Control of Calcium Signal Propagation to the Mitochondria by Inositol 1,4,5-Trisphosphate-binding Proteins*

Received for publication, October 12, 2004, and in revised form, December 27, 2004 Published, JBC Papers in Press, January 11, 2005, DOI 10.1074/jbc.M411591200

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Cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{C}) signals triggered by many agonists are established through the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) messenger pathway. This pathway is believed to use Ca\textsuperscript{2+}-dependent local interactions among IP\textsubscript{3} receptors (IP\textsubscript{3R}) and other Ca\textsuperscript{2+} channels leading to coordinated intracellular Ca\textsuperscript{2+} release from the endoplasmic reticulum throughout the cell and coupling Ca\textsuperscript{2+} entry and mitochondrial Ca\textsuperscript{2+} uptake to Ca\textsuperscript{2+} release. To evaluate the role of IP\textsubscript{3} in the local control mechanisms that support the propagation of [Ca\textsuperscript{2+}]\textsubscript{C} waves, store-operated Ca\textsuperscript{2+} entry, and mitochondrial Ca\textsuperscript{2+} uptake, we used two IP\textsubscript{3}-binding proteins (IP3BP): 1) the PH domain of the phospholipase C-like protein, p130 (p130PH); and 2) the ligand-binding domain of the human type-I IP3R (IP3R\textsubscript{24-605}). As expected, p130PH-GFP and GFP-IP3R\textsubscript{24-605} behave as effective mobile cytosolic IP\textsubscript{3} buffers. In COS-7 cells, the expression of IP3BP had no effect on store-operated Ca\textsuperscript{2+} entry. However, the IP\textsubscript{3}-linked [Ca\textsuperscript{2+}]\textsubscript{C} signal appeared as a regenerative wave and IP3BP's slowed down the wave propagation. Most importantly, IP3BP's largely inhibited the mitochondrial [Ca\textsuperscript{2+}]\textsubscript{C} signal and decreased the relationship between the [Ca\textsuperscript{2+}]\textsubscript{C} and mitochondrial [Ca\textsuperscript{2+}]\textsubscript{C} signals, indicating disconnection of the mitochondria from the [Ca\textsuperscript{2+}]\textsubscript{C} signal. These data suggest that IP\textsubscript{3} elevations are important to regulate the local interactions among IP3Rs during propagation of [Ca\textsuperscript{2+}]\textsubscript{C} waves and that the IP\textsubscript{3}-dependent synchronization of Ca\textsuperscript{2+} release events is crucial for the coupling between Ca\textsuperscript{2+} release and mitochondrial Ca\textsuperscript{2+} uptake.

Inositol 1,4,5-trisphosphate (IP\textsubscript{3})-induced Ca\textsuperscript{2+} liberation from intracellular stores results in a [Ca\textsuperscript{2+}]\textsubscript{C} signal that controls a wide spectrum of cell functions, including energy metabolism, gene transcription, and cell proliferation. Appropriate exposure of the effectors to Ca\textsuperscript{2+} throughout the cell is supported by several mechanisms that include the propagation of Ca\textsuperscript{2+} release throughout the cell without attenuation, the recruitment of Ca\textsuperscript{2+} entry, and efficient delivery of the [Ca\textsuperscript{2+}]\textsubscript{C} signal into organelles such as the nucleus and mitochondria (1).

Spreading of the [Ca\textsuperscript{2+}]\textsubscript{C} signal is facilitated by regenerative mechanisms of Ca\textsuperscript{2+} mobilization, which may derive from interactions between adjacent Ca\textsuperscript{2+} release sites. Non-metabolizable IP\textsubscript{3} analogs have been found to evoke [Ca\textsuperscript{2+}]\textsubscript{C} oscillations, providing support to the idea that a constant [IP3] is sufficient to trigger regenerative Ca\textsuperscript{2+} release via binding to and activating IP3Rs and ryanodine receptors (RyR) (2). Based on IP\textsubscript{3} microinjection and uncaging studies, the regenerative Ca\textsuperscript{2+} release during [Ca\textsuperscript{2+}]\textsubscript{C} waves was also claimed to be largely independent of [IP3] (3, 4). Along this line, IP\textsubscript{3}R-mediated [Ca\textsuperscript{2+}]\textsubscript{C} waves were observed in the absence of any stimulated IP\textsubscript{3} formation (5). However, released Ca\textsuperscript{2+} may also promote phospholipase C (PLC) activation and, in turn, increase IP\textsubscript{3}, providing a potential amplification mechanism for IP3R-mediated Ca\textsuperscript{2+} release waves (6–8). Ca\textsuperscript{2+} may activate the PLC coupled to the agonist receptor to stimulate cleavage of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) in the plasma membrane. The localization of phospholipase C enzyme isoforms (9, 10) and PIP\textsubscript{2} (11) in intracellular membranes has also been documented. IP3 formed at the plasma membrane may rapidly diffuse throughout the cytoplasm (12), but the presence of PIP\textsubscript{2} and PLC in the vicinity of the IP3R could also provide for a local IP3 feedback. Thus, the role of IP\textsubscript{3} fluctuations in Ca\textsuperscript{2+} wave propagation requires further investigation.

Recharging of the ER Ca\textsuperscript{2+} stores during the agonist-induced [Ca\textsuperscript{2+}]\textsubscript{C} signal involves Ca\textsuperscript{2+} entry mediated by so-called store-operated Ca\textsuperscript{2+}-entry mechanisms that might be mediated by the canonical transient receptor potential channel family (13). One of the proposed mechanisms for activation of Ca\textsuperscript{2+} entry during Ca\textsuperscript{2+} release is a conformational coupling between IP3Rs and store-operated Ca\textsuperscript{2+} channels (14). Although store-operated Ca\textsuperscript{2+} entry is activated by agents that directly target the ER Ca\textsuperscript{2+} store (15, 16), IP\textsubscript{3} binding to the IP3R has been response ratio; RyR, ryanodine receptor; PLC, phospholipase C; ER, endoplasmic reticulum; p130, 130-kDa phospholipase C-like protein; GABA\textsubscript{A}, \gamma-aminobutyric acid, type A; pericam; mt, mitochondrial; BSA, bovine serum albumin; Tg, thapsigargin; ECM, extracellular matrix; EGF, epidermal growth factor; ICM, intracellular medium; [Ca\textsuperscript{2+}]\textsubscript{in}, mitochondrial matrix [Ca\textsuperscript{2+}]; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate.

