Luminal Calcium Regulates the Inositol Trisphosphate Receptor of Rat Basophilic Leukemia Cells at a Cytosolic Site†

John H. Horne and Tobias Meyer*

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Received March 15, 1995; Revised Manuscript Received July 3, 1995®

ABSTRACT: Hormones, growth factors, and other stimuli can generate Ca^{2+} spikes and waves by activation of the phosphoinositide (PI) pathway. The sources of these Ca^{2+} signals are inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} stores. Here we use a rapid perfusion apparatus to measure the release of $^{45}Ca^{2+}$ from permeabilized rat basophilic leukemia (RBL) cells to investigate the regulation of IP_3-mediated Ca^{2+} release by cytosolic and luminal Ca^{2+}. At 200 nM IP_3, Ca^{2+} release was potentiated by an increase in the cytosolic Ca^{2+} concentration. This potentiation by Ca^{2+} was nearly absent at 500 nM IP_3. Previous studies in smooth muscle cells and neurons showed an inhibition of Ca^{2+} release above 300 nM Ca^{2+}. In contrast, no such inhibition was observed in RBL cells. When assayed at low cytosolic Ca^{2+} concentrations, IP_3-mediated release was steeply dependent upon luminal Ca^{2+} concentration. At high luminal Ca^{2+} concentration, 1 μM IP_3 released most of the stored Ca^{2+} even in the complete absence of cytosolic Ca^{2+}. However, at high cytosolic Ca^{2+} concentrations (890 nM), IP_3-mediated release was no longer steeply dependent upon the luminal Ca^{2+} concentration. Furthermore, high concentrations of BAPTA inhibited IP_3-mediated release in the absence of cytosolic Ca^{2+}. This suggests that a rapid and local luminal Ca^{2+} feedback is generated by luminal Ca^{2+} ions binding to cytosolic sites of the same channel or closely associated channels. This "luminal Ca^{2+} feedback" can be initiated by an increase in the concentration either of IP_3, of cytosolic Ca^{2+}, or of luminal Ca^{2+}. It is likely that "luminal Ca^{2+} feedback" is exploited by cells in both the initiation and termination of Ca^{2+} spikes.

Activation of the phosphoinositide (PI) pathway is initiated by receptor-mediated tyrosine phosphorylation of phospholipase C-γ (PLC-γ) or G-protein stimulation of PLC-β. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate and generates the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol in turn activates protein kinase C, and IP_3 opens IP_3-gated Ca^{2+} channels in the endoplasmic reticulum (Berridge, 1993). The IP_3-mediated increase in intracellular Ca^{2+} concentration regulates a variety of cellular processes including restructuring of the cytoskeleton, motility, secretion, and transcription. In rat basophilic leukemia cells (RBL cells), the mast cell tissue culture line used in these studies, antigen-mediated cross-linking of IgE receptors leads to tyrosine phosphorylation of PLC-γ (Metzger et al., 1986; Eiseman & Bolen, 1992). The role of the PI pathway in mast cells is to mediate exocytosis of secretory granules, synthesis of prostaglandins and leukotrienes, and expression of cytokines (Plaut et al., 1989).

Measurements of Ca^{2+} signals in single RBL cells, hepatocytes, and many other cell types have shown that the IP_3-mediated increase in Ca^{2+} concentration is not a gradual process but rather exhibits unique spatiotemporal patterns. Ca^{2+} signaling has been shown to occur as a series of transient increases in Ca^{2+} concentration, and the frequency but not the amplitude of transients was found to increase with an increase in the concentration of hormone (Woods et al., 1986; Millard et al., 1989). This temporal pattern of intracellular Ca^{2+} transients has been referred to as Ca^{2+} spiking. Ca^{2+} spiking may offer a unique form of temporal regulation which is advantageous for several cellular processes including Cl^- transport, hormone secretion, and activation of kinases (Kasai & Augustine, 1990; Tse et al., 1993; Hanson et al., 1994). Various theoretical models have been proposed describing the regulatory processes necessary to generate Ca^{2+} spikes and Ca^{2+} waves. A common feature of these models is a positive feedback parameter in which Ca^{2+} can activate its own release, thereby generating the explosive rise in Ca^{2+} concentration observed at the beginning of a Ca^{2+} spike or a Ca^{2+} wave. Candidates for this positive feedback that have been proposed include the Ca^{2+} activation of PLC, Ca^{2+}-induced Ca^{2+} release from IP_3-insensitive Ca^{2+} stores, and the co-requirement of Ca^{2+} and IP_3 for the opening of IP_3-gated Ca^{2+} channels [for a review of different models, see Berridge (1993) and Meyer and Stryer (1991)].

IP_3 receptors show structural as well as functional similarities with the ryanodine receptor, a family of Ca^{2+} channels predominantly expressed in muscle cells and neurons. Ryanodine receptors mediate the release of Ca^{2+} from intracellular stores by a direct Ca^{2+}-induced Ca^{2+} release mechanism (Tsien & Tsien, 1990). Studies with brain microsomes and skinned smooth muscle cells have shown that the IP_3 receptor can also be regulated by Ca^{2+} (Bezprozvanny et al., 1991; Finch et al., 1991; Iino & Endo, 1992). The regulation was found to be biphasic. Increasing the free Ca^{2+} concentration up to 300 nM potentiates IP_3-

† This work was supported by a grant from the National Institute of General Medical Sciences (GM48113), and by a Fellowship of the David and Lucile Packard Foundation to T.M.

* Address correspondence to this author at the Department of Cell Biology, Box 3709, DUMC, Durham, NC 27710. Fax: (919) 681-7978. Telephone: (919) 681-8072.

® Abstract published in *Advance ACS Abstracts,* September 15, 1995.

0006-2960/95/0434-12738$09.00/0

mediated Ca^{2+} release. A further increase in the free Ca^{2+} concentration above 300 nM leads to the inhibition of Ca^{2+} release. Finch et al. proposed that the potentiation of Ca^{2+} release can be explained by Ca^{2+} and IP_3 functioning as co-agonists for channel opening (Finch et al., 1991). Recently, however, controversy has arisen as to the existence of this mode of regulation. Experiments by Combettes et al. suggested that the observed Ca^{2+} potentiation is the result of an EGTA artifact [Combettes et al., 1994; discussed in Combettes and Champeil (1994) and Finch and Goldin (1994)]. An additional level of complexity was added by the finding that the concentration of luminal Ca^{2+} significantly affects IP_3-mediated release. Missiaen et al. demonstrated that increased loading of the ER with Ca^{2+} reduces the amount of IP_3 required for half-maximal release of stored Ca^{2+} (Missiaen et al., 1992), and also that the potentiation of release by cytosolic Ca^{2+} is dependent upon the luminal Ca^{2+} concentration (Missiaen et al., 1994). In contrast, other studies have shown that partial depletion of Ca^{2+} stores by thapsigargin or ionomycin has no effect on the ability of IP_3 to release Ca^{2+} (Shuttleworth, 1992; Combettes et al., 1992). Furthermore, studies using brain microsomes in a planar bilayer assay demonstrated that submillimolar concentrations of Ca^{2+} on the luminal side of the channel have no effect upon IP_3-mediated channel opening (Bezprozvanny & Ehrlich, 1994).

We chose to investigate the role of cytosolic and luminal Ca^{2+} in the regulation of the IP_3 receptor in RBL cells in order to better understand the mechanism of antigen-induced Ca^{2+} spiking. We show that increases in both cytosolic Ca^{2+} and luminal Ca^{2+} concentrations can potentiate IP_3-gated Ca^{2+} release by functionally replacing each other as co-agonists for IP_3-mediated channel opening. At high luminal Ca^{2+} concentrations, a majority of the stored Ca^{2+} can be released in the complete absence of cytosolic Ca^{2+}. Under these conditions, Ca^{2+} release can be inhibited by the fast Ca^{2+} buffer BAPTA, suggesting that luminal Ca^{2+} has to bind to a cytosolic site to exert its regulatory role. This suggests that the potentiation by luminal Ca^{2+} is the result of a local positive feedback by which Ca^{2+} ions passing through the pore bind to a site close to the pore on the same channel or on closely associated channels.

