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All-or-None Activation of CRAC Channels by Agonist Elicits Graded Responses in Populations of Mast Cells

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In nonexcitable cells, receptor stimulation evokes Ca\(^{2+}\) release from the endoplasmic reticulin stores followed by Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels in the plasma membrane. In mast cells, store-operated entry is mediated via Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels. In this study, we find that stimulation of muscarinic receptors in cultured mast cells results in Ca\(^{2+}\)-dependent activation of protein kinase Ca and the mitogen activated protein kinases ERK1/2 and this is required for the subsequent stimulation of the enzymes Ca\(^{2+}\)-dependent phospholipase A\(_2\) and 5-lipoxygenase, generating the intracellular messenger arachidonic acid and the proinflammatory intercellular messenger leukotriene C\(_4\). In cell population studies, ERK activation, arachidonic acid release, and leukotriene C\(_4\) secretion were all graded with stimulus intensity. However, at a single cell level, Ca\(^{2+}\) influx was related to agonist concentration in an essentially all-or-none manner. This paradox of all-or-none CRAC channel activation in single cells with graded responses in cell populations was resolved by the finding that increasing agonist concentration recruited more mast cells but each cell responded by generating all-or-none Ca\(^{2+}\) influx. These findings were extended to acutely isolated rat peritoneal mast cells where muscarinic or P2Y receptor stimulation evoked all-or-none activation of Ca\(^{2+}\) entry but graded responses in cell populations. Our results identify a novel way for grading responses to agonists in immune cells and highlight the importance of CRAC channels as a key pharmacological target to control mast cell activation. The Journal of Immunology, 2007, 179: 5255–5263.

In eukaryotic cells, Ca\(^{2+}\) influx is a critical trigger for a diverse array of cellular responses including exocytosis, muscle contraction, gene transcription, and cell growth (1). In nonexcitable cells, Ca\(^{2+}\) entry occurs through store-operated and second messenger-gated Ca\(^{2+}\) channels in the plasma membrane (2, 3). Store-operated Ca\(^{2+}\) channels are activated following the emptying of intracellular Ca\(^{2+}\) stores, are regulated by a diverse range of agonists, and are found in a variety of nonexcitable cells. Electrophysiological evidence indicates that store-operated channels represent a heterogeneous family (4). The best characterized member is the Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel and the whole cell current flowing through CRAC channels is called I\(_{\text{CRAC}}\) (5, 6). Defective CRAC channel activity has been causally linked to inherited primary immunodeficiencies (7) and Ca\(^{2+}\) entry through CRAC channels regulates exocytosis (8), gene transcription (9), and cytoplastic enzymes such as NO synthase (10), adenylate cyclase (11), and Ca\(^{2+}\)-dependent activation of protein kinase Ca and the mitogen activated protein kinases ERK1/2 and this is required for the subsequent stimulation of the enzymes Ca\(^{2+}\)-dependent phospholipase A\(_2\) and 5-lipoxygenase, generating the intracellular messenger arachidonic acid and the proinflammatory intercellular messenger leukotriene C\(_4\). In cell population studies, ERK activation, arachidonic acid release, and leukotriene C\(_4\) secretion were all graded with stimulus intensity. However, at a single cell level, Ca\(^{2+}\) influx was related to agonist concentration in an essentially all-or-none manner. This paradox of all-or-none CRAC channel activation in single cells with graded responses in cell populations was resolved by the finding that increasing agonist concentration recruited more mast cells but each cell responded by generating all-or-none Ca\(^{2+}\) influx. These findings were extended to acutely isolated rat peritoneal mast cells where muscarinic or P2Y receptor stimulation evoked all-or-none activation of Ca\(^{2+}\) entry but graded responses in cell populations. Our results identify a novel way for grading responses to agonists in immune cells and highlight the importance of CRAC channels as a key pharmacological target to control mast cell activation.

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2 Address correspondence and reprint requests to Prof. Anant Parekh, Oxford University, Parks Road, Oxford, U.K. E-mail address: anant.parekh@dpag.ox.ac.uk

3 Abbreviations used in this paper: CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\); cPLA\(_2\), Ca\(^{2+}\)-dependent phospholipase A\(_2\); LTC\(_4\), leukotriene C\(_4\); InsP\(_3\), inositol 1,4,5-trisphosphate; 2-APB, 2-aminophenyl borate.

Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

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acutely isolated rat peritoneal mast cells. Our new results dem-
strate that increasing agonist concentration increases protein kinase Cα and ERK activation, cPLA₂ stimulation, and LTC₄ secretion in a graded manner. However, activation of IₑCa and Ca²⁺ influx remain essentially all-or-none processes. Responses appear graded with stimulus intensity because increasing agonist concentration recruits more cells in the population but each cell that responds does so by activating Ca²⁺ influx in an essentially all-or-none manner. Our results therefore identify a mechanism for eliciting graded responses in a multicellular system despite all-or-none ac-
tivation of CRAC channels by receptor stimulation.

