Dual Regulation of Calcium Mobilization by Inositol 1,4,5-Trisphosphate in a Living Cell

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abstract Changes in cytosolic free calcium ($[Ca^{2+}]_i$) often take the form of a sustained response or repetitive oscillations. The frequency and amplitude of $[Ca^{2+}]_i$ oscillations are essential for the selective stimulation of gene expression and for enzyme activation. However, the mechanism that determines whether $[Ca^{2+}]_i$ oscillates at a particular frequency or becomes a sustained response is poorly understood. We find that $[Ca^{2+}]_i$ oscillations in rat megakaryocytes, as in other cells, results from a Ca^{2+}-dependent inhibition of inositol 1,4,5-trisphosphate (IP_3)–induced Ca^{2+} release. Moreover, we find that this inhibition becomes progressively less effective with higher IP_3 concentrations. We suggest that disinhibition, by increasing IP_3 concentration, of Ca^{2+}-dependent inhibition is a common mechanism for the regulation of $[Ca^{2+}]_i$ oscillations in cells containing IP_3-sensitive Ca^{2+} stores.

key words: megakaryocyte • protein kinase C • pleckstrin • IP_3-5-phosphatase • platelets

INTRODUCTION

Calcium is a universal intracellular signaling agent involved in a myriad of processes from fertilization to cell death (Berridge et al., 1998). Changes in cytosolic free calcium ($[Ca^{2+}]_i$) are well documented for cells stimulated by many hormone and growth factor agonists that generate the second messenger inositol 1,4,5-trisphosphate (IP_3).[1] $[Ca^{2+}]_i$ signals can be a single transient or a sustained increase, but very often take the form of repetitive spikes or oscillations. The frequency and amplitude of $[Ca^{2+}]_i$ oscillations are essential for initiating numerous cellular processes, including selective stimulation of gene expression (Dolmetsch et al., 1998; Li et al., 1998) and the activation of specific enzymes (De Koninck and Schulman, 1998). It has been observed that as the concentration of agonist is increased $[Ca^{2+}]_i$ oscillations increase in frequency, eventually becoming a sustained $[Ca^{2+}]_i$ elevation (Jacob et al., 1988; Wakui et al., 1989; Heemskerk et al., 1993). A similar phenomenon has also been seen when cells are dialyzed with increasing concentrations of the nonmetabolized IP_3 analogue, inositol 1,4,5 trisphosphorothioate (Petersen et al., 1991). However, the mechanism by which the $[Ca^{2+}]_i$ oscillation frequency increases and how the response changes into a sustained $[Ca^{2+}]_i$ elevation is not understood.

Many models of agonist-induced $[Ca^{2+}]_i$ oscillations in nonexcitable cells require some form of Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} release as a fundamental component (Fewtrell, 1993). In these models, released Ca^{2+} feeds back to inhibit further release of Ca^{2+} by IP_3 (Payne et al., 1988; Ogden et al., 1990; Ilyin and Parker, 1994; Oancea and Meyer, 1996; Carter and Ogden, 1997). However, it is not clear how these models could explain the increase in the $[Ca^{2+}]_i$ oscillation frequency with increased agonist concentration described above. Or for that matter how the $[Ca^{2+}]_i$ oscillation changes into a sustained $[Ca^{2+}]_i$ elevation. A possible answer might come from in vitro studies, which have shown that the extent of Ca^{2+}-dependent inhibition may be regulated by the concentration IP_3. For example, the inhibition by Ca^{2+} of IP_3-induced Ca^{2+} release from cerebellar microsomes (Joseph et al., 1989; Combettes et al., 1994; Hannaert-Merah et al., 1995) and permeabilized A7r5 smooth muscle cells (Bootman et al., 1995) decreases as the IP_3 concentration is elevated. Likewise, a similar effect is seen at the level of the single IP_3-gated Ca^{2+} channel (Kaftan et al., 1997; Mak et al., 1998). Whether or not this decrease of Ca^{2+}-dependent inhibition as the IP_3 concentration is elevated occurs in intact cells is not known. The experiments described herein were designed to extend these in vitro findings to an intact cell, the rat megakaryocyte. We show for the first time, in an intact cell, that Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} release becomes progressively less effective with higher IP_3 concentrations.

METHODS

The methods used in these experiments have been fully described in previous publications (Tertyshnikova and Fein, 1997, 1998; Tertyshnikova et al., 1998; Lu et al., 1999). They are described briefly below.

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[1]*Abbreviations used in this paper:* IP_3, inositol 1,4,5-trisphosphate; IP_3-R, IP_3 receptor.

Rat Megakaryocytes

Bone marrow is obtained from the tibial and femoral bones of adult Wistar rats. After filtration through a 75-μm nylon mesh to eliminate large masses of cells, the bone marrow suspension is spun and washed twice before incubation in standard external solution containing (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4, supplemented by 0.1% BSA. Megakaryocytes are clearly distinguished from other bone marrow cells on the basis of their large size (25–50 μm) and multilobular nucleus (Uneyama et al., 1993; Kapural and Fein, 1997). All experiments are done within 2–6 h after preparation at room temperature (23–25°C).

Measurement of $[Ca^{2+}]_i$ and Photolysis of Caged Compounds

Megakaryocytes are viewed through a coverslip forming the bottom of the recording chamber using a Diaphot microscope equipped with a Fluor 100× 1.3 NA oil immersion lens (Nikon Inc.). Single cell fluorometry is accomplished using an Ionoptix photon-counting fluorescence subsystem with a dual excitation light source (designed by Dr. D. Tillotson; Ionoptix) using Oregon Green 488 BAPTA-1 (OGB488) as the $[Ca^{2+}]_i$ indicator. Fluorescence intensity is measured on-line using the Ionwizard program (IonOptix). For photolysis of caged compounds, pulses of ultra violet light (290–370 nm) are applied to the cell through the second channel of the dual excitation light source. Calibration of photolysis in the microscope was by measurement of the fluorescence change produced in the pH dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein by protons released during the photolysis of NPE-caged ATP (Fink et al., 1999). A 50-s flash uncaged ~50% of the caged ATP.

Chemicals

The "cell-permeant" AM ester and the "cell-impermeable" hexapotassium salt of OGB488 are obtained from Molecular Probes, Inc. Caged IP_3 and caged $GPIP_2$ [1-(alpha-glycerophosphoryl)-myo-inositol 4,5-diphosphate, P4(5)-1-(2-nitrophenyl) ethyl ester] are from Calbiochem Corp. GF109203X is from Biomol and 2,3-diphosphoglycerate (2,3-DPG) is from Sigma Chemical Co.

