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*J Immunol* published online 22 July 2015
http://www.jimmunol.org/content/early/2015/07/22/jimmunol.1500253

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/07/22/jimmunol.1500253.DCSupplemental

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Identification of a New Exo-Endocytic Mechanism Triggered by Corticotropin-Releasing Hormone in Mast Cells

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The key role of mast cells (MC), either in development of inflammatory pathologies or in response to environmental stress, has been widely reported in recent years. Previous studies have described the effects of corticotropin-releasing hormone (CRH), which is released from inflamed tissues by cellular stress signals, on MC degranulation, a process possibly driven by selective secretion of mediators (piecemeal degranulation). In this study, we introduce a novel granular exo-endocytic pathway induced by CRH on peritoneal MC. We found that CRH triggers substantial exocytosis, which is even stronger than that induced by Ag stimulation and is characterized by large quanta size release events. Membrane fluorescence increases during stimulation in the presence of FM1-43 dye, corroborating the strength of this exocytosis, given that discrete upward fluorescence steps are often observed and suggesting that secretory granules are preferentially released by compound exocytosis. Additionally, the presence of a depot of large tubular organelles in the cytoplasm suggests that the exocytotic process is tightly coupled to a fast compound endocytosis. This CRH-stimulated mechanism is mediated through activation of adenylate cyclase and an increase of cAMP and intracellular Ca²⁺, as evidenced by the fact that the effect of CRH is mimicked by forskolin and 8-bromo-cAMP. Thus, these outcomes constitute new evidence for the critical role of MC in pathophysiological conditions within a cellular stress environment and an alternative membrane trafficking route mediated by CRH. The Journal of Immunology, 2015, 195: 000–000.

Mast cells (MC) are connective tissue and mucosa cells, mostly positioned at the interface of the external environment and nerves or blood vessels. Therefore, it is not surprising that MC have been widely recognized as important effectors of the immune system, playing a pivotal role in both innate and acquired immune system processes, inflammation, and host–pathogen interactions (1–3). Moreover, MC are involved in numerous diseases that are affected by stress, such as asthma, inflammatory arthritis, inflammatory bowel disease, and chronic inflammation, and can significantly promote or suppress various aspects of the stress response (4–6). When MC are activated to degranulate, secretory granules (SG) release their contents, which include a diverse array of biologically active products, many of which mediate potential immunoregulatory effects (7, 8).

After MC activation, a degranulation process occurs when SG-containing mediators fuse with the plasma membrane by exocytosis in an exquisitely orchestrated cellular process (9). Studies using electrophysiological and electrochemical techniques have described this process in detail and established three exocytotic modes in secretory cells: full-collapse fusion, reversible fusion (kiss-and-run), and compound exocytosis. Each of these modes controls the rate and amount of granular content release and thus controls exocytosis strength. The three exocytotic modes are followed by different endocytotic routes (classical endocytosis, kiss-and-run, and bulk endocytosis), which ensure sufficient membrane recycling (10). Compound exocytosis consists of a fusion event of a giant SG preformed by granule–granule fusion or by fusion of an SG on an already fused but not collapsed SG. This mode of exocytosis has been well established in MC (11), and our group has previously reported that it can be followed by a mode of endocytosis called compound endocytosis (12). In addition to compound exocytosis, it has been shown that MC can release granular mediators by piecemeal degradation (PMD), a process in which small vesicles are budded off from SG and fuse with the plasma membrane, leading to selective content discharge. Following exocytosis, endocytosis of these small vesicles is initiated to retrieve them from the plasma membrane. Finally, the vesicles traverse the cytoplasm to fuse with SG (13–16). This type of exocytosis can be mediated by different stimuli such as IL-1 or IL-4 (17, 18).

The crosslinking of IgE bound to receptor (FceRI) by multi-valent Ags is traditionally known for being a potent MC activation stimulus that leads to anaphylactic degranulation (19, 20). However, MC can also be activated by non-Ag triggers such as neuropeptides, complement activation, and certain toxins (1, 8). Corticotropin-releasing hormone (CRH), a peptide that activates the hypothalamic/pituitary/adrenal axis under stress conditions, is also peripherally secreted and has proinflammatory effects (21) mediated through immune cells, including MC (22). Indeed, it has been shown that MC synthesize and secrete CRH, and there is evidence indicating that the main CRH receptors, CRH-R1 and CRH-R2, are functionally expressed by MC as well (23, 24).
Although the mechanism underlying Ag stimulation leading to anaphylactic degranulation is well understood, the effects of CRH on MC activation remain unclear. It has been shown that CRH induces MC degranulation and selective secretion of mediators stored in cytoplasmic SG, most likely through PMD (22, 24, 25). Even so, whether there are other exocytotic mechanisms involved in this process is still unknown. In this study, we stimulated peritoneal MC (PMC) and intestinal MC (IMC) with CRH. We applied imaging and electrochemical techniques to better understand and supplement our knowledge about CRH-triggered exocytosis and endocytosis. We observed that CRH increases the 5-hydroxytryptamine (5-HT, or serotonin) content of SG and the number of release events in PMC. Imaging of the exo- and endocytosis events during hormone stimulation in the presence of N-(3-triethylaminomunopyrrol-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM1-43) allowed us to identify multiple steps that reflect compound exocytosis. This process was tightly coupled to rapid compound endocytosis. We also found that CRH, acting on its membrane receptors, triggers a strong increase in intracellular calcium (Ca^{2+}), which was mimicked by both 8-bromo-cGMP and forskolin, indicating that cAMP might favor this response. The fact that CRH is able to trigger mainly large SG fusion indicates that MC release a large amount of cargo and differs from the selective secretion through anaphylactic degranulation (28).