Materials and Methods

Cell culture

Rat basophilic leukemia cells stably expressing the muscarinic type 3 re-
ceptor were a gift from Dr. J. Putney (National Institute of Environmental
Health Sciences, Research Triangle Park, NC). Cells were cultured (37°C,
5% CO₂) in MEM (10% FBS, 2 mM l-glutamine, penicillin-strep-
tomyacin, and 100 μM G-418, as described previously (12, 15). For Ca²⁺
ing imaging and patch clamp experiments, cells were passaged (using trypsin)
on glass coverslips and used 36 – 72 h after plating.

Isolation of rat peritoneal mast cells

The mast cells were isolated from female, Sprague-Dawley rats, weighing
~300 g. The animals were sacrificed according to “Schedule 1”, (carbon
oxide overdose and neck dislocation). Immediately, 100 – 150 ml of ster-
ile HEPES buffer (in mM: NaCl 150, KCl 5.6, HEPES 10, NaOH 1.5,
MgCl₂ 1, CaCl₂ 2, glucose 10 g/l BSA (pH 7.4)) was injected into the
peritoneal cavity. The abdomen was massaged for 2 min and then the buffer
removed. This was centrifuged at 200 × g for 10 min. The pellet was
resuspended in MEM, triturated, and plated onto glass coverslips (for
Ca²⁺ imaging and immunocytochemistry) or cell-culture dishes (for LTC₄
measurements).

Ca²⁺ imaging

Ca²⁺ imaging experiments were conducted using the IMAGO CCD cam-
era-based system from TILL Photonics, as described previously (12). Cells
were alternately excited at 356 and 380 nm (20-ms exposures) using a
Polychrome Monochromator and images were acquired every 2 – 3 s. Im-
ages were obtained using a Leica confocal microscope and anal-
yzed off-line using IGOR Pro. Cells were loaded with Fura-2-AM (2 μM)
for 40 min at room temperature in the dark and then washed three
times in standard external solution of composition (in mM) NaCl 145,
KCl 2.8, CaCl₂ 2, MgCl₂ 2, D-glucose 10 g/l BSA (pH 7.4) with NaOH.
Cells were left for 15 min in the dark to allow further deesterification. Ca²⁺
and Ba²⁺ signals are presented as the fluorescence ratio (356/380) ΔF.

Immunocytochemistry

After treatment with carbachol, the cells were fixed in 4% paraformalde-
yde in phosphate buffer, for 30 min at room temperature. All washes used
0.01% PBS, (PBS in mM: NaCl 137, KCl 2.7, Na₂HP0₄ 8, KH₂PO₄ 1). The
cells were blocked with 2% BSA and 10% goat serum for 2 h. Trans-
location of protein kinase Cα was visualized using a polyclonal rabbit IgG
Ab, (Santa Cruz Biotechnology). After blocking, the cells were washed and
incubated in antiPKCα (1/1000 in 0.2%BSA, 1% goat serum), overnight at
4°C. The cells were thoroughly washed and the secondary Ab (Alexfluor
568-conjugated goat anti-rabbit IgG) was applied at 1/2000 in PBS for 2 h
at room temperature. The cells were thoroughly washed and then incubated with
primary Ab for 1 h on an orbital shaker for 1.5 h at 37°C, as described previously (12). Cells were then washed twice with serum-free MEM to remove unincorporated [³H]arachidonic acid. Thapsigargin with 0.5% fatty acid-free BSA was added for different times (see text). Medium was collected and centrifuged at 25 g for 5 min to remove any floating cells. Radioactivity in the supernatant was measured. The amount of [³H]arachidonic acid released into the medium was ex-
pressed as a percentage of the total [³H]arachidonic acid uptake.

LTC₄ measurements

Following stimulation of attached cells with carbachol, the supernatant was
collected and LTC₄ levels were measured by enzyme immunoassay (Cay-
man Chemicals) as described previously (12). In brief, 50 μl of superna-
tant, leukotriene C₄ acetylcholinetransferase and leukotriene C₄ antisemur
were added to each enzyme immunoassay well. Following incubation for
18 h at room temperature, the wells were emptied and rinsed five times with
enzyme immunoassay washing buffer. Two hundred microliters of
Ellman’s reagent (prepared fresh) was added to each well and the plate was
placed on an orbital shaker for 1.5 h in the dark. Plate absorbance was
measured at a wavelength of 405 nm. Data is presented relative to basal
LTC₄ secretion from nonstimulated cells.