Cell Loading of Caged Compounds

The cell-permeant AM ester of OGB488 is dissolved in DMSO and stored at −20°C. For the experiments not using patch clamping, cells are transferred onto glass coverslips and incubated with 2.5–5 μM OGB488/AM for 30 min. For the experiment with caged calcium, the cells are first incubated with 10–30 μM caged calcium for at least 2 h. The final concentration of DMSO is always <0.1%. The coverslips with adherent cells are then washed several times with the standard external solution, and kept in the dark until use. For the other experiments, caged IP_3 or caged $GPIP_2$ together with OGB488 hexapotassium salt are included in the intrapipette solution at 100 and 200 μM, respectively [composition (mM): 20 KCl, 120 K-glutamate, 1 MgCl, 2 Na-GTP, 10 HEPES, pH 7.3]. Standard whole-cell patch-clamp recording techniques are used to voltage clamp and internally dialyze single megakaryocytes. Membrane current is monitored using an Axopatch-1D patch clamp amplifier (Axon Instruments). For most cells, 5–6 min is required for the OGB488 fluorescence signal to equilibrate in the patch-clamped cell.

Agonist Application

ADP or the mixture of ADP with GF109203X are dissolved in the standard external solution and applied directly to single megakaryocytes using a DAD-6 computer-controlled local superfusion system (ALA Scientific Instruments, Inc.). The output tube of the micromanifold (100 μm inside diameter) is placed within ~200 μm of the cell and the puff pressure is adjusted to achieve rapid agonist application while avoiding any mechanical disturbance of the cell.

RESULTS

To study Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} release, we performed paired-pulse experiments in rat megakaryocytes that are a convenient model for studying Ca^{2+} signaling in nonexcitable cells, because they express only an IP_3-sensitive Ca^{2+} store and lack a ryanodine-sensitive Ca^{2+} store (Uneyama et al., 1993). For the first pulse of IP_3, in a paired-pulse experiment, the intracellular increase of IP_3, resulting from photorelease from caged IP_3, causes a transient release of Ca^{2+} lasting a few hundred milliseconds. After the response to the first pulse, there is a period of desensitization lasting several seconds, during which responses to a second pulse of IP_3 are diminished in amplitude (see Fig. 2 A). The available evidence indicates that this period of desensitization is due to Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} release (Ogden et al., 1990; Payne et al., 1988, 1990; Ilyin and Parker, 1994; Oancea and Meyer, 1996; Carter and Ogden, 1997).

In rat basophilic leukemia cells, maximal desensitization of the response to the second pulse of IP_3 is observed for a first pulse of IP_3 that produced a $[Ca^{2+}]_i$ response of near maximal amplitude (Oancea and Meyer, 1996). Therefore, we began our experiments by first measuring the power dependence of IP_3-induced Ca^{2+} release (Fig. 1). In Fig. 1, B and C, we plot the normalized peak amplitude (R/R_{max}) of the IP_3-mediated $[Ca^{2+}]_i$ response as a function of the flash duration, which is directly proportional to IP_3 concentration. As can be seen in Fig. 1 C, the data are well fit with the Hill equation with a coefficient of $n = 7$. For the 10 cells in Fig. 1 C, the flash duration that produced a response of half the maximal amplitude was 203 ± 95 ms (mean ± SD).

We found that maximal desensitization was observed when the flash duration in a paired-pulse experiment produced a response just below that which gives a response of saturating amplitude. An example of such an experiment can be seen in Fig. 2 A for which, after the release of Ca^{2+} produced by the photorelease of IP_3, there is a period of desensitization during which a subsequent increase in IP_3 releases less calcium. As the time interval between the pulses of IP_3 increases, the response to the second pulse recovers back to that of the first. The desensitization is not due to emptying of the Ca^{2+} stores, because desensitization of the second response disappears if the duration of the second flash is increased threefold, thereby saturating the amplitude of the second response ($n = 6$ cells, data not shown). The desensitization also disappears if the duration of

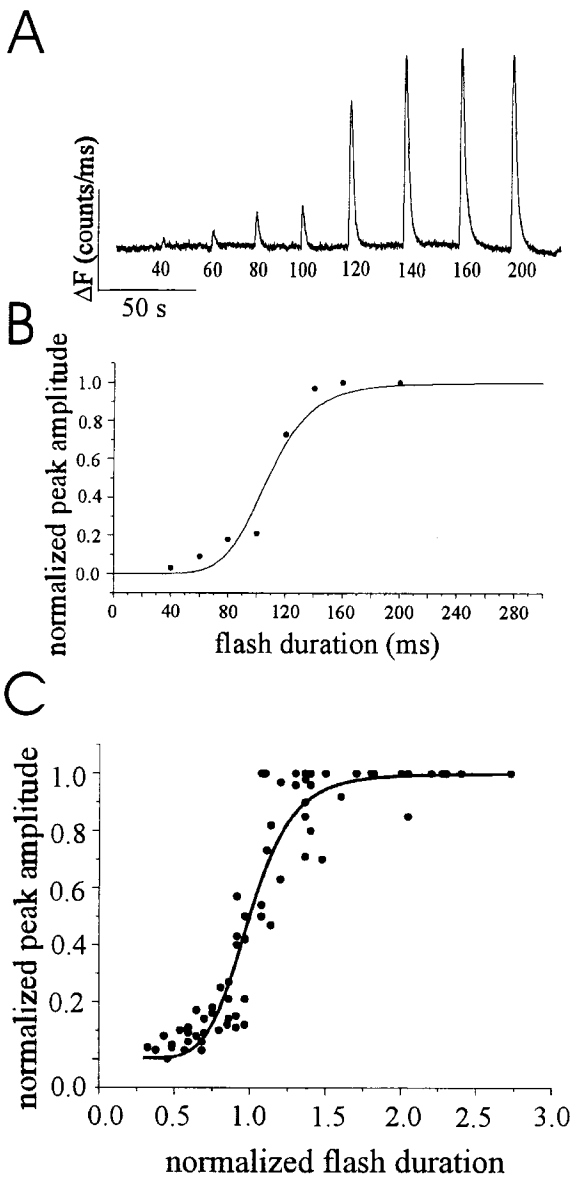

Figure 1. Dose–response curve for IP_3-mediated Ca^{2+} release. (A) IP_3-mediated $[Ca^{2+}]_i$ response for UV flashes of increasing duration. Caged IP_3 (100 μM) and OGB488 (200 μM) were included in the patch pipette solution. (B) Normalized peak amplitude (R/R_{max}) of the IP_3-mediated $[Ca^{2+}]_i$ response for the data in A as a function of the flash duration, where R_{max} is the maximum peak amplitude observed. (C) Normalized peak amplitude (R/R_{max}) of the IP_3-mediated $[Ca^{2+}]_i$ response for 10 cells as a function of the normalized flash duration. The data in B and C are well fit with the Hill equation $R/R_{max} = 1/[1 + (d_{1/2}/d)^n]$, with $n = 7$, where $d_{1/2}$ is the flash duration that gives a peak response of 1/2 the maximum amplitude. (B) $d_{1/2} = 108$ ms, (C) $d_{1/2} = 1$.

