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

Svetlana Tertyshnikova and Alan Fein

From the Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030-3505

Abstract: Changes in cytosolic free calcium ([Ca\(^{2+}\)]) often take the form of a sustained response or repetitive oscillations. The frequency and amplitude of [Ca\(^{2+}\)], oscillations are essential for the selective stimulation of gene expression and for enzyme activation. However, the mechanism that determines whether [Ca\(^{2+}\)], oscillates at a particular frequency or becomes a sustained response is poorly understood. We find that [Ca\(^{2+}\)], 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\(^{2+}\)], 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\(^{2+}\)]) 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\(^{2+}\)] 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\(^{2+}\)] 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\(^{2+}\)], oscillations increase in frequency, eventually becoming a sustained [Ca\(^{2+}\)] 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\(^{2+}\)], oscillation frequency increases and how the response changes into a sustained [Ca\(^{2+}\)] elevation is not understood.

Many models of agonist-induced [Ca\(^{2+}\)] 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\(^{2+}\)], oscillation frequency with increased agonist concentration described above. Or for that matter how the [Ca\(^{2+}\)], oscillation changes into a sustained [Ca\(^{2+}\)], 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; Hannan-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 (Kafian 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.

http://www.jgp.org/cgi/content/abstract/115/4/481

481 J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2000/04/481/9 $5.00
Volume 115 April 2000 481-489
**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$_2$, 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±2°C).

**Measurement of [Ca$^{2+}$], 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 (OG488) as the [Ca$^{2+}$] 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$^+$/9$^+$-(bis(carboxyethyl)-5(6)-carboxyfluorescein by protons released during the light source (Lawrence et al., 1993; Kapural and Fein, 1997). The final concentration of DMSO is all-0.1% The coverslips with adherent cells are then washed several times with the standard external solution, and kept in the temperature (23°C). For the experiments not using patch clamp- ing, cells are transferred onto glass coverslips and incubated with a second pulse of IP$_3$. In rat megakaryocytes, 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; Illyin 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$^{2+}$] 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$^{2+}$], 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

**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 photo-release 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; Illyin and Parker, 1994; Oancea and Meyer, 1996; Carter and Ogden, 1997).

**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$_2$, 2 NaGTP, 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.
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 IP₃ sensitivity was sufficient to explain the reduced amplitude of the response to the second pulse of IP₃, 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 Ca²⁺-dependent inhibition of IP₃-induced Ca²⁺ release becomes progressively less effective with higher IP₃ concentrations. For this purpose, we used procedures that would increase the lifetime of IP₃, by slowing down its hydrolysis. We began by comparing the time course for the recovery from desensitization produced by IP₃ injection with the time course for recovery from desensitization produced by injection of a hydrolysis-resistant analogue of IP₃, namely GPIP₂. GPIP₂ is a less potent but fully active analogue of IP₃ that is poorly metabolized, and the caged form of GPIP₂ has been used to mobilize Ca²⁺ from IP₃-sensitive Ca²⁺ stores (Berven and Barritt, 1994). As with IP₃, the flash duration when using caged GPIP₂ is set to give a response just below that which gives a response of saturating amplitude. After the release of Ca²⁺ 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 photorelease IP\(_3\) to a time course similar to that produced by photorelease 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.

\[
y = (1 - e^{-(t - 1.5)/\tau}). \tag{1}
\]

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 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 plekstrin, the major substrate of PKC in platelets, because phosphorylated plekstrin has been shown to activate the IP\(_3\)-5-phosphatase (Auethavekij 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 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 0.5 of 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 IP\(_3\) 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\(^{2+}\)], on further Ca\(^{2+}\) release and the removal of Ca\(^{2+}\) from the cytoplasm. That is, the hydrolysis of...
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$_{2+}$] 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.
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$_{\text{2+}}$]$_i$ 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 photorelease 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$_{\text{2+}}$]$_i$. Likewise a series of flashes that photorelease IP$_3$ produce a sustained elevation of [Ca$_{\text{2+}}$]$_i$, 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$_{\text{2+}}$]$_i$ (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$_{\text{2+}}$ by IP$_3$ and the disinhibition of Ca$_{\text{2+}}$-dependent inhibition of IP$_3$-mediated Ca$_{\text{2+}}$ release by increasing IP$_3$ concentration. We show this disinhibition as acting via calmodulin because recently published experiments have indicated that Ca$_{\text{2+}}$-dependent inhibition of IP$_3$-mediated Ca$_{\text{2+}}$ release for the type 1 IP$_3$ receptor (IP$_3$-R) is mediated by calmodulin (Michikawa et al., 1999) (see discussion).

**Figure 6.** Effect of the PKC inhibitor, GF109203X, on ADP-induced [Ca$_{\text{2+}}$]$_i$ oscillations in a rat megakaryocyte. Changes in [Ca$_{\text{2+}}$]$_i$ were monitored by measuring OGB488 fluorescence intensity and are expressed as $\Delta F$ (counts/ millisecond). GF109203X (15 $\mu$M) and ADP (100 $\mu$M) 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$_{\text{2+}}$ and OGB488 using the cell-permeant forms of each molecule as described in methods. [Ca$_{\text{2+}}$], spikes resulting from photorelease of caged Ca$_{\text{2+}}$ are shown superimposed for the purpose of comparison. GF 109203X had no effect on the time course of the fall in [Ca$_{\text{2+}}$]$_i$ after photolysis of caged Ca$_{\text{2+}}$. Cyclopiazonic acid (CPA) was applied to the cell to serve as a positive control for inhibition of Ca$_{\text{2+}}$ sequestration. GF109203X (30 $\mu$M) and CPA (5 $\mu$M) were applied to the cell via the local superfusion system as described in methods.