Statistical analysis

Data is presented as the mean ± SEM. Statistical significance was con-
cidered as p < 0.01, using Student’s t test, and is denoted by an asterisk (*).

Results

Ca²⁺ influx following muscarinic receptor stimulation activates

Whole cell patch clamp experiments were conducted as described (12, 15).
Synguard-coated, fire-polished patch pipettes filled with a solution that
contained 145 mM cesium glutamate, 8 mM NaCl, 1 mM MgCl₂, 2 mM
Mg-ATP, 10 mM HEPES, 10 mM EGTA, 4.6 mM CaCl₂ (pH 7.2) with
CsOH. Free Ca²⁺ was buffered at ~140 nM. Pipette resistance was ~5
MOhms when placed in an external solution containing 145 mM NaCl, 2.8
mM KCl, 10 mM CaCl₂, 2 mM MgCl₂, 10 mM CsCl, 10 mM D-glucose,
10 mM HEPES (pH 7.4) with NaOH. A correction of + 10 mV was
applied for the subsequent liquid junction potential that arose from the
glutamate-based pipette solution. IₑCa was measured at ~80 mV from
temperature ramps (~100 to + 100 mV lasting 50 msec) applied at 0.5 Hz,
FIGURE 1. Ca²⁺ influx following muscarinic receptor stimulation activates cPLA₂ and LTC₄ secretion via recruitment of ERK. A, Stimulation with 100 μM carbachol results in Ca²⁺ release followed by Ca²⁺ influx into the cells (averaged data from 63 cells is shown). In the absence of external Ca²⁺, only the Ca²⁺ release phase is seen (55 cells). B, Carbachol (100 μM) activates cPLA₂ and arachidonic acid release but only in the presence of external Ca²⁺. Arachidonic acid release in response to carbachol is similar in extent to that seen with thapsigargin, is not additive with thapsigargin and is blocked by the MEK inhibitor U0126 (10 μM; pretreated for 15 min). The abbreviation carb. refers to carbachol. C, Carbachol (100 μM) activates LTC₄ secretion, but only in the presence of external Ca²⁺. As with arachidonic acid release, carbachol-evoked LTC₄ secretion is similar in extent to that induced by thapsigargin, is not additive with thapsigargin and is suppressed by U0126. D, Muscarinic receptor activation (100 μM carbachol for 8 min) stimulates ERK phosphorylation, and to an extent similar to that seen following challenge with thapsigargin (2 μM for 8 min). ERK phosphorylation to carbachol was prevented by U0126.

(Fig. 1B). The extent of arachidonic acid release following muscarinic receptor activation was not additive with that seen following stimulation with the SERCA pump inhibitor thapsigargin (2 μM for 8 min), which empties stores and maximally activates CRAC channels (Fig. 1B). Arachidonic acid released by cPLA₂ activity is subsequently metabolized by the 5-lipoxygenase enzyme to form the potent proinflammatory paracrine signal LTC₄ (12). Muscarinic receptor stimulation in the presence, but not absence, of external Ca²⁺ resulted in secretion of LTC₄ and to an extent similar to that seen with thapsigargin (Fig. 1C). Again, there was no additivity between the two stimuli.

Stimulation with carbachol in the presence of external Ca²⁺ triggered phosphorylation (and hence activation) of the mitogen activated protein kinases ERK1 and 2 (Fig. 1D). Application of carbachol to wild-type RBL cells not transfected with muscarinic receptor failed to evoke any resolvable ERK activation (data not shown). Carbachol activated ERK to an extent similar to that seen following stimulation with thapsigargin (Fig. 1D). ERK1/2 are activated following dual phosphorylation of critical threonine and tyrosine residues by the upstream mitogen activated protein kinase kinases MEK1/2. The MEK1/2 inhibitor U0126 (19) suppressed ERK activation following muscarinic receptor stimulation (Fig. 1D). U0126 also suppressed cPLA₂ activation (Fig. 1B) and generation of LTC₄ (Fig. 1C) following carbachol stimulation. U0126 has no effect store-operated Ca²⁺ signals (13). Collectively, these results demonstrate that Ca²⁺ influx following stimulation of muscarinic receptors recruits the ERK pathway, which then results in the generation of the second messenger arachidonic acid as well as the paracrine signal LTC₄.