* This work was supported by grants from the National Institutes of Health (to G. H.) and by a grant of the Hungarian Science Foundation (OTKA T-034606) (to P. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP3R, IP\textsubscript{3} receptor; IP3BP, IP\textsubscript{3}-binding protein; PH, pleckstrin homology; FRAP, fluorescence recovery after photobleaching; \textit{t}\textsubscript{lag}, the lag time needed to reach half of the maximum cytosolic Ca\textsuperscript{2+} response; F\textsubscript{GFP}, GFP fluorescence; RFP, red fluorescence protein; YFP, yellow fluorescence protein; R\textsubscript{0}/R\textsubscript{p}, prestimulation ratio versus peak.
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claimed to have a role in activation of Ca$^{2+}$ entry (17, 18). Nonetheless, the question of whether an increase in [IP$_3$] is required for the channel activation remains elusive. IP$_3$-induced [Ca$^{2+}$]$_i$ spikes are also delivered to the mitochondria to control the activity of several enzymes that participate in ATP production as well as that of other proteins compartmentalized to the matrix space (19–21). Mitochondrial Ca$^{2+}$ uptake sites display low affinity toward Ca$^{2+}$ and appear to respond mostly to the large local [Ca$^{2+}$]$_i$, transients that occur in the vicinity of the activated IP$_3$Rs and RyRs (21–24), but a rapid mode of Ca$^{2+}$ uptake at relatively low [Ca$^{2+}$]$_i$ has also been documented (25). A local interaction between RyRs and mitochondrial Ca$^{2+}$ uptake sites may secure that even fundamental Ca$^{2+}$ release events ([Ca$^{2+}$]$_i$ sparks) induce a [Ca$^{2+}$]$_i$ increase in the neighboring mitochondrion (Ca$^{2+}$ mark) (26).

However, a substantially larger [Ca$^{2+}$]$_i$ rise occurs during a global [Ca$^{2+}$]$_i$ signal, suggesting that numerous Ca$^{2+}$ release events cooperate with each other to establish a [Ca$^{2+}$]$_i$ signal in a mitochondrion. At the ER-mitochondrial interface, great quantities of IP$_3$Rs have been visualized and enrichment in ER Ca$^{2+}$ pumps and mitochondrial Ca$^{2+}$ uniporters is probable (reviewed in Refs. 27 and 28). Morphology of the ER-mitochondrial associations and concentration of the Ca$^{2+}$ transporters at the interface may play a role in controlling coordinated activation of the individual Ca$^{2+}$ release events that give rise to the IP$_3$-linked [Ca$^{2+}$]$_i$ spikes. Thus, the role of IP$_3$ in coordination of Ca$^{2+}$ release events is of great interest in a variety of Ca$^{2+}$ signaling mechanisms. We reasoned that mobile IP$_3$ buffers should be useful to test the role of IP$_3$ in [Ca$^{2+}$]$_i$ waves propagation, activation of Ca$^{2+}$ entry, and in IP$3$R-dependent activation of mitochondrial Ca$^{2+}$ uptake.

An intensively investigated IP$_3$-binding module is the IP$_3$-binding domain of the IP$3$R. The N-terminal cytoplasmic region of the human type-1 IP$3$R (residues 1–604) binds IP$_3$ with comparable affinity to the full-length IP$3$R (29). Removal of residues 1–223 (suppressor region) further increases the affinity for IP$_3$ (30). Expression of IP$_3$-R$_{224–605}$ has been shown to attenuate the ATP-induced [Ca$^{2+}$]$_i$ signal in human embryonic kidney 293 and in COS-7 cells (31, 32). Another structurally unrelated IP$_3$-binding module is the PH domain of certain proteins. Some PH domains are known to bind IP$_3$ and/or PIP$_2$, unrelated IP$_3$-binding module is the PH domain of certain proteins. Some PH domains are known to bind IP$_3$ and/or PIP$_2$, and the (1,4,5)IP$_3$-binding domain (residues 224–605) of the human type-I (1,4,5)IP$_3$ receptor with cyan, green, or yellow fluorescent protein have been described previously (31). In addition, the PH domain of p130 and the R134L mutant were also inserted into a plasmid encoding a monomeric red fluorescent protein (RFP-p130PH and RFP-p130PH-R134L) (36). Ratiometric pericam targeted to the mitochondria (pericam-nt) has also been published previously (37). Bacterial expression of the GFP-fused protein domains (p130PH-GFP, GFP-IP$3$R$_{224–605}$ and GFP-PLC$\gamma$1PH R40L) was carried out as described previously (31).

Cell Culture—COS-7 cells (obtained from ATCC) were cultured in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in humidified air (5% CO$_2$) at 37 °C. RBL-2H3 cells were cultured as described previously (22). For imaging experiments, cells were plated onto poly-l-lysine-treated glass coverslips at a density of 20,000–25,000/cm$^2$ and were grown for 3–4 days. For cell suspension studies, cells were cultured for 4–6 days in 75-cm$^2$ flasks.

Transfection of Cells—Cells plated onto poly-l-lysine-coated coverslips were transfected with plasmid DNA (1.5 µg/ml) for 6–12 h using Lipofectamine (Invitrogen) and Opti-MEM I medium (Invitrogen) according to the manufacturer’s instructions. Cells were observed 24–36 h after transfection.

Measurement of [IP$_3$] in COS-7 Cells—Before use, the cells were preincubated in an extracellular medium (2% BSA/ECM) consisting of 121 mM NaCl, 5 mM NaHCO$_3$, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 10 mM glucose, and 2% BSA, pH 7.4. To monitor Ca$^{2+}$ entry (Mn$^{2+}$ quench), cells were loaded with 5 µM Fura-2/AM for 20–30 min in the presence of 200 µM Mn$^{2+}$ and 0.003% (w/v) pluronic acid at room temperature. Sulfonpyrazone was also present during the imaging measurements to minimize dye loss. To monitor Ca$^{2+}$ entry, cells were loaded with 5 µM rhod2/AM for 50 min in the presence of 0.003% (w/v) pluronic acid at 35 °C. After loading, cells were washed and imaged in the same medium containing 0.5% BSA but no 4,5'-edetracer was added. In some experiments, Ca$^{2+}$ was not added to 0.25% BSA/ECM and the final [Ca$^{2+}$]$_{ECM}$ was <1 µM.

Coverslips were mounted at the thermostated stage (35 °C) of a Olympus IX70 inverted microscope fitted with a ×40 (UAPo, NA 1.35) oil immersion objective. For simultaneous measurements of fura2, pericam, and IP3R immunofluorescence, Leica IRBP inverted microscope equipped with a motorized turret under computer control and fitted with the above described objective was used. Fluorescence images were collected using a cooled CCD camera (PXL, Photometrics or, for fast-imaging, a frame-transfer device, Pluto, PixelVision) (22, 31, 39). The following multi-parameter imaging protocols were applied: fura2 and GFP (fura2, 340- and 380-nm excitations or 380 nm for Mn$^{2+}$ quenching; enhanced GFP, 490-nm excitation, 510-nm longpass dichroic mirror, and a 520-nm longpass emission filter); CFP and YFP (CFP, 435-nm excitation, 480/40-nm emission; FRET, 435-nm excitation, 535/30-nm emission); and fura2, pericam, and RFP (fura2, 340- and 380-nm excitation; pericam, 414- and 495-nm excitation, 510-nm longpass dichroic mirror, and a 520-nm longpass emission filter; RFP, 545-nm excitation, 570-nm longpass dichroic mirror, and a 600/75-nm emission filter; CCD imaging system). In most of the experiments, a set of images was obtained every 3 s, whereas in recording of the waves 15 frames were obtained in each second, and in the measurements that involved a switch of the filter cube in each cycle, a set of images was obtained every 6 s.

