Reversible Desensitization of Inositol Trisphosphate-induced Calcium Release Provides a Mechanism for Repetitive Calcium Spikes

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Repetitive transient increases in cytosolic calcium concentration (calcium spikes or calcium oscillations) are a common mode of signal transduction in receptor-mediated cell activation. Repetitive calcium spikes are initiated by phospholipase C-mediated production of inositol 1,4,5-trisphosphate (InsP$_3$) and are thought to be generated by a positive feedback mechanism in which calcium potentiates its own release, a negative feedback mechanism by which calcium release is terminated, and a slow recovery process that defines the time interval between calcium spikes. The molecular mechanisms that terminate each calcium spike and define the spike frequency are not yet known. Here we show, in intact rat basophilic leukemia cells, that calcium responses induced by InsP$_3$ are diminished for a period of 30–60 s following an InsP$_3$-induced calcium spike. The sensitivity of calcium release for InsP$_3$ was probed by UV laser-mediated photorelease of InsP$_3$ and calcium responses were monitored by fluorescence calcium imaging. A maximal loss in sensitivity (desensitization) was observed for InsP$_3$ increases that resulted in a near maximal calcium spike and was expressed as an 80–100% reduction in the calcium response to an equal amount of InsP$_3$, released 10 s after the first UV pulse. When the amount of released InsP$_3$ in the second pulse was increased 2-3-fold, desensitization was overcome and a second calcium response of equal amplitude to the first was produced. A power dependence of 3.2 was measured between the amount of released InsP$_3$ and the amplitude of the triggered calcium response, explaining how a small decrease in InsP$_3$ sensitivity can lead to a nearly complete reduction in the calcium response. Desensitization was abolished by the addition of the calcium buffers BAPTA and EGTA and could be induced by microinjection of calcium, suggesting that it is a calcium-dependent process. Half-maximal desensitization was observed at a free calcium concentration of 290 nM and increased with a power of 3.7 with peak calcium concentration. These studies suggest that reversible desensitization of InsP$_3$-induced calcium release serves as a "saw-tooth" parameter that controls the termination of each spike and the frequency of calcium spikes.

Receptor-mediated activation of the phosphoinositide path-

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constants for half-maximal recovery after calcium-induced inactivation are 1 s in brain microsomes (9), 3 s in Xenopus oocytes (17), and 6 s in hepatocytes (18). These recovery times are significantly shorter than the time intervals between calcium spikes observed in RBL cells, hepatocytes, and many other cell types that were typically between 10 s and several minutes. Interestingly, one study in permeabilized hepatocytes reported that inactivation of the InsP3 receptor is mediated by phosphorylation and dephosphorylation (20) and all or none calcium release from multiple calcium stores with different sensitivities for InsP3 (21–23). It is conceivable that some of the proposed mechanisms of spike termination are cell-type specific and are caused by different ratios of calcium pumps and calcium channels, by the presence of different isoforms of InsP3 receptors, or by different regulatory proteins associated with InsP3 receptors. Alternatively, some of the previous studies in microsomes and permeabilized cells may not represent physiologically relevant conditions.

We used repetitive UV laser-mediated photorelease of InsP3 in intact RBL cells to investigate the termination of calcium spikes and the generation of interspike intervals. These tumor mast cells are known to exhibit repetitive calcium spikes following antigen stimulation with variable spike amplitudes that range from 200 to 700 nM. For stimulation with low doses of antigen, the time interval between spikes is also variable and typically ranges between 10 s and 1 min. We now show that calcium spikes with amplitudes as low as 200 nM lead to a gradual reduction in the sensitivity of calcium stores for InsP3. The time course for recovery from this desensitization is on the same time scale as the observed interspike interval during calcium spiking, suggesting that desensitization of InsP3-induced calcium release is a mechanism that contributes not only to the termination of individual calcium spikes but also to the setting of the interspike intervals during repetitive calcium responses.

### MATERIALS AND METHODS

#### Cell Culturing and Electroporation—

Rat basophilic leukemia cells (2H3 type, a tumor mast cell line) were grown in Dulbecco’s modified Eagles medium with 20% fetal bovine serum (LifeSciences, Inc.), 1 mM L-glutamine, 200 units of penicillin, and 0.2 mg/ml streptomycin (Sigma) at 37 °C and 5% CO2. Cells were plated on glass coverslips at least 3 h before each experiment. Adherent cells were washed twice with an extracellular buffer (135 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, 1.5 mM CaCl2, 1.5 mM MgCl2) followed by a wash with the same buffer lacking calcium and Mg2+ (electroporation buffer). A self-built field electroporation device was used for cell loading. Electroporation was typically performed for a field strength of 300 V/cm applied for a 10–50 ms period. For most experiments, the calcium indicator Fluo-3 (2 mM) and the caged InsP3 (4–8 mM) were used in the electroporation buffer. In some experiments, BAPTA or EGTA (50 mM) was added to this mixture. The applied field transiently permeabilized the cells, leading to the exchange of approximately 1% of the cytosolic volume with the extracellular applied sample as tested with fluorescent markers. After loading, cells were washed five times with extracellular buffer containing sulfonpyrazone (250 μM, to minimize the loss of indicator from the cell) and left for at least 5 min at 37 °C to recover.