Tight temporal correlation between ERK stimulation, cPLA₂ activation, and LTC₄ secretion

Fig. 2A shows that stimulation with carbachol (100 μM) resulted in a time-dependent activation of ERK (upper panel) and densitometric analysis of the gel is plotted in Fig. 2B. Generation of arachidonic acid and secretion of LTC₄ also increased with the duration of carbachol stimulation (Fig. 2, C and D, respectively). We normalized the extent of ERK activation, arachidonic acid generation, and LTC₄ secretion to the value measured after 12 min stimulation and the corresponding time courses are superimposed in Fig. 2E. All three parameters showed similar kinetics of development, consistent with a tight temporal correlation between them.

Graded dependence on stimulus intensity

To establish the relationship between ERK stimulation, cPLA₂ activation, and LTC₄ secretion with stimulus intensity, we examined their extent of activation following different levels of muscarinic receptor occupancy. Fig. 3A shows that increasing agonist concentration resulted in graded activation of ERK in cell population measurements. The corresponding dose-response curve is plotted in Fig. 3B. Activation of cPLA₂ (Fig. 3C) and secretion of LTC₄ (Fig. 3D) were also graded with stimulus intensity. Normalized responses to ERK stimulation, cPLA₂ activation, and LTC₄ secretion are superimposed in Fig. 3E. Importantly, the curves were superimposable, indicating that each pathway was activated in a graded manner by carbachol and to similar relative extents.

The receptor-activated Ca²⁺ influx pathway that drives ERK stimulation involves CRAC channels

The finding that ERK activation, cPLA₂ stimulation, and LTC₄ secretion, which are all dependent on Ca²⁺ influx, were graded with agonist concentration is unexpected because agonist-dependent activation of Iₘ₇₃ by InsP₃ is essentially an all-or-none process (15). We considered various possibilities for this apparent discrepancy. For example, receptor activation could recruit additional Ca²⁺ entry pathways to Iₘ₇₃ and these could be activated in a graded manner. However, three pieces of evidence militate against this. First, whole cell patch clamp recordings demonstrated that carbachol application activated a nonvoltage-gated inwardly rectifying Ca²⁺ current with a reversal potential +60 mV (Fig. 4A), which was indistinguishable from Iₘ₇₃ activated by store depletion (thapsigargin or dialysis with 10 mM EGTA; Ref. 20). Second, mitochondrial depolarisation suppresses store-operated Ca²⁺ entry and this reflects, at least in part, enhanced Ca²⁺-dependent inactivation of CRAC channels due to the loss of mitochondrial Ca²⁺ buffering (21). Depolarisation of mitochondria with either the complex III respiratory chain inhibitor antimycin A...
(5 µM/ml) or collapse of the proton gradient with 2mM p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (both in the presence of oligomycin) suppressed carbachol-evoked Ca\(^{2+}\) influx, but without affecting Ca\(^{2+}\) release from the stores (22). Importantly, mitochondrial depolarization suppressed activation of cPLA\(_2\) (data not shown) and LTC\(_4\) secretion (Fig. 4D) following stimulation with carbachol. Finally, I\(_{\text{CRAC}}\) is suppressed by application of the CRAC channel blocker 2-aminoethylphenyl borate (2-APB) (40 µM; Ref. 2), 2-APB suppressed Ca\(^{2+}\) influx following stimulation with 100 µM carbachol (data not shown) and inhibited subsequent cPLA\(_2\) activation (Fig. 4C) and LTC\(_4\) secretion (Fig. 4D). We also considered the possibility that receptor stimulation might recruit different Ca\(^{2+}\) influx pathways in a concentration-dependent manner. It has been suggested in HEK293 cells for example that low concentrations of agonist recruit a nonstore-operated Ca\(^{2+}\) influx pathway gated by arachidonic acid that is 2-APB insensitive whereas higher agonist concentrations activate store-operated Ca\(^{2+}\) influx (23). However, 2-APB suppressed Ca\(^{2+}\) influx and both Ca\(^{2+}\)-dependent arachidonate release and LTC\(_4\) secretion following stimulation with a low concentration of carbachol (1 µM; Fig. 4, C and D), suggesting that Ca\(^{2+}\) influx even at low agonist concentrations in mast cells is via CRAC channels.

**Divalent cation entry following receptor stimulation is an apparent all-or-none process**

To see whether store-operated Ca\(^{2+}\) influx was supralinearly related to agonist concentration in intact cells, we applied different concentrations of carbachol in Ca\(^{2+}\)-free solution to fura 2-loaded cells and then added Ba\(^{2+}\) to the extracellular solution. Ba\(^{2+}\) permeates CRAC channels but, unlike Ca\(^{2+}\), is not transported out of the cytoplasm by Ca\(^{2+}\) ATPases and is therefore used as an indicator of unidirectional divalent cation entry (2). We measured the initial rate of Ba\(^{2+}\) entry as an indicator of the number of open CRAC channels and aggregate data from all cells used is summarized in Fig. 4E (n > 150 cells per point). The relationship between carbachol concentration and the rate of Ba\(^{2+}\) entry was clearly graded and correlated well with corresponding agonist-response curves in Fig. 3. However, this dose-response curve reflects all