**Materials and Methods**

**Cell culture**

Primary MC cultures were prepared from wild-type C57BL/6 mice or Wistar rats. All rodent studies were conducted under a protocol approved by the Ethical Committee of the University of Seville and Consejería de Agricultura y Pesca (Junta de Andalucía, Spain). MC were isolated from the peritoneal cavity of 3- to 4-mo-old mice following a procedure described in detail elsewhere (11, 12). The peritoneal wash fluid contains a large number of cells, a small fraction of which are MC. Using a Percoll (Sigma-Aldrich) gradient, fully differentiated connective tissue PMC were separated from other cells (28). Isolated PMC were plated on poly-d-lysine–treated coverslips and maintained in advanced RPMI 1640 medium (Life Technologies) supplemented with FBS (10% v/v), penicillin (50 U/ml), streptomycin (50 μg/ml), l-glutamine (1 mM), and N-acetyl-L-cysteine (1 mg/ml) and EDTA (2 mM) to remove fat removed, and then washed with NaCl (0.9%). The solutions used to return to the baseline amperometric recording. The latency of release that follows stimulation was measured as the length of time required for completion of 20% of the total secretory response (final cumulative Q value). The analysis of an individual exocytotic event was performed through the measurement of several parameters (see the graphic in Table II). Only well-resolved spikes with amplitudes >5 pA were used for our analysis. Each amperometric parameter was statistically analyzed by taking the mean value of the events from individual cells with >15 spikes during the recording. Amperometric spike characteristics reveal rich details about the intracellular movement of the secretory granule toward the cell membrane and fusion with it (34, 35). The analysis of a spike (spike Q) can be directly related to the number of molecules secreted from other cells (28).

**Epifluorescence imaging**

Exocytosis was imaged on an epifluorescence microscope system including an inverted microscope (Axiovert 200) equipped with a high-resolution CCD camera (ORCA-R2 C10600-10B; Hamamatsu Photonics). FM1-43 fluorescence was detected using a standard filter set (XFI115-2; Omega Optical). Images were acquired through a ×63 oil immersion objective (102 nm/image pixel). Images were acquired at 1 Hz with a 0.2-s exposure. Imaging experiments were performed with the equipment (4 μM FM1-43, drug solution containing cell) using a commercial patch-clamp amplifier (EPC-10 USB; HEKA Electronic). The electrode, placed in close proximity to a cell, was held at 700 mV versus an Ag/AgCl reference electrode (World Precision Instruments) using a commercial patch-clamp amplifier (EPC-10 USB; HEKA Electronic). At this voltage, 5-HT is the main secretory product detected in PMCs by amperometry (31, 32). The signal was digitized at 4 kHz and filtered with an internal low-pass Bessel filter at 2 kHz with the acquisition software PatchMaster (HEKA Electronic). The signal was displayed in real time and stored digitally.

**Analysis of amperometric data**

The amperometric data were analyzed in Igor Pro 6 (version 6.21; WaveMetrics) using an Igor procedure file designed for analysis of quantal release and developed by Sulzer and colleagues (33). The amperometric integral (cumulative Q) was calculated by subtracting the constant current level in the carbon fiber and integrating the whole amperometric recording. The latency of release that follows stimulation was measured as the length of time required for completion of 20% of the total secretory response (final cumulative Q value). The analysis of an individual exocytotic event was performed through the measurement of several parameters (see the graphic in Table II). Only well-resolved spikes with amplitudes >5 pA were used for our analysis. Each amperometric parameter was statistically analyzed by taking the mean value of the events from individual cells with >15 spikes during the recording. Amperometric spike characteristics reveal rich details about the intracellular movement of the secretory granule toward the cell membrane and fusion with it (34, 35). The analysis of a spike (spike Q) can be directly related to the number of molecules secreted from a cell. Spike Q is the ascending slope of spike calculated from 25 to 75% from the final membrane fluorescence. The analysis of a foot signal was performed through the measurement of several parameters (see the graphic in Table II). The foot frequencies were measured by counting the total number of feet with respect to the total number of spikes. The foot amplitude is proportional to the fusion pore conductance (36) and the foot duration is thought to reflect the stability of the fusion pore (37).
Live-cell confocal microscopy