#### Single Cell Calcium Measurement—

The calcium indicator Fluo-3 was excited at 488 nm, using an inverted Nikon Diaphot microscope and an Odyssey confocal imaging system (Noran Instruments Inc., WI). Fluorescence intensity traces from individual cells were obtained by monitoring the average overall intensity of the cell each 30–100 ms by using the Image-1 software (Universal Imaging Corp., West Chester, PA). UV Laser-mediated Photorelease of InsP3—Transient increases in InsP3 concentration were induced by computer-controlled UV laser pulses of different intensity and duration (3–1000 ms) (using a computer-controlled Uniblitz shutter). The UV light released active InsP3 from an inactive photoreleaseable InsP3 precursor. The UV laser was coupled through the fluorescence port of the microscope using a dichroic mirror, and the beam was expanded with a lens to a spot size of ~15 μm in the focal plane. The expansion of the UV laser beam enabled the illumination of the entire cell volume and a uniform photorelease of InsP3 across the cell.

Calibration of the Free Calcium Concentration—An apparent free calcium concentration was calculated from the equation [calcium] = Kd(Fm – F(t))/(Fm – F(0)), where F(t) is the fluorescence intensity of Fluo-3 and Fm was measured as the fluorescence intensity after the addition of ionomycin (~1 μM) in the presence of 1.5 mM extracellular calcium. Fm, was calculated as 0.05% Fm. The background autofluorescence was determined by measuring the fluorescence of a cell that was not loaded with indicator and was subtracted from Fm, and Fm,.

Piezoelectric Calcium Injection—Transient calcium increases in the cell were induced by direct microinjection of CaCl2 (2–10 mM). Cells were microinjected using a computer-controlled piezoelectric actuator (Physik Instrument, Germany) that can move a sharp glass pipette rapidly into and out of the cell. The distance of the pipette movement and the time it stayed inside the cell were adjustable (typically a 10-μm pipette movement was used together with a 30–60 ms injection time). A variable back pressure of 1–5 p.s.i. was used to avoid clogging of the pipette and to guarantee sufficient calcium injection.

#### RESULTS AND DISCUSSION

Reversible Down-regulation of InsP3-induced Calcium Responses—The mechanism of repetitive calcium spiking was investigated in adherent RBL cells loaded with the calcium indicator Fluo-3 and UV photoreleaseable InsP3, caged InsP3 (24). Calcium transients were triggered by photorelease of InsP3 using UV laser pulses of variable energy (adjusted by changing the pulse duration). Each UV pulse converted a small fraction of the inactive caged InsP3 into active InsP3, which then released calcium from intracellular stores. The increase in InsP3 concentration was transient since the half-life of InsP3 in the cytosol has been estimated to be on the time scale of seconds (11, 25). Calcium responses of stimulated cells were recorded using a brightness versus time function of the imaging software with a time resolution of 30 or 100 ms.

Fig. 1A shows an example of a series of UV pulses of equal energy (e) that were 10–15 s apart (a typical example of 16 repetitions is shown). A strong down-regulation of the calcium response could be observed for the second UV pulse triggered 10 s after the first one. Subsequent UV pulses showed a gradual recovery of the calcium response. The 100 times stronger UV pulse at the end of the protocol was used to determine the maximal amount of InsP3-releasable calcium (typically 400–700 nM). In the example shown, the calcium response to the second UV pulse was 3% of the first one and recovered to 45% of its original amplitude after 50 s. The incomplete recovery of the calcium response may be due to degradation of caged InsP3 by enzymatic hydrolysis and photorelease, toxicity caused by the released nitrophenyl group, persistent InsP3 receptor inactivation, depletion of internal calcium stores, or to pumping of calcium outside of the cell. To reduce these potential problems, protocols with fewer UV pulses were used in subsequent experiments.