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**Figure 2.** Time course of ERK activation, arachidonic acid release and LTC\(_4\) secretion following stimulation with carbachol. A, Western blots showing the extent of ERK phosphorylation after carbachol challenge (100 µM) for the times indicated. Upper panel depicts ERK1/2 phosphorylation and the lower panel depicts total ERK2, used as a control for constant gel loading. B, Data from three experiments as in A is summarized. C and D, Time course of arachidonic acid release (C) and LTC\(_4\) secretion (D) are shown. E, Time courses of ERK activation, arachidonic acid release and LTC\(_4\) secretion are superimposed to show the close temporal association.

**Figure 3.** Graded activation of ERK, arachidonic acid and LTC\(_4\) secretion following stimulation with different concentrations of carbachol. A, Western blots showing the relationship between agonist concentration and ERK phosphorylation. B, Aggregate data from three experiments is summarized. C and D, Corresponding dose-response curves for arachidonic acid release and LTC\(_4\) secretion. E, Dose-response curves for the three parameters are superimposed to show the similar dependence on agonist concentration.
cells in the population i.e., those that respond to carbachol as well as those that do not. Importantly, when we applied carbachol in Ca^{2+}-free solution, taking Ca^{2+} release as an indicator of receptor activation, we found that only a fraction of the cells responded to lower concentrations of agonist (Fig. 4F). Increasing carbachol concentration increased the number of cells that responded. The graded response for Ba^{2+} entry in Fig. 4E closely resembles that for agonist responsiveness in Fig. 4F, and therefore is not a true representation of Ba^{2+} influx. We therefore analyzed the rate of Ba^{2+} entry only for those cells that responded to carbachol. Results are shown in Fig. 4G. The rate of Ba^{2+} entry was now independent of agonist concentration for all responding cells. Although less straightforward to interpret due to cytoplasmic Ca^{2+} removal processes, we also compared the rate of Ca^{2+} entry following stimulation with low and high concentrations of carbachol.

The rate of Ca^{2+} entry to 1 μM carbachol was 84 ± 9% that seen in 100 μM carbachol and the small difference was not statistically significant (p > 0.3). If each cell responds to a given agonist concentration in an all-or-none way, then the fractional response in a cell population should be equal to the number of cells that are activated. Consistent with this, whereas virtually all cells respond to 100 μM carbachol, ~40% of the cells do so to 1 μM agonist (Fig. 4F). Relative to 100 μM carbachol, 1 μM activates ERK, ePLA2, and LTC4 to similar extents (39.7, 29.5, and 41.0%, respectively; Fig. 3).

Hence, the graded relationship between agonist concentration and divalent cation influx in Fig. 4E is misleading because it represents the likelihood of a cell responding to carbachol in an all-or-none manner rather than graded cation influx into each cell.

To examine this further, we constructed a carbachol dose-response curve for I_{CRAC} activation (Fig. 4H). The relationship was supralinear, consistent with a previous report in which adenosine receptors were activated (15). Increasing agonist concentration increased the number of cells that responded to carbachol but, even

FIGURE 4. Graded responses to carbachol despite all-or-none activation of I_{CRAC}. A, Carbachol (100 μM) activates I_{CRAC}. Inset shows the current-voltage relationship taken at steady state. B, Carbachol-evoked LTC4 secretion is impaired by depolarising mitochondria with antimycin A or FCCP (both with oligomycin alone) but not by oligomycin alone. C and D, The CRAC channel blocker 2-APB suppresses arachidonic acid release (C) and LTC4 secretion (D) to a submaximal (1 μM) and maximal (100 μM) dose of carbachol. E, Ba^{2+} entry rate through CRAC channels is graded with agonist concentration when all cells are analyzed. The fraction of cells that respond to carbachol increases with agonist concentration. Carbachol was applied in Ca^{2+}-free solution and the response measured was Ca^{2+} release from the stores. G, Ba^{2+} entry rate is independent of agonist concentration (above threshold) when only those cells that responded in panel F are analyzed. H, I_{CRAC} activation is steeply related to carbachol concentration.