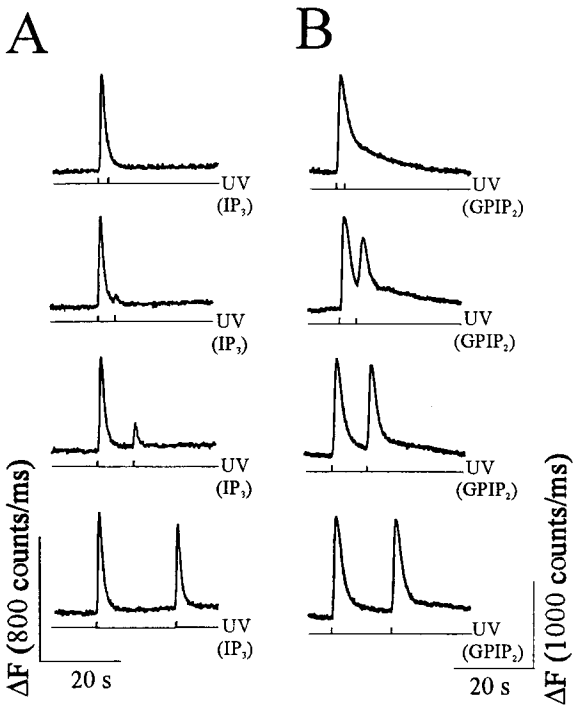

Figure 2. Paired pulse experiment to measure recovery from desensitization in rat megakaryocytes loaded with either caged-IP_3 or -$GPIP_2$. In each trace of A and B, the rise in $[Ca^{2+}]_i$ was monitored by measuring OGB488 fluorescence intensity and is expressed as ΔF (counts/millisecond). For each trace in A and B, the cell was stimulated by two flashes of UV light, which released IP_3 in A and $GPIP_2$ in B. For the cell in A, 100 μM caged IP_3 and 200 μM OGB488 were included in the patch pipette solution. For the cell in B, 100 μM caged $GPIP_2$ and 200 μM OGB488 were included in the patch pipette solution. See methods for further experimental details.

both flashes is increased three- to fourfold, thereby saturating the response amplitude of the response to each flash ($n = 3$ cells, data not shown). These findings are similar to what was found for rat basophilic leukemia cells (Oancea and Meyer, 1996), for which it was concluded that a two- to threefold decrease in IP_3 sensitivity was sufficient to explain the reduced amplitude of the response to the second pulse of IP_3, and we suggest that the same is true for rat megakaryocytes.

The experiments described above establish the basic conditions for measuring the time course of recovery in a paired-pulse experiment. Having established these conditions, we can now turn to the central question of this investigation, whether Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} release becomes progressively less effective with higher IP_3 concentrations. For this purpose, we used procedures that would increase the lifetime of IP_3, by slowing down its hydrolysis. We began by comparing the time course for the recovery from desensitization produced by IP_3 injection with the time course for recovery from desensitization produced by injection of a hydrolysis-resistant analogue of IP_3, namely $GPIP_2$. $GPIP_2$ is a less potent but fully active analogue of IP_3 that is poorly metabolized, and the caged form of $GPIP_2$ has been used to mobilize Ca^{2+} from IP_3-sensitive Ca^{2+} stores (Berven and Barritt, 1994). As with IP_3, the flash duration when using caged $GPIP_2$ is set to give a response just below that which gives a response of saturating amplitude. After the release of Ca^{2+} pro-

duced by the photorelease of $GPIP_2$ (Fig. 2 B), the cell recovers its sensitivity much faster than in Fig. 2 A. It would appear from the results in Fig. 2 that the recovery from desensitization accelerates when the rate of hydrolysis of IP_3 is slowed down. This is the opposite of what one would expect if the recovery from desensitization were following the time course for the hydrolysis of IP_3. We assume that the acceleration in the rate of recovery is due to the decreased rate of hydrolysis of $GPIP_2$ compared with IP_3. If this assumption is correct, then we should be able to accelerate the recovery from desensitization produced by photoreleased IP_3 to a time course similar to that produced by photoreleased $GPIP_2$ by inhibiting the IP_3-5-phosphatase, the enzyme which hydrolyses IP_3.

Accordingly, in Fig. 3, we compare the time course for recovery after photorelease of IP_3, in the presence and absence of 2,3-DPG (2,3-diphosphoglycerate), an inhibitor of the IP_3-5-phosphatase (Shears, 1989; Wood et al., 1990). In Fig. 3, we plot the ratio (A2/A1) as a function of the time interval between the pulses, where A2 is the peak amplitude of the response to the second pulse of IP_3 and A1 is the peak amplitude of the response to the first pulse of IP_3 (Fig. 2). Each group of recovery data in Fig. 3 was fit with Eq. 1 to obtain an estimate of the average time for recovery for each experimental condition.

$$y = (1 - e^{-(t-1.5)/\tau}). \quad (1)$$

In Eq. 1, the 1.5-s time delay is the approximate time to peak for the response to IP_3 or $GPIP_2$. For IP_3 alone, $\tau = 15$ s ($n = 8$ cells) and for IP_3 with 2,3-DPG, $\tau = 4.2$ s ($n = 6$ cells). Also included in Fig. 3 are recovery data for $GPIP_2$ that were fit with $\tau = 2.6$ s ($n = 5$ cells) and data for IP_3 in the presence of GF109203X, which were fit with $\tau = 5.4$ s ($n = 11$ cells).

GF109203X is a cell-permeable inhibitor of PKC that has been used effectively to inhibit PKC in platelets (Toullec et al., 1991). Inhibition of PKC in platelets causes an approximately threefold increase in IP_3 levels in thrombin-activated platelets (King and Rittenhouse, 1989). This is thought to occur via the inhibition of the phosphorylation of pleckstrin, the major substrate of PKC in platelets, because phosphorylated pleckstrin has been shown to activate the IP_3-5-phosphatase (Auethavekiat et al., 1997). Therefore, inhibition of PKC by GF109203X should inhibit the hydrolysis of IP_3 by the 5-phosphatase and consequently prolong the lifetime of IP_3. The experimental data in Figs. 2 and 3 clearly suggest that the recovery from desensitization is accelerated when the rate of hydrolysis of IP_3 is slowed down. This suggests that the extent of Ca^{2+}-dependent inhibition is diminished when the lifetime of IP_3 is increased.

To be certain that the findings in Fig. 3 are not somehow the result of an effect of GF109203X, 2,3-DPG, or

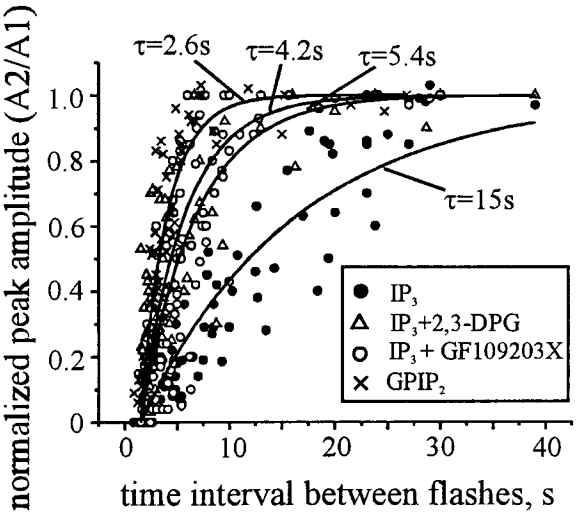

Figure 3. Time course of recovery from desensitization in rat megakaryocytes loaded with either caged IP_3 or $GPIP_2$. The data points were obtained from paired-pulse experiments similar to those in Fig. 2. Each data point represents the ratio (A2/A1) of the peak amplitude of the response to the second pulse of IP_3 (A2) to the peak amplitude of the response to the first pulse of IP_3 (A1) as a function of the time interval between the pulses (Fig. 2). We used 10 mM 2,3-DPG and 10 μM GF109203X in these experiments. Although a concentration of 10 mM for 2,3-DPG may seem high, it is actually quite reasonable as the K_i for inhibition of IP_3-5-phosphatase is in the millimolar range (for example, the $K_i = 4$ mM in squid photoreceptors; Wood et al., 1990). See methods for further experimental details and the text for an explanation of the solid curves and the τ values associated with them.