To quantify endocytosis, cells were incubated in an external solution containing FM1-43 (4 μM) and CRH (10 nM) or DNP (1 μg/ml) for 5 min and washed with standard external solution. Images were captured using an upright Olympus FV1000 confocal laser scanning microscope equipped with three visible wavelength lasers (argon-krypton laser with 488, 561, and 633 nm excitation lines) and a ×60 water-immersion objective (82 nm/image pixel, numerical aperture of 1.2). To illuminate FM1-43–labeled endocytotic events, cells were exposed to the 561-nm excitation laser. The bandwidth of emission spectrum was adjusted to 580–620 nm for acquiring images. Reconstruction of three-dimensional images from 1-μm Z-stacks were prepared using ImageJ (National Institutes of Health). To detect and quantify the fluorescent area on raw images from single slices from the Z-projection of each cell, HCImage software was used. We assumed that the fluorescent area could be simple (a single spot) or compound (two, three, or more fused spots, also referred as endocytotic organelles) in its equatorial plane and were selected based on threshold brightness and a minimum fluorescent area, discarding duplicated events found in more than one slice.

Recordings of Ca2+ signal

Changes in Ca2+ concentration in response to different drugs were monitored by dual excitation microfluorimetry in PMC incubated in an external solution containing fura 2-AM (5 μM) for 40 min at 37°C. Then, cells were washed twice in external solution without the probe at room temperature and used immediately for imaging. Fura 2-AM loading was usually uniform over the cytoplasm, and compartmentalization of the dye was never observed. Experiments were conducted using an Axiovert 200 inverted microscope equipped with a standard filter set (XF04-2; Omega Optical). Dye-loaded cells were given a long application of the stimuli and then washed. During recordings, cells were excited at 360/380-nm wavelength exposures (time, 0.5 s; data acquisition at 0.5 Hz) and fluorescence elicited was collected at 510 nm (ΔF ratio). Data were acquired and stored using HCImage software and exported to Igor Pro 6.21 to perform analysis. Time to peak represents the time to reach the peak maximum. The plateau phase (ΔF ratio plateau) was calculated as the difference between the final fluorescence after washing and the baseline fluorescence. All values were normalized to the basal fluorescence (baseline).

Solutions and reagents

All experiments were performed in an external solution that contained 140 mM NaCl, 10 mM HEPES, 3 mM KOH, 2 mM MgCl2, and 1 mM CaCl2. Locke’s buffer contained 140 mM NaCl, 5 mM KCl, 25 mM NaHCO3, 5 mM HEPES, and 11.1 mM glucose. FM1–43 (1 mM stock solution) and fura 2-AM (1 mM stock solution) were obtained from Life Technologies. CRH (5 μM stock solution), Ag DNP–human serum albumin (1 mg/ml stock solution) were obtained from Tocris Bioscience. All stock solutions were aliquoted and stored at −20°C to protect them from light.

Statistical analysis

Throughout this study, each variable of the treatment groups was compared using a Mann–Whitney U test unless otherwise specified. A Student t test was used to compare data groups when both passed the normality test (Kolmogorov–Smirnov). Data were considered significant at p ≤ 0.050 and are plotted as the means with SEM. All statistical analyses were carried out using SPSS Statistics 22 software (IBM).

Results

CRH induces a high number of fusion events with a large quantal size

Amperometry is a sensitive and very powerful method of measuring secretion by monitoring the release of electroactive secretory products, such as 5-HT, with a carbon-fiber microelectrode placed very close to the surface of an isolated cell. Following cell stimulation, a brief current in the form of spikes can be observed as a consequence of the oxidation of the transmitters released by SG (31, 38). This technique allows for the detection of differences in the amount of product secreted per SG and cell (39). Therefore, we studied the effects of CRH and Ag (DNP) on the characteristics of exocytotic events.

Fig. 1 shows the typical amperometric recording after a 5-min application of 1 μg/ml Ag (Fig. 1A) and 10 nM CRH (Fig. 1B). The temporal course shows that after stimulation, there is an initial latency phase before amperometric spikes appear. This period was longer under CRH than Ag stimulation (CRH, 4.95 ± 0.58 min, n = 16; DNP, 2.01 ± 0.30 min, n = 18; p = 0.001 by Student t test). Even so, the response phase is longer in CRH-treated cells (8.15 ± 0.72 min) than in Ag-treated cells (4.13 ± 0.57 min; p = 0.0001 by Student t test). Additionally, the number of successfully fused SG (the number of spikes; Fig. 1C) is higher in CRH- than in Ag-treated cells (CRH, 90 ± 19; DNP, 54 ± 18; p = 0.050, Fig. 1C). This difference in spike number was more marked when we measured the cumulative charge (Q) per cell (p = 0.0001, see Fig. 1D). The cumulative Q is larger in CRH-stimulated cells than in Ag-stimulated cells (CRH, 1.21 ± 0.17 nC; DNP, 0.42 ± 0.09 nC). These data indicate that the total amount of 5-HT released per cell is greater during exocytosis triggered by CRH. We observed that the average amperometric spike recorded after CRH stimulation (black trace) is larger than that recorded after Ag stimulation (gray trace) (Fig. 1E). CRH evoked spikes characterized by a large amplitude and large area under the spike. Taking into account that the spike area defines spike Q, we used log-transformed values for curve fitting of the Q individual spikes (CRH, n = 1437 spikes; DNP, n = 964 spikes) (Fig. 1F). We observed a shift in the centers of the two distributions (CRH, 0.50 pC; DNP, 0.23 pC), showing that spikes recorded during CRH stimulation contain more 5-HT than Ag-induced spikes.

