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$^{2+}$]) 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, result 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$^{2+}$]) are well documented for cells stimulated by many hormone and growth factor agonists that generate the second messenger inositol 1,4,5-trisphosphate (IP$_3$). [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; Hannaeht-Merah et al., 1995) and permeabilized A7r5 smooth muscle cells (Booian 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.
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 MgCl₂, 2 CaCl₂, 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 [Ca²⁺], 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 fluorescence is accomplished using an Ionoptix photon-counting fluorescence subsystem with a dual excitation monochromator (IonOptix). For photolysis of caged compounds, pulses of ultra violet light (290–370 nm) are applied to the cell through an Ionwizard probe, Inc. Caged IP₃ and Photolysis of Caged Compounds

The "cell-permeant" AM ester and the "cell-impermeable" hexapotassium salt of OGB488 are obtained from Molecular Probes, Inc. Caged IP₃ and caged GPIP₂ (1-[alpha-glycerophosphoryl]-myo-inositol 4,5-diphosphate, P4(5)-1-(2-nitrophenyl) ethyl ester) are from Calbiochem Corp. GF109203X is from Biocon 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 IP₃ or caged GPIP₂, 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 MgCl₂, 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.

Results

To study Ca²⁺-dependent inhibition of IP₃-induced Ca²⁺ release, we performed paired-pulse experiments in rat megakaryocytes that are a convenient model for studying Ca²⁺ signaling in nonexcitable cells, because they express only an IP₃-sensitive Ca²⁺ store and lack a ryanodine-sensitive Ca²⁺ store (Uneyama et al., 1993). For the first pulse of IP₃, in a paired-pulse experiment, the intracellular increase of IP₃, resulting from photorelease from caged IP₃, causes a transient release of Ca²⁺ 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 IP₃ are diminished in amplitude (see Fig. 2 A). The available evidence indicates that this period of desensitization is due to Ca²⁺-dependent inhibition of IP₃-induced Ca²⁺ 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 IP₃ is observed for a first pulse of IP₃ that produced a [Ca²⁺]i response of near maximal amplitude (Oancea and Meyer, 1996). Therefore, we began our experiments by first measuring the power dependence of IP₃-induced Ca²⁺ release (Fig. 1). In Fig. 1, B and C, we plot the normalized peak amplitude (R/Rₘₐₓ) of the IP₃-mediated [Ca²⁺]i response as a function of the flash duration, which is directly proportional to IP₃ 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 Ca²⁺ produced by the photorelease of IP₃, there is a period of desensitization during which a subsequent increase in IP₃ releases less calcium. As the time interval between the pulses of IP₃ increases, the response to the second pulse recovers back to that of the first. The desensitization is not due to emptying of the Ca²⁺ 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
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 GIP$_2$ (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 GIP$_2$ 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 GIP$_2$ 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.

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

(1)

In Eq. 1, the 1.5-s time delay is the approximate time to peak for the response to IP$_3$ or GIP$_2$. 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 GIP$_2$ 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 GIP$_2$ 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 GIP$_2$ 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 $108 \pm 39$ ms ($n = 8$ cells) for GIP$_2$, $155 \pm 54$ ms ($n = 5$ cells) for IP$_3$ in the presence of 2,3-DPG, and $121 \pm 44$ ms ($n = 8$ cells) for IP$_3$ in the presence of GF109203X. The flash duration for half-maximal amplitude for GIP$_2$ 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 GIP$_2$ 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
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\(^{2+}\)] 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\(^{2+}\)] oscillation, we examined the effect of GF109203X on ADP-induced [Ca\(^{2+}\)] 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\(^{2+}\)] 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\(^{2+}\)] 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\(^{2+}\)] after the flash-induced rise in [Ca\(^{2+}\)] should reflect the activity of Ca\(^{2+}\) sequestration and/or extrusion mechanisms (see Tertyshnikova et al., 1998). As can be seen in Fig. 7, photoreleased [Ca\(^{2+}\)] 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 IP₃, the rise in [Ca²⁺]i would become plateau-like when the hydrolysis of IP₃ is slowed down. Accordingly, in Fig. 8, we compare the responses to multiple flashes, which photorelease IP₃, 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 IP₃ 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 IP₃-5-phosphatase, a series of flashes that photorelease IP₃ produce a sustained elevation of [Ca²⁺]i. Likewise a series of flashes that photorelease IP₃ produce a sustained elevation of [Ca²⁺]i, in the presence of GF109203X (Fig. 8 D). Furthermore, a train of flashes that photorelease the hydrolysis-resistant IP₃-analogue GPIP₂ also produce a sustained elevation of [Ca²⁺]i (Fig. 8 C).

The simplified diagram in Fig. 9 summarizes our findings, emphasizing the dual regulation of calcium mobilization by IP₃. For the sake of simplicity, GPIP₂ has been left out of the figure. The heavy lines in Fig. 9 are meant to represent the release of Ca²⁺ by IP₃ and the disinhibition of Ca²⁺-dependent inhibition of IP₃-mediated Ca²⁺ release by increasing IP₃ concentration. We show this disinhibition as acting via calmodulin because recently published experiments have indicated that Ca²⁺-dependent inhibition of IP₃-mediated Ca²⁺ release for the type 1 IP₃ receptor (IP₃R) is mediated by calmodulin (Michikawa et al., 1999) (see discussion).

DISCUSSION

Our results demonstrate for the first time an important property of [Ca²⁺]i signaling in intact cells: an increase in the lifetime of IP₃ brings about a decrease in Ca²⁺-dependent inhibition. These findings suggest a mechanism by which high concentrations of intracellular IP₃ can cause cells to maintain an elevated level of [Ca²⁺]i. Indeed, this may explain the occurrence of sustained [Ca²⁺]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 IP₃ analogue inositol 1,4,5-trisphosphorothioate (Petersen et al., 1991). Our findings also suggest a possible mechanism for the regulation of the frequency of [Ca²⁺]i oscillations in cells containing IP₃-sensitive Ca²⁺ 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 [Ca²⁺]i signaling.

Since platelets express primarily the type 1 isoform of the IP₃-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 IP₃-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 IP₃-R, exhibit decreased Ca²⁺-dependent inhibition at elevated IP₃ concentrations. Moreover, single channel recordings from the cerebellar type 1 IP₃-R (Koftan et al., 1997) and a similar receptor found...
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$^{2+}$] 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 dishibit 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$^{2+}$] oscillation into a more sustained [Ca$^{2+}$] 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$^{2+}$] oscillation to become a more sustained [Ca$^{2+}$] 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 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$^{2+}$ release in megakaryocytes is the highly nonlinear depen-
idence 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 Capiol, 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 (Irving, 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 the major substrate of PKC in platelets, in regulating Ca$^{2+}$ mobilization in platelets also; in as much as megakaryocytes, they should be relevant to calcium mobilization in megakaryocytes.

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REFERENCES

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