$GPIP_2$ on the power dependence of IP_3-induced Ca^{2+} release, we carried out the experiment presented in Fig. 4. The data in Fig. 4 clearly show that the power dependence for $GPIP_2$- and IP_3-induced Ca^{2+} release in the presence of GF109203X or 2,3-DPG are no different than the power dependence of IP_3-induced Ca^{2+} release itself. The flash duration that produced a response of half the maximal amplitude was 108 ± 39 ms ($n = 8$ cells) for $GPIP_2$, 155 ± 54 ms ($n = 5$ cells) for IP_3 in the presence of 2,3-DPG, and 121 ± 44 ms ($n = 8$ cells) for IP_3 in the presence of GF109203X. The flash duration for half-maximal amplitude for $GPIP_2$ and IP_3 in the presence of GF109203X are significantly different than that for IP_3 at the $P = 0.05$ level using the unpaired Student's t test. However, the flash duration for half-maximal amplitude for IP_3 in the presence of 2,3-DPG is not significantly different than that for IP_3. Hence the findings in Fig. 3 are consistent with our suggestion that the extent of Ca^{2+}-dependent inhibition is diminished when the lifetime of IP_3 is increased.

Based on the data in Figs. 2 and 3, we predict that the falling phase of the response to the uncaging of $GPIP_2$ should be dominated by the inhibitory effect of elevated $[Ca^{2+}]_i$ on further Ca^{2+} release and the removal of Ca^{2+} from the cytoplasm. That is, the hydrolysis of

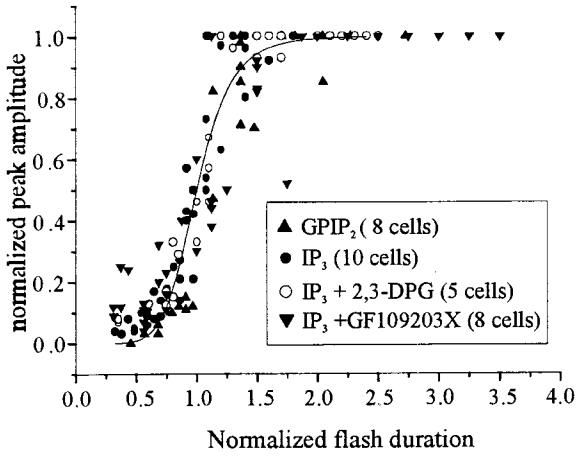

Figure 4. Dose–response curve for $GPIP_2$-mediated Ca^{2+} release and IP_3-mediated Ca^{2+} release in the presence of 2,3-DPG or GF109203X. OGB488 (200 μM) together with either caged IP_3 (100 μM) or $GPIP_2$ (100 μM) were included in the patch pipette solution. We used 10 mM 2,3-DPG and 10 μM GF109203X in these experiments. As in Fig. 1 C, we plot the normalized peak amplitude (R/R_{max}) of the IP_3- or $GPIP_2$-mediated $[Ca^{2+}]_i$ response as a function of the normalized flash duration, where R_{max} is the maximum peak amplitude of the $[Ca^{2+}]_i$ response. Also included in this figure is the data from Fig. 1 C. The data are well fit with the Hill equation $R/R_{max} = 1/[1 + (d_{1/2}/d)^n]$, with $n = 7$ and $d_{1/2} = 1$.

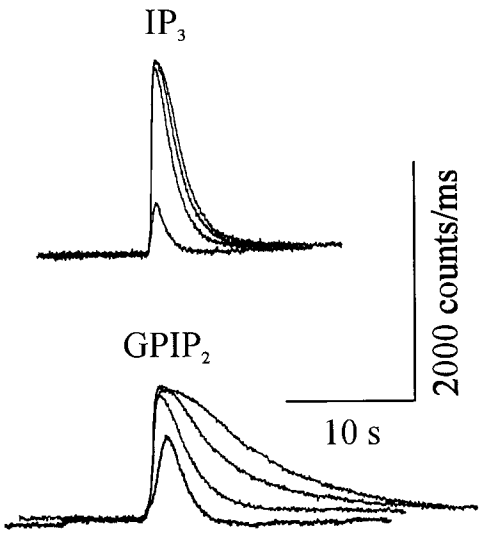

Figure 5. Comparison of the time course of the $[Ca^{2+}]_i$ response to the uncaging of IP_3 or $GPIP_2$ in rat megakaryocytes. Flashes of 150-, 300-, 1,000-, and 2,000-ms duration were used for uncaging with each cell. Changes in $[Ca^{2+}]_i$ were monitored by measuring OGB488 fluorescence intensity and are expressed as ΔF (counts/millisecond). Results similar to those shown were seen in two other cells each.

$GPIP_2$ by the 5-phosphatase should have minimal effect on the falling phase of the response. Accordingly, the falling phase of the response to the uncaging of $GPIP_2$ should be greatly prolonged when compared with that for the uncaging of IP_3, especially as the amount of IP_3 or $GPIP_2$ uncaged is increased. In Fig. 5, we compare the time course of the $[Ca^{2+}]_i$ response to the uncaging of IP_3 with that for the uncaging of $GPIP_2$. As the duration of the uncaging flash is increased from 150 to 2,000 ms, it can be seen that the falling phase of the response to $GPIP_2$ is greatly prolonged when compared with that for IP_3. Results similar to those in Fig. 5 were seen in two additional cells each.

As mentioned above, Ca^{2+}-dependent inhibition of IP_3-mediated Ca^{2+} release is thought to play a central role in the generation of $[Ca^{2+}]_i$ oscillations. Also, megakaryocytes exhibit $[Ca^{2+}]_i$ oscillations when exposed to ADP (Tertyshnikova and Fein, 1997; Uneyama et al., 1993). To examine how the lessening of Ca^{2+}-dependent inhibition will affect an agonist-induced $[Ca^{2+}]_i$ oscillation, we examined the effect of GF109203X on ADP-induced $[Ca^{2+}]_i$ oscillations. As shown in Fig. 6, in the presence of GF109203X, ADP causes a plateau-like rise in $[Ca^{2+}]_i$ ($n = 3$ cells).The experiment in Fig. 6 was carried out in a Ca^{2+}-free external solution in presence of 1 mM BAPTA, to rule out the possibility that the effect of GF109203X was on Ca^{2+} influx. Results similar to those in Fig. 6 were obtained when the experiment was performed in standard external solution that contains 2 mM calcium ($n = 4$ cells, data not shown). GF109203X is also a less potent inhibitor of cAMP-PK; however, the effect of GF109203X on rat megakaryocytes is entirely different from what we have found when inhibiting cAMP-PK in these cells (Tertyshnikova and Fein, 1998).