To gain insight into a single exocytotic event, we quantified several parameters of individual spikes (Table I). Spikes triggered by both stimuli were no different in kinetic parameters (t1/2 and fall time) with the exception of Riseon, which was longer in CRH-evoked exocytosis. However, in line with our previous observation, there were differences in quantitative parameters between the two groups. Spikes recorded after CRH stimulation showed larger Imax and spike Q than did those recorded after Ag stimulation, suggesting that CRH triggers exocytosis of large quantal size SG. We next examined foot signals preceding amperometric spikes because parameters of these amperometric feet (e.g., their duration, amplitude, and charge) provide information about properties of the exocytotic event (34). Foot frequency indicates that ~50% of CRH-induced spikes had a prespike foot. We observed that foot duration was no different between CRH- and Ag-treated cells. In contrast, foot amplitude and foot Q are significantly different (Table II) between the two stimuli, further showing that CRH does affect the amount of 5-HT release.

Altogether, our electrochemical measurements indicate that the intense exocytosis observed after CRH stimulation in PMC is stronger than the Ag-induced response and is a consequence of a higher number of SG fused with the plasma membrane and a large amount of transmitter released by an exocytotic event.

FM1–43 fluorescence pattern suggests that preferentially CRH induces compound exocytosis

We next sought to verify the strength of the exocytosis evoked by CRH using a fluorescent dye. We visualized the exocytosis triggered by CRH (10 nM) and Ag (1 μg/ml) using the fluorescent probe FM1–43 (4 μM) (Fig. 2, Supplemental Videos 1, 2). FM1–43 and similar amphipathic styryl dyes have been used for imaging exo-endocytosis pathways in different types of cells. These dyes can reversibly stain membranes and are more fluorescent when bound to membranes than when in solution (40). In PMC, both the membrane and the dense core of SG are stained with FM1–43, making the release of SG visible as a bright diffraction of limited spots (12).

Ag stimulation generally showed a gradual rising in the fluorescent trace, without discrete upward steps (nos. 2 and 3 in
However, CRH stimulation resulted in upward steps that corresponded with the presence of large fluorescent spots (nos. 2 and 3 in Fig. 2B). The examination of the fluorescent spot area corroborated that CRH induces exocytosis of giant SG (CRH, 3.29 ± 0.40 μm², n = 26 cells; DNP, 1.62 ± 0.08 μm², n = 48 cells) (Fig. 2C). Additionally, we measured the fluorescence intensity increase (ΔF) by subtracting the basal membrane fluorescence (no. 1 in Fig. 2A, 2B) to the final fluorescence (no. 4 in Fig. 2A, 2B). The analysis showed that exocytosis induced by CRH (1073 ± 131 arbitrary units [a.u.], n = 26) is stronger than that evoked by Ag (775 ± 95 a.u., n = 48), (Fig. 2D). Altogether, these experimental data suggest that the strong exocytosis triggered by CRH is likely due to a compound exocytosis process (41).

CRH stimulation leads to the formation of large endosome-like structures

We next examined whether CRH and/or Ag response could also induce a compensatory endocytosis coupled to the observed exocytosis. To test this, we performed live cell confocal microscopy for a quantitative assessment of the total endocytosed probe (see Materials and Methods). As indicated above, FM1-43 can be used as a reliable reporter of exocytosis in PMC. After washing, the internalized fluorescence observed in cells is a consequence of an endocytotic process. A 5-min exposure to both stimuli (10 nM CRH or 1 μg/ml Ag) resulted in a rapid compensatory endocytotic response (Fig. 3). However, CRH-stimulated cells took up a greater amount of the dye than did Ag-stimulated cells, indicating that CRH triggers a more intense endocytosis (Fig. 3A). Indeed, in CRH-stimulated cells, the total area occupied by the endocytotic events per cell was larger (16.24 ± 1.95 μm², n = 38) than in Ag-stimulated cells (11.57 ± 2.63 μm², n = 42) (Fig. 3B). To test whether these spots represent a single endocytotic event or a compound event (multiple fused SG), single sections gradually obtained in the Z plane from a CRH-stimulated cell were analyzed (Fig. 3C). A detailed view of the data allowed us to describe the presence of large tubular endocytotic organelles that have a several-fold higher area than a single spot (Fig. 3C, inset). Remarkably, this type of tubular endocytotic structure was not often observed after Ag stimulation. On the whole, the size of the endocytotic events are larger in CRH-stimulated cells (4.38 ± 1.57 μm², n = 38) than

