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Ca\textsuperscript{2+} Waves Initiate Antigen-Stimulated Ca\textsuperscript{2+} Responses in Mast Cells\textsuperscript{1}

Roy Cohen,* Alexis Torres,* Hong-Tao Ma,† David Holowka,\textsuperscript{2}* and Barbara Baird* 

Ca\textsuperscript{2+} mobilization is central to many cellular processes, including stimulated exocytosis and cytokine production in mast cells. Using single cell stimulation by IgE-specific Ag and high-speed imaging of conventional or genetically encoded Ca\textsuperscript{2+} sensors in rat basophilic leukemia and bone marrow-derived rat mast cells, we observe Ca\textsuperscript{2+} waves that originate most frequently from the tips of extended cell protrusions, as well as Ca\textsuperscript{2+} oscillations throughout the cell that usually follow the initiating Ca\textsuperscript{2+} wave. In contrast, Ag conjugated to the tip of a micropipette stimulates local, repetitive Ca\textsuperscript{2+} puffs at the region of cell contact. Initiating Ca\textsuperscript{2+} waves are observed in most rat basophilic leukemia cells stimulated with soluble Ag and are sensitive to inhibitors of Ca\textsuperscript{2+} release from endoplasmic reticulum stores and to extracellular Ca\textsuperscript{2+}, but they do not depend on store-operated Ca\textsuperscript{2+} entry. Knockdown of transient receptor potential channel (TRPC)1 and TRPC3 channel proteins by short hairpin RNA reduces the sensitivity of these cells to Ag and shifts the wave initiation site from protrusions to the cell body. Our results reveal spatially encoded Ca\textsuperscript{2+} signaling in response to immunoreceptor activation that utilizes TRPC channels to specify the initiation site of the Ca\textsuperscript{2+} response. 


Ca\textsuperscript{2+} waves were first characterized in Xenopus oocyte fertilization (4), and they have since been identified in excitable (5) and nonexcitable cell types, including hepatocytes (6), HeLa cells (7), and neutrophils (8). In myocytes, Ca\textsuperscript{2+} nonexcitable cell types, including hepatocytes (6), HeLa cells (7), and neutrophils (8). In myocytes, Ca\textsuperscript{2+} waves were shown to initiate from elementary Ca\textsuperscript{2+} events called “Ca\textsuperscript{2+} sparks” (9), and they are thought to propagate through the cytosol by calcium-induced calcium release from endoplasmic reticulum stores (10). Ca\textsuperscript{2+} waves are frequently initiated by activation of plasma membrane receptors that stimulate Ca\textsuperscript{2+}-dependent signaling within the cell (11). Similar mechanisms may be involved in stimulating Ca\textsuperscript{2+} puffs and maintaining the propagation of Ca\textsuperscript{2+} waves in nonexcitable cells (3, 12). However, Ca\textsuperscript{2+} waves in response to immunoreceptor signaling have not been previously reported.

Ca\textsuperscript{2+} oscillations have been characterized in many cell types, including RBL-2H3 mast cells (13, 14), where they have been temporally correlated with degranulation events (15, 16). Ca\textsuperscript{2+} oscillations are sustained by store-operated Ca\textsuperscript{2+} entry (SOCE),\textsuperscript{3} other ion channels, as well as membrane potential (17). Mast cells play key roles in the inflammatory process in both innate and adaptive immune responses (18). In the latter, binding of multivalent Ag to receptor-associated IgE aggregates this receptor, FceRI, which causes mast cell activation, resulting in Ca\textsuperscript{2+} mobilization and consequent exocytotic release of mediators of allergy and inflammation (19). RBL-2H3 cells are immortalized mucosal mast cells that have been utilized for extensive biochemical and cell biological investigations of mast cell function (20–22).

In the present study, we used high-speed confocal imaging to investigate cytoplasmic Ca\textsuperscript{2+} dynamics activated via FceRI in rat basophilic leukemia (RBL) cells and in rat bone marrow-derived mast cells (BMMCs), which are also mucosal in character (23). We find that Ca\textsuperscript{2+} responses to soluble Ag initiate in the form of a wave that begins most frequently at the tip of an extended cell protrusion and propagates throughout the entire cell in several seconds. In contrast, localized delivery of Ag attached to the tip of a micropipette results in repetitive, localized Ca\textsuperscript{2+} puffs that infrequently develop into propagated waves. Our results provide evidence that Ca\textsuperscript{2+} wave initiation from extended protrusions depends on Ca\textsuperscript{2+} influx via transient receptor potential channels (TRPCs), leading to the onset of SOCE-dependent Ca\textsuperscript{2+} oscillations and mast cell activation.

Materials and Methods

cDNA plasmids

The GCaMP2 construct (24) was provided by Dr. M. Kotlikoff, Cornell University College of Veterinary Medicine. Small hairpin RNA (shRNA) plasmids targeting TRPCs (TRPC1, TRPC3, TRPC5, TRPC7, and GFP control) were characterized in RBL cells as previously described (25).

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\textsuperscript{4} Abbreviations used in this paper: SOCE, store-operated Ca\textsuperscript{2+} entry; BMMC, bone marrow-derived mast cell; BSS, buffered salt solution; CRAC, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+}; DNP, 2,4-dinitrophenyl; PLC, phospholipase C; RBL, rat basophilic leukemia; ROL, region of interest; shRNA, small hairpin RNA; 2-ABP, 2-aminoethyldiphenylborate; TG, thapsigargin; TRPC, transient receptor potential channel.
Chemicals and reagents
Fluo-4 AM and Fluo-5F AM were purchased from Invitrogen/Molecular Probes. U73122, n-sphingosine, thapsigargin, A23187, 2-aminoethylidiphenyl borate (2-APB), and GdCl₃ were from Sigma-Aldrich. N,N,N-dimethylsphingosine (DM-sphingosine) was from Avanti Polar Lipids.

Cells
RBL-2H3 cells (26) were maintained in monolayer culture in MEM supplemented with 20% FBS (Atlanta Biologicals) and 10 μg/ml gentamicin sulfate. All tissue culture reagents were obtained from Invitrogen unless otherwise noted. Rat BMMCs were differentiated from femur-derived stem cells of Lewis strain rats by culturing for 14–28 days in the presence of rat stem cell factor (50 ng/ml) and rat IL-3 (100 ng/ml) as previously described (27). Cells were harvested 3–5 days after passage and plated overnight in MatTek coverslip dishes in complete medium for experiments with Fluo-5F AM loading. For experiments with GCaMP2 as the Ca²⁺ indicator, 5 × 10⁶ RBL-2H3 cells were electroporated in 0.5 ml of cold electroporation buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/ml glucose, 20 mM HEPES (pH 7.4)) with 5–8 μg of plasmid DNA at 280 V and 950 μF using Gene Pulser X (Bio-Rad), then plated onto MatTek dishes (~500,000 cells/plate). Cells were sensitized with 0.5 μg/ml anti-2,4-dinitrophenyl (DNP) IgE (28) during overnight cell culture incubation. Under these conditions, the cell transfection efficiency with GCaMP2 is typically 20–30%.