The results in Fig. 6 are very similar to those obtained by examining the effect of another PKC inhibitor, staurosporine, on ATP-induced $[Ca^{2+}]_i$ oscillations monitored as a calcium-activated potassium current oscillation (Uneyama et al., 1993). These workers (Uneyama et al., 1993) speculated that the effect on $[Ca^{2+}]_i$ oscillations, of inhibiting PKC with staurosporine, resulted from an inhibition of the Ca^{2+} pump. To investigate whether GF109203X affects Ca^{2+} uptake and/or extrusion, we used caged Ca^{2+} for the experiment in Fig. 7. The time course of the fall in $[Ca^{2+}]_i$ after the flash-induced rise in $[Ca^{2+}]_i$ should reflect the activity of Ca^{2+} sequestration and/or extrusion mechanisms (see Tertyshnikova and Fein, 1998; Tertyshnikova et al., 1998). As can be seen in Fig. 7, photoreleased $[Ca^{2+}]_i$ declined at the same rate in the presence and absence of GF109203X. In the experiment shown in Fig. 7, cyclopiazonic acid, an inhibitor of the smooth endoplasmic reticulum calcium ATPase in platelets (Papp et al., 1993), was used as a positive control for inhibition of Ca^{2+} sequestration. Similar results as those in Fig. 7 were seen in two other cells. The results in Fig. 7 appear to convincingly rule out inhibition of the Ca^{2+} pump as an explanation for the findings in Fig. 6.

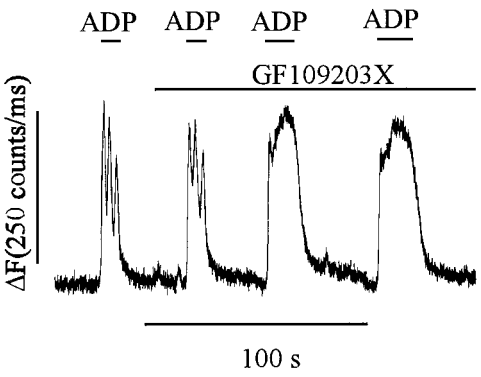

Figure 6. Effect of the PKC inhibitor, GF109203X, on ADP-induced $[Ca^{2+}]_i$ oscillations in a rat megakaryocyte. Changes in $[Ca^{2+}]_i$ were monitored by measuring OGB488 fluorescence intensity and are expressed as ΔF (counts/millisecond). GF109203X (15 μM) and ADP (100 μM) were applied to the cell via the local superfusion system. The cell was loaded with OGB488 using the cell permeable AM ester of OGB488. Similar results were seen in five other cells. See methods for further experimental details.

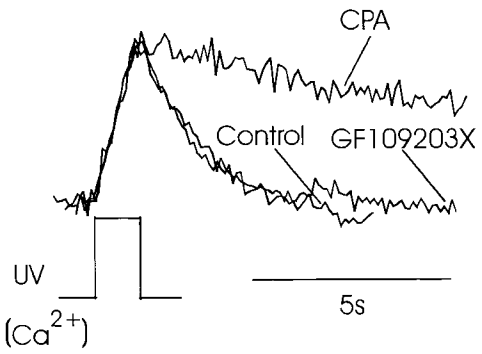

Figure 7. The PKC inhibitor GF109203X does not affect the rate of calcium removal from the cytoplasm. The cell was loaded with caged Ca^{2+} and OGB488 using the cell-permeant forms of each molecule as described in methods. $[Ca^{2+}]_i$ spikes resulting from photorelease of caged Ca^{2+} are shown superimposed for the purpose of comparison. GF 109203X had no effect on the time course of the fall in $[Ca^{2+}]_i$ after photolysis of caged-Ca^{2+}. Cyclopiazonic acid (CPA) was applied to the cell to serve as a positive control for inhibition of Ca^{2+} sequestration. GF109203X (30 μM) and CPA (5 μM) were applied to the cell via the local superfusion system as described in methods.

Based on the data of Figs. 2, 3, and 6, we would expect that in response to multiple injections of IP_3, the rise in $[Ca^{2+}]_i$ would become plateau-like when the hydrolysis of IP_3 is slowed down. Accordingly, in Fig. 8, we compare the responses to multiple flashes, which photorelease IP_3, in the presence and absence of 2,3-DPG. As can be seen in Fig. 8 A, the response to the first flash that photoreleases IP_3 is large, and the responses to subsequent flashes are greatly reduced in amplitude. Based on the results presented in Figs. 2 and 3, the finding in Fig. 8 A is as expected. In contrast, in the experiment of Fig. 8 B, in which 10 mM 2,3-DPG was included in the patch pipette to inhibit the IP_3-5-phosphatase, a series of flashes that photorelease IP_3 produce a sustained elevation of $[Ca^{2+}]_i$. Likewise a series of flashes that photorelease IP_3 produce a sustained elevation of $[Ca^{2+}]_i$ in the presence of GF109203X (Fig. 8 D). Furthermore, a train of flashes that photorelease the hydrolysis-resistant IP_3-analogue $GPIP_2$ also produce a sustained elevation of $[Ca^{2+}]_i$ (Fig. 8 C).

The simplified diagram in Fig. 9 summarizes our findings, emphasizing the dual regulation of calcium mobilization by IP_3. For the sake of simplicity, $GPIP_2$ has been left out of the figure. The heavy lines in Fig. 9 are meant to represent the release of Ca^{2+} by IP_3 and the disinhibition of Ca^{2+}-dependent inhibition of IP_3-mediated Ca^{2+} release by increasing IP_3 concentration. We show this disinhibition as acting via calmodulin because recently published experiments have indicated that Ca^{2+}-dependent inhibition of IP_3-mediated Ca^{2+} release for the type 1 IP_3 receptor (IP_3-R) is mediated by calmodulin (Michikawa et al., 1999) (see discussion).

DISCUSSION

Our results demonstrate for the first time an important property of $[Ca^{2+}]_i$ signaling in intact cells: an increase in the lifetime of IP_3 brings about a decrease in Ca^{2+}-dependent inhibition. These findings suggest a mechanism by which high concentrations of intracellular IP_3 can cause cells to maintain an elevated level of $[Ca^{2+}]_i$. Indeed, this may explain the occurrence of sustained $[Ca^{2+}]_i$ elevations at high agonist concentrations (Jacob et al., 1988; Wakui et al., 1989; Heemskerk et al., 1993) and when cells are dialyzed with high concentrations of the nonmetabolized IP_3 analogue inositol 1,4,5 trisphosphorothioate (Petersen et al., 1991). Our findings also suggest a possible mechanism for the regulation of the frequency of $[Ca^{2+}]_i$ oscillations in cells containing IP_3-sensitive Ca^{2+} stores. One test of the value of our findings will come from future studies that extend these observations to other cell types and incorporate these mechanisms into mathematical models of $[Ca^{2+}]_i$ signaling.