The down-regulation of the calcium response was examined by varying the UV pulse energy. A series of UV pulses of identical energy were made 10, 30, 70, and 150 s after a first UV pulse (Fig. 1, B and C). Interestingly, UV pulses that triggered small calcium responses did not lead to down-regulation (n = 14; Fig. 1B). When the UV pulse energy was increased, the initial calcium responses became larger and down-regulation of the following responses became more pronounced.
Down-regulation of InsP₃-induced calcium responses in intact cells. Traces of the fluorescence intensity of individual RBL cells loaded with the calcium indicator Fluo-3 and photoreleasable InsP₃, are shown as a function of time. A, a series of UV pulses of identical energies (e) were made every 10–15 s (e was used as a relative energy unit that was different for each cell). The initial calcium response induced a down-regulation of InsP₃-mediated calcium release that can be observed in the smaller response to subsequent UV pulses. InsP₃-induced calcium response gradually recovered over time. A 100 times stronger UV pulse (100e) was used at the end to determine the amount of stored calcium that could be released by InsP₃. B, a protocol with less frequent UV pulses than in A was used. Identical pulses were made at 10, 30, 70, and 150 s following a first pulse. No measurable desensitization of InsP₃-induced calcium release was observed when calcium responses were smaller than 30% of the maximal amplitude (measured by the 100e pulse). C, the same protocol as in B showed that maximal desensitization of InsP₃-induced calcium release could be observed when the initial calcium response was nearly maximal (estimated by the 100e pulse). The amplitude of the second calcium response was then 20% and 20% of the original amplitude, and peak amplitude, of subsequent pulses typically recovered to 50–80% of its initial value.

Maximal down-regulation could be observed following UV pulses that produced a calcium response that was near its maximal amplitude (n = 23; Fig. 1C). For this optimal UV pulse energy, the amplitude of the second calcium response was reduced by 80–100%. When the UV pulse energy in such a protocol was further increased, the second calcium response became larger while the amplitude of the first response remained nearly the same (data not shown). Thus, maximal down-regulation of calcium release can only be observed in a narrow range of InsP₃ and calcium concentration.

Down-regulation of InsP₃-induced Calcium Release Can Be Explained by a Lowering in InsP₃ Sensitivity—The observation that down-regulation was less prominent for pairs of supra-maximal InsP₃ pulses suggested that down-regulation is more likely a desensitization than an inactivation mechanism. Supporting evidence for a desensitization mechanism was obtained from two different protocols using pairs of UV pulses. In the first protocol, the energy of the initial UV pulse was chosen to give maximal down-regulation, and a second pulse of equivalent or higher energy was triggered 10 s later. Fig. 2A shows three pairs of pulses from different cells in which the second UV pulse had one, two, and three times more energy than the first one. The ratio of the amplitude of the second calcium response to the first one was then plotted as a function of the relative increase in UV energy of the second pulse (Fig. 2B). Using this protocol, we found that down-regulation can be described as a shift in InsP₃ sensitivity that can be overcome in all cells by the release of two to three times more InsP₃ (n = 63). In the case of a typical inactivation mechanism, one would have expected that down-regulation could not be overcome by higher InsP₃ concentrations.

A second protocol investigated the down-regulation mechanism by comparing the rise times of calcium spikes in paired pulse protocols. A first pulse that induced maximal desensitization was followed 10 or 60 s later by a seven times stronger pulse (Fig. 2C and D). In these protocols, the amplitudes of the first and second calcium responses were nearly the same. If inactivation of the InsP₃ receptor would be responsible for the observed down-regulation, fewer InsP₃ channels should be opened at saturating InsP₃ concentration, and a slower rate of calcium release should be observed during down-regulation. A half-rise time, τ₁⁄₂, was defined as the time required for calcium to increase between 10 and 60% of its peak amplitude and was chosen as an indirect measure of the number of open calcium channels during the calcium rise. We found that the τ₁⁄₂ for the first calcium spike in each pair of pulses ranged from 50 to 400 ms. Independent of this cell to cell variability, the τ₁⁄₂ for the second UV pulse was nearly identical to the first one for both time intervals, τ₁⁄₂UV/τ₁⁄₂V = 0.75 ± 0.4 for the 10-s delay (n = 11, example shown in Fig. 2C) and τ₁⁄₂UV/τ₁⁄₂V = 1.2 ± 0.4 for the 60-s delay (n = 11, example shown in Fig. 2D). In the case of an inactivation mechanism, one would have expected this ratio to be much smaller for the 10-s delay when compared with the 60-s delay. Thus, the observed nearly identical rise times in calcium concentration before and after down-regulation further suggest that desensitization and not inactivation explain the down-regulation of InsP₃-induced calcium release. This analysis also excludes another possible mechanism for down-regulation. The nearly maximal rate of calcium release observed for the initial UV pulse suggests that most InsP₃ receptors were already opened during the first pulse that induced maximal desensitization. This observation makes it unlikely that the first pulse would lead to the inactivation of high affinity InsP₃ receptors and that the second pulse, generating a higher InsP₃ concentration, would release calcium from subpopulations of InsP₃ receptors with lower affinities.