For experiments with TRPC shRNA constructs, plasmids containing these sequences (Origene Technologies) were co-electroporated with that for GCaMP2 using 20 μg of TRPC shRNA plasmid and 8 μg of GCaMP2 plasmid for 5 × 10⁶ cells. No effect of these plasmids on cell viability was detected.

For characterization of rat BMMCs, differentiated cells were labeled with 2 μg/ml Alexa 488-IgE (29) for 2 h at 37°C, then washed and fixed in 4% (w/v) p-formaldehyde and 0.1% (w/v) glutaraldehyde for 20 min at room temperature. Fixed cells were further labeled in 10 mg/ml BSA/PBS/0.01% Na₃ with 3 μg/ml monoclonal anti-α-galactosyl GD1b, ganglioside AA₄₄ (30), followed by 10 μg/ml Alexa 555-goat anti-mlgG (Invitrogen). Washed, labeled cells were imaged as previously described (31).

Live cell calcium imaging
Twenty-four hours after transfection, cells were washed with buffered salt solution (BSS; 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml glucose, 20 mM HEPES (pH 7.2–7.4), 1 mg/ml BSA) and imaged using a heated (37°C) ×63 (NA of 1.4) oil immersion objective on a Zeiss Meta 510 confocal microscope. GCaMP2, Fluor-4, or Fluor-5F was excited using the green-line of a krypton/argon laser and viewed with a 505–530 band-pass filter. In experiments using Fluor-4 AM or Fluor-5F AM, cells were labeled by incubating with 0.5 μM (RBL cells) or 2.5 μM (rat BMMCs) of the indicator in BSS in the presence of 2.5 mM sulfinpyrazone for 30 min in 37°C (5 min for rat BMMCs), then washed and imaged in BSS.

Cells expressing GCaMP2 or loaded with Fluor dyes were approached with an ~5-μm-diameter pulled glass capillary, typically positioned within 100 μm from the cell and prefilled with stimulating solution. Cells were imaged at 10–30 Hz while applying a puff of 1.7 μg/ml Ag (DNP-BSA). Pressure and duration of stimulation was controlled by a Picospritzer III (FMI Medical Instruments). Pharmacological reagents were added to the dishes just before initiating data collection (see Results). For some experiments, DNP-BSA (Ag) or unmodified BSA was covalently coupled to the micropipette surface using chemical treatments and procedure previously described for covalent attachment of proteins to patterned surfaces (32).

Data analysis
Images of individual cells are representative from multiple experiments. Offline image analysis was conducted using Zeiss LSM image analysis software and ImageJ (National Institutes of Health). Changes in fluorescence (F) were normalized by the initial fluorescence (F₀) and are expressed as ΔF/F₀. Unless stated otherwise, cells that displayed any type of Ca²⁺ elevation including Ca²⁺ puffs upon stimulation were counted as responding cells. Wave occurrence was calculated as the percentage of cells with a detectable Ca²⁺ wave, compared with the total number of responding cells in a given experimental condition. The lag time before Ca²⁺ wave initiation was measured from the initiation of the pulse of Ag or other stimulus. Statistical analysis of Ca²⁺ oscillations was conducted by counting and averaging the peaks during a 10 min segment starting from 2 min of stimulation including the initiating wave. Wave propagation velocity was measured as the time (number of frames × time per frame) for spreading of the wavefront across a cell of measured dimension. Unless otherwise stated, all data are presented as mean ± SEM. For each of the experiments, the number of cells analyzed (n) is presented in the text. Data were processed and plotted using Origin (OriginLab) and Excel (Microsoft). Statistical comparisons between experiments were performed using Student’s t test.

Results
Ca²⁺ mobilization events in individual RBL mast cells
To observe the earliest steps in the responses of RBL mast cells to stimulation of IgE receptors by multivalent Ag, we delivered a short (10 s) pulse of Ag via a micropipette placed within 100 μm of the cell while imaging changes in cytosolic Ca²⁺ (Fig. 1A). For most experiments, we used the genetically encoded Ca²⁺ indicator, GCaMP2, which has a moderate Kd (~350 nM) with rapidly reversible kinetics (33). GCaMP2 is brighter than wild-type GFP,
maintains a 5-fold dynamic range, displays very low pH sensitivity, and is fully functional at 37°C (24, 33).

Using high imaging rates of 10–30 frames per second, we observe two major types of spatially and temporally resolved Ca\(^{2+}\) mobilization dynamics in individual cells as illustrated in Fig. 1B and supplemental Movie 1.\(^4\) Stimulation of cells with a saturating concentration of Ag (1.7 \(\mu g/ml\) in the pipette) resulted in \(\geq 90\%\) of the cells responding with a Ca\(^{2+}\) wave that initiated a global Ca\(^{2+}\) response. This initiating Ca\(^{2+}\) wave followed a lag period of \(\approx 20\ s\), on average, after starting the Ag pulse (Table I). As illustrated in Fig. 1B and as discussed below, the Ca\(^{2+}\) wave usually initiates from the tip of an extended cell protrusion and propagates down the length of the cell body. The site of wave initiation does not detectably correlate with the location of the micropipette relative to any particular part of the cell. Ca\(^{2+}\) waves also initiated most frequently from the tips of extended protrusions in response to bath addition of Ag, but the lag time for wave initiation was more variable under these conditions (data not shown).

In most cells the Ca\(^{2+}\) wave is followed by a second manifestation of calcium dynamics, that is, regenerative Ca\(^{2+}\) oscillations that occur throughout the cell body as previously described for these cells (13, 14, 16). Although not seen in the cell shown in Fig. 1B, Ca\(^{2+}\) oscillations often extend throughout the length of the cell, including protrusions. Spatial and temporal resolution of these two processes are shown in Fig. 1C as time traces for the tip of the protrusion (red trace and red region of interest (ROI) in inset) and the cell body (black trace, yellow ROI in inset) for the cell in Fig. 1B. In some cells, we observed localized Ca\(^{2+}\) puffs before the initiation of waves; however, these were infrequent under conditions of optimal stimulation (see below).