Since platelets express primarily the type 1 isoform of the IP_3-R (O'Rourke et al., 1995; Quinton and Dean, 1996) and megakaryocytes are the precursors of platelets, our findings may directly reflect properties of the type 1 IP_3-R. Remember that, as mentioned in the introduction, cerebellar microsomes (Joseph et al., 1989; Combettes et al., 1994; Hannaert-Merah et al., 1995) and permeabilized A7r5 smooth muscle cells (Bootman et al., 1995), which contain primarily the type 1 isoform of the IP_3-R, exhibit decreased Ca^{2+}-dependent inhibition at elevated IP_3 concentrations. Moreover, single channel recordings from the cerebellar type 1 IP_3-R (Kaftan et al., 1997) and a similar receptor found

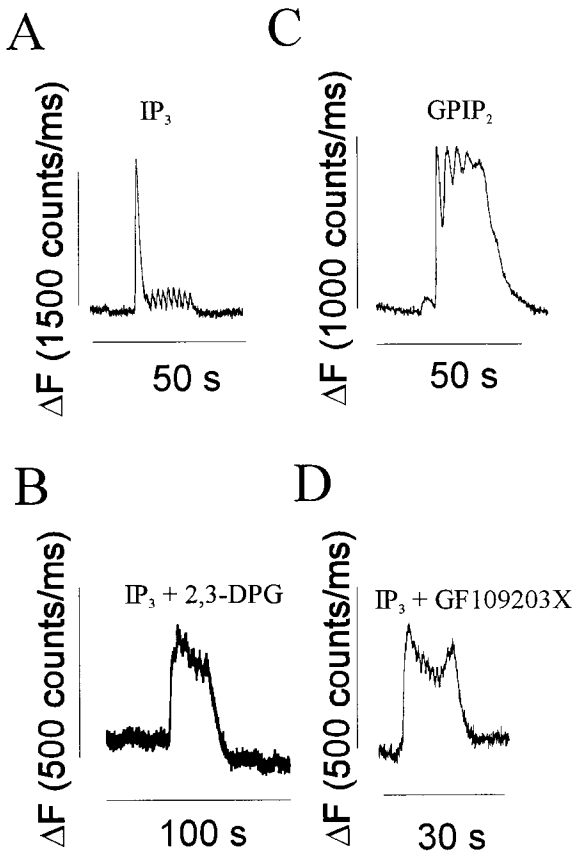

Figure 8. Elevation of $[Ca^{2+}]_i$ resulting from multiple flashes that photorelease caged IP_3 or $GPIP_2$. (A) Caged IP_3, (B) caged IP_3 in the presence of 10 mM 2,3-DPG, (C) caged $GPIP_2$, and (D) caged IP_3 in the presence of 5 μM GF109203X. Either caged IP_3 (100 μM) and OGB488 (200 μM), or caged $GPIP_2$ (100 μM) and OGB488 (200 μM) were included in the patch-pipette solution. The cell in A was stimulated with 11 flashes spaced 1.8-s apart, the cell in B was stimulated with 6 flashes spaced 3.6-s apart, the cell in C was stimulated with 16 flashes spaced 1.2-s apart, and the cell in D was stimulated with 11 flashes spaced 1.2-s apart. Similar results as those in A–D were seen in at least three to five other cells each.

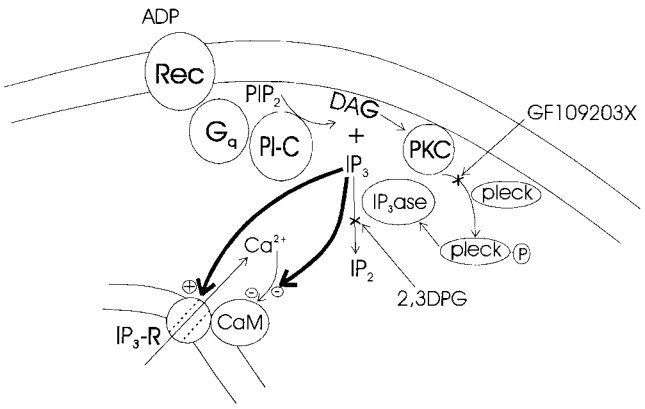

Figure 9. Simplified diagram illustrating disinhibition of Ca^{2+}-dependent inhibition of IP_3-mediated Ca^{2+} release with higher IP_3 concentrations (heavy arrow). ADP is shown as activating phospholipase C (Pl-C) via the GTP-binding protein (G_q) to form diacylglycerol (DAG) and IP_3. IP_3 is shown as causing the release of Ca^{2+} (heavy arrow) and DAG is shown as activating PKC, which in turn phosphorylates pleckstrin (pleck). Phosphorylated pleckstrin is shown as activating the IP_3-5 phosphatase (IP_3ase), which inactivates IP_3 by hydrolyzing it to inositol 1,4-bisphosphate (IP_2). GF109203X and 2,3DPG are shown as inhibiting PKC and the IP_3ase, respectively. Released Ca^{2+} is shown as feeding back via calmodulin (CaM) to inhibit further release of Ca^{2+} by the IP_3-R.

in *Xenopus* oocytes (Mak et al., 1998) indicate that the open probability remains high in the presence of a saturating level of IP_3, even if $[Ca^{2+}]_i$ is raised to high concentrations. It should be kept in mind that IP_3 binding to the purified cerebellar type 1 IP_3-R is not inhibited by Ca^{2+} and it was proposed that inhibition by Ca^{2+} required an accessory protein (Supattapone et al., 1988; Benevolensky et al., 1994), which was recently shown to be calmodulin (Michikawa et al., 1999) (Fig. 9).

The observation that Ca^{2+}-dependent inhibition of the type 1 IP_3-R is mediated by calmodulin implies that inhibition of calmodulin should disinhibit Ca^{2+}-dependent inhibition of IP_3-mediated Ca^{2+} release (Michikawa et al., 1999). Based on our findings, we would predict that such a disinhibition would transform a $[Ca^{2+}]_i$ oscillation into a more sustained $[Ca^{2+}]_i$ elevation (for example, see Fig. 6). This experiment has in fact already been done in rat megakaryocytes, where it was found that the calmodulin inhibitors W-7 and trifluoperazine caused the agonist-induced $[Ca^{2+}]_i$ oscillation to become a more sustained $[Ca^{2+}]_i$ elevation (Uneyama et al., 1993). Note that W-7 is the same calmodulin inhibitor used in the study of Michikawa et al. (1999). One test of the worthiness of our interpretation of these findings will come from the extension of these observations to other cell types.