MATERIALS AND METHODS

Perfusion Apparatus. Perfusion bottles were pressurized with nitrogen gas to provide a constant flow rate of 23 mL/min. Teflon tubing (i.d. 0.9 mm) was used to connect the pressure bottles to a series of zero dead volume y-valves (LFYA series solenoid valves, Lee Co.). These valves were controlled by a Basic program and a relay board (DAS-8, Keithley-Metrabyte). The setup allowed one to switch between seven perfusion bottles. The effluent from the y-valves was connected to a cell chamber, which was constructed of Plexiglass and served as a holder for two filters. The bottom filter (MSI AcetatePlus) had a 0.65 μm opening in order to retain cells in the cell chamber. The top filter was made of hardened crepe paper with openings of 20−30 μm (#520-B, Schleicher & Schuell). This filter was used to restrict the movement of permeabilized cells due to turbulent flow in the filter. The total volume of the cell chamber with filters in place was 400 μL. The approximate exchange time of the chamber was measured with a short pulse of rhodamine and was found to be ~1 s. The effluent from the cell chamber was collected in a Gilson FC203 fraction collector. In the experiments presented, a collection rate of 1 fraction per 1.2 s was used.

Loading and Permeabilization. RBL cells were grown in 75 cm^2 tissue culture flasks using D-MEM plus 10% fetal bovine serum, 4 mM L-glutamine, and 10 μg/mL gentamycin. Cells were harvested by a 3 min incubation at 37 °C with HBSS supplemented with 0.05% trypsin and 0.53 mM EDTA. Cells were then washed with extracellular buffer (125 mM NaCl, 5 mM KCl, 20 mM HEPES at pH 7.0, and 0.1 mM EGTA), suspended at 2×10^6 cells/mL of extracellular buffer, and added to one of the pressure bottles. Approximately 0.8×10^6 cells were perfused into the cell chamber for each experiment. The cells were then washed for 30 s with extracellular buffer. Subsequently, trapped cells were perfused with a permeabilization-loading solution which consisted of intracellular buffer (125 mM KCl, 5 mM NaCl, 20 mM HEPES at pH 7.0, 10 μM EGTA, 1 mM $MgCl_2$, 1 mM ATP, 1.0−5.0 μCi of $^{45}CaCl_2$, and 20 μg/mL digitonin) which has been run over a Ca^{2+} sponge column (Meyer et al., 1990). The concentration of digitonin was chosen by determining the minimal concentration of digitonin that fully permeabilized RBL cells as measured by staining with trypan blue. Cells were allowed to permeabilize and load with $^{45}Ca^{2+}$ in this buffer for 120 s. Following this loading step, cells were perfused with an intracellular Ca^{2+}-EGTA buffer (120 mM KCl, 5 mM NaCl, 20 mM HEPES at pH 7.0, 20 mM EGTA, and equimolar Ca^{2+}-EGTA). This buffer was used to remove digitonin and $^{45}Ca^{2+}$ and to clamp the free cytosolic Ca^{2+} level to the desired concentration. Cells remained permeabilized for at least 10 min after removal of digitonin, as assayed by trypan blue (data not shown). Digitonin was removed because control experiments showed that maintaining digitonin in subsequent perfusion steps significantly reduced the fraction of Ca^{2+} stores that could be released by IP_3. The Ca^{2+}-EGTA wash step was maintained for 50 s, before the stimulus protocol for a particular experiment was initiated. Release of stored $^{45}Ca^{2+}$ was measured by collection of 1.2 s fractions. The $^{45}Ca^{2+}$ content of each fraction was measured using a Beckman scintillation counter.

Ca^{2+} Buffers. The concentration of free Ca^{2+} in the perfusing solutions was clamped by addition of an equimolar concentration of Ca^{2+}-EGTA to a buffer containing 20 mM EGTA. With this protocol, the free EGTA concentration remained constant for all buffers. This condition was chosen because an earlier study indicated that changing the concentration of free EGTA may alter IP_3-dependent Ca^{2+} release (Combettes et al., 1994). Specifically, the free Ca^{2+} concentration was titrated by addition of a 1:1 Ca^{2+}−EGTA mixture (500 mM each) to intracellular buffer that contained 125 mM KCl, 5 mM NaCl, 20 mM HEPES at pH 7.0, and 20 mM EGTA (Tsien & Pozzan, 1989). All solutions were calibrated by using a small amount of the Ca^{2+} indicator Indo-1 in a fluorometer.

The free Ca^{2+} concentration that was present during the loading step was difficult to control because of the low concentration of EGTA in the loading solution (10 μM). The low EGTA concentration was necessary to reduce the amount of radioactive Ca^{2+} used for each experiment. For this reason, the free [Ca^{2+}] present during loading was directly measured by collecting the effluent during the loading step and measuring the free [Ca^{2+}] with Indo-1. The free Ca^{2+}

concentration during the loading step was increased by addition of $^{45}Ca^{2+}$ at a constant concentration of cold Ca^{2+} (Figure 5b,c), or by addition of cold Ca^{2+} at constant concentration of $^{45}Ca^{2+}$ (Figure 5a).

In order to achieve the high concentrations of BAPTA necessary to rapidly bind Ca^{2+}, it was necessary to make the osmolarity of all buffers used in the experiments identical. Experiments using 100 mM BAPTA had the following buffer concentrations: 100 mM BAPTA tetrapotassium salt, 20 mM K^+-HEPES at pH 7.0, and 5 mM NaCl. The osmolarity of this buffer was measured using an osmometer and found to be 360 mmol/kg. Other buffers used for this experiment were adjusted to 360 mmol/kg by addition of KCl. The 50 mM BAPTA buffer contained 50 mM BAPTA tetrapotassium salt, 20 mM HEPES at pH 7.0, 5 mM NaCl, and 20 mM EGTA. The EGTA control buffer contained 120 mM KCl, 5 mM NaCl, 20 mM HEPES, and 20 mM EGTA.

RESULTS

IP_3-Releasable Calcium Stores Are Intact in the Perfusion System. The mechanism of regulation of the IP_3 receptor by Ca^{2+} was investigated with a computer-controlled perfusion apparatus that allowed us to measure the release of $^{45}Ca^{2+}$ from permeabilized cells. Rapid exchange of the perfusate between different solutions was achieved by computer-controlled Y-valves. Up to seven different pressurized bottles could be connected to the cell chamber. Two filters were used in the cell chamber for the retention of cells (0.6 μm for the second filter). The experimental protocol was initiated by perfusing intact cells into the cell chamber. Trapped cells were then permeabilized by changing the perfusate to a buffer containing digitonin. Intracellular Ca^{2+} stores were loaded by addition of $^{45}Ca^{2+}$ and ATP to the perfusate. Excess $^{45}Ca^{2+}$ was removed 2 min later by perfusion with a Ca^{2+}-EGTA buffer. The time course of IP_3-mediated $^{45}Ca^{2+}$ release was then measured by addition of IP_3 to the perfusate and the subsequent collection of fractions every 1.2 s.