For evaluation of [Ca$^{2+}$]$_i$, fura2 fluorescence was calculated for the total area of individual cells and the background fluorescence obtained over regions of each image was subtracted. Photobleaching of the fluorescence ratios. In the cells expressing high amounts of fluorescent proteins, detectable GFP/pericam fluorescence was obtained at the excitation wavelengths used for fura2 (40). The GFP-related fluorescence was not affected by changes in [Ca$^{2+}$]. Although a comparison of the fura2 fluorescence between fluorescent protein-expressing cells and non-transfected cells was used to visualize the kinetics of the signal in each image sequence, to avoid underestimation of the
fluorescence ratios reflecting the changes in [Ca\textsuperscript{2+}]. GPN versus p130PH-GFP or GFP-IP\textsubscript{R224–605} and RFP-N versus RFP-p130PH were also analyzed in all of the comparisons. For evaluation of [Ca\textsuperscript{2+}]\textsubscript{i}, the perisynaptic signal was masked. Recordings obtained from all of the transfected cells on the field were averaged for comparison in each experiment. Experiments were carried out with at least four different cell preparations, and 20–60 cells were monitored in each experiment. Significance of differences from the relevant controls was calculated by Student’s t test.

**Confocal Imaging of Fluorescence Recovery after Photobleaching (FRAP) Measurements in COS-7 Cells—**COS-7 cells were transfected with GPN, GFP-IP\textsubscript{R224–605}, p130PH-GFP, mito-YFP, or PLC\textsubscript{G}, PH-GFP. Experiments were performed in 0.25% BSA at 35 °C. A Bio-Rad Radiance 2100 confocal laser-scanning system coupled to an Olympus IX70 inverted microscope with a ×40 (U-Apo, NA 1.35) oil immersion objective was used to record image series. The 488-nm line of a krypton/argon laser was used to excite the GFP. For the FRAP measurements, images were taken continuously at 512 × 512 resolution at a high digital zoom (0.1 μm/pixel) with a frame rate of 0.3 Hz. Two 64 μm\textsuperscript{2} (80 × 80 pixels) regions were selected for bleaching. Two images were recorded pre-bleach followed by five bleaching images with the selected regions illuminated at 16.7 (GPN-N) and 25 times (GFP-IP\textsubscript{R224–605}, p130PH-GFP, and mito-YFP) the normal scanning intensity. The cell was then refilled for 4 min. Cell permeabilization was evaluated by trypan blue exclusion, and after a 5-min incubation, 95% cells were trypan blue-positive. Compartmentalized fura2FF has been shown to occur in the cytosol. Fluorescence was monitored in a multi-wavelength-excitation dual wavelength-emission fluorimeter (Delta RAM, IX70 inverted microscope with a 488-nm line of a krypton/argon laser was used to excite the GFP. For the FRAP measurements, images of COS-7 cells transfected with constructs encoding the PH domain of the p130, the N-terminal ligand-binding domain of the IP\textsubscript{R}224–605, or the PH domain of the PLC\textsubscript{G}, fused to GFP. Images were recorded before (upper panel) and 90 s after the addition of 50 μm ATP (lower panel). B, confocal images from a FRAP experiment with COS-7 cells transiently transfected with p130PH-GFP (i, ii, iv, and vii), GFP-IP\textsubscript{R224–605} (iii and iv), and YFP targeted to the mitochondrial matrix via a fusion with the targeting sequence of cytochrome c oxidase subunit VIII (mito-GFP, iv and viii). Pre-bleach is shown in the upper row, and immediately post-bleach (3.3 s) is shown in the lower row. The bleaching regions are marked by a red box.

**RESULTS AND DISCUSSION**

**Cellular Localization of IP\textsubscript{3} and Inositol Lipid-Binding Domains Fused to GFP—**We first visualized by confocal microscopy the subcellular localization of the IP\textsubscript{3}-binding domains, IP\textsubscript{3}\textsubscript{R224–605} and p130PH, in COS-7 cells, which were expressed as GFP fusion proteins. GPN was used as a control for cytosolic distribution (Fig. 1A, i), whereas PLC\textsubscript{G}, PH-GFP that binds to PIP\textsubscript{2} through its PH domain was used as a reference for plasma membrane localization (Fig. 1A, iv). As indicated by the GFP fluorescence, GFP-IP\textsubscript{R224–605} and p130PH-GFP were found to be present in the cytosol (Fig. 1A, ii and iii). The cells were stimulated next with ATP that induces PLC-mediated cleavage of PIP\textsubscript{2} to enhance IP\textsubscript{3} formation. In ATP-stimulated COS-7 cells, no change was observed in the distribution of GFP-N, GFP-IP\textsubscript{R224–605}, and p130PH-GFP (Fig. 1A, i, vi, and vii). The ATP-induced hydrolysis of PIP\textsubscript{2} was confirmed by the partial translocation of PLC\textsubscript{G}, PH-GFP from the membrane to the cytosol, which appeared as a fluorescence decrease at the plasma membrane and an increase in the cytosol (Fig. 1A, viii).

FRAP studies were used to assess the mobility of the IP3BP's in the cytosol (Fig. 1B). When a large area of a p130PH-GFP-expressing cell was illuminated, an essentially homogeneous decrease in fluorescence appeared throughout the cell within 3 s (Fig. 1B, i) versus vi), suggesting fast cytosolic distribution of the fluorescent protein. Furthermore, when small regions of the GFP-IP\textsubscript{R224–605} and p130PH-GFP-expressing cells were photobleached, the post-bleach fluorescence in these regions was not different from the pre-bleach signal, indicating that the fluorescence was recovered completely within 3 s (Fig. 1B, ii versus vii and iii versus vii). As a positive control, GFP targeted to the mitochondrial matrix, mito-GFP, which shows slower redistribution than the cytosolic GFP (42), was also photobleached. In this case, the loss of fluorescence in the region of bleaching was apparent in the post-bleach image (Fig. 1B, iv versus viii; 29 ± 3%, n = 12). Although the rate of image acquisition was too low to determine the half-recovery time for GFP-IP\textsubscript{3}\textsubscript{R224–605} and p130PH-GFP, it is likely to be in the
subsecond range, similarly to the half-recovery Time measured for a freely diffusible cytosolic PH domain-GFP construct (PLCδ1PH/R40L-GFP, 0.2s) (43). Collectively, the above data suggest that the GFP-IP3,R224–605 and GFP-PH-IP3 are highly mobile cytosolic proteins.