We used Fluo-4 and Fluo-5F to confirm our main results for these Ca\(^{2+}\) dynamics. Fluo-4 and GCaMP2 have similar affinities for Ca\(^{2+}\), but the fluorescence response kinetics are approximately an order of magnitude slower for the GFP-based sensor. As shown in supplemental Fig. S1, despite this slower response time for GCaMP2, Ca\(^{2+}\) wave velocities measured using GCaMP2 were significantly higher than those measured with Fluo-4, indicating that GCaMP2 kinetics are not a limiting factor in our measurements. The percentage of cells exhibiting Ca\(^{2+}\) waves detected by Fluo-4 is smaller, and the observed waves initiate after a longer lag time and exhibit slower propagation velocities. Interestingly, fewer Ca\(^{2+}\) oscillations were detected with both Fluo-4 and Fluo-5F compared with GCaMP2, possibly due to Ca\(^{2+}\) buffering effects of these organic dyes, which are difficult to load into cells at controlled concentrations. Overall, we found GCaMP2 to be most useful for our experiments.

Although there are cell-to-cell differences in appearance and characteristics of Ag-stimulated Ca\(^{2+}\) waves and subsequent oscillations in the RBL cells, we found that several characteristics are highly consistent for a particular concentration of Ag. These include the lag time to initiation of the first Ca\(^{2+}\) peak, the wave velocity, and the frequency (number observed in 2 min) of the oscillations (Table I).

**Cellular protrusions are the primary initiation sites for Ca\(^{2+}\) waves in RBL cells**

As summarized in Fig. 2B, \(\approx 90\%\) of the cells responded to Ag (1.7 \(\mu g/ml\)) with a propagating Ca\(^{2+}\) wave, whereas for the remaining \(\approx 5\%\) of the responding cells we detected a nondirectional, global elevation in cytoplasmic Ca\(^{2+}\). Although stimulated influx of extracellular Ca\(^{2+}\) is required for cellular degranulation, we observed wave initiation with similar frequency in the presence and absence of extracellular Ca\(^{2+}\), suggesting that Ca\(^{2+}\) influx is not necessary for Ca\(^{2+}\) wave generation and propagation. However, a clear difference in the spatial localization of wave initiation is observed, depending on the presence or absence of extracellular Ca\(^{2+}\). As illustrated in Fig. 2A and supplemental Movie 2, and summarized in Fig. 2B, wave initiation in the absence of extracellular Ca\(^{2+}\) occurs most frequently at a site in the cell body, with

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\(^4\) The online version of this article contains supplemental material.

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Table I. Summary of Ca\(^{2+}\) responses parameters with GCaMP2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Response (% of cells)</th>
<th>Ca(^{2+}) Waves (% of cells)</th>
<th>Waves from Protrusions (% of waves)</th>
<th>Oscillationsb</th>
<th>Wave Velocity (m/s)</th>
<th>Lag Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>1700 ng/ml</td>
<td>96.3</td>
<td>90</td>
<td>65</td>
<td>4.5 ± 0.3</td>
<td>42.6 ± 4</td>
</tr>
<tr>
<td></td>
<td>170 ng/ml</td>
<td>94</td>
<td>87</td>
<td>54</td>
<td>4.9 ± 0.4</td>
<td>37.3 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>17 ng/ml</td>
<td>90.5</td>
<td>81</td>
<td>62</td>
<td>5 ± 0.4</td>
<td>18.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>1.7 ng/ml</td>
<td>53.8</td>
<td>78</td>
<td>86</td>
<td>3.7 ± 0.9</td>
<td>15.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>0 [Ca(^{2+})]</td>
<td>98.6</td>
<td>89</td>
<td>31</td>
<td>1.5 ± 0.1</td>
<td>25.8 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>50 mM KCl</td>
<td>91</td>
<td>73</td>
<td>27.2</td>
<td>5.7 ± 0.5</td>
<td>27.8 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>2 mM Ba(^{2+})</td>
<td>64</td>
<td>78</td>
<td>72</td>
<td>1.6 ± 0.3</td>
<td>46.9 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>2 mM Sr(^{2+})</td>
<td>90</td>
<td>89</td>
<td>75</td>
<td>1.7 ± 0.2</td>
<td>44.4 ± 3.6</td>
</tr>
<tr>
<td>TG</td>
<td>76.2</td>
<td>43.7</td>
<td>71</td>
<td>30.7</td>
<td>1.7 ± 0.2</td>
<td>30.3 ± 16.4</td>
</tr>
<tr>
<td>A23187</td>
<td>73.9</td>
<td>44.8</td>
<td>30.7</td>
<td>2.4 ± 0.2</td>
<td>19.9 ± 5.3</td>
<td>17.9 ± 2.7</td>
</tr>
</tbody>
</table>

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\(^a\) Unless otherwise stated Ag was used at 1700 ng/ml; number of cells in each experiment was 16–80 as specified in text or figure legends.

\(^b\) During first 2 min of response. Data are means ± SEM.
Extended protrusions are common morphological features of RBL mast cells that are readily observed at low cell densities. They develop several hours after cell attachment and define an axis of cell migration (J. Lee and D. Holowka, unpublished results). Similar protrusions are frequently observed with mucosal mast cells imaged in vivo (for examples, see Refs. 34–37).

We found that substitution of extracellular Ca\(^{2+}\) with equimolar concentrations of Ba\(^{2+}\) or Sr\(^{2+}\) before stimulation did not markedly alter either the percentage of cells with Ag-stimulated waves or initiation from elongated protrusions (Table I). The velocity of wave propagation was reduced by ~40% in the absence of extracellular Ca\(^{2+}\), whereas substitution of Ca\(^{2+}\) by Ba\(^{2+}\), which is not taken up into stores by sarco-endoplasmic Ca\(^{2+}\)-ATPase (SERCA) pumps (38), does not alter wave velocity (Table I). These results are consistent with wave initiation from protrusions by a Ca\(^{2+}\)-influx component and propagation by Ca\(^{2+}\)-dependent Ca\(^{2+}\) release from intracellular stores (10, 39). Interestingly, we find that the lag time for wave appearance following Ag stimulation was longer in the absence of extracellular Ca\(^{2+}\) (Table I), but was not changed when Ca\(^{2+}\) was substituted with Ba\(^{2+}\) or Sr\(^{2+}\). This delay could be attributed to a reduced "ignition" capacity of the calcium response in the absence of a Ca\(^{2+}\) influx and is further discussed below.

In addition to the observed effects on Ca\(^{2+}\) waves, the absence of extracellular Ca\(^{2+}\) substantially decreases the frequency of subsequent Ca\(^{2+}\) oscillations (Fig. 2C), as expected from the dependence of these oscillations on sustained, store-operated Ca\(^{2+}\) influx (17). Substitution of Ba\(^{2+}\) or Sr\(^{2+}\) for extracellular Ca\(^{2+}\) markedly reduces the frequency of oscillations (Table I), consistent with the high selectivity of Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels to Ca\(^{2+}\) over Ba\(^{2+}\) or Sr\(^{2+}\)(40, 41).