The results of a typical experiment using this assay system are shown in Figure 1a ($n = 9$). Permeabilized cells were loaded and washed before the free Ca^{2+} concentration of the perfusate was stepped to 470 nM. Fraction collection was initiated, and a base line of $^{45}Ca^{2+}$ release was established. A background of $^{45}Ca^{2+}$ release was always observed in the assay system. Cells were then perfused with an identical Ca^{2+}-EGTA buffer containing 800 nM IP_3. Immediately following perfusion with 800 nM IP_3, the rate of $^{45}Ca^{2+}$ release increased 10-fold above background (Figure 1a). Finally, the amount of $^{45}Ca^{2+}$ remaining in the stores was determined by perfusion with 1% Triton X-100. The percentage of the stored $^{45}Ca^{2+}$ that was released by IP_3 was determined by comparing the area under the peaks resulting from perfusion with IP_3 and with Triton. In this experiment, 800 nM IP_3 released 70% of the total stored $^{45}Ca^{2+}$.

This protocol was repeated at different IP_3 concentrations, while maintaining the cytosolic Ca^{2+} concentration constant at 470 nM, and the data were normalized to the percentage of $^{45}Ca^{2+}$ released by 1600 nM IP_3 ($n = 9$). The means and standard deviations of three such experiments are shown in Figure 1b. IP_3-mediated Ca^{2+} release was half-maximal at 187 ± 38 nM (SD, $n = 3$). The observation that 1 μM IP_3 releases 70−80% of stored $^{45}Ca^{2+}$, and that the concentration

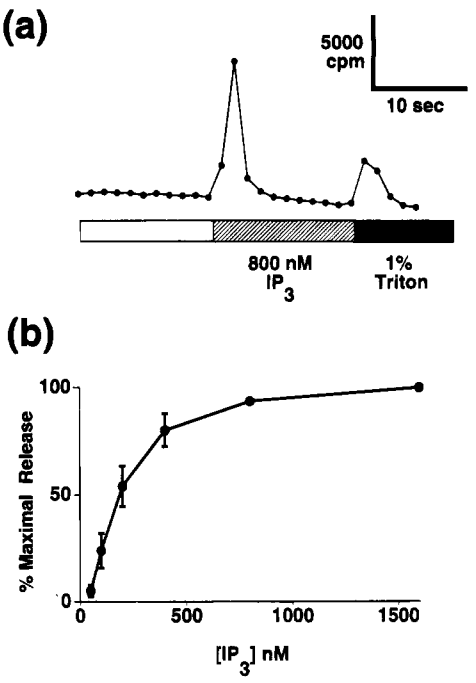

FIGURE 1: Measurement of IP_3-mediated release of stored Ca^{2+}. (a) Cells were permeabilized, loaded with $^{45}Ca^{2+}$, and washed for 50 s with a Ca^{2+}-EGTA buffer clamped at 45 nM free Ca^{2+}. The perfusate was switched to 470 nm free Ca^{2+} 10 s prior to fraction collection. After establishing a base line of $^{45}Ca^{2+}$ release, the perfusate was switched to an identical buffer with the addition of 800 nM IP_3. The amount of calcium that remained in the store was determined by perfusion with 1% Triton. The perfusion protocol is outlined by the stimulus bar. Data points represent cpm per 1.2 s fraction (representative trace, $n = 9$). (b) The percentage of Ca^{2+} released is determined as a function of the IP_3 concentration ($n = 9$). Data were normalized to the percentage of $^{45}Ca^{2+}$ released by 1600 nM IP_3 (100% is the sum of the IP_3 and Triton peaks). The mean and standard deviation of three experiments are shown.

for half-maximal release was in the 200 nM range, indicates that Ca^{2+} stores remain remarkably intact using this assay system. Previous studies using brain microsomes typically released only 6% of the stored Ca^{2+} at 10 μM IP_3 (Finch et al., 1991).

Direct Ca^{2+}-Induced Ca^{2+} Release Is Not Significant in RBL Cells. In order to study the regulation of the IP_3 receptor by Ca^{2+}, it was necessary to first determine the significance of direct Ca^{2+}-induced Ca^{2+} release in permeabilized RBL cells. Such a release process has been shown in cells that contain ryanodine receptors (Tsien & Tsien, 1990). We tested for this by asking whether a stepwise increase in the Ca^{2+} concentration of the perfusate would result in release of stored $^{45}Ca^{2+}$. Permeabilized cells were loaded with $^{45}Ca^{2+}$ and washed with a Ca^{2+}-EGTA buffer clamped at 45 nM. The cytosolic Ca^{2+} concentration was then stepped to 470 nM (Figure 2a, $n = 8$) or 2000 nM (Figure 2b, $n = 8$). Finally, the amount of $^{45}Ca^{2+}$ remaining in the stores was determined by perfusion with 1% Triton X-100. No appreciable release of stored $^{45}Ca^{2+}$ could be observed following an increase in cytosolic $[Ca^{2+}]$ to either 470 nM or 2 μM. This suggests that direct Ca^{2+}-induced Ca^{2+} release is not significant in this cell system.

Cytosolic Ca^{2+} Potentiates IP_3-Mediated Release Only at Low IP_3 Concentrations. The Ca^{2+} dependence of IP_3-mediated Ca^{2+} release was measured at a fixed IP_3 concentration in the presence of different cytosolic Ca^{2+} concentrations. The Ca^{2+} concentration in the perfusate was varied

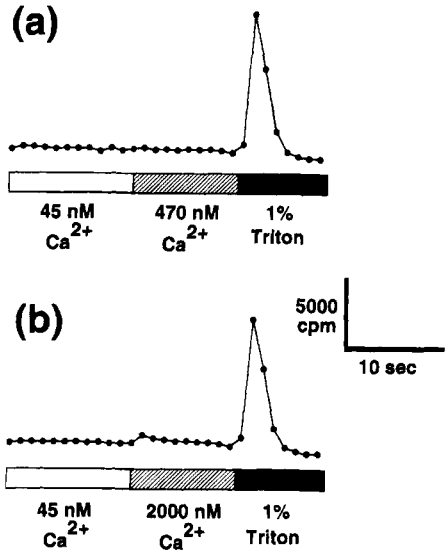

FIGURE 2: Absence of direct Ca^{2+}-induced Ca^{2+} release in RBL cells. Cells were permeabilized, loaded with $^{45}Ca^{2+}$, and washed for 60 s by perfusion with a Ca^{2+}-EGTA buffer clamped at 45 nM free Ca^{2+}. Collection was initiated with the free Ca^{2+} clamped at 45 nM (a and b). The perfusate was then switched to a Ca^{2+}-EGTA buffer clamped at 470 nM free Ca^{2+} (a, single trace shown, $n = 8$) or 2000 nM free Ca^{2+} (b, single trace shown, $n = 8$). At the end of each experiment, the perfusate was switched to 1% Triton. The perfusion protocols are outlined by the stimulus bars. The calibration bars apply to both (a) and (b). The data points represent cpm per 1.2 s fraction.

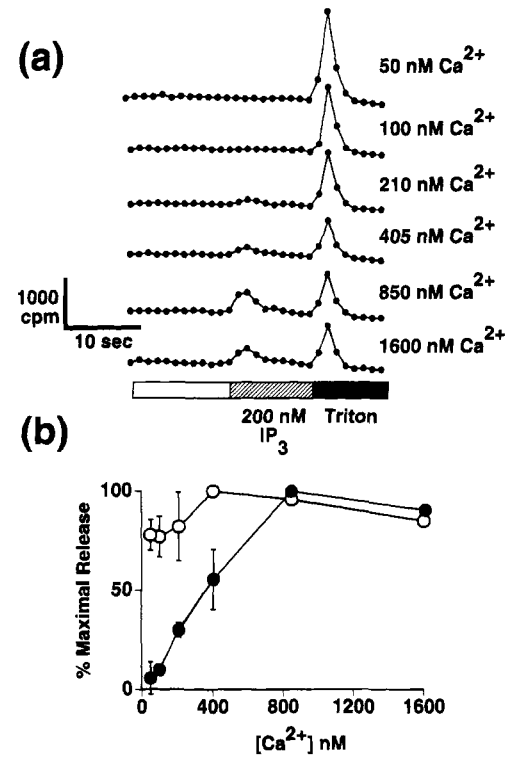

FIGURE 3: Regulation of IP_3-mediated Ca^{2+} release by cytosolic Ca^{2+}. (a) Cells were permeabilized, loaded, and washed at 45 nM free Ca^{2+} for 50 s. The perfusate was then switched to a Ca^{2+}-EGTA buffer set to free Ca^{2+} concentrations between 50 nM and 1.6 μM. Fraction collection was initiated 10 s later, and a base line of Ca^{2+} release was established. The perfusate was then switched to an identical buffer containing 200 nM IP_3. The perfusion protocol is outlined by the stimulus bar. Data points represent cpm per 1.2 s fraction ($n = 6$). (b) The percentage of stored $^{45}Ca^{2+}$ released by 200 nM IP_3 (filled circles, mean and SD, $n = 2$) or by 500 nM IP_3 (open circles, mean and SD, $n = 3$) is shown as a function of the cytosolic free Ca^{2+} concentration present during release. Data were normalized to the maximal percent released [percent released at a cytosolic free Ca^{2+} concentration of 850 nM (filled circles) or 405 nM (open circles)].