Power Dependence of InsP₃-mediated Calcium Release—How can a shift in InsP₃ sensitivity by a factor of 2–3 in the desensitized state reduce calcium responses by up to 100%? We addressed this question by measuring peak amplitudes and calcium release rates in response to a series of UV pulses with increasing energy. Fig. 3A shows a typical fluorescence intensity trace used for the analysis. The amplitude of each calcium response was converted to an approximate free calcium concentration using ionomycin at the end of the experiment. The free calcium concentration was then graphed as a function of the relative amount of released InsP₃ (Fig. 3B). An approximate power dependence between the relative amount of InsP₃ produced, which is proportional to the relative pulse energy (e/e₀), and the relative increase in calcium concentration (ΔCa) was determined using a simplified allosteric equation,

\[
\Delta Ca = \Delta Ca_0 (e/e_0)^{a/m + a/m}
\]

(Eq. 1)
where $\Delta C_{a_n}$ was determined as the maximal relative calcium amplitude and $a_n$ and $m$ were determined from a root mean square fit. The best fit in Fig. 3B was obtained for a power dependence of $m = 4$, suggesting that calcium release is tightly controlled by the cytosolic InsP$_3$ concentration. Averaged over six similar experiments, the optimal power dependence was $m = 3.2 \pm 1.3$. On the same graph, the initial rate of release, measured as the slope between 10 and 60% of the amplitude of the calcium response, was plotted as a function of the relative amount of InsP$_3$ released for each response. Analysis using an equation similar to Equation 1 demonstrated that the power dependence was $m = 2.6 \pm 0.5$ (average of five experiments, $m = 3$ in the graph shown). The molecular basis for the observed nonlinear dependence shown in Fig. 3B is likely mediated by a requirement for the binding of several InsP$_3$ molecules to the InsP$_3$ receptor before the channel can open (12) and by an amplification of calcium release by calcium-mediated positive feedback (7–9). Thus, a strong suppression of the InsP$_3$-mediated calcium responses can be fully explained by a small shift in InsP$_3$ sensitivity combined with a steep power dependence between InsP$_3$ and calcium released.

Significance of InsP$_3$ Degradation for the Termination of InsP$_3$-Induced Calcium Spikes—The experimental protocols used in Figs. 1 and 2 do not reveal whether the rapid decline of calcium levels after each UV pulse is the result of the degradation of InsP$_3$ or of a down-regulation of InsP$_3$-mediated calcium release. To address this question, we loaded cells with the photoreleasable caged InsP$_3$ analog, caged GIP2. Compared with InsP$_3$, GIP2 has been shown to be degraded 10 times slower inside the cell (26). Fig. 4A shows a typical experiment in which a UV pulse was used to photorelease GIP2. The time constant for calcium reuptake ($t_r$) was measured as the time during which calcium was lowered between 75 and 25% of the maximal amplitude. Following the photorelease of GIP2, $t_r$ was $2.95 \pm 0.64$ s ($n = 18$), which was in the same range as $t_r$ measured for InsP$_3$ ($t_r = 2.52 \pm 0.51$ s, $n = 16$) or antigen-mediated calcium spikes ($t_r = 2.5 \pm 0.38$ s, $n = 20$). This suggests that degradation of InsP$_3$ did not have a major contribution in the rapid return to the basal calcium concentration observed in previous experiments with caged InsP$_3$. Furthermore, the observed termination of calcium spikes in the presence of GIP2 provides evidence that calcium concentration can return to base line in the presence of an elevated InsP$_3$ concentration.

**Fig. 2.** Experimental evidence that desensitization and not inactivation accounts for the down-regulation of InsP$_3$-mediated calcium release. A and B, down-regulation of the InsP$_3$-mediated calcium response can be overcome by increasing the InsP$_3$ concentration. For the three pairs of UV pulses shown in A, the duration of the second pulse was, respectively, one, two, and three times longer than the first pulse. B, plot of the ratio of the two amplitudes in each pair of pulses versus the ratio of the UV pulse energies that generated them. Approximately three times as much InsP$_3$ was needed for a second pulse to fully overcome a maximal desensitization induced by a first pulse. C and D, analysis of the rise times of the calcium signals during and after down-regulation. A second UV pulse with a 7-fold higher energy followed the initial UV pulse. The initial pulse was chosen to generate a maximal calcium response. In C, the second pulse was made in the down-regulated state, about 10 s after the first one. In the example shown, the rise times of the two calcium responses (the time required for a calcium increase from 10 to 60% of its amplitude) were nearly the same, 95 and 120 ms, respectively (see text for further analysis). In D, the second pulse was made after recovery from down-regulation, about 60 s after the first one. In the example shown, the rise times for the two calcium responses were 90 and 75 ms, respectively. These results suggest that the maximal rate of InsP$_3$-mediated calcium release is not significantly affected by the down-regulation.