We compared the dynamic features of Ag-stimulated Ca\(^{2+}\) responses in RBL cells to those observed upon stimulation with either the SERCA inhibitor thapsigargin (TG) or the Ca\(^{2+}\)-ionophore A23187. As illustrated by individual time traces in Fig. 2D and summarized in Fig. 2F, the oscillatory dynamics of Ag responses are largely absent from responses to TG and A23187. For TG at 1 \(\mu M\) in the pipette, a transient increase in cytoplasmic Ca\(^{2+}\) was typically observed, followed by a more sustained increase that depends on extracellular Ca\(^{2+}\). As summarized in Fig. 2E, Ca\(^{2+}\) waves are less frequently observed with TG stimulation, but, when observed, waves usually initiate from extended protrusions. For stimulation by A23187 at 20 \(\mu M\) in the pipette, Ca\(^{2+}\) responses initiate as a wave in only ~40% of the cells, and this occurs very infrequently from extended protrusions (Fig. 2E). Furthermore, when the response to A23187 does initiate as a Ca\(^{2+}\) wave, the wave velocity is substantially slower than observed in cells stimulated by high concentration of Ag or TG (see Table I). Similar to TG, A23187-mediated elevation in cytoplasmic Ca\(^{2+}\) does not cause oscillatory behavior in most of the stimulated cells (Fig. 2, D and F).

In summary, elevations in cytoplasmic Ca\(^{2+}\) caused by either ionophore or TG-induced release from stores can also initiate Ca\(^{2+}\) waves from discrete sites along the plasma membrane, but with lower probability. These results further indicate that cytoplasmic Ca\(^{2+}\) oscillations such as those initiated by Ag can be uncoupled from Ca\(^{2+}\) wave initiation caused by these nonreceptor means of cell activation.

**Characterization of the Ca\(^{2+}\) response in rat BMMCs**

To evaluate whether the characteristics of Ca\(^{2+}\) responses that we observe for RBL-2H3 cells are general for mast cells, BMMCs were differentiated from Lewis rats as previously described (27). When plated overnight on glass slides in MatTek dishes, these
cells attach firmly and frequently exhibit extended protrusions, similar in morphology to RBL cells. We confirmed their identity by labeling with Alexa 488-IgE and AA4 mAb that labels a mast cell-specific ganglioside (42, 43) (supplemental Fig. S4), as well as by staining granules with Alcian blue (44) (data not shown). BMMCs sensitized with IgE and labeled with Fluo-5F were stimulated at t = 0 with a puff of 1.7 μg/ml DNP-BSA (right panel). Changes in Ca\(^{2+}\) concentration in response to stimulation are depicted, where brighter colors represent higher Ca\(^{2+}\) concentration. Time line analysis highlights the Ca\(^{2+}\) elevation initiating in the tip of the cellular protrusion and propagating through the cell body. B. Traces of Fluo-5F intensity changes over time as calculated for the same cell imaged in A. Black and red traces in right panel represent changes in Ca\(^{2+}\) concentration in cell body (yellow ROI) and in cell protrusion (red ROI), respectively, in the left panel.

**FIGURE 3.** Ca\(^{2+}\) dynamics in individual rat BMMCs. A. Confocal image of a representative rat BMMC sensitized with anti-DNP IgE and loaded with Fluo-5F (left panel), and time line analysis of indicated ROI for this cell stimulated at t = 0 with a puff of 1.7 μg/ml DNP-BSA (right panel). Changes in Ca\(^{2+}\) concentration in response to stimulation are depicted, where brighter colors represent higher Ca\(^{2+}\) concentration. Time line analysis highlights the Ca\(^{2+}\) elevation initiating in the tip of the cellular protrusion and propagating through the cell body. B. Traces of Fluo-5F intensity changes over time as calculated for the same cell imaged in A. Black and red traces in right panel represent changes in Ca\(^{2+}\) concentration in cell body (yellow ROI) and in cell protrusion (red ROI), respectively, in the left panel.

**Ag dose dependence and spatial localization**

To assess further the nature and function of the Ca\(^{2+}\) waves, we characterized the dose dependence of Ag-stimulated Ca\(^{2+}\) responses in RBL cells detected with GCaMP2 (supplemental Fig. S2). More than 90% of the cells respond to Ag doses in the range of 17–1700 ng/ml in the pipette, and >50% of the cells respond at the lowest dose tested, 1.7 ng/ml (supplemental Fig. S2A). Ca\(^{2+}\) waves were observed in a similarly high percentage of the responding cells (≥80%) at all doses tested, but Ca\(^{2+}\) waves initiate more frequently at extended protrusions at the lowest Ag dose (supplemental Fig. S2C). Wave velocity was found to be dependent on the concentration of Ag (supplemental Fig. S2B), and longer lag times between Ag addition and the first Ca\(^{2+}\) response were observed at lower Ag doses (supplemental Fig. S2D), but the frequency of Ca\(^{2+}\) oscillations changes little over the four orders of magnitude of Ag concentration (supplemental Fig. S2E).

Ca\(^{2+}\) puffs are localized and transient elevations in cytoplasmic Ca\(^{2+}\) that may serve as a priming mechanism for wave initiation and subsequent oscillations (39, 45, 46). Stimulated Ca\(^{2+}\) puffs are observed less often than Ca\(^{2+}\) waves at higher doses of Ag, but they appear more often at the lowest concentration of Ag tested (supplemental Fig. S2F). Consistent with these observations, Ca\(^{2+}\) puffs may represent subthreshold activation of the cells and production of insufficient amounts of IP\(_3\) to generate a propagated Ca\(^{2+}\) elevation and a global Ca\(^{2+}\) response.

Interestingly, when Ag is covalently coupled to the tip of a micropipette and delivered locally by cell contact, we usually observe a series of stimulated Ca\(^{2+}\) puffs that frequently occur near the point of contact. As illustrated in Fig. 4, two different responses are typical: in one case, repetitive puffs are observed at relatively regular time intervals, often over several minutes of contact, but they fail to propagate as waves or global cellular Ca\(^{2+}\) elevation for more than several micrometers (Fig. 4A). Alternatively, Ca\(^{2+}\) puffs initiated by local contact of the Ag-coated pipette occasionally propagate as waves that traverse the length of the cell (Fig. 4B). Under these conditions of stimulation, contact at a cell protrusion did not significantly increase the probability of wave propagation. Ca\(^{2+}\) waves or waves were observed in >85% of cells contacted by DNP-BSA-coated tips in both the presence and absence of extracellular Ca\(^{2+}\) (Fig. 4C) and only rarely in cells contacted by unconjugated micropipettes or those conjugated with unmodified BSA (Fig. 4C). Moreover, Ca\(^{2+}\) waves were just as frequent when cells were locally stimulated with DNP-BSA-conjugated micropipettes in the absence of extracellular Ca\(^{2+}\) (Fig. 4C). These results suggest that the localized responses are mediated by release of Ca\(^{2+}\) from internal stores, rather than by Ca\(^{2+}\) influx. Moreover, localized activation of FcεRI can stimulate spatially restricted Ca\(^{2+}\) responses, and, in this situation, the site of Ca\(^{2+}\) response initiation is determined by the location of receptor stimulation.