FIGURE 5. Muscarinic receptor stimulation evokes cytoplasmic Ca^{2+} signals and triggers LTC4 secretion in primary rat peritoneal mast cells. A, Typical response to carbachol (100 μM) from a fura 2-loaded primary rat peritoneal mast cell. Carbachol was applied in Ca^{2+}-free solution and then 2 mM Ca^{2+} was readmitted (arrow). B, Carbachol dose-response curve to LTC4 secretion from primary peritoneal mast cells. *p < 0.05 and **p < 0.01 indicate significance relative to control.
at lower agonist concentrations, if a cell responded to carbachol it did so by generating maximal I_{CRAC}. With 1 µM carbachol, 5 of 9 cells responded but for the cells that did, I_{CRAC} was -1.9 ± 0.4 pA/pF. With 100 µM carbachol, in contrast, 10 of 11 cells responded and I_{CRAC} had a similar amplitude (-2.3 ± 0.2 pA/pF; p > 0.1). No I_{CRAC} developed following application of 0.1–0.2 µM carbachol (Fig. 4H), and such concentrations failed to evoke detectable ERK activation, arachidonic acid generation, or LTC4 secretion (Fig. 3).

**Muscarinic receptor-evoked responses in acutely isolated rat peritoneal mast cells**

The preceding results were obtained using cultured mast cells, raising the question of whether the findings are of physiological relevance. To examine this, we isolated peritoneal mast cells from rats and then designed experiments to see whether varying agonist concentration activated cells in a nonlinear way.

We first explored the classical Ag-IgE-FCεRI pathway, which links into the InsP3 signaling cascade. However, responses to Ag are extremely variable in primary mast cells and even a maximal agonist concentration evokes a diverse pattern of Ca^{2+} signals. Indeed, we observed a range of responses to a fixed Ag concentration, including a transient Ca^{2+} spike, sinusoidal Ca^{2+} oscillations, and a sustained Ca^{2+} signal. Stimulation in Ca^{2+}-free solution followed by Ca^{2+} readmission failed to trigger a consistent Ca^{2+} influx signal, with some cells responding by generating Ca^{2+} oscillations, others a Ca^{2+} plateau, and some not responding to Ca^{2+} readmission at all. With such variability, we were unable to dissect out the Ca^{2+} influx component and hence we turned to other agonists.

Stimulation of muscarinic receptors with 100 µM carbachol resulted in the generation of cytoplasmic Ca^{2+} signals consisting of Ca^{2+} release followed by store-operated Ca^{2+} influx (Fig. 5A), which unlike Ag could be easily separated. Lowering agonist concentration to 10 or 1 µM evoked similar patterns of response, although the percentage of cells responding fell (70% for 1 µM carbachol compared with 99% for 100 µM agonist). Muscarinic receptor stimulation also resulted in graded LTC4 secretion (Fig. 5B). To examine the relationship between agonist concentration and cell responsiveness at the single cell level, we used protein kinase C translocation to the plasma membrane as an indicator of cell activation for two reasons. First, translocation of the kinase is a key early step for cPLA2 activation and subsequent LTC4 secretion and hence is of considerable physiological significance. Second, we

**FIGURE 6.** Stimulating muscarinic receptors in rat peritoneal mast cells activates protein kinase Ca in a nonlinear manner. A, Distribution of protein kinase Ca in control (left panel) cells and after stimulation with thapsigargin (2 µM) for 4 min. B, Aggregate data from single cell image analysis from several experiments is shown. C–F, All-points histograms plotting the extent of protein kinase Ca translocation (normalized to the mean response seen in 100 µM carbachol; called % Max. response) against the frequency of observing such a response (expressed as %). C shows the control profile, and D–F the corresponding pattern for 1, 10 and 100 µM carbachol. G, Carbachol concentration is plotted against % maximum response, with the latter reflecting the responses of all cells. H, The histogram compares signals between control cells and those exposed to 1 and 10 µM carbachol after those cells that responded by evoking protein kinase C translocation (normalized to the mean % Max. response) against the frequency of observing such a response (expressed as %).
were able to quantify protein kinase Ca movement with confidence because the mAb we used was very specific. Down-regulation of protein kinase Ca (following overnight exposure to phorbol ester) resulted in complete loss of staining (data not shown). As with RBL cells, stimulation of CRAC channels following exposure to thapsigargin resulted in robust migration of protein kinase Ca to the plasma membrane in primary peritoneal mast cells (Fig. 6A). Images were analyzed on a cell-by-cell basis and aggregate data is summarized in Fig. 6B. We then applied different concentrations of carbachol for four minutes and measured the extent of protein kinase Ca translocation. Histograms were constructed and the pattern of response between control (nonstimulated) cells (Fig. 6C) and those exposed to 1, 10, and 100 μM carbachol compared (Fig. 6, D–F). Compared with the control response profile, raising the carbachol concentration increased the frequency of observing large responses. Aggregate data from all cells is plotted as a dose-response curve in Fig. 6G, and includes cells that responded and those that did not. When all cells are included, the relationship is clearly graded. Fig. 6H plots the data in another way. In this study, we removed all those cells that responded by generating maximal protein kinase Ca translocation (>95% of response seen to 100 μM carbachol) and then averaged the remaining responses. There was no significant difference between control cells and those exposed to 1 or 10 μM carbachol. Hence, raising carbachol concentration recruited more cells but each cell that responded did so maximally.