Whether or not these properties of the type I receptor also belong to the type II and III IP_3-Rs is problematic. Recent single-channel bilayer recordings from the type II and III receptors indicate that they do not exhibit Ca^{2+}-dependent inhibition (Hagar et al., 1998; Ramos-Franco et al., 1998); however, in bilayer recordings, essential accessory proteins may have been lost. On the other hand, Ca^{2+}-dependent inhibition has been observed, using other techniques, in some cell types that contain primarily the type II and III receptors (Taylor, 1998); however, these studies are complicated by the presence of other receptor subtypes. Further experimental work will be needed to determine the extent to which the findings presented here are exemplary of cells that contain primarily the type II and III IP_3-Rs. It may be that cells contain mixtures of the different isoforms of the IP_3-R to combine properties specific to each type of receptor.

One of the striking features of IP_3-mediated Ca^{2+} release in megakaryocytes is the highly nonlinear depen-

dence between IP_3 and peak Ca^{2+} (Figs. 1 and 4). In other cell types, the dependence is not as steep (Khodakhah and Ogden, 1995; Oancea and Meyer, 1996; Carter and Ogden, 1997; Ogden and Capiod, 1997); for example, in rat basophilic leukemia cells, the Hill coefficient is 3.2, as compared with 7 for megakaryocytes. There are two factors that would be expected to contribute to the nonlinear dependence between IP_3 and peak Ca^{2+}. First is a requirement for the binding of several IP_3 molecules to the IP_3-receptor before the channel can open, and second is an amplification of Ca^{2+} release by positive feedback mediated by Ca^{2+} (for example, see Iino, 1990; Bezprozvanny et al., 1991). It may be that there are additional unknown factors at work in megakaryocytes, which are responsible for the exceptionally steep dependence found in these cells.

It might be argued that as the result of inhibition of the 5-phosphatase by 2,3-DPG, more IP_3 is converted by the IP_3-3-kinase to inositol 1,3,4,5-tetrakisphosphate (IP_4). IP_4 has been shown to enhance the amount of Ca^{2+} mobilized by submaximal concentrations of IP_3 in the L1210 cell line (Loomis-Husselbee et al., 1996, 1998). If such a phenomenon were to occur in megakaryocytes, it could possibly explain our findings with 2,3-DPG; however, to our knowledge, it is not known whether such a phenomenon occurs in megakaryocytes or for that matter in cells other than L1210 cells. Furthermore, such a mechanism would not be able to explain our findings with $GPIP_2$. Moreover, it should be kept in mind that it is still controversial whether or not IP_4 plays any role in Ca^{2+} signaling (Irvine, 1992; Putney and Bird, 1993).

Although the findings reported here were obtained in megakaryocytes, they should be relevant to calcium mobilization in platelets also; in as much as megakaryocytes are the precursors of platelets. Specifically, we speculate that our findings suggest a role for pleckstrin, which is a major substrate for PKC in platelets, in regulating $[Ca^{2+}]_i$ oscillations by regulating the lifetime of IP_3.

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REFERENCES

Auethavekiat, V., C.S. Abrams, and P.W. Majerus. 1997. Phosphorylation of platelet pleckstrin activates inositol polyphosphate 5-phosphatase I. *J. Biol. Chem.* 272:1786–1790.

Benevolensky, D., I.I. Moraru, and J. Watras. 1994. Micromolar calcium decreases affinity of inositol trisphosphate receptor in vascular smooth muscle. *Biochem. J.* 299:631–636.

Berridge, M.J., M.D. Bootman, and P. Lipp. 1998. Calcium—a life and death signal. *Nature.* 395:645–648.

Berven, L.A., and G.J. Barritt. 1994. A role for a pertussis toxin–sensitive trimeric G-protein in store-operated Ca^{2+} inflow in hepatocytes. *FEBS Lett.* 346:235–240.

Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature.* 351: 751–754.

Bootman, M.D., L. Missiaen, J.B. Parys, H. De Smedt, and R. Casteels. 1995. Control of inositol 1,4,5-trisphosphate–induced Ca^{2+} release by cytosolic Ca^{2+}. *Biochem. J.* 306:445–451.

Carter, T.D., and D. Ogden. 1997. Kinetics of Ca^{2+} release by $InsP_3$ in pig single aortic endothelial cells: evidence for an inhibitory role of cytosolic Ca^{2+} in regulating hormonally evoked Ca^{2+} spikes. *J. Physiol.* 504:17–33.

Combettes, L., Z. Hannaert-Merah, J.F. Coquil, C. Rousseau, M. Claret, S. Swillens, and P. Champeil. 1994. Rapid filtration studies of the effect of cytosolic Ca^{2+} on inositol 1,4,5-trisphosphate–induced $^{45}Ca^{2+}$ release from cerebellar microsomes. *J. Biol. Chem.* 269:17561–17571.

De Koninck, P., and H. Schulman. 1998. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science.* 279:227–230.

Dolmetsch, R.E., K. Xu, and R.S. Lewis. 1998. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature.* 392:933–936.

Fewtrell, C. 1993. Ca^{2+} oscillations in non-excitable cells. *Annu. Rev. Physiol.* 55:427–454.

Fink, C.C., B. Slepchenko, and L.M. Loew. 1999. Determination of time-dependent inositol-1,4,5-trisphosphate concentrations during calcium release in a smooth muscle cell. *Biophys. J.* 77:617–628.

Hagar, R.E., A.D. Burgstahler, M.H. Nathanson, and B.E. Ehrlich. 1998. Type III $InsP_3$ receptor channel stays open in the presence of increased calcium. *Nature.* 396:81–84.

Hannaert-Merah, Z., L. Combettes, J.F. Coquil, S. Swillens, J.P. Mauger, M. Claret, and P. Champeil. 1995. Characterization of the co-agonist effects of strontium and calcium on myo-inositol trisphosphate-dependent ion fluxes in cerebellar microsomes. *Cell Calc.* 18:390–399.

Heemskerk, J.W., P. Vis, M.A. Feijge, J. Hoyland, W.T. Mason, and S.O. Sage. 1993. Roles of phospholipase C and $Ca^{(2+)}$-ATPase in calcium responses of single, fibrinogen-bound platelets. *J. Biol. Chem.* 268:356–363.

Iino, M. 1990. Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig *taenia caeci*. *J. Gen. Physiol.* 95:1103–1122.

Ilyin, V., and I. Parker. 1994. Role of cytosolic Ca^{2+} in inhibition of $InsP_3$-evoked Ca^{2+} release in *Xenopus* oocytes. *J. Physiol.* 477:503–509.

Irvine, R.F. 1992. Is inositol tetrakisphosphate the second messenger that controls Ca^{2+} entry into cells? *Adv. Second Messenger Phosphoprotein Res.* 26:161–185.

Jacob, R., J.E. Merritt, T.J. Hallam, and T.J. Rink. 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature.* 335:40–45.

Joseph, S.K., H.L. Rice, and J.R. Williamson. 1989. The effect of external calcium and pH on inositol trisphosphate-mediated calcium release from cerebellum microsomal fractions. *Biochem. J.* 258:261–265.

Kaftan, E.J., B.E. Ehrlich, and J. Watras. 1997. Inositol 1,4,5-trisphosphate ($InsP_3$) and calcium interact to increase the dynamic range of $InsP_3$ receptor-dependent calcium signaling. *J. Gen. Physiol.* 110:529–538.