**FIGURE 1.** Effects of CRH and Ag (DNP) on secretory response of individual MC. (A) Representative amperometric trace and cumulative Ψ (black trace) obtained during a 5-min application (arrow) of 1 μg/ml DNP. The split line represents the final period in which amperometric spikes were not detected. (B) Representative amperometric trace and cumulative Ψ (black trace) recorded during a 5-min application of 10 nM CRH (arrow). Calibration for time, amperometric current, and cumulative Ψ are indicated with solid bars. (C) The bar histogram shows the number of spikes pooled from cells stimulated with CRH (filled) and DNP (open). *p < 0.05, Mann–Whitney U test. (D) The bar histogram shows the average of total amount of 5-HT released per cell (cumulative Ψ) stimulated with CRH (filled) or DNP (open). **p < 0.001, Mann–Whitney U test. (E) Average spikes generated from the data from Tables I and II, obtained from stimulation with CRH (black spike) and DNP (gray spike). (F) Frequency histograms of the log-transformed values of individual spikes Ψ from CRH-treated cells. The solid line represents the Gaussian function fitted to the data from CRH-treated cells. The dashed line represents the Gaussian function fitted to the data from DNP-treated cells. Data in (C) and (D) are represented as the means ± SEM. All data shown are representative of four independent cultures (DNP, n = 18 cells; CRH, n = 15 cells).
in Ag-stimulated cells (1.55 ± 0.24 μm², n = 42) (Fig. 3D). Overall, the presence of these large endocytic organelles suggests that CRH evokes a compound exocytosis that is tightly coupled to the compound endocytosis of several SG by a single fission event and results in a rapid and efficient compensation of the membrane excess caused by intense exocytosis.

**CRH and Ag stimulation trigger different Ca²⁺ signals**

It has long been apparent that the elevation of Ca²⁺ is important in the regulation of MC degranulation (42). Indeed, it has been shown that activation by Ag stimulation induces an increase of Ca²⁺ (19, 20). Activation of CRH-R can elevate Ca²⁺ in different cells (43), including MC, and there is some evidence that Ca²⁺ increases through CRH-R1 and hence leads to a degranulation process (44). We measured and compared Ca²⁺ signals evoked by a long application of both stimuli (10 nM CRH and 1 μg/ml Ag) using the fura 2-AM probe (45) (Fig. 4). CRH addition caused a rapid peak increase in Ca²⁺ followed by a decrease to a stable plateau phase (Fig. 4A, black trace). Ag stimulation also evoked an increase but at a slower rate and with a higher plateau phase (Fig. 4A, gray trace). The amplitude of the Ca²⁺ signal (ΔF ratio) was no different between the two stimuli (CRH, 0.128 ± 0.009 a.u., n = 49; DNP, 0.127 ± 0.005 a.u., n = 103) (Fig. 4B). In contrast, the plateau phase was lower in CRH-stimulated cells (0.025 ± 0.001 a.u.)

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**Table I. Effects of 10 nM CRH and 1 μg/ml DNP on secretory spike parameters**

<table>
<thead>
<tr>
<th></th>
<th>I_{max} (pA)</th>
<th>Rise_{m} (pA/ms)</th>
<th>Spike Q (pC)</th>
<th>t½ (ms)</th>
<th>Fall Time (ms)</th>
<th>Cells (n)</th>
<th>Spikes (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP</td>
<td>40.1 ± 5.5</td>
<td>3.8 ± 0.9</td>
<td>2.6 ± 0.2</td>
<td>63.7 ± 5.0</td>
<td>64.4 ± 5.8</td>
<td>18</td>
<td>964</td>
</tr>
<tr>
<td>CRH</td>
<td>70.0 ± 6.9</td>
<td>5.8 ± 0.9</td>
<td>5.5 ± 0.6</td>
<td>75.7 ± 12.4</td>
<td>75.0 ± 12.9</td>
<td>16</td>
<td>1437</td>
</tr>
<tr>
<td>p Value</td>
<td>0.003</td>
<td>0.050</td>
<td>0.001</td>
<td>0.825</td>
<td>0.932</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Parameters of secretory spikes elicited by a 5-min application of 10 nM CRH or 1 μg/ml DNP (Ag) in MC. The following parameters were analyzed from the detected spikes: fall time, total decay time; Rise_{m}, ascending slope of spike calculated from 25 to 75% from I_{max}; spike Q, area of spike or spike net charge; t½, spike width at half height. Data are presented as means ± SEM. The pairs of data sets were compared using a Mann–Whitney U test (p value).

