juice Induces Increase in \([\text{Ca}^{2+}]\), and Depolarization of DRG Neurons**

The \([\text{Ca}^{2+}]\) response to bath application of MMC juice was measured on cultured DRG neurons loaded with Fluo-4. This MMC juice was prepared from the supernatant of C48/80-activated mast cell cultures (see MATERIALS AND METHODS) and undoubtedly contained a large variety of mediators, such as histamine, serotonin, neutral proteases, cytokines, and chemokines (24). On the basis of the measurements in MMC juice (ELISA; see ELISA Measurements), the final bath concentrations of histamine and serotonin were estimated at 0.6 \(\mu\)M each. MMC juice obtained by CGRP or SP stimulation was not used to avoid direct activation of DRG neurons by these neuropeptides.

A typical example of sequential Fluo-4 fluorescence images demonstrating the response of DRG neurons to MMC juice is depicted in Fig. 7A. Application of MMC juice induced in the soma of DRG neurons a rapid increase in \([\text{Ca}^{2+}]\), followed by a slow exponential decrease toward the baseline. The \([\text{Ca}^{2+}]\), responses of the DRG somata to MMC juice were accompanied by comparable increases in \([\text{Ca}^{2+}]\), in their neurites. Only the soma responses were analyzed. The calcium signals of four representative DRG neurons are depicted in Fig. 7B. We found that all (57/57; 5 cultures) DRG neurons responded to MMC juice application with a marked increase in \([\text{Ca}^{2+}]\). The mean amplitude (RF) of the response was 1.9 ± 0.4 \((n = 5)\), and the
The mean lag time was 5 ± 2 s. The distribution of the lag times is shown in Fig. 8A. Activation of DRG neurons by MMC juice was often accompanied by a \([Ca^{2+}]\) response of the (Fluo-4 loaded) fibroblasts present in the culture dish (example in Fig. 7A).

To confirm that C48/80-induced MMC degranulation indeed activates extrinsic primary afferent neurons, we performed intracellular recordings to measure the effects of pressure-pulse application of MMC juice on the membrane potential of neurons belonging to this particular population. The primary afferent neurons were identified both by their electrophysiological properties (as described in DRG neuron cultures in RESULTS) and by the CGRP-IR of the impaled cells (Fig. 3C). All afferent neurons (resting membrane potential: \(-64 ± 8\) mV; \(n = 19\)) responded to MMC juice application with a long-lasting depolarization (duration range: 1–10 min; Fig. 9A). Depolarizations had an amplitude of \(12 ± 6\) mV (\(n = 19\)) and were accompanied by a decrease in membrane input resistance to \(46 ± 13%\) (98 ± 28 MΩ; \(n = 4\)) of controls (213 ± 58 MΩ; \(n = 4\); Fig. 9B). The neurons did not respond to application of C48/80 alone (Fig. 9C; \(n = 13\)). These results demonstrate that the application of MMC juice evokes a membrane depolarization of extrinsic primary afferent neurons.

We further measured the \([Ca^{2+}]\) response of Fluo-4-loaded DRG neurons to degranulation of (nonloaded) MMC, which were added to the DRG culture dish immediately before the
onset of the experiment (final concentration: 10^5 MMC/100 μl). Because CGRP and SP are known to directly activate neurons (15, 22), only C48/80 was used for MMC degranulation. All DRG neurons (20/20 in 2 cultures) responded to application of C48/80 (10 μg/ml), with a rise in [Ca^{2+}]_i (Fig. 8B). The mean amplitude of these responses (RF) was 1.7 ± 0.4 (n = 2). The mean lag time (25 ± 10 s; n = 2) was much larger (P < 0.0001; Fig. 8A) than that of the DRG neural responses directly induced by MMC juice (5 ± 2 s; n = 5). This finding is in accordance with the measured lag periods of the process of MMC degranulation itself (47 s). The lag period of the fibroblast responses was found to be 29 ± 9 s (20 fibroblasts; n = 2), which is not different (P = 0.64) from that of the DRG neurons (25 s). These results demonstrate that the fast increase in [Ca^{2+}]_i in DRG neurons, indicating activation, is evoked by the degranulation products released from C48/80-stimulated MMC.