from 50 nM to 1.6 μM, which includes the range of Ca^{2+} concentrations observed during Ca^{2+} spiking. The free Ca^{2+} concentration in each Ca^{2+} buffer was measured with the Ca^{2+} indicator Indo-1 in a fluorometer. Buffers were made at a constant free EGTA concentration by addition of variable amounts of equimolar Ca^{2+}-EGTA to a buffer containing 20 mM EGTA. Collection was initiated during perfusion with Ca^{2+}-EGTA buffers. Release of stored $^{45}Ca^{2+}$ was then measured in response to a step increase to 200 nM or 500 nM IP_3 while maintaining the same free Ca^{2+} concentration. Under these conditions, the amount of Ca^{2+} stored in the lumen of the ER was the same for each experiment.

Following a step to 200 nM IP_3, release of $^{45}Ca^{2+}$ was nearly absent at 50 nM free Ca^{2+} but increased at higher cytosolic Ca^{2+} levels, reaching a maximum at 850 nM free Ca^{2+} (Figure 3a, $n = 2$). The percentage of stored Ca^{2+} released by 200 nM IP_3 is represented in Figure 3b as a function of the free Ca^{2+} concentration (closed circles, $n = 2$). IP_3-mediated Ca^{2+} release is strongly Ca^{2+}-dependent, and the mean Ca^{2+} concentration necessary for half-maximal release was 400 nM. However, when probed with 500 nM IP_3, the Ca^{2+} dependence is much less significant (Figure 3b, open circles, $n = 3$). These measurements show that an increase in the cytosolic Ca^{2+} concentration potentiates the release of stored Ca^{2+} only at submaximal IP_3 concentrations.

Cytosolic Ca^{2+} Does Not Inactivate the IP_3 Receptor in RBL Cells. One aspect of our results in RBL cells significantly differs from those in brain microsomes and skinned smooth muscle cells. In these cells, a marked inhibition of IP_3-mediated Ca^{2+} release was observed at free Ca^{2+} concentrations between 300 nM and 1 μM. This type of inhibition, which occurs at a concentration of cytosolic Ca^{2+} achieved during a Ca^{2+} spike, has been suggested to play an integral role in the termination of Ca^{2+} spikes. At concentrations of cytosolic Ca^{2+} from 300 nM to 1.6 μM, which includes the concentration range observed during antigen-induced Ca^{2+} spiking, no significant inhibition of IP_3-mediated Ca^{2+} release was observed in permeabilized RBL cells (Figure 3b, $n = 5$). To ensure the accuracy of the Ca^{2+}-EGTA buffers, each buffer was calibrated with Indo-1. These observations suggest that another mode of spike termination, other than inactivation by Ca^{2+}, is present in RBL cells.

Cytosolic Ca^{2+} Is Not Absolutely Required as a Co-agonist for IP_3-Mediated Ca^{2+} Release. In order to test whether Ca^{2+} is required as a co-agonist along with IP_3 for channel opening as has been proposed by Finch et al. (1991), IP_3-mediated release of $^{45}Ca^{2+}$ was measured in the absence of cytosolic Ca^{2+}. The free Ca^{2+} concentration was reduced to very low levels by addition of 20 mM EGTA to the perfusate after an initial wash step at 45 nM free Ca^{2+}. The fraction of stored Ca^{2+} that can be released by 1 μM IP_3 was then determined in the continued presence of 20 mM EGTA (Figure 4a, $n = 10$). For this protocol, cells were loaded under physiological conditions ($[Ca^{2+}]_{load} = 300$ nM), and the concentration of Ca^{2+} in the lumen is the same for each run. Strikingly, 55% of the stored Ca^{2+} could be released in the absence of cytosolic Ca^{2+}. In the presence of 405 nM free

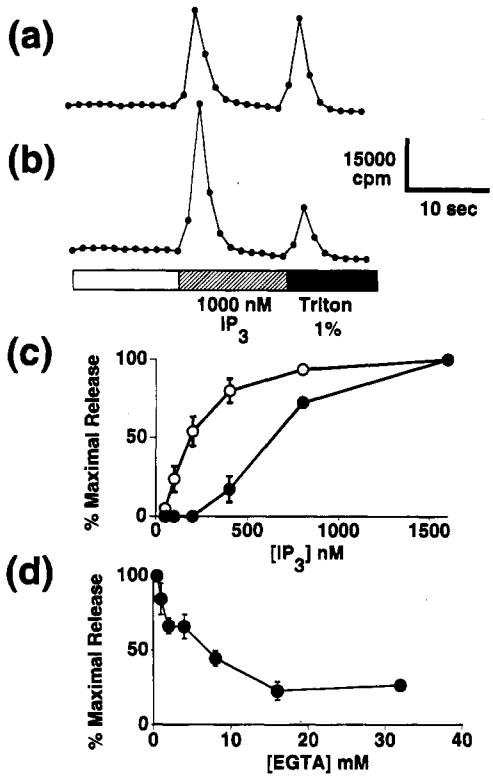

FIGURE 4: IP_3 releases a significant amount of Ca^{2+} in the absence of cytosolic Ca^{2+}. Cells were permeabilized, loaded, and washed at 45 nM free Ca^{2+} for 50 s. The perfusate was then switched to a buffer without Ca^{2+} (a, 20 mM EGTA, $n = 10$) or to a buffer at 405 nM free Ca^{2+} (b, $n = 10$). Fraction collection was initiated 10 s later, and subsequently the perfusate was switched to identical buffers containing 1 μM IP_3. The protocol is outlined by the stimulus bar. Data points represent cpm per 1.2 s fraction. Calibration bars apply for (a) and (b). (c) The same protocol was repeated at different IP_3 concentrations, and the data were normalized to the percent of maximal release (percent released by 1600 nM IP_3). The mean and standard deviation of three experiments assayed for release in EGTA (filled circles) or at 405 nM free Ca^{2+} (open circles) are shown as a function of the IP_3 concentration. (d) Control experiment. The percentage of maximal released by 400 nM IP_3 (normalized to release at 0.5 mM EGTA) is shown as a function of the concentration of EGTA present during release (mean and SD, $n = 2$).

Ca^{2+}, the fraction of Ca^{2+} released by 1 μM IP_3 increased only to 76% (Figure 4b, $n = 10$). This indicates that cytosolic Ca^{2+} is not absolutely required as a co-agonist for Ca^{2+} release in RBL cells.