All-or-none Ca\(^{2+}\) influx following purinoceptor activation in primary mast cells

Mast cells express P2Y receptors (24), which couple to phosphoinositide P1 turnover, which can then activate phospholipase C to produce the second messengers diacylglycerol and inositol 1,4,5-triphosphate (InsP3) (24). ATP acts as a strong agonist in mast cells, Ca\(^{2+}\)-free solution on CRAC channel activation. Following stimulation (4 min) with different concentrations of ATP in Ca\(^{2+}\)-free solution, we applied Ba\(^{2+}\) extracellularly (Fig. 7A) and measured the rate of divalent cation entry. Fig. 7B plots the rate of Ba\(^{2+}\) influx against ATP concentration for all cells exposed to agonist. The relationship is clearly graded. However, as with muscarinic receptor stimulation, not all cells responded to ATP particularly at the lower concentrations. Moreover, even if a cell responded to a low dose of ATP (like 1 μM in Fig. 7A), Ba\(^{2+}\) entry was often barely resolvable and similar to the background Ba\(^{2+}\) influx seen in the absence of agonist. We therefore plotted background-corrected Ba\(^{2+}\) entry only in those cells that generated a Ca\(^{2+}\) release signal to ATP. Aggregate data is summarized in Fig. 7C. Regardless of ATP concentration, if a cell responded it did so in an essentially all-or-none manner.

The overall extent and kinetics of the Ca\(^{2+}\) signal is sculpted both by Ca\(^{2+}\) release/influx into and Ca\(^{2+}\) removal from the cytoplasm. To see whether different concentrations of ATP affected the rate of removal of Ca\(^{2+}\) from the cytosol, we measured the half-time (t\(_{1/2}\)) of decay of the initial Ca\(^{2+}\) transient (in Ca\(^{2+}\)-free solution). For 5 μM, 100 μM, and 1 mM ATP, t\(_{1/2}\) of decay was 26 ± 2, 24 ± 1.6, and 28 ± 1.3 s and these values were not significantly different from each other. The amplitude of the Ca\(^{2+}\) transients was similar for the different concentrations. Hence, Ca\(^{2+}\) clearance is not altered by ATP over the range 5 μM - 1 mM.

We repeatedly failed to see clear translocation of protein kinase Ca to the plasma membrane over a range of ATP concentrations (data not shown). We attribute this to the transient production of diacylglycerol, due to rapid receptor desensitization. Consistent with this, we did not observe a detectable increase in LTC\(_4\) production even at an ATP concentration of 100 μM (data not shown).

Discussion

In immune cells, a major route for Ca\(^{2+}\) influx is provided by CRAC channels (18, 25). In mast cells, CRAC channel-dependent Ca\(^{2+}\) influx triggers exocytosis (8) as well as generation of the proinflammatory molecule LTC\(_4\) (13). In T lymphocytes, the importance of CRAC channels is underscored by the development of a severe combined immunodeficiency in patients with a mutation in Orai1 (26), a protein that contributes to formation of the CRAC channel pore (26–29). In this study, we have found that stimulation of two different cell surface receptors in mast cells evokes all-or-none CRAC channel activation. Despite this, graded Ca\(^{2+}\)-dependent stimulation of ERK, cPLA\(_2\), and LTC\(_4\) secretion was obtained in populations of RBL and primary mast cells. This phenomenon was resolved by the finding that responses were graded with stimulus intensity because increasing agonist concentration recruited more cells in the population but each cell that responded did so by generating maximal I\(_{CRAC}\) and Ca\(^{2+}\) influx. Whereas virtually all cells responded to 100 μM carbachol by developing a cytoplasmic Ca\(^{2+}\) rise and protein kinase Ca translocation to the plasma membrane, ~40% of the cells did so to 1 μM agonist. This proportion correlated well with the functional responses: relative to 100 μM carbachol, 1 μM carbachol activated ERK, cPLA\(_2\), and LTC\(_4\) to similar extents (39.7, 29.5, and 41.0%, respectively).