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**Table II. Parameters of the foot signal from spikes induced by 10 nM CRH and 1 μg/ml DNP**

<table>
<thead>
<tr>
<th></th>
<th>Foot Amplitude (pA)</th>
<th>Foot Q (pC)</th>
<th>Foot Duration (ms)</th>
<th>Foot Frequency (%)</th>
<th>n Foot</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP</td>
<td>2.9 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>48.4 ± 7.8</td>
<td>31.5 ± 3.2</td>
<td>305</td>
</tr>
<tr>
<td>CRH</td>
<td>3.6 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>62.0 ± 8.1</td>
<td>46.2 ± 4.3</td>
<td>654</td>
</tr>
<tr>
<td>p Value</td>
<td>0.015</td>
<td>0.011</td>
<td>0.168</td>
<td>0.001</td>
<td>---</td>
</tr>
</tbody>
</table>

The analyzable prespikes foot parameters were as follows: foot amplitude, maximum oxidation current; foot frequency, percentage of spikes that presented a foot preceding the spike; foot Q, foot area or foot net charge. Data are presented as means ± SEM. All values obtained were compared using a Mann–Whitney U test (p value).
induced by Ag stimulation but faster and with a lower plateau phase.

**Effects of 8-bromo-cAMP and forskolin on Ca^{2+}, levels in MC**

CRH binding to CRH-R typically activates adenylate cyclase, leading to increased intracellular concentrations of cAMP (46). Hence, we investigated whether the Ca^{2+} increase is mediated by CRH-induced cAMP. We tested whether the cell-permeable cAMP analog 8-bromo-cAMP and the adenylate cyclase activator forskolin could increase Ca^{2+} in PMC (Fig. 5). PMC were stimulated with 10 nM CRH (Fig. 5A), 1 mM 8-bromo-cAMP (Fig. 5B), and 10 μM forskolin (Fig. 5C). We found that both 8-bromo-cAMP and forskolin mimicked Ca^{2+} signals induced by CRH. The addition of the drugs produced a remarkably similar peak increase in Ca^{2+} as was obtained with CRH stimulation (8-bromo-cAMP, 98 ± 5%, n = 33; forskolin, 89 ± 14%, n = 12) (Fig. 5E). We then stimulated PMC with CRH in the presence of astressin, a nonspecific CRH-R antagonist. The effect of CRH on Ca^{2+} increase was completely abolished with astressin at the concentration of 1 μM (Fig. 5D, 5E). These observations suggest that CRH induces a rise in Ca^{2+} through CRH-R–dependent activation of adenylate cyclase and increased cAMP. These results were also confirmed in rat PMC.

CAMP alone is sufficient to elicit exocytosis in PMC

We next determine whether cAMP elevation is sufficient to elicit exocytosis in PMC. We labeled PMC with FM1-43 and stimulated them with 8-bromo-cAMP (Fig. 6). We compared the cAMP-induced exocytosis with the cellular response to CRH. We found that ΔF under 8-bromo-cAMP stimulation is similar to that obtained with CRH (Fig. 6) (CRH, 1098 ± 144 a.u., n = 8; 8-bromo-cAMP, 1075 ± 108 a.u., n = 21). To further confirm that compound fusion is the preferential mode of cAMP-induced exocytosis, we also examined the fluorescent area of single spots. Our analysis revealed that the average spot area is not significantly different between CRH-treated cells (CRH, 3.29 ± 0.40 μm^2) and 8-bromo-cAMP-treated cells (8-bromo-cAMP, 3.07 ± 0.71 μm^2; p = 0.374). We next compared the effects of astressin on exocytosis by direct observation of FM1-43 fluorescence in CRH-stimulated cells. We found that exocytosis is completely abolished when CRH-R are blocked, suggesting that this hormone triggers exocytosis through CRH-R (Fig. 6) (CRH plus astressin, 53 ± 22 a.u., n = 6).

**FM1-43 imaging suggests that CRH evokes PMD in IMC**

Because it has been shown that CRH leads to selective secretion of vascular endothelial growth factor by PMD (24), we wanted to determine whether the type of degranulation induced by CRH (piecemeal or complete exocytosis) might simply depend on phenotypical differences between connective tissue MC from the peritoneal cavity and mucosal MC from the intestinal lamina propria. We therefore cultured IMC and imaged exocytosis in the presence of 4 μM FM1-43 (Fig. 7). The scheme in Fig. 7A represents different images acquired during a representative experiment with CRH-stimulated IMC (no. 1 basal state, nos. 2 and 3 exocytotic, and no. 4 endocytotic fluorescence). The time course of FM1-43 fluorescence is represented in Fig. 7B. The increase in fluorescence was gradual, and after 10 min of recording, ΔF reached an average value of 674 ± 116 a.u. This ΔF is lower than that obtained in PMC (1073 ± 131 a.u., p = 0.030). After washing off the extracellular dye, the internalized fluorescence can be observed as tiny spots whose area is 0.27 ± 0.09 μm^2 (Fig. 7C).