Effect of recombinant p130PH-GFP on IP3-induced Ca2+ release in permeabilized COS-7 cells—To evaluate the extent to which the GFP-fused IP3-binding domains can buffer IP3 in the cytoplasm, we analyzed the effect of recombinant p130PH-GFP on IP3-induced Ca2+ release in permeabilized COS-7 cells (Fig. 2A). The change of [Ca2+]i in the cytosolic buffer ([Ca2+]o) was monitored by fura2. First, a Ca2+ pulse (750 nM CaCl2) was added to the permeabilized cells. The added Ca2+-induced [Ca2+]i rise and subsequent rapid decay were not affected by the presence of p130PH-GFP (250 nM), indicating that cytoplasmic Ca2+ buffering and the ER Ca2+ uptake were not influenced by p130PH-GFP (Fig. 2A, red versus black line). After a steady state [Ca2+]i was attained, submaximal IP3 was added in three steps, 100 nM IP3 each, followed by the addition of a supramaximal dose (7.5 μM). The three additions of 100 nM IP3 triggered a dose-dependent increase in [Ca2+]i, and the supramaximal IP3 evoked an additional large [Ca2+]i increase (Fig. 2A). When recombinant p130PH-GFP was present, the [Ca2+]i rise elicited by the suboptimal IP3 was largely attenuated but the [Ca2+]i change in response to maximum IP3 dose was not affected (Fig. 2A, red versus black line). On average, >50% inhibition of the [Ca2+]i signal evoked by 200 nM IP3 was obtained in the presence of p130PH-GFP (250 nM), whereas no inhibition was caused by the GFP fusion protein of PLCδ, PH R40L, a PLCδ, PH mutant that does not bind IP3 (Fig. 2B, left) (31). The dose response relation also showed highly effective inhibition of the IP3-induced [Ca2+]i signal by p130PH-GFP (Fig. 2B, right). Similar to p130PH-GFP, GFP-IP3,R224–605 (50 nM) also inhibited the [Ca2+]i signal induced by 100 nM IP3 (40% inhibition) and failed to affect the [Ca2+]i response elicited by 7.5 μM IP3 (not shown, n = 2). These results suggest that p130PH-GFP and GFP-IP3,R224–605 were able to compete with the IP3R for IP3 until IP3 was available for saturation of both the IP3R and the recombinant IP3BPs. As compared with IP3, Tg (2 μM), an inhibitor of the ER Ca2+ pumps, induced a slow but progressive [Ca2+]i rise that was mediated by Ca2+ leak from the ER (Fig. 2C). p130PH-GFP did not affect the Tg-induced [Ca2+]i signal, suggesting that the ER Ca2+ leak was not controlled by p130PH-GFP (Fig. 2C). Thus, IP3-binding domains attenuated selectively the IP3R-mediated Ca2+ release from the ER.

Effect of IP3-binding Domains on the [Ca2+]i Signal Evoked by IP3-linked Agonists in Intact Cells—To further analyze the effect of p130PH and IP3,R224–605 on Ca2+ signaling, agonists activating PLCδ or PLCγ were added to intact COS-7 cells expressing either p130PH-GFP or GFP-IP3,R224–605. Microscopic imaging of GFP fluorescence (490-nm excitation) simultaneously with fura2 fluorescence (340- and 380-nm excitation) allowed us to evaluate the agonist-induced [Ca2+]i signal both in transfected and non-transfected (control) cells (Fig. 3). ATP (50 μM) that activates PLCδ through P2-purinergic receptors elicited a robust and rapid global [Ca2+]i elevation in control cells (Fig. 3, A and B, 50- versus 66-s image). In contrast, the GFP-positive cells (Fig. 3, A and B, GFP image) did not exhibit a [Ca2+]i rise or displayed only a delayed response (Fig. 3, A, GFP-IP3,R224–605 120-s image, and B, p130PH-GFP, 81-s image). The mean response of the control cells showed a steep and
To determine the effect of the IP3-binding domain on the IP3R224–605 cells and p130PH-GFP cells, we measured the lag time of the [Ca\textsuperscript{2+}] signal in control cells, which was delayed as compared with the ATP-induced [Ca\textsuperscript{2+}] signal (Fig. 3, panels A–C) or p130PH-GFP (Fig. 3, D) resulted in further extension of the lag time and smaller magnitude of the EGF-stimulated [Ca\textsuperscript{2+}] signal (Fig. 3, D). This result is likely to reflect a bleed through the bright FGFP to p130PH-GFP-expressing cells. However, both F\textsubscript{GFP} and ΔR were larger in GFP-N cells than in GFP-IP3R224–605 or p130PH-GFP-expressing cells (Table I), suggesting that the IP3-binding domains caused a decrease in the magnitude of the [Ca\textsuperscript{2+}] signal. Table I also shows that the ratio before stimulation (R\textsubscript{0}), the change in R\textsubscript{F380} during the [Ca\textsuperscript{2+}] signal (ΔR), and the mean GFP fluorescence (F\textsubscript{GFP}) (Table I). In addition to the cells that did not express GFP-IP3R224–605 or p130PH-GFP, cells transfected with GFP-N were also used as a control. Both the fraction of cells that did not respond to agonist and the lag time of the [Ca\textsuperscript{2+}] signal, were similar in GFP-N cells to that in non-transfected cells (Table I). Notably, R\textsubscript{0} and ΔR for any of the agonists were smaller in GFP-N cells than in non-transfected cells (Table I). This result is likely to reflect a bleed through the bright FGFP to the records of the fura2 fluorescence (in particular to F\textsubscript{380}) in the GFP-N cells (Table I). Thus, the cross-talk between GFP and fura2 fluorescence could also contribute to the small ΔR obtained in GFP-IP3R224–605 or p130PH-GFP-expressing cells. However, both F\textsubscript{GFP} and ΔR were larger in GFP-N cells than in GFP-IP3R224–605 or p130PH-GFP cells (Table I), suggesting that the IP3-binding domains caused a decrease in the magnitude of the [Ca\textsuperscript{2+}] signal. Table I also shows that the [Ca\textsuperscript{2+}] signal, evoked by bradykinin, another agonist of the PLC\textbeta pathway, was also delayed and decreased by GFP-IP3R224–605, whereas the [Ca\textsuperscript{2+}] rise induced by Tg was not affected by GFP-IP3R224–605 (Table I). When compared with non-transfected cells, the GFP-IP3R224–605 or p130PH-GFP-expressing cells showed an apparent attenuation of both the initial and the
sustained phases of the ATP- and EGF-induced [Ca\(^{2+}\)]\(_c\) signal (Fig. 3, A, B, D, and E). However, the difference in the sustained phase is not a reflection of the inhibition of Ca\(^{2+}\) entry because no difference in the sustained phase was observed when the GFP-IP3R224–605 or p130PH-GFP-expressing cells were compared with GFP-expressing cells (see below, Fig. 6, A and B). Taken together, these data show that the expression of IP3-binding domains suppress [Ca\(^{2+}\)]\(_c\) signaling that is dependent on IP3 formation and IP3R-mediated Ca\(^{2+}\) mobilization. By contrast, no effect appeared on [Ca\(^{2+}\)]\(_c\) elevation that was dependent on non-IP3R-mediated discharge of the ER.