Analysis of the fraction of Ca^{2+} released, as a function of the IP_3 concentration, showed that the sensitivity of the IP_3 receptor for IP_3 increases with an increase in the cytosolic Ca^{2+} concentration. IP_3 titrations are done in the absence of Ca^{2+} (20 mM EGTA, $n = 6$), and in the presence of 470 nM free Ca^{2+} ($n = 6$). The data were normalized to the fraction of stored Ca^{2+} released by 1600 nM IP_3. The mean and standard deviation of three such experiments are plotted in Figure 4c. Increasing cytosolic [Ca^{2+}] from very low levels (20 mM EGTA, closed circles) to 470 nM free Ca^{2+} (open circles) decreased the half-maximal IP_3 concentration required for release approximately 3-fold (the EC_{50} for IP_3 in the presence of EGTA is 630 $\pm$ 26 nM, $n = 3$; the EC_{50} at 470 nM free Ca^{2+} is 187 $\pm$ 38 nM, $n = 3$). Thus, at a constant concentration of Ca^{2+} in the lumen, an increase in the cytosolic Ca^{2+} concentration effectively lowers the concentration of IP_3 required for IP_3-mediated Ca^{2+} release.

In a control experiment, we wanted to ensure that there was sufficient Ca^{2+} buffering in the perfusion system in order to clamp the cytosolic Ca^{2+} concentration to very low levels. It is unlikely that residual Ca^{2+} in the 20 mM EGTA buffer would be sufficient to bind to the IP_3 receptor and mediate the release of 55% of the stored Ca^{2+}. However, it is conceivable that a significant amount of EGTA is necessary to clamp the Ca^{2+} released by IP_3. We addressed this concern because cellular Ca^{2+} buffers are most likely washed out during the perfusion steps and the amount of Ca^{2+} released by IP_3 is significant when permeabilized cells are concentrated on the filter. We determined the concentration of EGTA necessary to clamp the released Ca^{2+} by measuring the percentage of the store released by 400 nM IP_3 at an increasing concentration of EGTA (Figure 4d, $n = 2$). We assumed that buffering by EGTA was sufficient when a doubling in the EGTA concentration did not further reduce IP_3-mediated Ca^{2+} release. This concentration was reached at 16 mM. For this reason, Ca^{2+}-EGTA buffers with a 20 mM excess of EGTA over Ca^{2+} were used in order to tightly clamp the free Ca^{2+} concentration.

The measurements in Figure 4 demonstrate that cytosolic Ca^{2+} is not absolutely required for IP_3-mediated Ca^{2+} release. However, increasing cytosolic [Ca^{2+}] lowers the concentration of IP_3 necessary for opening of IP_3-gated channels. Furthermore, Figure 4a demonstrates that a significant fraction of the stored Ca^{2+} can be released in the absence of cytosolic Ca^{2+}. One possible explanation of this observation would be that luminal Ca^{2+} coming through the channel is acting locally, binding to the cytosolic regulatory site and thereby replacing the requirement for cytosolic Ca^{2+}. Therefore, we went on to investigate the role of luminal Ca^{2+} in the regulation of the IP_3 receptor in our system, more specifically, whether the concentration of Ca^{2+} in the lumen could regulate the cytosolic Ca^{2+} requirement of the IP_3 receptor.

Increasing Luminal Ca^{2+} Can Functionally Replace the Regulatory Requirement for Cytosolic Ca^{2+}. Ca^{2+} stores were loaded to different levels by varying the free Ca^{2+} concentration during loading, while keeping the time of loading and the ATP concentration constant. The concentration of luminal Ca^{2+} was determined as a function of the free Ca^{2+} concentration present during loading ($n = 3$, with variable loading conditions). A representative curve is shown in Figure 5a, which demonstrates that stores are loaded to a low level in the presence of 100 nM free Ca^{2+} and are maximally loaded in the presence of 500 nM free Ca^{2+}. In Figure 5b, cells were loaded in the presence of 120 nM or 520 nM free Ca^{2+}. Cells were then challenged with 1 μM IP_3 in the presence of 20 mM EGTA. For cells loaded to a low luminal Ca^{2+} concentration, 1 μM IP_3 released only 16% of the stored $^{45}Ca^{2+}$ (top panel, $n = 6$). In contrast, if the Ca^{2+} stores are loaded to a higher level, 1 μM IP_3 released 53% of the stored $^{45}Ca^{2+}$ (bottom panel, $n = 6$). Although we were not able to obtain statistics on the relationship of loading and release (because the free Ca^{2+} present during loading is not reproduced between experiments), Figure 5c summarizes the data from four separate experiments. These measurements show that the fraction of the store that can be released in EGTA is dependent on the concentration of Ca^{2+} in the lumen, especially for cells loaded between 50 and 200 nM free Ca^{2+} (filled circles). When release is assayed in the presence of 405 nM free Ca^{2+} (open circles), the percentage

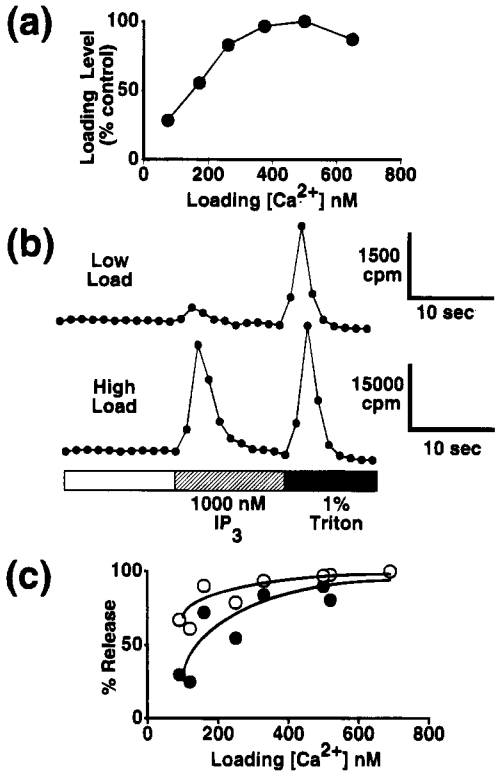

FIGURE 5: Regulation of IP_3-mediated Ca^{2+} release by luminal Ca^{2+}. (a) Calibration experiment. The concentration of luminal $^{45}Ca^{2+}$ is shown as a function of the free Ca^{2+} concentration present during loading. The amount of luminal $^{45}Ca^{2+}$ was determined by perfusion with 1% Triton. The measurements were corrected for the ratio of $^{45}Ca^{2+}$ to cold Ca^{2+} and normalized to the maximal amount of Ca^{2+} that could be loaded (representative trace, $n = 3$). (b) Cells were permeabilized with digitonin and loaded in the presence of 120 nM free Ca^{2+} (low load, $n = 6$) or in the presence of 520 nM free Ca^{2+} (high load, $n = 6$). Cells were then washed at 45 nM free Ca^{2+} for 50 s, and the perfusate was switched to 20 mM EGTA. Fraction collection was initiated 10 s later, and the perfusate was switched to an identical buffer containing 1 μM IP_3. The protocol is outlined by the stimulus bar. Data points represent cpm released per 1.2 s fractions. The free Ca^{2+} concentration during loading was increased from 120 to 520 nM by adding more $^{45}Ca^{2+}$ to the loading mixture. This explains the large difference in $^{45}Ca^{2+}$ released. (c) Ca^{2+} stores were loaded at a range of free Ca^{2+} concentrations between 100 and 700 nM. The percentage of stored $^{45}Ca^{2+}$ that could be released by 1 μM IP_3 in the absence of cytosolic Ca^{2+} (20 mM EGTA, closed circles) or in the presence of 405 nM free Ca^{2+} (open circles) is shown as a function of the free Ca^{2+} concentration present during loading. All the data from four separate experiments are plotted.

of stored Ca^{2+} that could be released by IP3 was much less dependent upon the concentration of Ca^{2+} in the lumen. This suggests that luminal Ca^{2+} is an important regulator for IP_3-mediated Ca^{2+} release only at low cytosolic Ca^{2+} concentrations, and that luminal Ca^{2+} can, at least in part, replace the requirement for cytosolic Ca^{2+} as a co-agonists for IP_3-mediated Ca^{2+} release.