Time Course for Recovery from Desensitization—Can reversible desensitization of InsP$_3$-induced calcium release define the calcium spike frequency? The time interval between calcium spikes in RBL cells was found to be between 10 s and >1 min following activation by low doses of antigen (data not shown). The protocols used in Fig. 1, A and C, have shown that the time needed for full recovery from desensitization is variable between cells and is typically longer than 30 s. We measured the half-time for recovery, $\Delta t$, more accurately by using a protocol with three identical UV pulses ($n = 59$; Fig. 4B). The first pulse was chosen to induce maximal desensitization, a second pulse of equal energy was triggered after a variable delay time, $\Delta t$, and a third identical pulse was used to measure recovery from desensitization (triggered 120 s after the second one). The desensitization was measured as the ratio of the amplitudes of the second to the third calcium response, $A_2/A_3$, and is shown in Image 1.
Reversible Desensitization of InsP3-Mediated Calcium Release

**Fig. 3. Measurement of the InsP3 sensitivity of calcium stores.**

A and B, a series of calcium responses with increasing amplitudes was generated by increasing the amount of InsP3, photoreleased by each UV pulse. The examples in C show six pulses with energies of ε, 1.3ε, 2ε, 2.7ε, 4ε, and 6.7ε. The amplitude of the response to ionomycin was used to approximately calibrate the peak free calcium concentration. D, shows an analysis of the peak calcium concentration and of the initial rate of calcium release as a function of the relative amount of InsP3 released (which is proportional to the relative UV pulse energy). The initial rate of release was measured for each calcium response as the slope between 10 and 60% of its maximal amplitude. The resulting plots were fit by Equation 1 (see "Results and Discussion").

Fig. 4C as a function of the delay time, Δt. Each point on the graph was calculated as the average of 8–16 experiments. Ratios larger than 1 were reached since the amount of caged InsP3 in the cell was reduced following the 120-s period between the second and third UV pulse. Recovery from desensitization was strikingly nonlinear, and half-maximal recovery was reached after approximately 30 s. The large size of the error bars is due to significant cell to cell variability in the recovery time. The nonexponential increase in the amplitudes of the test pulses during the recovery process was fit by the equation,

\[ A_2/A_3 = \alpha \times (\Delta t)^m \times \left(1 - (\Delta t)^n \right)^m \]  

(Eq. 2)

where \( \alpha \) was chosen as the averaged maximal ratio \( A_2/A_3 \). The best fit for the graph in Fig. 3C was for \( m = 4 \) and \( \Delta t_{50} = 32 \) s. It should be noted that the underlying recovery process may actually be linear with time and that the nonlinear dependence observed in this graph may be the result of the steep InsP3 sensitivity observed in Fig. 2D. The slow recovery process is consistent with the hypothesis that desensitization of InsP3-induced calcium release leads to a delay in the triggering of subsequent calcium transients during calcium spiking.

Is Desensitization a Calcium or an InsP3-mediated Process?—Our previous studies induced desensitization by simultaneously raising InsP3 and calcium concentrations. To investigate whether calcium is necessary for desensitization, we co-electroporated caged InsP3, Fluo-3, and the calcium buffers EGTA and BAPTA into cells. While calcium buffers at 1 mM and higher totally abolished calcium responses to InsP3, in the presence of lower concentrations of calcium buffers (50–200 μM), calcium transients could still be triggered by higher InsP3 concentrations, but the induced calcium transients were relatively small and significantly broader. In this range of concentrations of calcium buffers, no desensitization of InsP3-mediated calcium release could be observed (Fig. 5A, \( n = 8 \) for BAPTA and \( n = 15 \) for EGTA). This suggests that desensitization of InsP3-induced calcium release requires free cytosolic calcium concentration to increase above a critical threshold.