After showing that the IP3R-mediated Ca\(^{2+}\) mobilization is effectively inhibited by the recombinant IP3BPs (Fig. 2), their effect on the IP3 formation was tested (Fig. 4). Because only a small fraction of the transfected cells expressed GFP-IP3R224–605 (<20% efficacy) and no single-cell IP3 assay was available, we determined the basal and agonist-stimulated total [IP3] in cells transfected with p130PH-GFP (~50% efficiency) or its mutant form unable to bind IP3. The basal and ATP-stimulated [\[^{3}H\]IP3] levels were ~20 and 40% higher, respectively, in the p130PH-GFP-transfected cells (Fig. 4). After correction for transfection efficiency, the p130PH-GFP-expressing cells showed ~40 and 80% increase in the basal and ATP-stimulated [\[^{3}H\]IP3] levels, respectively. This result suggests that the buffering effect of p130PH-GFP brought about a compensatory increase in total IP3, enabling the cells to keep the basal-free IP3 levels close to normal and to show an [IP3] signal during stimulation by an agonist. Although the total IP3 formation was increased, the IP3BPs would have suppressed the free [IP3] rise to attenuate the IP3-linked [Ca\(^{2+}\)] signal.

Based on the above results, the cytosolic IP3BPs may serve as mobile cytosolic IP3-buffers, providing a novel means to study the role of [IP3] elevation in calcium signaling phenomena that have been proposed to depend on regenerative activation of the IP3Rs (e.g., [Ca\(^{2+}\)] wave propagation) or to depend on local interactions between IP3R and other Ca\(^{2+}\) channels (e.g., activation of store depletion-induced Ca\(^{2+}\) entry and Ca\(^{2+}\) signal delivery to the mitochondria).

### Table 1

|          | Control | GFP-N | p130PH-GFP | GFP-IP3R224–605 |
|----------|---------|-------|------------|-----------------|
| ATP +Ca\(^{2+}\) | [\[^{3}H\]IP3] formation (a.u.) | 5266 | 4750 | 3113 |
| ATP -Ca\(^{2+}\) | R0/Rp | 0.64/1.96 | 0.47/1.01 | 0.47/0.75 | 0.55/0.97 |
| ATP -Ca\(^{2+}\) | R0/Rp | 0.69/1.39 | 0.57/0.95 | 0.56/0.86 | 0.6/1.0 |
| BK | R0/Rp | 0.63/1.68 | 0.51/0.87 | 0.63/0.83 |
| Tg +Ca\(^{2+}\) | R0/Rp | 0.38/0.5 | 0.49/0.7 |
| Tg -Ca\(^{2+}\) | R0/Rp | 0.59/1.05 | 0.55/0.71 | 0.53/0.72 |

**Fig. 4. Effect of the IP3BPs on the basal and ATP-stimulated [\[^{3}H\]IP3] formation.** Time course of total cellular [\[^{3}H\]IP3 in cells transfected with p130PH-GFP or GFP-p130PH-R134L (control). The data are representative of experiments carried out in duplicates and repeated twice.
**Fig. 5.** Effect of GFP-IP$_3$R$_{224-605}$ on propagation of IP$_3$-linked [Ca$^{2+}$]$_i$ waves through COS-7 cells. Cells were transfected with GFP-IP$_3$R$_{224-605}$ and loaded with fura2 as described in Fig. 3 legend. To evaluate the spatial pattern of [Ca$^{2+}$]$_i$, waves evoked by ATP (50 μM), images were acquired at 15 frames/s at 350-nm excitation. The purple overlays show the fluorescence decrease at each time point and thus represent the spatial localization of the [Ca$^{2+}$]$_i$ increases within the cells. In the upper right image, arrows indicate the directions of wave propagation. In addition, an image was acquired at 490-nm excitation to visualize the GFP-IP$_3$R$_{224-605}$-expressing cells (upper left). In the graphs on the right, time courses of the changes in F$_{350}$ are shown at subregions of the cells (marked by purple numbers) selected along the direction of the wave propagation for each cell. Upper graph, the velocity of wave propagation (left) and the rate of [Ca$^{2+}$]$_i$ rising phase (right) are shown (mean ± S.E. n = 6).

GFP-IP$_3$R$_{224-605}$ (lower panel). The traces show that both the rising phase of the [Ca$^{2+}$]$_i$ spike at each subcellular region and the rate of traveling of the [Ca$^{2+}$]$_i$ signal from one region to the next were relatively slow in the GFP-IP$_3$R$_{224-605}$-expressing cell. Summary of data obtained in a series of similar experiments shows that the rate of [Ca$^{2+}$]$_i$ rise and wave velocity were smaller in GFP-IP$_3$R$_{224-605}$-expressing cells than in non-transfected as well as in GFP-N-expressing cells (Fig. 5, bar charts; p < 0.001; n = 6). These results suggest that the regenerative mechanism of the [Ca$^{2+}$]$_i$ waves must involve an elevation of [IP$_3$] in COS-7 cells stimulated with IP$_3$-linked agonists. Previous work (44) has shown that varying concentrations of agonists elicit varying magnitudes of [H]IP$_3$ formation but induce [Ca$^{2+}$]$_i$ spikes that show a constant height and trigger [Ca$^{2+}$]$_i$ waves that exhibit a constant rate of progress in hepatocytes. When COS-7 cells were sequentially stimulated with a suboptimal (0.5 μM) and a maximally effective dose of ATP (200 μM), the lag time of the [Ca$^{2+}$]$_i$ wave was affected by the stimulus strength (6.3 ± 0.9 versus 3.0 ± 0.8 s, p < 0.03, n = 5), whereas the amplitude was stable (∆F/F$_{0}$ = 0.58 ± 0.02 versus 0.56 ± 0.01, n = 6) similar to the results obtained in hepatocytes. However, the wave velocity was affected by the concentration of the agonist (32.3 ± 7.2 versus 55.8 ± 4.6 μm/s, p < 0.02, n = 6), indicating that in COS-7 cells the [Ca$^{2+}$]$_i$ wave velocity seems to be controlled by the agonist-induced IP$_3$ production. Thus, dampening of the cellular [IP$_3$] increase and modification of the timing of the rapid IP$_3$ diffusion may mediate the effect of GFP-IP$_3$R$_{224-605}$ on the regenerative mechanism of [Ca$^{2+}$]$_i$ wave propagation. Interestingly, a wave of IP$_3$ production has been recently claimed to underlie the fertilization Ca$^{2+}$ wave in Xenopus oocytes (45). Because PIP$_2$ may exert an inhibition on the IP3R Ca$^{2+}$ channel (46), cleavage of PIP$_2$ in the vicinity of the IP3Rs would not only provide IP$_3$ for channel activation but it could also relieve an inhibition elicited by PIP$_2$. The effect of IP3BPs may also involve attenuation of the effect of IP$_3$ close to the IP3Rs.