These results show that the localized Ca\(^{2+}\) responses can be initiated by localized receptor stimulation, and these responses are apparently independent of Ca\(^{2+}\) influx. Overall, these results show that Ca\(^{2+}\) responses to soluble Ag begin most frequently as Ca\(^{2+}\) waves that are propagated from initiation sites near the tips of extended protrusions, whereas those stimulated by locally applied micropipette-conjugated Ag are initiated at the site of stimulation and are more commonly manifested as repetitive Ca\(^{2+}\) puffs that less frequently evolve into Ca\(^{2+}\) waves.

**Pharmacologic characterization of Ca\(^{2+}\) dynamics**

In initial efforts to identify molecular components of Ag-stimulated waves and oscillations, we investigated the effects of several different inhibitors of IP\(_3\) production and modulators of Ca\(^{2+}\) influx. As summarized in Fig. 5 and in Table I, the phospholipase C (PLC) inhibitor U73122 reduces the percentage of cells showing Ca\(^{2+}\) responses with a sharp dose dependence that is half maximal between 2 and 2.5 μM. U73122 also reduces the percentage of cells contacted by unconjugated micropipettes or those conjugated with unmodified BSA (Fig. 4C). Moreover, Ca\(^{2+}\) waves were just as frequent when cells were locally stimulated with DNP-BSA-conjugated micropipettes in the absence of extracellular Ca\(^{2+}\) (Fig. 4C). These results suggest that the localized responses are mediated by release of Ca\(^{2+}\) from internal stores, rather than by Ca\(^{2+}\) influx. Moreover, localized activation of FcεRI can stimulate spatially restricted Ca\(^{2+}\) responses, and, in this situation, the site of Ca\(^{2+}\) response initiation is determined by the location of receptor stimulation.

These results show that the localized Ca\(^{2+}\) responses can be initiated by localized receptor stimulation, and these responses are apparently independent of Ca\(^{2+}\) influx. Overall, these results show that Ca\(^{2+}\) responses to soluble Ag begin most frequently as Ca\(^{2+}\) waves that are propagated from initiation sites near the tips of extended protrusions, whereas those stimulated by locally applied micropipette-conjugated Ag are initiated at the site of stimulation and are more commonly manifested as repetitive Ca\(^{2+}\) puffs that less frequently evolve into Ca\(^{2+}\) waves.
inhibited with a similar dose dependence, consistent with IP₃-dependent intracellular Ca²⁺ increase as a necessary precursor to SOCE (48).

2-APB was initially described as a noncompetitive antagonist of IP₃ receptor (IP₃R)-mediated Ca²⁺ release by micromolar preparations (49), and it was subsequently shown to inhibit SOCE in T cells at concentrations in the range of 10–50 μM (50). In RBL cells, 2-APB at concentrations up to 40 μM has no effect on Ag-stimulated Ca²⁺ release from stores in the absence of extracellular Ca²⁺, indicating that it fails to inhibit IP₃R-mediated Ca²⁺ release from stores in these cells (D. Holowka, unpublished results). As summarized in Fig. 5, addition of 20 μM 2-APB before Ag stimulation does not significantly reduce the percentage of responding cells (Fig. 5A) or the percentage of cells that exhibit wave initiation (Fig. 5B). In contrast, 20 μM 2-APB strongly inhibits Ca²⁺ oscillations subsequent to wave initiation (Fig. 5C), consistent with its inhibitory effect on SOCE that contributes to Ca²⁺ oscillations in these cells (51–53).

A previous study by Penner and colleagues (54) identified both d-sphingosine and N,N-dimethylsphingosine (DMS) as effective inhibitors of IP₃ and TG-elicited CRAC channel activation in RBL cells. As summarized in Fig. 5B and Table I, we found that 8 μM d-sphingosine or 8 μM DMS strongly inhibits Ca²⁺ wave initiation. Earlier studies suggested that DMS prevents Ca²⁺ responses by inhibition of sphingosine kinase (55, 56). However, this mechanism is unlikely for the inhibition we observe because d-sphingosine is a substrate of this enzyme family. In other studies, we have evidence that these basic amphiphiles inhibit Ca²⁺ responses by electrostatically neutralizing negatively charged phospholipids at the inner leaflet of the plasma membrane (Ref. 57 and N. Smith et al., manuscript in preparation). The small percentage of cells that do respond in the presence of d-sphingosine appear to have oscillation frequencies similar to untreated cells (Fig. 5C), suggesting that this aspect of SOCE is less potently inhibited than wave initiation. These results identify these sphingosine derivatives as effective inhibitors of the earliest manifestations of Ca²⁺ responses to Ag in these cells.

We further tested the effects of inhibitors of dihydrophydine-sensitive Ca²⁺ channels on Ag-stimulated Ca²⁺ wave initiation in RBL cells (58). As summarized in Fig. 5, we observed a modest (~30%) reduction of wave initiation from extended protrusions by 10 μM nifedipine, but no effects of other L channel inhibitors such as Bay K⁺ (+) were detected (data not shown), suggesting that the effect of nifedipine observed may be due to nonspecific inhibition of K⁺ channels resulting in cell depolarization (59, 60). To investigate further a possible contribution of CRAC conductance to wave generation, we examined the effects of Gd³⁺ on Ca²⁺ responses to Ag. Pre-addition of 10 μM Gd³⁺ strongly inhibits Ca²⁺ oscillations in response to Ag (Fig. 5C), consistent with inhibition of SOCE (61), but it causes only a small reduction in the percentage of waves initiating from extended protrusions (Fig. 5B). These results are consistent with those obtained with 2-APB that indicate little or no contribution of CRAC channels to Ag-stimulated wave initiation. Because Ca²⁺ influx contributes to the spatial localization of Ca²⁺ wave initiation from protrusions (Fig. 2), these results suggest that Ca²⁺ channels other than CRAC or voltage gated channels contribute to this process.