Activation of protein kinase Ca and ERK, which are critical for LTC\(_4\) production in mast cells, are largely independent of even large rises in global cytoplasmic Ca\(^{2+}\) (data not shown; W. C. Chang, 2007).
C. Nelson, J. Di Capite, V. Halse, A. Parekh, submitted), instead being tightly coupled to local Ca\(^{2+}\) influx through CRAC channels (12). Activation of the protein kinase C/ERK/cPLA\(_2\) cascade is therefore accomplished primarily through a rise in subplasmalemmal Ca\(^{2+}\) concentration. Because the main determinant of subplasmalemmal Ca\(^{2+}\) concentration is CRAC channel activity, which develops in an all-or-none manner macroscopically, then here would not necessarily result in all-or-none generation of all plasmalemmal Ca\(^{2+}\) (12). Activation of the protein kinase C/ERK/cPLA\(_2\) cascade is coexpressed with CRAC channels and gated in a graded manner. This contributes to responses in other immune cells? Several studies have reported all-or-none Ca\(^{2+}\) responses in lymphocytes. Interaction of single helper T cells with their physiological ligand, antigenic peptide bound to MHC molecules on APCs, resulted in all-or-none Ca\(^{2+}\) responses and this was independent of Ag concentration (30). As is the case with our results from mast cells, increasing Ag concentration recruited more T cells in the population to respond, but each cell retained its all-or-none Ca\(^{2+}\) response. Cytoplasmic Ca\(^{2+}\) responses to Ag in CTLs are also thought to be an all-or-none process (31). Naive CD\(^{4}\) T cells generated all-or-none Ca\(^{2+}\) responses following activation by B cells (32). Ca\(^{2+}\)-dependent T cell proliferation in response to IL-2 is thought to be an all-or-none process (33, 34). Similarly, phorbol ester-induced activation of the MAP kinase JNK is an all-or-none event (35). Low concentrations of phorbol ester evoke full activation of JNK in a fraction of Jurkat T lymphocytes but no activation at all in the rest of the population. Similarly, all-or-none Ca\(^{2+}\) responses have been observed in neutrophils following stimulation with complement C5a (36) or insoluble immune complex (37).

Despite all-or-none activation of CRAC channels, cells have the ability to produce graded Ca\(^{2+}\) responses. This can be accomplished by varying the membrane potential and therefore electrical driving force for Ca\(^{2+}\) influx or by subsequent inactivation of the CRAC channels by a variety of intracellular signals (reviewed in Ref. 2). Alternatively, nonstore-operated Ca\(^{2+}\) channels could be coexpressed with CRAC channels and gated in a graded manner. In addition to store-operated Ca\(^{2+}\) influx, neutrophils, T and B lymphocytes all express nonstore-operated plasmalemmal Ca\(^{2+}\)-permeable pathways including Ca\(^{2+}\)-activated Ca\(^{2+}\)-permeable nonselective channels (38), TRPM2 (39), TRPV6 (40), and InsP\(_3\)-gated channels (41). Finally, the cytoplasmatic Ca\(^{2+}\) signal will be shaped not only by the rate of Ca\(^{2+}\) entry through CRAC channels but also the rate of Ca\(^{2+}\) removal from the cytoplasm. The latter reflects the concerted actions of plasma membrane transporters, mitochondrial Ca\(^{2+}\) uptake through the uniporter and sequestration into the stores by SERCA pumps (1), all of which are subject to regulation by intracellular signals (42).

In one previous study on human lung-derived mast cells, a low dose of Ag evoked a smaller cytoplasmatic Ca\(^{2+}\) rise than a 20-fold higher one (43). It was suggested that Ca\(^{2+}\) signals were thus graded with stimulus intensity rather than being all-or-none events. In that study however, the relative contributions of Ca\(^{2+}\) release and Ca\(^{2+}\) influx to the overall Ca\(^{2+}\) signal were not dissected out. Because low levels of stimulus intensity can trigger some Ca\(^{2+}\) release without subsequent store-operated Ca\(^{2+}\) influx (2, 15), the submaximal concentration of agonist used (43) might not have generated enough InsP\(_3\) to ensure stores emptied sufficiently for CRAC channels to activate. Indeed, this was the case with a low concentration of ATP (1 \(\mu\)M) in our experiments (Fig. 7A). Despite clear Ca\(^{2+}\) release, cation entry did not occur. Ca\(^{2+}\) signals to ATP were graded with stimulus intensity because 1 \(\mu\)M ATP evoked only Ca\(^{2+}\) release whereas the higher concentrations triggered both Ca\(^{2+}\) release and influx.

What might be the relevance of our findings to mast cell function? Mast cells are known to secrete strongly and can release their entire secretory contents upon stimulation. Hide et al. (44) have found that ionomycin, a potent activator of CRAC channels through its ability to empty stores rapidly (4), triggers all-or-none degranulation in primary rat mast cells. From a physiological perspective, such a process seems essential because mast cells are generally found in tissues in small numbers and hence need to secrete extensively to affect their immediate environment.

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References