High Concentrations of BAPTA Inhibit the Regulation of the IP_3 Receptor by Luminal Ca^{2+}. The potentiation of Ca^{2+} release at high luminal Ca^{2+} concentration could be mediated by luminal Ca^{2+} passing through the channel and binding to the same cytosolic regulatory site, thereby replacing the requirement for cytosolic Ca^{2+}. Alternatively, luminal Ca^{2+} could also be regulating the IP_3 receptor at a site on the luminal side of the channel as has been proposed previously (Missiaen et al., 1992, 1994). An experiment which could

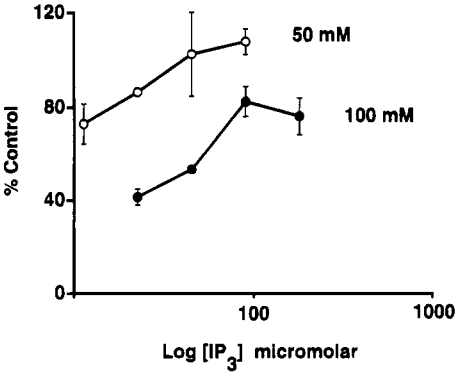

FIGURE 6: Fast Ca^{2+} buffer BAPTA can reduce IP_3-meditated Ca^{2+} release. The protocol for this experiment was similar to the one used for the IP_3 titrations in Figure 2. IP_3-mediated calcium release was measured in the presence of 50 mM BAPTA (open circles) or in the presence of 100 mM BAPTA (filled circles) for a series of IP_3 concentrations. For each IP_3 concentration, the percentage of Ca^{2+} released was calibrated by perfusion with 1% Triton and then normalized to control measurements (Ca^{2+} released by 2 μM IP_3 in 20 mM EGTA). All points show the mean and standard deviation ($n = 2$, except for the highest IP_3 concentration in each trace, $n = 4$).

differentiate between these two scenarios is the rapid buffering of luminal Ca^{2+} passing through the channel before it has a chance to bind to potential cytosolic regulatory sites. If luminal Ca^{2+} regulates the IP_3 receptor at a cytosolic site, then IP_3-mediated Ca^{2+} release in the absence of cytosolic Ca^{2+} should be reduced in the presence of a fast cytosolic Ca^{2+} buffer.

In order to buffer Ca^{2+} at a close distance from the channel, it is necessary to use a Ca^{2+} buffer with a fast on-rate. EGTA has a slow on-rate because it is protonated in the Ca^{2+}-free form (Tsien, 1980). It has been estimated that the time required for Ca^{2+} binding to EGTA is longer than 30 μs in the presence of 20 mM EGTA. During this time, Ca^{2+} can travel a distance of about 200 nm (Stern, 1992). The same author estimates that 100 mM of the faster Ca^{2+} buffer BAPTA is required to buffer Ca^{2+} within a distance of 18 nm from a Ca^{2+} channel. Because BAPTA is a competitive inhibitor of IP_3 binding (Richardson & Taylor, 1993), we decided to use the maximal percentage of Ca^{2+} that can be released by IP_3 as a measure of whether BAPTA can interfere with Ca^{2+} release. Decreases in the maximal percentage of Ca^{2+} that can be released by IP_3 in the presence of BAPTA would not reflect competitive inhibition, but instead represent an effect of fast Ca^{2+} buffering.

IP_3 was titrated in the presence of 50 and 100 mM BAPTA at constant osmolarity (Figure 6). The addition of 50 mM BAPTA (open circles) increased the half-maximal concentration of IP_3 necessary for release; however, it had no significant effect on the maximal fraction of release as compared to control ($n = 2$ except for $[IP_3] = 90$ μM, $n = 4$). In contrast, 100 mM BAPTA (filled circles) significantly decreased the percentage of the store which can be released in the absence of cytosolic Ca^{2+} ($n = 2$ except for $[IP_3] = 180$ μM, $n = 4$). The data shown were normalized to control measurements in which the percentage of Ca^{2+} released by 2 μM IP_3 was measured at 20 mM EGTA. The observed inhibition of Ca^{2+} release by 100 mM BAPTA, and not by 50 mM BAPTA, provides additional support for the hypothesis that luminal Ca^{2+} positively regulates the IP_3 receptor at a cytosolic site.

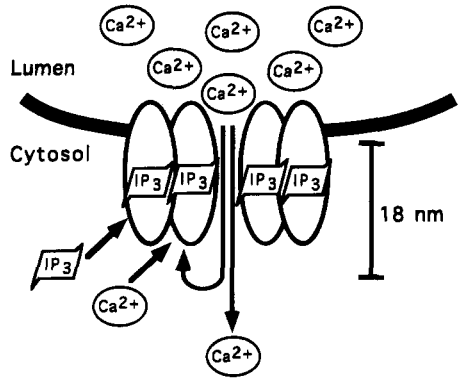

FIGURE 7: Regulation of the IP_3 receptor by luminal and cytosolic Ca^{2+}. Schematic representation of the regulation of a single tetrameric IP_3 receptor. A key feature of the model is that the regulation by luminal Ca^{2+} is mediated by the binding of luminal Ca^{2+} to cytosolic regulatory sites. The arrows indicate that "luminal Ca^{2+} feedback" by which luminal Ca^{2+} potentiates its own release can be initiated by raising $[IP_3]$, by raising cytosolic $[Ca^{2+}]$, or by increasing the concentration of Ca^{2+} in the lumen. Once a channel is open, release is maintained by luminal Ca^{2+} that comes through a pore and binds to the cytosolic regulatory sites of the same channel or closely associated channels. The scale bar indicates a calculated range of action for the cloud of high Ca^{2+} surrounding an open channel.

DISCUSSION

Model for the Regulation of the IP_3 Receptor by Cytosolic and Luminal Calcium. Our measurements show that cytosolic and luminal Ca^{2+} are both important regulators of the IP_3 receptor. The release of stored Ca^{2+} by 200 nM IP_3 was markedly potentiated by increasing the cytosolic Ca^{2+} concentration between 100 nM and 1 μM (Figure 3a,b). This Ca^{2+} dependence was much less significant at 500 nM IP_3 (Figure 3b). In contrast to previous studies using brain microsomes and skinned smooth muscle cells, RBL cells did not show an inhibition of IP_3-mediated Ca^{2+} release when cytosolic Ca^{2+} was elevated above 300 nM (Figure 3b). However, IP_3-mediated Ca^{2+} released had a steep dependence upon the luminal Ca^{2+} concentration when assayed at low cytosolic Ca^{2+} concentrations (Figure 5c). When the concentration of luminal Ca^{2+} was high, 1 μM IP_3 released a majority of the stored Ca^{2+} in the complete absence of cytosolic Ca^{2+} (Figure 5b). However, when assayed at a high cytosolic Ca^{2+} concentration (405 nM), IP_3-mediated Ca^{2+} release was much less dependent upon the concentration of Ca^{2+} in the lumen (Figure 5c). These results demonstrate that, in the presence of IP_3, an increase of either cytosolic or luminal Ca^{2+} is sufficient to initiate release of stored Ca^{2+}. Therefore, both luminal and cytosolic Ca^{2+} can act as co-factors for IP_3-mediated Ca^{2+} release. The similar co-factor function of luminal and cytosolic Ca^{2+} combined with the observation that Ca^{2+} release is inhibited in the presence of high concentrations of the Ca^{2+} buffer BAPTA (Figure 6) supports the hypothesis that luminal and cytosolic Ca^{2+} exert their role by binding to the same cytosolic regulatory site. However, we cannot entirely exclude the possibility that luminal Ca^{2+} exerts its regulatory role at a luminal site.