This spot area is smaller than that obtained in Ag-stimulated PMC (Fig. 3D, 1.55 ± 0.24 μm^2, p = 0.0001 by Student t test) and IMC.
stimulated with 100 μg/ml C48/80 (Fig. 7C, 1.19 ± 0.23 μm²). A plausible explanation for these observations, based on background literature, could be that IMC undergo PMD, whereas PMC complete exocytosis in response to CRH.

Discussion

It has been suggested that CRH-induced activation of MC causes differential release by PMD (24, 47). This mechanism has been proposed as a general secretory mechanism for the slow release of
bioactive materials of granulated secretory cells (14) and produces ultrastructural changes characterized by empty or partially empty SG chambers that do not fuse with the plasma membrane (14, 48, 49). The aim of the present research was to understand how CRH stimulates MC and evokes transmitter release using electrochemical and fluorescent techniques. Surprisingly, CRH treatment leads to a large and explosive 5-HT release and intense degranulation in PMC that does not agree with PMD. Our results indicate that CRH induces secretion of the SG contents by extrusion via exocytosis. The number of exocytotic events and the total amount of 5-HT are larger than the Ag-stimulation response. The increase in 5-HT is caused by a gain in the granular content. Table I shows that $Q$ increases by 50%. The effect of CRH on SG charge occurs within 2 to 5 min. A comparison of the data from Fig. 1 indicates that the increase in the total amount of 5-HT secretion comes mainly from a net enlargement in $Q$, not from the frequency of firing. The rapid changes observed in granule content are unlikely to be caused by an increase in 5-HT synthesis. However, the augmentation of the granular content could be a result of compound fusion (i.e., two or more SG that fuse before exocytosis) (50). Previous analysis of amperometric spikes has indicated that different modes of exocytosis could be reflected in several characteristics, such as the spike half-width or spike rise (39, 51, 52). Moreover, a previous study suggested that amperometric feet correspond to SG with higher content (35), similar to what we observed in our study (Table II). In either case, this method cannot conclusively address the changes observed in spike shape to compound fusion. However, live-cell imaging offers the possibility of identifying different modes of exocytosis and endocytosis (40, 53). Thus, during CRH stimulation in the presence of the fluorescent dye FM1-43 we observed large steps reflecting multiple SG that exocytose through a single fusion event (41) (Fig. 2). SG that undergo compound exocytosis could appear as giant spherical shape events, explaining the large increase in fluorescence observed.

When FM1-43 is washed from the bath, some fluorescent signals remain associated to the same location as the previous spots, indicating the retrieval of FM1-43–stained SG dense cores (Fig. 3). The estimation of the size of the internalized membrane (area of endocytotic organelles) is larger in CRH-treated cells than in Ag-treated cells. Endocytosis occurs rapidly after CRH-induced stimulation, as evidenced by the ability to retain FM dye into SG cores just 5 min after exocytosis. An endocytotic mechanism that can explain the rapid and extensive membrane retrieval in MC is compound endocytosis (12). Such a mechanism is a rapid process because fused SG are not obliged to flatten but are retrieved nearly intact. This mechanism also results in a massive internalization of the membrane because it involves the recapture of the compound cavity formed by the fused SG. Therefore, compound exocytosis should be followed by compound endocytosis in PMC under CRH stimulation. As a result, large endocytic cavities with reduced contents should appear when MC are stimulated by CRH. It is curious that PMD can be imaged by electron microscopy as a uniform decrement of granule electron density. Additionally, during the process of PMD, SG sometimes maintain their original size but more often become enlarged (48, 54). Because PMD identification is dependent on ultrastructural criteria, the possibility cannot be ruled out that the rapid exo-

**FIGURE 5.** Calcium signals evoked by 8-bromo-cAMP and forskolin. (A–D) Representative traces, normalized at baseline, of the $\text{Ca}^{2+}$ signals evoked by 10 nM CRH (A), 1 mM 8-bromo-cAMP (8-Br-cAMP) (B), 10 µM forskolin (Forsk) (C), and 1 µM astressin (Ast)-treated cells stimulated with 10 nM CRH (D). (E) ΔF ratio measured in cells treated with 8-Br-cAMP, Forsk, and CRH plus Ast in relationship to CRH response. Significant differences were only found between CRH and CRH plus Ast. Data in (E) are represented as the means ± SEM. All data shown are representative of two independent cultures (CRH, n = 28 cells; 8-Br-cAMP, n = 33 cells; Forsk, n = 12; CRH plus Ast, n = 39). ***$p < 0.001$, Mann–Whitney $U$ test.
endocytosis mode we have identified after CRH exposure is responsible for the previously reported PMD phenotype. In contrast, we observed that CRH induces a gradual increase in fluorescence in IMC (Fig. 7) by the appearance of many tiny spots, suggesting the fusion of small vesicles. Two scenarios could, in principle, be envisioned for these data. First, CRH leads to differential release by PMD, as previously described. Second, CRH induces complete exocytosis, as we observed in PMC. Given that the size of the fluorescent events are more discrete than those obtained during complete fusion, a likely explanation is that IMC perform PMD in response to CRH, which would support the scenario of PMD. Hence, the measurement of membrane fluorescence using FM dyes can be used as an in vivo technique that allows for the study of PMD. Indeed, our experimental data show that after this exocytotic mechanism is induced, a fast and efficient membrane retrieval event occurs.