To determine if an increase in InsP3 is required for desensitization, a transient increase in free calcium concentration was generated by microinjection of calcium using a piezoelectric actuator. Before each calcium injection, the optimal UV pulse energy for desensitization was determined in a 2-pulse protocol. The calcium was injected at least 70 s after the test pulses, and the sensitivity of calcium stores to InsP3 was immediately probed by a series of UV pulses of equal energy (\( n = 31 \); Fig. 5B). In the trace shown, three UV pulses were made at 10, 30, and 70 s after the injection. Desensitization was observed fol-
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**Fig. 5.** Desensitization can be induced by an increase in calcium concentration alone. **A,** the importance of the peak calcium amplitude for desensitization was tested by introducing additional calcium buffers into the cytosol. Cells were loaded with caged InsP\(_3\), Fluo-3, and with either one of the calcium buffers BAPTA or EGTA (resulting in 50–200 \(\mu\)M calcium buffer inside the cell). At this concentration of calcium buffers, calcium responses to InsP\(_3\) could still be observed but were significantly attenuated and broadened. To test whether desensitization can occur in cells loaded with calcium buffers, a protocol with repetitive UV pulses, similar to the protocol in Fig. 1B, was used. Buffering of calcium by 50–200 \(\mu\)M BAPTA or EGTA was sufficient to abolish the desensitization of InsP\(_3\)-mediated calcium responses. **B,** the importance of InsP\(_3\) for desensitization was tested by raising the intracellular calcium concentration without increasing the InsP\(_3\)-concentration. At the beginning of the protocol, two identical UV pulses were made to determine the fluorescence amplitude needed for maximal desensitization. More than 60 s later, the cell was injected for a variable time period (typically 30–50 ms) using a piezoelectric actuator that controlled a glass micropipette filled with a 10 mM calcium solution. The calcium transient generated by microinjection of calcium had a similar amplitude and duration as the initial InsP\(_3\)-mediated calcium response. The calcium injection was followed by a series of UV pulses with the same energy as the initial one. As shown by the subsequent calcium responses, desensitization can be induced by microinjection of calcium into the cytosol without requiring an increase in InsP\(_3\) concentration.

![Diagram](image)

The calcium injection was followed by a series of UV pulses showing that an increase in calcium concentration is sufficient to induce desensitization at basal InsP\(_3\) concentration. It should be noted, however, that desensitization induced by calcium injection was slightly smaller than that following an InsP\(_3\)-mediated calcium transient. In a direct comparison, InsP\(_3\)-mediated calcium responses triggered 10–20 s after either a calcium injection or an InsP\(_3\)-mediated calcium response were, respectively, 34 ± 16% (n = 20) and 24 ± 16% (n = 23) of the maximally recovered calcium responses. This does not exclude the possibility that InsP\(_3\) can act as a co-factor for desensitization. It should also be noted that these experiments do not exclude the possibility that the increase in calcium leads to a direct increase in InsP\(_3\) concentration by activation of phospholipase C. However, such a mechanism is not very likely since calcium appears to act as a co-factor on already activated phospholipase C and much less as a direct activator of the enzyme (27).

**Dependence of Desensitization on the Peak Calcium Concentration**—We investigated the dependence of desensitization on the peak calcium concentration using a paired UV pulse protocol. The two UV pulses had the same energy and were 12 s apart. Several such pairs with increasing UV pulse energy were made in the same cell (Fig. 6A). As the energies of the two UV pulses in each pair were increased, the amplitude of the second calcium response in each pair decreased when compared with the first one. The protocol was stopped when maximal desensitization was observed. Ionomycin was added at the end of the protocol to calculate the approximate free calcium concentrations. Desensitization was measured as the ratio between the amplitude of the second calcium response (A\(_2\)) and the first one (A\(_1\)), and the ratio A\(_2\)/A\(_1\) was plotted against the calculated peak calcium concentration for each first pulse (n = 7; Fig. 6B). The results were fit to the equation,

\[
\frac{A_2}{A_1} = \alpha \times K_2^{m_2} / ([Ca]^{m_1} + K_{12}^{m_2})
\]

(Eq. 3)

with \(\alpha\) determined as the maximal ratio A\(_2\)/A\(_1\). Half-maximal desensitization occurred at 281 ± 98 \(nM\) peak calcium concentration and had an average power dependence that ranged between 2 and 6 in different experiments (average of m = 3.7 ± 1.5). Desensitization of InsP\(_3\)-induced calcium release is therefore steeply dependent on calcium concentration and occurs within the range of peak calcium concentration observed during antigen-mediated calcium spiking.