FIGURE 4. Stimulation of RBL cells by contact with Ag-coated micropipette. A and B, Representative cells expressing GCaMP2, sensitized with IgE and stimulated with DNP-BSA conjugated micropipettes. A, Micropipette (indicated by yellow arrowhead) contacting the cell at the tip of a protrusion elicits a train of spatially restricted Ca²⁺ puffs, each traveling no more than 30 μm along the protrusion. B, Contact stimulation at the cell body results repetitive Ca²⁺ puffs in the cell body that sometimes propagate as a wave to the protrusion. Left panels, Images with ROIs defined; right panels, Ca²⁺ concentration changes in ROIs of corresponding color. Black arrow indicates initiation of contact between micropipette and cell. C, Histogram showing percentage of cells responding with local Ca²⁺ puffs only (light blue) or more global Ca²⁺ elevation (dark blue) due to contact with DNP-BSA conjugated micropipettes. n/fixed BSA-conjugated micropipettes in the presence of extracellular Ca²⁺.
were cotransfected with the Ca\(^{2+}\) indicator GCaMP2 and one of the TRPC targeting shRNA plasmids as described in Materials and Methods. We found that transfection with TRPC1 shRNA and, to a lesser extent, with TRPC3 shRNA reduces the responsiveness of the cells to a low concentration of Ag compared with cells expressing a mock shRNA sequence, and these effects are particularly notable if cells responding with Ca\(^{2+}\) puffs are not included in the count (Fig. 6A). As shown in supplemental Fig. S3, we observe ~50% reduction in TRPC1 expression due to shRNA transfection in these cells, similar to previous results (25). This value represents a lower limit to the knockdown of TRPC1-cell-expressing GCaMP2, which is cotransfected with the shRNA constructs. Under these knockdown conditions, we did not observe a significant reduction in the percentage of cells responding to Ag in cells transfected with TRPC5 or TRPC7. In addition to reduction in cell responsiveness, we found that transfection with TRPC1 or TRPC3 shRNA prolongs the lag time from Ag addition to the onset of the Ca\(^{2+}\) response compared with cells transfected with TRPC5, TRPC7, or the control shRNA (Fig. 6B). These results suggest that TRPC1 and TRPC3 play a significant role in the initiating Ca\(^{2+}\) wave phase of the cellular response to Ag stimulation.

Most notably, expression of TRPC1 and TRPC3 shRNA results in decreased frequency of Ca\(^{2+}\) wave initiation from cell protrusions compared with expression of mock shRNA (Fig. 6C). Expression of TRPC7 shRNA also decreases the frequency of Ca\(^{2+}\) wave initiation from cell protrusions, even though this construct does not significantly alter the capacity of the cells to respond or the lag time of the response. Measurements of Ca\(^{2+}\) wave velocity showed only marginal differences for cells expressing the TRPC shRNA constructs, suggesting that these channels are not involved in Ca\(^{2+}\) wave propagation (data not shown). These results also indicate that reduction in the capacity of cells to respond to Ag stimulation after the TRPC1 or TRPC3 shRNA expression is caused by perturbation of wave initiation of the Ca\(^{2+}\) response, rather than in later steps involved in propagating the Ca\(^{2+}\) signal, which have been shown to be inhibited by TRPC5 shRNA (25).

**Discussion**

IgE receptor-mediated Ca\(^{2+}\) mobilization plays key roles in mast cell activation and consequent exocytotic release of inflammation and allergy-related mediators. Previous studies characterized dramatic Ca\(^{2+}\) oscillations stimulated by Ag in these cells (15) that correlate temporally to exocytotic events (13, 15). In the present study we used high-speed confocal imaging to characterize rapid, early events in stimulated Ca\(^{2+}\) mobilization with high spatial resolution. Our principal new finding is that the initial increase in cytoplasmic Ca\(^{2+}\) in response to Ag stimulation in RBL mast cells is highly spatially organized, initiating at specific regions of the cell and propagating through the cytosol as a Ca\(^{2+}\) wave. The existence and nature of Ca\(^{2+}\) waves in hematopoietic cells has been controversial, and the study characterizing high-speed, circumferential waves in neutrophils (63) could not be confirmed (64). Lee and Oliver previously described evidence for a Ca\(^{2+}\) influx pathway in RBL cells that precedes Ca\(^{2+}\) release from intracellular stores (65). The Ca\(^{2+}\) waves stimulated via FceRI that we have characterized in the present study are similar in several aspects to Ca\(^{2+}\) waves observed in certain excitable and other nonhematopoietic cells (6, 7, 66), but they have not been previously detected in Ca\(^{2+}\) responses stimulated by multichain immune recognition receptors.

In certain cell types, elementary Ca\(^{2+}\) events such as Ca\(^{2+}\) puffs can lead to initiation and propagation of a Ca\(^{2+}\) wave, and this has...
been shown to be regulated by a number of factors, including spatial organization of intracellular Ca$^{2+}$ release sites (67–70). In RBL mast cells, the high incidence of wave initiation from elongated cellular protrusions suggests that these protrusions contain specialized structures that ignite the cellular response. Interestingly, the percentage of stimulated Ca$^{2+}$ waves that initiate from protrusions increases at weak stimulation, indicating that protrusions are particularly sensitive to Ag. A survey of the literature indicates that such protrusions are a common feature of mucosal mast cells in tissue sites (34, 36, 37, 71), consistent with an important role for these protrusions in mast cell physiology. The high sensitivity of wave initiation in protrusions that we observe could result from locally enhanced coupling of the IgE receptor and the machinery responsible for Ca$^{2+}$ release from stores. We do not detect enhanced expression of FcεRI in these protrusions, but we find that initiation of Ca$^{2+}$ waves in protrusions is selectively reduced in the absence of extracellular Ca$^{2+}$, suggesting a facilitating Ca$^{2+}$ influx pathway at these protrusions. As discussed below, our results point to Ca$^{2+}$-dependent potentiation of IP$_3$ receptors close to sites of Ca$^{2+}$ influx at protrusions, rendering these receptors more sensitive and therefore more probable sites for Ca$^{2+}$ wave initiation.

Ca$^{2+}$ oscillations following initial waves exhibit a global, non-directional increase in Ca$^{2+}$ concentration that contrasts with directional increase in Ca$^{2+}/$H$^{+}$, suggesting a facilitating Ca$^{2+}$ influx pathway at these protrusions. As discussed below, our results point to Ca$^{2+}$-dependent potentiation of IP$_3$ receptors close to sites of Ca$^{2+}$ influx at protrusions, rendering these receptors more sensitive and therefore more probable sites for Ca$^{2+}$ wave initiation.