Kapural, L., and A. Fein. 1997. Changes in the expression of voltage-gated K^+ currents during development of human megakaryocytic cells. *Biochim. Biophys. Acta.* 1326:319–328.

Khodakhah, K., and D. Ogden. 1995. Fast activation and inactivation of inositol trisphosphate-evoked Ca^{2+} release in rat cerebel-

lar Purkinje neurones. *J. Physiol*. 487:343–358.

King, W.G., and S.E. Rittenhouse. 1989. Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol trisphosphates and tetrakisphosphate in human platelets exposed to thrombin. *J. Biol. Chem*. 264:6070–6074.

Li, W., J. Llopis, M. Whitney, G. Zlokarnik, and R.Y. Tsien. 1998. Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature.* 392:936–941.

Loomis-Husselbee, J.W., P.J. Cullen, U.E. Dreikausen, R.F. Irvine, and A.P. Dawson. 1996. Synergistic effects of inositol 1,3,4,5-tetrakisphosphate on inositol 2,4,5-triphosphate-stimulated Ca^{2+} release do not involve direct interaction of inositol 1,3,4,5-tetrakisphosphate with inositol triphosphate-binding sites. *Biochem. J.* 314:811–816.

Loomis-Husselbee, J.W., C.D. Walker, J.R. Bottomley, P.J. Cullen, R.F. Irvine, and A.P. Dawson. 1998. Modulation of Ins(2,4,5)P_3-stimulated Ca^{2+} mobilization by Ins(1,3,4, 5)P_4: enhancement by activated G-proteins, and evidence for the involvement of a GAP1 protein, a putative Ins(1,3,4,5)P_4 receptor. *Biochem. J.* 331: 947–952.

Lu, X., A. Fein, M.B. Feinstein, and F.A. O'Rourke. 1999. Antisense knock out of the inositol 1,3,4,5-tetrakisphosphate receptor $GAP1^{(IP4BP)}$ in the human erythroleukemia cell line leads to the appearance of intermediate conductance K(Ca) channels that hyperpolarize the membrane and enhance calcium influx. *J. Gen. Physiol*. 113:81–96.

Mak, D.O.D., S. McBride, and J.K. Foskett. 1998. Inositol 1,4,5-trisphosphate activation of inositol tris-phosphate receptor Ca^{2+} channel by ligand tuning of Ca^{2+} inhibition. *Proc. Natl. Acad. Sci. USA*. 95:15821–15825.

Michikawa, T., J. Hirota, S. Kawano, M. Hiraoka, M. Yamada, T. Furuichi, and K. Mikoshiba. 1999. Calmodulin mediates calcium-dependent inactivation of the cerebellar type 1 inositol 1,4,5-trisphosphate receptor. *Neuron*. 23:799–808.

Oancea, E., and T. Meyer. 1996. Reversible desensitization of inositol trisphosphate-induced calcium release provides a mechanism for repetitive calcium spikes. *J. Biol. Chem.* 271:17253–17260.

Ogden, D., and T. Capiod. 1997. Regulation of Ca^{2+} release by $InsP_3$ in single guinea pig hepatocytes and rat Purkinje neurons. *J. Gen. Physiol*. 109:741–756.

Ogden, D.C., T. Capiod, J.W. Walker, and D.R. Trentham. 1990. Kinetics of the conductance evoked by noradrenaline, inositol trisphosphate or Ca^{2+} in guinea-pig isolated hepatocytes. *J. Physiol.* 422:585–602.

O'Rourke, F., E. Matthews, and M.B. Feinstein. 1995. Purification and characterization of the human type 1 Ins(1,4,5)P_3 receptor from platelets and comparison with receptor subtypes in other normal and transformed blood cells. *Biochem. J.* 312:499–503.

Papp, B., K. Paszty, T. Kovacs, B. Sarkadi, G. Gardos, J. Enouf, and A. Enyedi. 1993. Characterization of the inositol trisphosphate–sensitive and insensitive calcium stores by selective inhibition of the endoplasmic reticulum-type calcium pump isoforms in isolated platelet membrane vesicles. *Cell Calc.* 14:531–538.

Payne, R., T.M. Flores, and A. Fein. 1990. Feedback inhibition by calcium limits the release of calcium by inositol trisphosphate in *Limulus* ventral photoreceptors. *Neuron*. 4:547–555.

Payne, R., B. Walz, S. Levy, and A. Fein. 1988. The localization of calcium release by inositol trisphosphate in *Limulus* photoreceptors and its control by negative feedback. *Philos. Trans. R. Soc. Lond. B Biol. Sci*. 320:359–379.

Petersen, C.C., E.C. Toescu, B.V. Potter, and O.H. Petersen. 1991. Inositol triphosphate produces different patterns of cytoplasmic Ca^{2+} spiking depending on its concentration. *FEBS Lett.* 293:179–182.

Putney, J.W., Jr., and G.S. Bird. 1993. The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocr. Rev.* 14:610–631.

Quinton, T.M., and W.L. Dean. 1996. Multiple inositol 1,4,5-trisphosphate receptor isoforms are present in platelets. *Biochem. Biophys. Res. Commun*. 224:740–746.

Ramos-Franco, J., M. Fill, and G.A. Mignery. 1998. Isoform-specific function of single inositol 1,4,5-trisphosphate receptor channels. *Biophys. J.* 75:834–839.

Shears, S.B. 1989. Metabolism of the inositol phosphates produced upon receptor activation. *Biochem. J.* 260:313–324.

Supattapone, S., P.F. Worley, J.M. Baraban, and S.H. Snyder. 1988. Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J. Biol. Chem.* 263:1530–1534.

Taylor, C.W. 1998. Inositol trisphosphate receptors: Ca^{2+}-modulated intracellular Ca^{2+} channels. *Biochim. Biophys. Acta*. 1436:19–33.

Tertyshnikova, S., and A. Fein. 1997. $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves in rat megakaryocytes. *Cell Calc*. 21:331–344.

Tertyshnikova, S., and A. Fein. 1998. Inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cAMP-dependent protein kinase in a living cell. *Proc. Natl. Acad. Sci. USA.* 95:1613–1617.

Tertyshnikova, S., X. Yan, and A. Fein. 1998. cGMP inhibits IP_3-induced Ca^{2+} release in intact rat megakaryocytes via cGMP- and cAMP-dependent protein kinases. *J. Physiol.* 512:89–96.

Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771–15781.

Uneyama, H., C. Uneyama, and N. Akaike. 1993. Intracellular mechanisms of cytoplasmic Ca^{2+} oscillation in rat megakaryocyte. *J. Biol. Chem*. 268:168–174.

Wakui, M., B.V. Potter, and O.H. Petersen. 1989. Pulsatile intracellular calcium release does not depend on fluctuations in inositol trisphosphate concentration. *Nature.* 339:317–320.

Wood, S.F., E.Z. Szuts, and A. Fein. 1990. Metabolism of inositol 1,4,5-trisphosphate in squid photoreceptors. *J. Comp. Physiol. [B]*. 160:293–298.