**Evaluation of Potential Molecular Mechanisms for Desensitization**—The observed mechanism of desensitization cannot be explained by the previously described calcium-mediated inactivation of the InsP\(_3\) receptor in brain microsomes and Xenopus oocytes. The latter down-regulation mechanism recovers more rapidly (<3 s) and involves a reduction in the opening probability of the InsP\(_3\) receptor. In contrast, the mechanism of down-regulation observed here has a much longer time constant than that of calcium-mediated inactivation and can be explained by a lowering in InsP\(_3\) binding affinity. We investigated several possible mechanisms by which calcium could mediate desensitization. It has been shown that InsP\(_3\) receptors from brain are phosphorylated in vitro by protein kinase A, protein kinase C, and calcium/calcmodulin-dependent protein kinase II (28). Among these three kinases, only the last two are activated by calcium. A recent report (20) used purified InsP\(_3\) receptor from rat cerebellum and showed that calcineurin, a calcium/calcmodulin-dependent phosphatase, is physically associated with the InsP\(_3\) receptor-FKBP12 complex and is able to modulate the phosphorylation state and calcium flux properties of the type I InsP\(_3\) receptor. They proposed that calcineurin, anchored to InsP\(_3\) receptor via FKBP12, dephosphorylates the site on the InsP\(_3\) receptor that was previously phosphorylated by protein kinase C. In the dephosphorylated state, the calcium flux through the InsP\(_3\) receptor was found to be reduced. Because calcium/calcmodulin is an activator of calmodulin kinase II, as well as of calcineurin, we tested the significance of calmodulin for calcium-mediated desensitization by application of the calmodulin inhibitor calmidazolium (29) to the bath (~10 \(\mu\)M, data not shown). Using the same protocol as shown in Fig. 1C, desensitization was not altered by
chondrial calcium uptake by carbonyl cyanide p-chlorophenylhydrazine (1 μM, n = 6) and antimycin A (0.1 μM, n = 5) also had no effect on desensitization. In addition, we addressed whether the activity of calcium pumps may be up-regulated for a sustained time period after each calcium spike. Addition of the calcium pump blocker thapsigargin allowed us to measure the leak rate of calcium. A sustained up-regulation of the calcium pump would be reflected in a higher leak rate after a desensitizing pulse. However, we found that, following thapsigargin addition, the rate of calcium leakage out of the stores was nearly the same before and after the desensitizing pulse and was 30–100 times slower than the rate of calcium release generated by the photorelease of InsP₃. In conclusion, desensitization of InsP₃-induced calcium release has to be mediated by a mechanism other than the prolonged up-regulation of calcium pump activity.

How Does the Desensitization of InsP₃-mediated Calcium Release Relate to the Previously Observed Incremental InsP₃ Sensitivity of Calcium Stores or Quantal Calcium Release? Previous studies in permeabilized cells have shown that submaximal increases in InsP₃ concentration lead to a biphasic calcium release (30–32). This biphasic release can be repeated by successive incremental increases in InsP₃ concentration (31). This phenomenon has been explained by either the presence of InsP₃ receptors with different affinities, by a possible desensitization mechanism, or by all or none calcium release from clusters of InsP₃ receptors. We believe that the termination of calcium release for submaximal InsP₃ pulses observed in Fig. 3 is, in part, the result of such an incremental InsP₃ sensitivity of calcium stores. However, it is not likely that the incremental sensitivity of InsP₃ receptors relates to the desensitization mechanism observed here since desensitization is only observed for InsP₃ pulses that generate nearly maximal calcium responses. In contrast, incremental sensitivity in permeabilized cells could be best observed for very small InsP₃ concentrations and submaximal calcium release rates. Furthermore, incremental InsP₃ sensitivity appears to be largely independent of calcium concentration while desensitization requires calcium. Our earlier studies suggest that quantal calcium release is the result of the synchronized opening of small groups or clusters of InsP₃ receptors (14) and is not directly related to the desensitization mechanism observed here.

Implications for the Mechanism of Calcium Spiking in RBL Cells—Our experiments present evidence for the existence of a reversible desensitization mechanism of InsP₃-mediated calcium release. This desensitization was observed in intact cells, occurred for physiological calcium concentrations, and lasted for a time period similar to the interspike intervals observed in RBL cells. Half-maximal desensitization was induced at peak calcium concentrations of 280 nM, and half-maximal recovery occurred in 32 s. It has been argued that repetitive calcium spikes result from (i) a positive feedback mechanism that triggers calcium release as soon as a critical threshold is reached, (ii) a negative feedback mechanism by which calcium is lowered back to base line (this mechanism would also determine the peak amplitude of each calcium response), and (iii) a mechanism that generates the interspike interval by keeping the cell quiescent in the presence of elevated levels of InsP₃ (2, 16). The reversible desensitization characterized in our study can explain the latter two points.