**DISCUSSION**

The present study shows that application of the mast cell degranulator C48/80 and of the neuropeptides CGRP and SP evokes an increase in [Ca^{2+}]_i in cultured MMC. This finding demonstrates that CGRP and SP cause degranulation of MMC, which, in the case of CGRP, was found to be G_i protein mediated. The other important finding is that stimulation with MMC de-
shown that the increase in \([\text{Ca}^{2+}]\) after C48/80 application was found to be 47 s, which is in line with previous reports on antigenic degranulation of RBL cells [38 s (30); range 10–50 s (44)]. The variety of oscillatory activation patterns observed in C48/80-stimulated MMC corresponds with the oscillations reported in RBL studies [\([\text{Ca}^{2+}]\) fingerprinting (25, 30)]. Although the mechanism underlying the oscillations in \([\text{Ca}^{2+}]\) was not investigated in MMC, it seems most likely that C48/80 induces a biphasic increase in \([\text{Ca}^{2+}]\) in MMC as was reported for the RBL cells (30) and that fluctuations in the filling state of the internal \([\text{Ca}^{2+}]\) stores are involved in these \([\text{Ca}^{2+}]\) oscillations (30). Because only 57% of the MMC in the culture dishes reacted to C48/80 stimulation, probably not all the cultured mast cells were sufficiently mature to respond. The low relative maturity of part of the mast cells or of their secretory granules (36) is in agreement with our observation that all MMC responding to C48/80 reached relatively high RF values (>4), whereas the other MMC showed no rise in \([\text{Ca}^{2+}]\) at all.

**MMC degranulation by SP and CGRP**

Our finding that application of CGRP (10^{-7} M) or SP (10^{-7} M) to MMC evokes a rise in \([\text{Ca}^{2+}]\), led us to conclude that CGRP and SP are able to degranulate MMC. This conclusion is in accordance with measurements of SP-induced histamine secretion from MMC (40); however, CGRP-induced degranulation of MMC has not been reported before. Almost all information on degranulation of mast cells by SP (9, 21, 37, 40, 49) or CGRP (37, 49) originates from studies on CTMC, a functionally different type of mast cells (13, 26). The working concentration of CGRP and SP (10^{-7} M), based on the dose-response curves, is in line with previous reports on CTMC (40, 49), indicating that in vitro, relatively high doses of neuropeptides are required to induce mast cell degranulation. The concentrations of CGRP and SP were chosen in such a way that ~50% degranulation was expected compared with C48/80, as was actually observed during the calcium experiments. Low concentrations of neuropeptides (i.e., SP) are able to prime mast cells for activation at picomolar concentrations (17, 43). Thus, under in vivo conditions, degranulation of mast cells is most likely induced by lower concentrations than those used in the present study.

Unlike C48/80 stimulation, stimulation with CGRP and SP did not result in \([\text{Ca}^{2+}]\) oscillations, which led to the interpretation that the neuropeptides do cause a release of mediators from MMC (17, 43), rather than an anaphylactic shock-type degranulation. C48/80 is known to be able to directly activate intracellular G_i proteins in CTMC by a mechanism referred to as the peptidergic pathway of mast cell activation (8, 29). This activation pathway involves membrane-assisted receptor-independent stimulation of G_i-like proteins and appears to be non-specific in that it can also be used by neuropeptides, such as SP (4). Our finding that PT, a blocker of G_i proteins, completely inhibits the MMC response to CGRP, clearly demonstrates that the effect of CGRP is G_i protein mediated. Possible differences in the mechanism by which neuropeptides and C48/80 are translocated to the intracellular environment of the mast cell might explain why the lag time for neuropeptides (CGRP, 33 s; SP, 21 s) is smaller than that for C48/80 (47 s).
Given the reported receptor-mediated degranulation of CTMC by neuropeptides (43, 45), it should be mentioned that our experiments do not exclude the possibility that CGRP receptors are present on the MMC surface.