Ca$^{2+}$$^{2+}$ elevation seen for the waves. Because of this spatial distinction and the pharmacological differences, we conclude that waves and oscillations are two discrete but coupled Ca$^{2+}$ events in IgE receptor-mediated signaling. We suggest that Ca$^{2+}$ waves are the initiating event of mast cell activation, established by the unique spatial distribution of Ca$^{2+}$ influx pathways in these cells, involving TRPC channels that may be preferentially expressed in the elongated protrusions, rendering these to be more sensitive to Ag stimulation. Subsequent to the waves are Ca$^{2+}$ oscillations that are thought to result from IP$_3$-induced release of Ca$^{2+}$ from endoplasmic reticulum (72). These recurring Ca$^{2+}$ elevations were suggested to play a role in prolonging the duration of the Ca$^{2+}$ signal, enhancing its efficacy (16), and also in providing temporal encoding for downstream events such as degranulation (15). Interestingly, we could occasionally detect Ca$^{2+}$ oscillations in cells that did not demonstrate a Ca$^{2+}$ wave upon Ag stimulation, suggesting that the propagation of Ca$^{2+}$ through the cytoplasm during the wave period is not required for the onset of Ca$^{2+}$ oscillations.

In addition to Ca$^{2+}$ waves and oscillations, we detect Ca$^{2+}$ puffs in cells stimulated by Ag under certain conditions. Of particular interest are the repetitive Ca$^{2+}$ puffs that are elicited by local application of Ag with a DNP-BSA-coated micropipette, demonstrating that the molecular machinery capable of initiating local Ca$^{2+}$ responses is distributed throughout the cell and is not restricted to...
the protrusions. Ca\textsuperscript{2+} puffs stimulated by Ag-coated micropipette contact are similarly frequent in the absence of extracellular Ca\textsuperscript{2+}, indicating that the generation of spatially restricted Ca\textsuperscript{2+} elevations in these cells involves Ca\textsuperscript{2+} release from stores and does not require Ca\textsuperscript{2+} influx. However, the periodic nature of these puffs suggests a complex mechanism for their regulation that may be related to the periodic nature of more global Ca\textsuperscript{2+} oscillations elicited by soluble Ag. It will be interesting to determine whether these localized Ca\textsuperscript{2+} puffs are sufficient to elicit local exocytosis in these cells.

Pharmacologic characterization of IgE receptor-mediated Ca\textsuperscript{2+} waves yields evidence for PLC activation in this mechanism, whereas store-operated Ca\textsuperscript{2+} entry does not appear to play an important role in this early phase of the Ca\textsuperscript{2+} response. PLC\textgamma-dependent IP\textgamma production is important for generating Ca\textsuperscript{2+} responses to Ag in mast cells (73), and it is possible that a specific IP\textgamma receptor family member is involved in Ca\textsuperscript{2+} wave initiation. The greater sensitivity of IP\textgamma,R-3 to Ca\textsuperscript{2+} release by IP\textgamma (74) suggests this family member as a strong candidate for initiation of an IP\textgamma-dependent Ca\textsuperscript{2+} wave at protrusions. Ca\textsuperscript{2+} waves can be triggered by alternate means of Ca\textsuperscript{2+} mobilization, including Ca\textsuperscript{2+} ionophore and thapsigargin, but these agents cause less frequent Ca\textsuperscript{2+} waves (Table I). Furthermore, initiation of Ca\textsuperscript{2+} waves by Ca\textsuperscript{2+} ionophore occurs only infrequently at extended protrusions. Taken together, these results suggest that Ca\textsuperscript{2+} responses initiated by the IgE receptor are more spatially regulated than those caused by nonreceptor-mediated cell activation.

A recent study by Ma et al. provided evidence that TRPC channels contribute to stimulated Ca\textsuperscript{2+} influx in RBL cells (25), suggesting their possible involvement in the Ag-stimulated Ca\textsuperscript{2+} influx pathway that initiates Ca\textsuperscript{2+} waves from extended protrusions. Our results with shRNA-mediated knockdown of TRPC proteins provide evidence that decreased expression of TRPC1, and, to lesser extent, TRPC3, reduces the responsiveness of these cells to low concentration of Ag (Fig. 6, A and B) and decreases the frequency of wave initiation from extended protrusions (Fig. 6C). At higher concentrations of Ag, TRPC1 channel knockdown is less effective in decreasing the responsiveness of the cells (data not shown), indicating that this channel plays an important role in Ca\textsuperscript{2+} responses only under more physiological conditions of IgE receptor stimulation. Other TRPC channels may also contribute to Ca\textsuperscript{2+} wave initiation, and our failure to detect significant effects of TRPC5 knockdown, or the smaller effects of TRPC3 and RTPC7 knockdown, may be limited by the efficiency of their reduction under our experimental conditions.

These results suggest that Ca\textsuperscript{2+} influx via TRPC channels, particularly TRPC1, is likely to participate in the initial steps of mast cell activation by enhancing the sensitivity of these cells to threshold Ag stimulation. Although the mechanism for this enhancement is not yet understood, it is likely that a Ca\textsuperscript{2+} influx-dependent increase in IP\textgamma,R activation is involved. Two possible mechanisms are indicated in Fig. 4D: in one, local accumulation of TRPC at protrusions could enhance receptor-stimulated influx at this region; alternatively, enhanced physical coupling of the TRPC channels to IP\textgamma,R (75), PLC\textgamma (76), or FceRI in this region could mediate local Ca\textsuperscript{2+} influx to initiate wave propagation.

Our observations that rat BMMCs exhibits similar morphologies as RBL mast cells and initiate Ca\textsuperscript{2+} responses to Ag in the form of waves that begin in extended protrusions show that these stimulated waves are a general feature of Fc\textepsilonRI-mediated Ca\textsuperscript{2+} signaling. Both rat BMMCs (23) and RBL mast cells (20) have marker characteristics of mucosal mast cells, and it is not yet clear whether this wave response is a specific feature of this subset of mast cells. Mouse BMMCs undergo chemotactic responses on vitronectin (77), and it may be possible to observe stimulated Ca\textsuperscript{2+} waves under these conditions of cell attachment.

Our data establish Ca\textsuperscript{2+} waves originating from a specific cellular site as a common feature of the Ca\textsuperscript{2+} response to Ag in RBL mast cells and rat BMMCs, but the physiological function of these waves and the molecular basis for their site of initiation are not completely understood. Regulated Ca\textsuperscript{2+} signaling across the cell is a fundamental mechanism for information transfer between organelles or cellular regions that convey direction and temporal information. In this regard, we have preliminary evidence that direction of RBL cell migration correlates with the site of Ag-stimulated wave initiation (R. Cohen and J. Lee, unpublished results), suggesting a strategic localization of the Ca\textsuperscript{2+} signaling machinery to communicate directional information in these cells. Localized degranulation from extended protrusions might also be mediated by this machinery under certain physiological conditions.