These results suggest the following model for the regulation of the IP_3 receptor (Figure 7): Opening of the IP_3-gated channel requires binding of IP_3 as well as Ca^{2+} to cytosolic sites. The bound Ca^{2+} can originate from the cytosolic side, or luminal Ca^{2+} ions can cross the channel and bind to cytosolic sites before they equilibrate with cytosolic Ca^{2+} buffers. These luminal Ca^{2+} ions sustain Ca^{2+} release by occupying Ca^{2+} binding sites on the same channel and potentiate IP_3-mediated opening of closely associated channels. Through this mechanism of Ca^{2+} potentiation, luminal Ca^{2+} can generate a rapid and local positive feedback loop by which a single channel is kept persistently open and a group of associated IP_3-gated channels can be opened in a synchronous manner. In the complete absence of cytosolic Ca^{2+}, the positive feedback may be initiated by a small residual Ca^{2+} current. Such a current has been observed in a planar bilayer assay using IP_3 receptors from reconstituted brain microsomes (Bezprozvanny et al., 1991). This rapid and local positive feedback by which luminal Ca^{2+} potentiates its own release will be referred to as "luminal Ca^{2+} feedback". A recent theoretical study closely predicted the results of previous Ca^{2+} release studies using a postulated Ca^{2+} feedback by which luminal Ca^{2+} ions bind to a second regulatory Ca^{2+} binding site not accessible from the cytosol located within the channel pore (Swillens et al., 1994). Although there are important differences between our data and the Swillens' model, specifically we do not observe a Ca^{2+}-dependent inhibition, it will be interesting to learn whether the different assumptions in the model proposed in our study would lead to similar predictions as those calculated by Swillens et al.

Relationship to Other Studies on the Calcium Regulation of the IP_3 Receptor. Some of the controversies in the literature concerning the regulation of the IP_3 receptor by luminal and cytosolic Ca^{2+} can be addressed in the context of our measurements. According to our studies and the previous observations of Missiaen et al. (1994), the dependence of IP_3-mediated release on cytosolic Ca^{2+} can be more or less significant depending upon the amount of Ca^{2+} in the lumen. Furthermore, we noticed in our filter system that a significant amount of EGTA (20 mM) was required to sufficiently buffer the Ca^{2+} released from the store (Figure 4d). Both of these factors must be considered in order to determine the significance of Ca^{2+} potentiation of release in each cellular system. For example, the Ca^{2+} dependence of release in Figure 3 would have been much less prominent if assayed at a lower concentration of EGTA. These considerations may explain the differences between the observations of Finch and Goldin (1994), who reported a significant dependence on cytosolic Ca^{2+} in brain microsomes, and the observations of Combettes and colleagues (Combettes et al., 1994), who found no Ca^{2+} dependence using a similar preparation. It is conceivable that the luminal Ca^{2+} concentration and the concentration of EGTA required for sufficient buffering may have been different in these two assay systems.

Our studies may also explain some of the differences between the observations of Missiaen et al. (1992), who found that partial depletion of Ca^{2+} stores in permeabilized A7r5 smooth muscle cells renders them less sensitive to IP_3, and the observations of Shuttleworth (1992), Combettes et al. (1992), and Bezprozvanny and Ehrlich (1994), who reported that store loading had no effect on IP_3-mediated Ca^{2+} release. The differences in the latter studies could be in part due to a high level of store loading at which the effect of luminal Ca^{2+} is less significant (Figure 5c). Our results and those of Missiaen et al. (1994) indicate that the dependence of luminal Ca^{2+} is more significant at low cytosolic Ca^{2+} concentration and would be much less

apparent if assayed at higher cytosolic Ca^{2+} concentrations.

Missiaen et al. (1992) measured the IP_3 dependence of Ca^{2+} release at different loading levels and found that more IP_3 is required to release 50% of the releasable Ca^{2+} at lower loading levels than at higher ones, and that the potentiation of IP_3-mediated Ca^{2+} release by cytosolic Ca^{2+} is loading-dependent (Missiaen et al., 1994). They proposed that luminal Ca^{2+} binds to a luminal site, thereby increasing the IP_3 sensitivity of the receptor and replacing the requirement for cytosolic Ca^{2+} (Missiaen et al., 1992, 1994). This interpretation is not consistent with the inhibition of Ca^{2+} release in the presence of BAPTA (Figure 6). Our measurements also suggest that the change in the IP_3 requirement observed by Missiaen et al. (1992) could result from a higher occupancy of the cytosolic Ca^{2+} regulatory site at higher luminal Ca^{2+} concentration and not from a shift in the IP_3 binding affinity mediated by a luminal Ca^{2+} regulatory site.

The spatial range of luminal Ca^{2+} feedback can be approximated by examining the Ca^{2+} binding properties of BAPTA and EGTA. The primary difference between BAPTA and EGTA is that BAPTA is not protonated in the physiological pH range (Tsien, 1980). Therefore, BAPTA has a significantly faster on-rate for Ca^{2+} binding than the partially protonated EGTA. A recent study calculated the time required for Ca^{2+} ions coming through the pore of the channel to bind to Ca^{2+} buffers (Stern, 1992). Stern's calculations can be used to estimate that the 20 mM EGTA used in our experiment should buffer Ca^{2+} only after it diffuses a distance of approximately 200 nm from the pore. Furthermore, these calculations predict that 100 mM BAPTA is required to buffer a 1 pA local Ca^{2+} flux approximately 18 nm from the pore. Parys et al. (1993) observed that addition of 10 mM BAPTA had no effect on IP_3-mediated release; however, our results and these calculations predict that it is necessary to use higher concentrations of Ca^{2+} buffer in order to inhibit the local feedback of luminal Ca^{2+} onto the IP_3 receptor. The observation that 100 mM BAPTA can inhibit Ca^{2+} release, while 50 mM BAPTA has little effect, suggests that the binding site for luminal Ca^{2+} should be within approximately 20 nm of the channel pore. This is consistent with the model that luminal Ca^{2+} feedback is mediated by Ca^{2+} binding to cytosolic binding sites on the same channel or on closely associated channels.

Significance of Ca^{2+} Regulation of the IP_3 Receptor for Ca^{2+} Spiking and Ca^{2+} Waves. Luminal Ca^{2+} feedback is well suited to be an important mechanism for the generation of Ca^{2+} spikes and Ca^{2+} waves. In *Xenopus* oocytes, localized Ca^{2+} release microdomains have been observed by confocal microfluorometry following a rapid increase in the IP_3 concentration (Parker & Ivorra, 1993). Our measurements support the hypothesis that such microdomains of Ca^{2+} release are mediated by luminal Ca^{2+} feedback, which results in the synchronized opening of a group of IP_3-gated channels and generates a local Ca^{2+} pulse. These local Ca^{2+} pulses are likely to be the building blocks of Ca^{2+} spikes and Ca^{2+} waves.

Luminal Ca^{2+} feedback may also explain the phenomenon of "quantal" Ca^{2+} release. Previous studies have shown that IP_3-mediated Ca^{2+} release is a biphasic process (Muallem et al., 1989) and that small incremental increases in IP_3 concentration lead to short pulses of Ca^{2+} release (Meyer & Stryer, 1990). Quantal Ca^{2+} release has been observed in many cell systems and can also be observed in Figure 3a of this study. Immediately following the perfusion of 200 nM IP_3, Ca^{2+} is released at a fast rate; however, the release rate rapidly drops to near base line, even though IP_3 is still present and the Ca^{2+} stores are still loaded to high levels. It is interesting to consider whether the observations of quantal Ca^{2+} release can be explained by IP_3-mediated initiation and termination of luminal Ca^{2+} feedback. Indeed, luminal Ca^{2+} feedback would be triggered as soon as the IP_3 concentration increased above a critical threshold level and subsequently terminated when luminal Ca^{2+} levels fall below a critical level, at which point the luminal Ca^{2+} feedback cannot be sustained. The transient triggering of luminal Ca^{2+} feedback would generate a short Ca^{2+} pulse in response to a submaximal increase in IP_3 concentration which would only lower the luminal Ca^{2+} concentration but not empty the Ca^{2+} stores.