Activation of DRG Neurons by Mast Cell Juice

MMC juice was shown to activate all functional types of DRG neurons, as indicated by an increase in [Ca$^{2+}$]$_i$. The time course and shape of the calcium responses of DRG neurons to MMC juice are comparable with those of the activating depolarization induced by inflammatory mediators, such as histamine (12) and serotonin (11) in enteric neurons. Our coexperimental setup with loaded DRG neurons and unloaded MMC disclosed that C48/80-mediated MMC degranulation was able to activate DRG neurons. The duration of the neural calcium responses obtained by this MMC-mediated activation was much longer (>4 min) than that obtained by direct activation of DRG neurons by MMC juice (~100 s). Accordingly, the lag times of the neural responses to indirect activation were much longer (25 s) than that measured after application of MMC juice (5 s). This finding is quantitatively in accordance with our observation that >90% of the MMC show a lag time for
C48/80-induced degranulation (>20 s (Fig. 5). In this study, our particular interest was in the communication between MMC and the CGRP-containing extrinsic afferent neurites in the mouse ileum. The results of the electrophysiological experiments on identified (see RESULTS) neurons unequivocally demonstrate that MMC juice activates this particular population of ileum-innervating afferents.

**Bidirectional communication between MMCs and DRG neurons.** Intestinal schistosomiasis in the mouse ileum is accompanied by a mastocytosis and a concomitant increase in density...
of CGRP-IR extrinsic primary afferent nerve fibers (5, 6). The observed close apposition of MMC to the neural network (5) led to the hypothesis that a bidirectional communication between MMC and CGRP-IR nerve fibers is involved in the regulation of the inflammatory response. CGRP has chemotactic properties (10) and is thought to be implicated in the induction of mastocytosis. The finding that CGRP is also able to directly activate MMC suggests a functional importance of CGRP as a neural messenger in the signal pathway from these particular afferent neurites to the MMC. Activation of the afferent neurites by MMC degranulate confirms the functional importance of the mast cell’s inflammatory mediators in the signal pathway from MMC to the afferent nerve fibers.

In conclusion, the present study has provided evidence that MMC in culture are activated and degranulate in response to stimulation with C48/80, CGRP, and SP. In addition, MMC degranulate is able to activate DRG neurons. Taken together, these findings confirm our hypothesis that MMC activation and degranulation occur as a direct response to the release of CGRP from extrinsic primary afferent nerve fibers and that extrinsic primary afferent neurites can be activated by mast cell degranulatory compounds. More generally, our results provide support for the notion that mast cells and sensory nerve fibers are important neuroimmune components in axon-reflex pathways involved in tissue defense against injury and noxious stimuli (2, 41, 42).

ACKNOWLEDGMENTS

The authors thank the technical staff of all laboratories for excellent assistance. In particular, we wish to thank Prof. Dr. R. Blust (University of Antwerp) for the use of the ELISA plate reader and Dr. R. Nuydens (Janssen Research Foundation) for the technical and practical guidelines regarding the DRG cultures. We thank K. Verstraeten for expert technical assistance with the cell culturing. We are indebted to Prof. Dr. T. Frieling for valuable comments on the manuscript.

GRANTS

This study was supported by the InterUniversity Attraction Pole project P5/20, an Institute for the Promotion of Innovation by Science and Technology in Flanders fellowship from the Flemish Government (SB1146; to F. De Jonge), and by grants from the Wellcome Trust. A. De Laet is holder of a research fellowship of the Born Bunge Foundation. A. B. A. Kroese is holder of a guest professorship at the University of Antwerp.

Fig. 8. [Ca^{2+}]i response of DRG neurons to direct and indirect activation. A: distribution of the lag times of the responses of directly and indirectly activated DRG neurons. For direct activation, MMC juice was applied to a dish containing Fluo-4-loaded DRG neurons; for indirect activation, C48/80 was applied to a dish containing MMC as well as Fluo-4-loaded DRG neurons. Indirectly activated DRG neurons exhibited larger lag times (25 ± 10 s) than directly activated neurons (5 ± 2 s). B: RF of an indirectly stimulated neuron is depicted as a function of time. The relatively long lag time and long duration of the [Ca^{2+}]i responses are in accordance with the indirect nature of the stimulus, which involves degranulation of the MMC.

Fig. 9. Effect of pressure-pulse application of MMC juice (A and B) and C48/80 (C) on the membrane potential of afferent DRG neurons. Arrows indicate consecutive pressure-pulse applications with increasing durations (50–550 ms). Scale bar in B also applies to C. A: application of MMC juice evokes a longlasting depolarization of the membrane potential. B: the depolarization is accompanied by a decrease in membrane input resistance as revealed by the decreased amplitude of the voltage response to intrasomal injection of constant amplitude hyperpolarizing pulses (−0.1 nA, 60-ms duration with 0.5-s intervals). C: pressure-pulse application of C48/80 on the same neuron as in A did not evoke any response.
REFERENCES