In summary, we show that the response of RBL and rat BMMC to Ag stimulation initiates with a propagating Ca\textsuperscript{2+} wave, most often beginning at the tips of cellular protrusions in a Ca\textsuperscript{2+} influx-dependent manner. These waves initiate with TRPC1-dependent Ca\textsuperscript{2+} influx, then propagate by Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} release from stores, followed by extracellular Ca\textsuperscript{2+}-dependent oscillations. Spatial localization of wave initiation sites to extended protrusions suggests a link between this signaling response and polarized cell motility. Our results indicate that when mast cells encounter physiological levels of Ag that are insufficient to directly activate global Ca\textsuperscript{2+} mobilization, activation of Ca\textsuperscript{2+} influx mediated by TRPC1 channels in extended cell protrusions can ignite a response that is propagated and amplified. In this manner, the initiation of a physiological cellular response is both spatially and temporally controlled.

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**Disclosures**

The authors have no financial conflicts of interest.

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SUPPLEMENTAL MATERIALS

Supplemental Figure S1. Ca\textsuperscript{2+} mobilization monitored in RBL cells waves with genetically encoded GCaMP2 (n=80), as compared to indicator dyes Fluo-4 (n=40) and Fluo-5F (n=17). A) Images of cells sensitized with anti-DNP IgE, expressing GCaMP2 (top) or loaded with Fluo-4 (middle) or Fluo-5F (bottom), and stimulated with a puff of 1.7 µg/ml DNP-BSA. Right panels show time line analysis of the stimulated cells and changes in Ca\textsuperscript{2+} concentration occurring over time in the segments defined in the left panels. Changes in Ca\textsuperscript{2+} concentration are indicated by relative changes in brightness, where brighter colors represent higher Ca\textsuperscript{2+} concentrations. B) Percentage of indicator-labeled cells responding to stimulus with Ca\textsuperscript{2+} waves, averaged over multiple experiments ± SD; *P < 0.05 vs. cells expressing GCaMP2. C) Average velocity of Ca\textsuperscript{2+} waves measured with specified Ca\textsuperscript{2+} indicator. F) Average number of oscillations within 2 min after Ag stimulation. The first oscillation corresponds to the originating Ca\textsuperscript{2+} wave (dotted line). Error bars correspond to standard error of the mean (SEM).

Supplemental Figure S2. Sensitivity of Ca\textsuperscript{2+} responses to different doses of Ag: 1.7 ng/ml (n=25); 17 ng/ml (n=26); 170 ng/ml (n=26); 1.7 µg/ml (n=80). RBL cells expressing GCaMP2 were sensitized with anti-DNP IgE and stimulated with a puff from a pipette containing indicated concentrations of Ag. A) Percentage of cells responding with measurable Ca\textsuperscript{2+} elevation upon stimulation with Ag. B) Average velocity of Ca\textsuperscript{2+} wave. C) Bar graph showing percentage of cells responding to Ag stimulus with Ca\textsuperscript{2+} waves. Bar height shows total % waves; orange portion represents % waves originating in protrusions. D) Average lag time after Ag stimulation for initiation of Ca\textsuperscript{2+} wave. E) Average number of oscillations within 2 min after Ag stimulation. The first oscillation corresponds to the originating Ca\textsuperscript{2+} wave (dotted line). F) Percentage of
cells responding with Ca$^{2+}$ puffs. Error bars correspond to SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**Supplemental Figure S3.** Quantification of TRPC1 knock-down by shRNA expression. Western blot analysis of whole cell lysates from of 1.5x10$^6$ cells expressing shRNA targeting TRPC1 or a mock sequence. A) Expression levels of TRPC1 in knockdown cells or control cells expressing the mock shRNA sequence. B) Densitometry quantification of A, showing 50% reduction in TRPC1 expression (band a; blue bar) after shRNA treatment compared to control cells (band a; green bar). Protein expression levels as seen in nonspecific Ab labeling (band b) were unchanged by shRNA plasmids.

**Supplemental Figure S4.** Identification of rat BMMC by labeling with Alexa488-IgE and mAb AA4. Rat stem cells differentiated with IL3 and stem cell factor were plated overnight in MatTek wells and labeled as described in Materials and Methods. Representative field shows two cells, each labeled with Alexa488-IgE (green) and the mast cell-specific anti-ganglioside AA4 (red). Note polarized morphologies that are common for these cells after several hours on glass surfaces. Scale bar = 10 μm.
Supplementary movie 1. Ag stimulated Ca$^{2+}$ waves in RBL-2H3 cells. Ca$^{2+}$ wave in RBL cell expressing GCaMP2 sensitized with anti-DNP IgE and stimulated with a puff from a pipette containing 1.7µg/ml DNP-BSA (as depicted in Figure 1). Extracellular buffer is BSS with 2mM Ca$^{2+}$ (as in Figure 2A, top panel). Note wave initiates from extended cellular protrusion. Image rate = 21 Hz  (47.15 ms/frame). Movie =X3 real time.

Supplementary movie 2. Ca$^{2+}$ waves in the absence of extracellular Ca$^{2+}$. Ca$^{2+}$ wave in RBL cell expressing GCaMP2 sensitized with anti-DNP IgE and stimulated with a puff from a pipette containing 1.7µg/ml DNP-BSA. Extracellular buffer is BSS without Ca$^{2+}$ (as in Figure 2A, bottom panel). Note wave initiates from cell body. Image rate = 17 Hz  (59.6 ms/frame). Movie =X3 real time.
Fig S1

Ca\textsuperscript{2+} mobilization monitored in RBL cells waves with genetically encoded GCaMP2.
Fig S2

Sensitivity of Ca^{2+} responses to different doses of Ag.

A. Ca^{2+} Response (% of Cells) vs [Ag] (ng/ml)

B. Wave Velocity (μm/sec) vs [Ag] (ng/ml)

C. Graph showing % of Cells for different [Ag] (ng/ml) concentrations:
   - 1.7 ng/ml
   - 17 ng/ml
   - 170 ng/ml
   - 1700 ng/ml

D. Ca^{2+} Response Lag Time (sec) vs [Ag] (ng/ml)

E. Ca^{2+} Oscillations vs [Ag] (ng/ml)

F. Ca^{2+} Puffs (% of Cells) vs [Ag] (ng/ml)
Fig S3

Quantification of TRPC1 knock-down by shRNA expression.

A  shTRPC1  Control

B

TRPC1 Knock Down (% of Control)

0  10  20  30  40  50  60  70
Fig S4
Identification of rat BMMC by labeling with Alexa488-IgE (green) and mAb AA4 (red)