These considerations suggest that IP_3-dependent Ca^{2+} stores are a source of local and short Ca^{2+} pulses that are initiated by luminal Ca^{2+} feedback and terminated when the feedback can no longer be sustained. A macroscopic Ca^{2+} spike is then the result of the synchronized triggering of many such local Ca^{2+} pulses in an individual cell, and a Ca^{2+} wave is the result of the sequential triggering of neighboring Ca^{2+} pulses across a cell or from one cell to another. How can local Ca^{2+} pulses by synchronized? Our measurements suggest that luminal Ca^{2+} feedback can be triggered by increasing the concentration of IP_3, increasing the concentration of cytosolic Ca^{2+}, or increasing the concentration of Ca^{2+} in the lumen.

One mechanism by which an increase in IP_3 concentration could mediate synchronization of Ca^{2+} release has been proposed previously by one of the authors. According to this model, Ca^{2+} that is released by IP_3 increases PLC activity, leading to the production of more IP_3 (Meyer & Stryer, 1988). The current study supports the possibility of a second mode of synchronization mediated by the diffusion of released Ca^{2+} to neighboring Ca^{2+} stores that are not yet activated. The direct binding of Ca^{2+} to cytosolic sites on these inactive stores could initiate a secondary luminal Ca^{2+} feedback. Synchronization by Ca^{2+} diffusion and Ca^{2+} binding to cytosolic sites of the IP_3 receptor was originally suggested by Finch et al. (1991). Our studies also predict the possibility of a third mode of synchronization. After diffusion, Ca^{2+} could be pumped into an active store, and the increase in luminal Ca^{2+} would then initiate luminal Ca^{2+} feedback. These three mechanisms of synchronization have different kinetic properties. For instance, Ca^{2+} diffusion is significantly slower than the diffusion of IP_3 (Albritton et al., 1992). Furthermore, Ca^{2+} activation of PLC and Ca^{2+} loading into stores are both thought to be relatively slow processes, whereas the binding of Ca^{2+} to a cytosolic regulatory site is a rapid process. The positive feedback mechanisms mediated by Ca^{2+} activation of PLC, by Ca^{2+} activation of the IP_3 receptor, and by increasing luminal Ca^{2+} are mathematically equally well suited to explain the triggering of Ca^{2+} spikes and the propagation of Ca^{2+} waves. It will be interesting to learn whether all three synchronization mechanisms are used for different types of Ca^{2+} signals and for different cell types.

ACKNOWLEDGMENT

We thank Drs. G. J. Augustine, C. Nicchitta, B. Goldsmith, and E. C. Murphy for simulating discussions. We also thank

D. Poe for technical assistance in construction of the perfusion apparatus.

REFERENCES

Allbritton, N. L., Meyer, T., & Stryer, L. (1992) *Science 258*, 1812–1815.
Berridge, M. J. (1993) *Nature 361*, 315–325.
Bezprozvanny, I., & Ehrlich, B. E. (1994) *J. Gen. Physiol. 104*, 821–856.
Bezprozvanny, I., Watras, J., & Ehrlich, B. E. (1991) *Nature 351*, 751–754.
Blondel, O., Takeda, J., Janssen, H., Seino, S., & Bell, G. I. (1993) *J. Biol. Chem. 268*, 11356–11363.
Boitano, S., Dirksen, E. R., & Sanderson, M. J. (1992) *Science 258*, 292–295.
Combettes, L., & Champeil, P. (1994) *Science 265*, 813–815.
Combettes, L., Claret, M., & Champeil, P. (1992) *FEBS Lett. 301*, 287–290.
Combettes, L., Hannaert-Merah, Z., Coquil, J. F., Rousseau, C., Claret, M., Swillens, S., & Champeil, P. (1994) *J. Biol. Chem. 269*, 17561–17571.
Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S., & Smith, S. J. (1990) *Science 247*, 470–473.
Eiseman, E., & Bolen, J. B. (1992) *Nature 355*, 78–80.
Ferris, C. D., Huganir, R. L., Supattapone, S., & Snyder, S. H. (1989) *Nature 342*, 87–89.
Finch, E. A., & Goldin, S. M. (1994) *Science 265*, 813–815.
Finch, E. A., Turner, T. J., & Goldin, S. M. (1991) *Science 252*, 443–446.
Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., & Mikoshiba, K. (1989) *Nature 342*, 32–38.
Gilkey, J. C., Jaffee, L. F., Ridgway, E. B., & Reynolds, G. T. (1978) *J. Cell Biol. 76*, 448–466.
Hanson, P. I., Meyer, T., Stryer, L., & Schulman, H. (1994) *Neuron 12*, 943–956.
Iino, M., & Endo, M. (1992) *Nature 360*, 76–78.
Kasai, H., & Augustine, G. J. (1990) *Nature 348*, 735–738.
Lechleiter, J., Girard, S., Peralta, E., & Clapham, D. (1991) *Science 252*, 123–126.
Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., & Mikoshiba, K. (1991) *J. Biol. Chem. 266*, 1109–1116.
Metzger, H., Alcaraz, G., Hohman, R., Kinet, J. P., Pribluda, V., & Quarto, R. (1986) *Annu. Rev. Immunol. 4*, 419–470.
Meyer, T. (1991) *Cell 64*, 675–678.
Meyer, T., & Stryer, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5051–5055.
Meyer, T., & Stryer, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3841–3845.
Meyer, T., & Stryer, L. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 153–174.
Meyer, T., Wensel, T., & Stryer, L. (1990) *Biochemistry 29*, 32–37.
Millard, P. J., Ryan, T. A., Webb, W. W., & Fewtrell, C. (1989) *J. Biol. Chem. 264*, 19730–19739.
Missiaen, L., De Smedt, H., Droogmans, G., & Casteels, R. (1992) *Nature 357*, 599–602.
Missiaen, L., De Smetdt, H., Parys, J. B., & Cateels, R. (1994) *J. Biol. Chem. 269*, 7238–7242.
Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S., & Mikoshiba, K. (1992) *Science 257*, 251–255.
Muallem, S., Pandol, S. J., & Beeker, T. G. (1989) *J. Biol. Chem. 264*, 205–212.
Parker, I., & Ivorra, I. (1993) *J. Physiol. 461*, 133–165.
Parys, J. B., Missiaen, L., De Smedt, H., & Casteels, R. (1993) *J. Biol. Chem. 268*, 25206–25212.
Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., & Paul, W. E. (1989) *Nature 339*, 64–67.
Richardson, A., & Taylor, C. W. (1993) *J. Biol. Chem. 268*, 11528–11533.
Sanderson, M. J., Charles, A. C., & Dirksen, E. R. (1990) *Cell Regul. 1*, 585–596.
Shuttleworth, T. J. (1992) *J. Biol. Chem. 267*, 3573–3576.
Stern, M. D. (1992) *Cell Calcium 13*, 183–192.
Sudhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., & Mignery, G. A. (1991) *EMBO J. 10*, 3199–3206.
Swillens, S., Combettes, L., & Champeil, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10074–10078.
Tse, A., Tse, F. W., Almers, W., & Hille, B. (1993) *Science 260*, 82–84.
Tsien, R. Y. (1980) *Biochemistry 19*, 2396–2404.
Tsien, R. Y., & Pozzan, T. (1989) *Methods Enzymol. 172*, 231–262.
Tsien, R. W., & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol. 6*, 715–760.
Woods, N. M., Cuthbertson, K. S., & Cobbold, P. H. (1986) *Nature 319*, 600–602.

BI9505878