**DISCUSSION**

Our results demonstrate for the first time an important property of [Ca$_{\text{2+}}$]$_i$ signaling in intact cells: an increase in the lifetime of IP$_3$ brings about a decrease in Ca$_{\text{2+}}$-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$_{\text{2+}}$]. Indeed, this may explain the occurrence of sustained [Ca$_{\text{2+}}$]$_i$ 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$_{\text{2+}}$]$_i$ oscillations in cells containing IP$_3$-sensitive Ca$_{\text{2+}}$ 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$_{\text{2+}}$]$_i$ 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$_{\text{2+}}$-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
Tertyshnikova and Fein 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\(_{\text{i}}\)] 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\(_{\text{i}}\) and it was proposed that inhibition by Ca\(_{\text{i}}\) 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\(_{\text{i}}\)-dependent inhibition of the type 1 IP\(_3\)-R is mediated by calmodulin implies that inhibition of calmodulin should disinhibit Ca\(_{\text{i}}\)-dependent inhibition of IP\(_3\)-mediated Ca\(_{\text{i}}\) release (Michikawa et al., 1999). Based on our findings, we would predict that such a disinhibition would transform a [Ca\(_{\text{i}}\)] oscillation into a more sustained [Ca\(_{\text{i}}\)] 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\(_{\text{i}}\)] oscillation to become a more sustained [Ca\(_{\text{i}}\)] 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\(_{\text{i}}\)-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\(_{\text{i}}\)-dependent inhibition has been observed, using other techniques, in some cell types that contain primarily the type II and III IP\(_3\)-Rs (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\(_{\text{i}}\) 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 Caiqiod, 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 GPIP2. 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$_3$.

We thank Drs. L. Jaffe, R. Shaafi, M. Terasaki, and J. Watras for their constructive criticisms of an earlier version of this manuscript.

Submitted: 29 September 1999
Revised: 22 February 2000
Accepted: 28 February 2000

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.J. 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.

Bever, L.A., and G.J. Barr. 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, J.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. Misaela, J.B. Parys, H. De Smedt, and R. Castells. 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. Coquill, S. Rousseau, M. Claret, S. Swillens, and P. Champel. 1994. Rapid filtration studies of the effect of cytosolic Ca$^{2+}$ on inositol 1,4,5-trisphosphate-induced 45Ca$^{2+}$ 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, L., Z. Combettes, J.F. Coquill, S. Swillens, J.P. Mauger, M. Claret, and P. Champel. 1995. Characterization of the co-agonist effects of strontium and calcium on myo-inositol trisphosphate-dependent ion fluxes in cerebellar microsomes. Cell Calcium 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 Xanopus 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.

Kafan, 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-
Oancea, E., and T. Meyer. 1996. Reversible desensitization 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 InsP3 ester shows that Ca2+ 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-tetra- and 1,4,5-trisphosphate on inositol 2,4,5-trisphosphate-stimulated Ca2+ release do not involve direct interaction of inositol 1,3,4,5-tetrakisphosphate with inositol trisphosphate-binding sites. Biochim. 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)P3-stimulated Ca2+ mobilization by Ins(1,3,4,5)P4 and enhancement by activated G-proteins, and evidence for the involvement of a GAP1 protein, a putative Ins(1,3,4,5)P4 receptor. Biochim. 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 (...). Biochim. J. 331:81–96.

Mak, D.O.D., S. McBride, and J.K. Foskett. 1998. Inositol 1,4,5-trisphosphate activation of inositol trisphosphate receptor Ca2+ channel by ligand tuning of Ca2+ inhibition. Proc. Natl. Acad. Sci. USA. 95:15821–15825.

Michikawa, T., J. Hirota, S. Kawano, M. Hiraoka, M. Yamada, T. Furutchi, 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 Ca2+ release by InsP3 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. Tretham. 1990. Kinetics of the conductance evoked by noradrenaline, inositol trisphosphate or Ca2+ 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)P3 receptor from platelets and comparison with receptor subtypes in other normal and transformed blood cells. Biochim. 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 trisphosphate produces different patterns of cytoplasmic Ca2+ 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.

RamosFranco, 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. Biochim. 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: Ca2+-modulated intracellular calcium channels. Biochim. Biophys. Acta. 1436:19–33.

Tertyshnikova, S., and A. Fein. 1997. [Ca2+]i oscillations and [Ca2+]i waves in rat megakaryocytes. Cell Calc. 21:331–344.

Tertyshnikova, S., and A. Fein. 1998. Inhibition of inositol 1,4,5-trisphosphate-induced Ca2+ 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 IP3-induced Ca2+ release in intact rat megakaryocytes via cGMP- and cAMP-dependent protein kinases. J. Physiol. 512:89–96.

Tolley, D., P. Pianetti, H. Coste, P. Bellvergue, 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 Ca2+ 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.