**Effect of Cytosolic IP3BPs on Store-operated Ca$^{2+}$ Entry—Comparison of the [Ca$^{2+}$]$_i$ signal of the GFP-IP$_3$R$_{224-605}$ or p130PH-GFP-expressing cells with that of cells expressing GFP alone showed that the IP3BP-dependent inhibition of the [Ca$^{2+}$]$_i$ signal was confined to the initial phase (2–5 min) of the stimulation by both ATP and EGF (Fig. 6, A and B). To separate Ca$^{2+}$ entry from Ca$^{2+}$ mobilization, the cells were placed next into a Ca$^{2+}$-free medium stimulated with ATP and Ca$^{2+}$ was added back at 5 min of the stimulation (Fig. 6C). Also, the IP3BP was fused to monomeric RFP to minimize the bleed through to the fura2 fluorescence and Tg was added together with ATP to result in similar Ca$^{2+}$ store depletion in each condition. The non-transfected and the RFP-alone transfected cells showed a robust ATP + Tg-induced [Ca$^{2+}$]$_i$ spike and, in response to the Ca$^{2+}$ back addition, a rapid and massive elevation of [Ca$^{2+}$]$_i$ (Fig. 6C, black and red traces). The RFP-p130PH-expressing cells exhibited a slow and relatively small ATP + Tg-induced [Ca$^{2+}$]$_i$ spike, but the effect of the Ca$^{2+}$ back addition was as prompt and large as it was in the non-trans-
Fig. 6. IP3BPs fail to affect the ATP-stimulated Ca$^{2+}$ influx. A, time course of the [Ca$^{2+}$], signal induced by 50 μM ATP in GFP- and in GFP-IP3R224–605-expressing cells recorded with fura2. *Traces* show the mean response of the entire transfected cell population in the imaging field (8–15 cells) from 6–7 experiments. B, time course of the [Ca$^{2+}$], signal induced by 100 ng/ml EGF in GFP- and in p130PH-GFP-expressing cells recorded with fura2. *Traces* represent the mean response of the entire transfected cell population in the imaging field (8–15 cells) from 6–7 experiments. C, ATP-stimulated Ca$^{2+}$ entry in non-transfected cells (black), RFP-expressing cells (red), and RFP-p130PH-expressing cells (green). At a 5-min stimulation with 50 μM ATP and 2 μM Tg, Ca$^{2+}$ influx was initiated by the addition of 2 mM CaCl$_2$. If Ca$^{2+}$ was added back to non-stimulated cells, no substantial [Ca$^{2+}$] signal was detected (gray). *Traces* represent the mean response calculated for the entire transfected and non-transfected cell population in the imaging field (6–10 transfected and 7–15 non-transfected cells, respectively) from four different experiments.

Fig. 7. GFP-IP3R224–605 fails to affect Tg-induced Ca$^{2+}$ entry in COS-7 cells. A and B, measurement of Ca$^{2+}$ entry. Cells transfected with GFP-N or GFP-IP3R224–605 were loaded with fura2 and were incubated in Ca$^{2+}$-free extracellular medium. Time course of [Ca$^{2+}$], response elicited by sequential addition of 2 μM Tg and 1 mM CaCl$_2$ is shown. Change in the fluorescence ratio of fura2 (excited at 340/380 nm) was normalized to the maximal change evoked by Tg. *Traces* represent the mean response of control versus GFP-N and control versus GFP-IP3R224–605 cells. C, t$_{1/2}$ of Ca$^{2+}$ entry in control, GFP-N, and GFP-IP3R224–605 cells. t$_{1/2}$ referred to the time needed to reach half of the maximal [Ca$^{2+}$] rise. Data were the mean ± S.E. of values from the entire cell population in the imaging field (20–40 cells) from 4–5 different experiments. D and E, measurement of Mn$^{2+}$ quench. Activation of the plasma membrane Ca$^{2+}$ channel achieved by pre-incubating the cells with 2 μM Tg for 300 s was monitored by Mn$^{2+}$-induced (100 μM) quench of cytosolic fura2. The rate of quench reflected penetration of Mn$^{2+}$ into the fura2-containing cytosol.
fected or RFP-expressing cells (Fig. 6C, green trace). Based on these results, the IP3BPs do not appear to affect the Ca2+ entry evoked by the IP3-linked agonists.

Store-operated Ca2+ entry can be activated by ER Ca2+ depletion both in the presence and absence of a rise in total cellular [IP3]. Recent studies have suggested that a portion of the IP3-sensitive Ca2+ store is closely associated with the plasma membrane and interact with the store-operated Ca2+ channels in an IP3-dependent manner to stimulate Ca2+ entry (conformational coupling, reviewed in Ref. 14). IP3BPs could have failed to inhibit the Ca2+ influx in the ATP-stimulated cells if the agonist-induced IP3 signal was supramaximal for the Ca2+ entry. To determine whether any fluctuations in IP3 are involved in the control of the coupling between Ca2+ release and entry, we investigated the Ca2+ entry in GFP-IP3R224–605-expressing cells using two fura2-imaging approaches. In the first protocol, cells were incubated in the absence of extracellular Ca2+, the ER Ca2+ store was discharged using Tg, and then Ca2+ was added back to initiate Ca2+ entry (Fig. 7, A and B). The Tg-induced Ca2+ release appeared as a gradually rising and decaying [Ca2+]i signal, whereas Ca2+ entry gave rise to a rapid and massive [Ca2+]i signal (Fig. 7, A and B). The time course of the [Ca2+]i signal resulting from intracellular Ca2+ mobilization and Ca2+ entry was not changed by the expression of GFP-N or GFP-IP3R224–605 in the cells (Fig. 7, A and B, thick lines). As a measure of the Ca2+ entry, we calculated the lag time of the Ca2+ addition-induced Ca2+ signal (Fig. 7C). There was no significant difference among control (6.99 ± 0.28 s), GFP-N (6.86 ± 0.32 s), and GFP-IP3R224–605 (7.13 ± 0.33 s). In the second approach, we used Mn2+ as a surrogate for Ca2+ to characterize unidirectional ion flux through store depletion-activated Ca2+ channels (Fig. 7, D and E). The rate of Mn2+ transport through this pathway was shown by the quench of fura2 fluorescence measured at 360 nm, a Ca2+-independent excitation wavelength. Specifically, cells were pretreated with Tg for 300 s to deplete the ER Ca2+ store and to activate Ca2+ entry and then MnCl2 was added (Fig. 7, D and E). The time course of Mn2+ quench was not affected by the presence of IP3 (Fig. 8, A versus B). These results show that buffering of IP3 does not interfere with the activation of Ca2+ entry during depletion of the ER Ca2+ store. Notably, we tested Ca2+ entry only after complete store depletion had been reached; therefore, the results do not exclude the possibility that the kinetic of the activation of Ca2+ entry is modulated by IP3 (reviewed in Ref. 47).