We examined the Ca\(^{2+}\) signals triggered by CRH in PMC (Fig. 4). Within seconds, CRH evokes a rapid increase in Ca\(^{2+}\), followed by a decrease to a long-lasting plateau. Compared to CRH, the addition of DNP induced a rapid peak increase in Ca\(^{2+}\) at \(\sim 25\) s with a similar maximum value of Ca\(^{2+}\) but an elevated plateau phase. Urocortin, a peptide that is structurally related to CRH, has previously been reported to induce a similar Ca\(^{2+}\) signaling pattern in rat lung MC (44). The rapid peak increase in Ca\(^{2+}\) concentration observed after CRH administration does not

**FIGURE 7.** The exocytotic response induced by CRH in IMC measured by FM1-43 fluorescence. (A) Raw fluorescent images of an IMC bathed in 4\(\mu\)M FM1-43 (upper panels, no. 1), during stimulation with 10 nM CRH (nos. 2 and 3), and after washing (no. 4). Scale bar, 5 \(\mu\)m. (B) Trace of FM1-43 fluorescence of the cell shown in the upper panel images. Black arrow in trace indicates the beginning of drug application. (C) Analysis of the fluorescent spot area observed after CRH (filled bar) and 100 \(\mu\)g/ml C48/80 applications (open bar). Significant differences were found for \(\Delta F\) (C) evoked by CRH (filled bar) and C48/80 (open bar). Data in (C) are represented as the means \(\pm\) SEM. All data shown are representative of three independent cultures (CRH, \(n = 15\) cells; C48/80, \(n = 14\) cells). **\(p < 0.005\), Student \(t\) tests.

**FIGURE 6.** cAMP increases FM1-43 fluorescence in MC. (A) Raw fluorescent images of a MC bathed in 4\(\mu\)M FM1-43 and stimulated with 10 nM CRH (upper panel), 1 mM 8-bromo-cAMP (8-Br-cAMP, middle panel), or 10 nM CRH plus astressin (1 \(\mu\)M) (CRH + Ast) (lower panel). Left panels (no. 1) show an initial stage of recording, and right panels (no. 2) show a late stage of recording. Scale bar, 5 \(\mu\)m. (B) Average membrane fluorescence shows that there is no significant difference between the CRH and 8-bromo-cAMP response. Astressin completely inhibited the degranulation induced by CRH. PMC were incubated for 10 min with 1 \(\mu\)M astressin and then stimulated with 10 nM CRH. Data in (B) are represented as the means \(\pm\) SEM. All data shown are representative of two independent cultures (CRH, \(n = 8\) cells; 8-Br-cAMP, \(n = 21\) cells; CRH + Ast, \(n = 6\) cells). ***\(p < 0.001\), Mann–Whitney \(U\) test.
correlate with MC degranulation. However, this early Ca\(^{2+}\) signal may account for the fusion between unit SG to generate larger SG with high amounts of material able to fuse with the plasma membrane and release their contents to the external medium. This mechanism would provide reliable communication machinery for adaptation to environmental insults. Increases in Ca\(^{2+}\) and its sensor for vesicle fusion, synaptotagmin, are required for compound fusion (55) and other proteins such as Rab5 and VAMP8, are known to play a role in homotypic secretory granule fusion in MC (56).

In MC from peripheral tissues, the physiological actions of CRH involve coupling of both CRH-R1 and CRH-R2 to G\(_\text{q}\) proteins that stimulate cAMP-mediated signaling cascades (46). In chromaffin cells (50) and lactotrophs (57), CAMP modulates exocytotic kinetics and increases the size of exocytotic events and quantal size. We found that CRH-induced compound exocytosis is dependent on CAMP because 8-bromo-cAMP and forskolin mimic Ca\(^{2+}\) transitory kinetics and increases the size of exocytotic events and involves coupling of both CRH-R1 and CRH-R2 to G\(_\text{q}\) (56).

Mast cells are known to play a role in homotypic secretory granule fusion in endocytosis (10). Therefore, it is also possible that the Ca\(^{2+}\) increase. Ca\(^{2+}\) is dependent on cAMP because 8-bromo-cAMP and forskolin mimic Ca\(^{2+}\) release in intact mast cells. J. Gen. Physiol. 95: 397–409.