Our model predicts that, after hormone or antigen stimulation, the InsP₃ concentration has to increase to a level at which it can occupy a significant fraction of the InsP₃ receptors in order for repetitive calcium spikes to be triggered. The increase in cytosolic calcium concentration during the rising phase of an individual spike then leads to a rapid desensitization of InsP₃-desensitizable calcium stores.

Reversible Desensitization of InsP₃-Mediated Calcium Release

Fig. 6. Desensitization increases as a function of the peak calcium concentration. A, the dependence of desensitization on the peak calcium concentration was determined using a paired UV pulse protocol. The two UV pulses in each pair had the same energy and were 12 s apart. The relative energy in each pulse pair was varied to obtain the full range of peak calcium amplitudes for the first of the two pulses. Ionomycin was added at the end of the experiment to approximately calibrate the free calcium concentration. Ionomycin was added at the end of the experiment to approximately calibrate the free calcium concentration. B, desensitization was measured as the ratio between the amplitude of the second pulse, A₂, and the first pulse, A₁, in each pair and was plotted against the calculated peak calcium concentration of the first pulse. The dependence of desensitization on the peak calcium concentration was then fit using Equation 3 (see "Results and Discussion").
induced calcium release (Fig. 7). Combined with the previously observed increase in calcium uptake into stores at elevated calcium concentration (31), the reduction in calcium efflux leads to a rapid return of the free calcium concentration back to base line. Immediately after a calcium spike, recovery from desensitization begins with a time constant of ~30 s. During the initial time for recovery, calcium efflux from stores cannot compete with reuptake, and the basal calcium level is maintained. The triggering of the next calcium spike requires a threshold number of activatable calcium channels to be reached (shown by the bottom dashed lines in Fig. 7). Our analysis of the rate of calcium release in Fig. 2, C and D, suggests that this threshold number of InsP3 receptors has to be a significant fraction of the total number of InsP3 receptors (the top dashed lines in each panel depict the maximal number of activatable calcium channels). Once a critical number of InsP3-gated calcium channels has recovered, the positive feedback, by which calcium promotes the opening of InsP3 receptors, is initiated and the next calcium spike is triggered (Fig. 7, A and B). This model can explain that low stimulus intensities trigger calcium spikes with low frequencies (Fig. 7A) and that higher stimulus intensities trigger calcium spikes with higher frequencies (Fig. 7B). At higher InsP3 concentrations (Fig. 7B), a larger fraction of the desensitized InsP3 receptors remains active and a smaller fraction of the desensitized InsP3 receptors has to recover rather than at low InsP3 concentrations. Thus, while the recovery time constant is the same in both cases, the next calcium spike is triggered earlier at an initially high rather than low InsP3 concentration. This suggests that desensitization can serve a mechanism that defines the calcium spike frequency. When the initial InsP3 concentration increases even further, most InsP3 receptors remain activatable, explaining why plateau levels of calcium responses are typically observed for maximal antigen stimulation (Fig. 7C).

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Fig. 7. Model for frequency dependence of InsP3-mediated calcium spiking. Schematic representation of calcium signals for low, medium and high receptor stimuli. The top trace in each panel shows a step increase in InsP3 concentration. The middle trace in each panel shows the resulting changes in intracellular free calcium concentration. The bottom part in each panel depicts the number of activatable InsP3 receptors/calium channels. It is assumed that stimuli that trigger calcium spikes generate a nearly maximal or supramaximal initial InsP3 concentration. A, the initial increase in InsP3 concentration triggers a calcium spike that is terminated by desensitization of the InsP3 receptor and by the pumping of calcium back into the intracellular stores. When the calcium concentration is back at base line, the subsequent calcium spike is suppressed because an insufficient number of InsP3 receptors can participate in a positive feedback for calcium release. The recovery has a time constant of ~30 s and proceeds until a critical threshold number of InsP3 receptors again become activatable by calcium. At this point, the next calcium spike is triggered. The threshold and maximal number of activatable calcium channels are shown as dashed lines in each panel. B, for higher initial InsP3 concentrations, the recovery occurs with the same time constant. However, the higher InsP3 concentration enables most desensitized InsP3 receptors to still participate in a positive feedback, and a lower number of InsP3 receptors has to recover before the next positive feedback is triggered. Thus, at an increased InsP3 concentration, calcium spikes with the same amplitude but higher frequency are triggered when compared with low InsP3 concentrations. C, if InsP3 levels increase further, most InsP3 receptors remain activatable, calcium remains elevated at a plateau level of calcium concentration, and repetitive calcium spikes cannot occur.