Effect of Cytosolic IP3BPs on the Propagation of [Ca2+]i Signals into the Mitochondria—In a wide variety of cell types, the IP3-induced Ca2+ mobilization is effectively propagated to the mitochondria (for a summary see Table I in Ref. 48). Mitochondria form local interactions with subdomains of the ER (24), and at the sites of interaction, mitochondrial Ca2+ uptake is tightly coupled to IP3R-mediated Ca2+ release reminiscent of the organization of synaptic transmission (22). However, the functional organization of the local communication remains elusive. Using the IP3BPs, we tested the IP3 requirements for the ER-mitochondrial Ca2+ coupling. We first used a permeabilized cell model that allowed fluorometric measurement of [Ca2+]i simultaneously with [Ca2+]m during IP3-induced Ca2+ mobilization (22, 49). As we noted for the [Ca2+]i signal (Fig. 2), the [Ca2+]m elevations evoked by the addition of a submaximal IP3 were also suppressed in the presence of p130PH-GFP (125 nM) (Fig. 8, upper left). Importantly, the suppression of the [Ca2+]m signal was large compared with the attenuation of the [Ca2+]i signal (mean ± S.D. responses are shown in the bar charts). We have shown earlier that the IP3 dose response for [Ca2+]m rise is shifted rightward compared with the IP3 dose response for Ca2+ release (22). We speculated that the difference between [Ca2+]i and [Ca2+]m dose response relations may
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Contribute to the relatively large inhibition of the IP$_3$-induced [Ca$^{2+}$]$_{m}$ rise by p130PH-GFP. By lowering the dose of IP$_3$, we found that 125 nm IP$_3$ evoked a [Ca$^{2+}$]$_{m}$ signal identical to that of 200 nm IP$_3$ in the presence of 125 nm p130PH-GFP (Fig. 8). Furthermore, 125 nm IP$_3$ elicited a [Ca$^{2+}$]$_{m}$ rise that was comparable to the effect of 200 nm IP$_3$ in the presence of 125 nm p130PH-GFP (Fig. 8). Based on these data, IP3BPs suppress the Ca$^{2+}$ release mediated by the IP3Rs that provide Ca$^{2+}$ for mitochondrial Ca$^{2+}$ uptake. Furthermore, suppression of Ca$^{2+}$ release results in attenuation of the [Ca$^{2+}$]$_{m}$ signal more than the [Ca$^{2+}$]$_{i}$ rise. An important clue to this point is that a second-order relationship exists between [Ca$^{2+}$]$_{m}$ and the mitochondrial Ca$^{2+}$ uptake (for review see Ref. 50).

To evaluate in intact cells the calcium signal propagation to the mitochondria, we carried out measurements of [Ca$^{2+}$]$_{m}$ with ratiometric pericam-mt (37). Because the fluorescence spectrum of pericam-mt is similar to the spectrum of enhanced GFP, p130PH was introduced to the cells fused to RFP. Simultaneous recording of F$_{\text{RFP}}$, F$_{\text{pericam-mt}}$, and F$_{\text{fura2}}$ was achieved by combination of a filter wheel with a motorized turret, which permitted the selection of the appropriate excitation filters and filter cubes containing the dichroic reflectors and emission filters. Although this design allowed us to minimize the crosstalk between fluorophores, F$_{\text{pericam-mt}}$ was still detectable at 380-nm excitation, one of the excitation wavelengths used for fura2. Because pericam-mt was always excluded from the nucleus, the nuclear area was selected in each cell to calculate [Ca$^{2+}$]$_{i}$. As shown in Fig. 9, most of the cells expressing either RFP-N or RFP-p130PH also expressed pericam-mt (upper two rows of images) and the subcellular distribution of pericam-mt was similar to the distribution of enhanced GFP targeted to the mitochondria (Fig. 1B, iv). Furthermore, during stimulation with ATP, the cells expressing RFP alone displayed a substantial change in pericam-mt fluorescence, and the change in fluorescence followed distribution of the mitochondria. In contrast, the RFP-p130PH-expressing cells showed little or no change in pericam-mt fluorescence (Fig. 8, third row of images). To quantify [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{i}$ for every cell, the fura2 and pericam ratios were obtained and then the cell population average was calculated (Fig. 9, graphs). In RFP-N cells, the ATP-induced [Ca$^{2+}$]$_{m}$ rise (left panel, blue trace) was followed by a large [Ca$^{2+}$]$_{m}$ transient with a hardly noticeable delay (left panel, red trace). In the RFP-p130PH-expressing cells, the onset of the [Ca$^{2+}$]$_{m}$ rise was somewhat delayed and the peak was smaller (right panel, blue trace) but the [Ca$^{2+}$]$_{m}$ signal was almost abolished (right panel, red trace). Similar observations were made when GFP-IP3R$_{224–605}$ was expressed in cells and [Ca$^{2+}$]$_{i}$ was monitored with rhod2 compartmentalized to the mitochondria (n = 3, data not shown). Thus, in intact cells, despite the small decrease in the [Ca$^{2+}$]$_{i}$ signal, the suppression of the [Ca$^{2+}$]$_{m}$ signal by the IP3BPs was almost complete. Because individual mitochondria appear to utilize discrete paths of local communication to access the Ca$^{2+}$ released from the ER, subpopulations of mitochondria could be less sensitive to the increase in IP$_3$-buffering capacity than others. We acquired images at higher spatial resolution to evaluate the ATP-induced [Ca$^{2+}$]$_{m}$ signal in several small groups of mitochondria in individual RFP-p130PH-expressing cells (Fig. 10). The large [Ca$^{2+}$]$_{m}$ spike was not associated with a considerable [Ca$^{2+}$]$_{m}$ increase in any of the mitochondria (Fig. 10, red traces).

To further investigate the relationship between the agonist-induced Ca$^{2+}$ mobilization and the [Ca$^{2+}$]$_{m}$ signal, the [Ca$^{2+}$]$_{i}$ and [Ca$^{2+}$]$_{m}$ rise was calculated for the individual cells expressing either RFP or RFP-p130PH and the [Ca$^{2+}$]$_{m}$ was plotted over a range of [Ca$^{2+}$]$_{i}$ values attained in different single cells (Fig. 11). The distribution of the points representing the RFP-p130PH cells suggests that these cells show a relatively small [Ca$^{2+}$]$_{m}$ rise at any given [Ca$^{2+}$]$_{i}$ signal (Fig. 11, upper panel). As an example, the mean [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{i}$ responses were calculated for the RFP and RFP-p130PH cells that displayed 50–150% increase in the fura2 ratio. The mean [Ca$^{2+}$]$_{m}$ signal was similar in both the control and the IP$_3$ buffer-expressing cells, but the [Ca$^{2+}$]$_{i}$ rise was reduced by >80% in the RFP-p130PH cells (Fig. 11, lower panel). Thus, the [Ca$^{2+}$]$_{i}$ versus [Ca$^{2+}$]$_{m}$ relationship is altered in the presence of the IP3BPs.

Collectively, these data showed that the IP$_3$-induced [Ca$^{2+}$]$_{m}$ signal in the permeabilized cells and the agonist-induced [Ca$^{2+}$]$_{m}$ signal in the intact cells are largely suppressed in the presence of IP3BPs. Both in permeabilized and in intact cells, the [Ca$^{2+}$]$_{m}$ is more sensitive to the IP3BPs than the global [Ca$^{2+}$]$_{i}$ signal that can be considered as a measure of the amount of mobilized Ca$^{2+}$. The high sensitivity of the [Ca$^{2+}$]$_{m}$ signal to the IP3BPs can be attributed to a non-linear relation-
ship between \([\text{Ca}^{2+}]_{c}\) and mitochondrial \([\text{Ca}^{2+}]_{m}\), which has been documented for both isolated mitochondria (for review see Ref. 50) and permeabilized cells (22, 23). The \([\text{Ca}^{2+}]_{c}\) dependence of the \([\text{Ca}^{2+}]_{m}\) is apparently unaffected by IP3BPs in the permeabilized cells, because in this model, both the \([\text{Ca}^{2+}]_{c}\) and \([\text{Ca}^{2+}]_{m}\) signals evoked by a given dose of IP3 in the presence of an IP3BP could be reproduced by the addition of a lower dose of IP3 alone (Fig. 8). However, in the intact cells, the mitochondria were effectively dissociated from the global \([\text{Ca}^{2+}]_{c}\) signal by the IP3BPs (Figs. 9 and 10) and depression of the \([\text{Ca}^{2+}]_{m}\) signal was also shown (Fig. 11). The key to this point seems to be that the \([\text{Ca}^{2+}]_{m}\) uptake of the mitochondria depends on a local perimitochondrial \([\text{Ca}^{2+}]_{c}\) rise mediated by the adjacent IP3Rs (21, 22, 24). We have shown that activation of a cluster of \([\text{Ca}^{2+}]_{m}\) sites is sufficient to evoke a \([\text{Ca}^{2+}]_{m}\) signal (“\([\text{Ca}^{2+}]_{m}\) mark”) (26) but that optimal activation of the mitochondrial \([\text{Ca}^{2+}]_{m}\) uptake sites requires coordinated activation of the IP3Rs that deliver \([\text{Ca}^{2+}]_{m}\) to the mitochondria (22). Such coordinated activation of the IP3Rs is expected to be largely affected in the presence of an IP3 buffer, resulting in suppression of the local \([\text{Ca}^{2+}]_{c}\) signal even if the total \([\text{Ca}^{2+}]_{m}\) release and the cellular average \([\text{Ca}^{2+}]_{m}\) signal show little or no attenuation. Thus, the relationship between the global \([\text{Ca}^{2+}]_{c}\) and \([\text{Ca}^{2+}]_{m}\) in intact cells because the relationship between the local, perimitochondrial \([\text{Ca}^{2+}]_{c}\), and the global \([\text{Ca}^{2+}]_{c}\) is altered. We reason that the \([\text{Ca}^{2+}]_{m}\) signal was more sensitive to IP3BPs in intact cells than in permeabilized cells, because IP3 was added as a bolus to the permeabilized cells, whereas it was produced by PLCs in intact cells, enabling the IP3BPs to decrease the rate of \([\text{IP3}]_{c}\) rise.

**CONCLUSIONS**

The most important observation of this study is that the propagation of the \([\text{Ca}^{2+}]_{c}\) signal to the mitochondria is strikingly sensitive to the IP3-buffering power of the cytoplasm. Thus, the kinetics and spatial distribution of the \([\text{IP3}]_{c}\) rise appear to control the recruitment of the mitochondrial \([\text{Ca}^{2+}]_{m}\) buffering and ATP production to the \([\text{Ca}^{2+}]_{c}\) signal. Our work also evaluated for the first time, the effect of IP3 buffers on \([\text{Ca}^{2+}]_{m}\) wave propagation and on store-operated \([\text{Ca}^{2+}]_{m}\) entry and has provided evidence that the rapid increase in \([\text{IP3}]_{c}\) is important for the regenerative mechanism of the \([\text{Ca}^{2+}]_{c}\) wave but that rapid fluctuations in \([\text{IP3}]_{c}\) may not be required for the optimal activation of \([\text{Ca}^{2+}]_{m}\) entry by store depletion.

The IP3 signal has been envisioned to originate from the Gq- or tyrosine kinase-stimulated activation of PLC and hydrolysis of PIP2 at the plasma membrane and to utilize rapid diffusion of IP3 throughout the cells. However, recent studies have indicated that \([\text{Ca}^{2+}]_{m}\)-induced IP3 formation may also occur at intra-
cellular membranes (9–11). Furthermore, IP$_3$-binding proteins are expressed in several cell types and may also contribute to the shaping of the [IP$_3$] signal (e.g. p130) (29–33). Thus, the [IP$_3$] signal may display a complex spatio-temporal pattern. Overexpression of IP$_3$-binding proteins provided us with a tool to change the temporal pattern of the [IP$_3$] rise and to interfere with spatially confined fluctuations in [IP$_3$]. Our data indicate that the kinetics of the [IP$_3$] signal affects both the initiation and the propagation of the [Ca$^{2+}$] signal. Along this line, regional differences in IP$_3$ production and buffering may converge with regional differences in the Ca$^{2+}$ feedback on the IP3R/RyR to generate regional differences in [Ca$^{2+}$], wave velocity, which have been observed in several models. If an increase in IP$_3$ buffering leads to slower propagation of the [Ca$^{2+}$] wave but at each location the size of the [Ca$^{2+}$] rise is relatively stable, the activation of cytoplasmic Ca$^{2+}$ effectors may remain unaffected. Also, IP$_3$ buffers fail to modulate the store depletion-induced Ca$^{2+}$ entry, providing evidence that neither a global nor a local increase in IP$_3$ production is involved in the coupling between massive ER Ca$^{2+}$ depletion and store-operated Ca$^{2+}$ entry. By contrast, the presence of IP$_3$ buffers results in a dramatic inhibition of mitochondrial Ca$^{2+}$ accumulation during the agonist-induced [Ca$^{2+}$] signal. The large effect of the IP$_3$ buffer on mitochondrial Ca$^{2+}$ uptake could be partly explained by its non-linear dependence on extramitochondrial [Ca$^{2+}$]. However, we demonstrate that the relationship between the [Ca$^{2+}$]$_m$ and [Ca$^{2+}$]$_i$ signals is also suppressed when the IP$_3$ buffering is enhanced. A clue to this problem is that coordinated activation of the IP3Rs seems to be important for mitochondrial Ca$^{2+}$ uptake (22) and that IP$_3$ buffers may influence the rate of global [IP$_3$] increase or attenuate a local [IP$_3$] signal to compromise synchronized opening of the IP3Rs.

Taken together, the present results show IP3BPs as a valuable tool in the study of the mechanisms of local calcium signaling and indicate that a dynamic local interplay between Ca$^{2+}$ and IP$_3$ is of great significance for the propagation of the calcium signal throughout the cytosol and into the mitochondria. Furthermore, based on their relatively large effect on the [Ca$^{2+}$]$_m$ signal, IP3BPs may provide a tool to selectively attenuate the mitochondrial effects of the [Ca$^{2+}$] signal and can be useful to determine the mitochondrial contribution in a range of signaling cascades.

Acknowledgments—We thank David Weaver for help with confocal imaging. We are grateful to Dr. Roger Y. Tsien for the monomeric red fluorescent protein.

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