Regulation of Ca^{2+} Release by $InsP_3$ in Single Guinea Pig Hepatocytes and Rat Purkinje Neurons

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ABSTRACT The repetitive spiking of free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) during hormonal activation of hepatocytes depends on the activation and subsequent inactivation of $InsP_3$-evoked Ca^{2+} release. The kinetics of both processes were studied with flash photolytic release of $InsP_3$ and time resolved measurements of $[Ca^{2+}]_i$ in single cells. $InsP_3$ evoked Ca^{2+} flux into the cytosol was measured as $d[Ca^{2+}]_i/dt$, and the kinetics of Ca^{2+} release compared between hepatocytes and cerebellar Purkinje neurons. In hepatocytes release occurs at $InsP_3$ concentrations greater than 0.1–0.2 μM. A comparison with photolytic release of metabolically stable 5-thio-$InsP_3$ suggests that metabolism of $InsP_3$ is important in determining the minimal concentration needed to produce Ca^{2+} release. A distinct latency or delay of several hundred milliseconds after release of low $InsP_3$ concentrations decreased to a minimum of 20–30 ms at high concentrations and is reduced to zero by prior increase of $[Ca^{2+}]_i$, suggesting a cooperative action of Ca^{2+} in $InsP_3$ receptor activation. $InsP_3$-evoked flux and peak $[Ca^{2+}]_i$ increased with $InsP_3$ concentration up to 5–10 μM, with large variation from cell to cell at each $InsP_3$ concentration. The duration of $InsP_3$-evoked flux, measured as 10–90% risetime, showed a good reciprocal correlation with $d[Ca^{2+}]_i/dt$ and much less cell to cell variation than the dependence of flux on $InsP_3$ concentration, suggesting that the rate of termination of the Ca^{2+} flux depends on the free Ca^{2+} flux itself. Comparing this data between hepatocytes and Purkinje neurons shows a similar reciprocal correlation for both, in hepatocytes in the range of low Ca^{2+} flux, up to 50 $\mu M \cdot s^{-1}$ and in Purkinje neurons at high flux up to 1,400 $\mu M \cdot s^{-1}$. Experiments in which $[Ca^{2+}]_i$ was controlled at resting or elevated levels support a mechanism in which $InsP_3$-evoked Ca^{2+} flux is inhibited by Ca^{2+} inactivation of closed receptor/channels due to Ca^{2+} accumulation local to the release sites. Hepatocytes have a much smaller, more prolonged $InsP_3$-evoked Ca^{2+} flux than Purkinje neurons. Evidence suggests that these differences in kinetics can be explained by the much lower $InsP_3$ receptor density in hepatocytes than Purkinje neurons, rather than differences in receptor isoform, and, more generally, that high $InsP_3$ receptor density promotes fast rising, rapidly inactivating $InsP_3$-evoked $[Ca^{2+}]_i$ transients.

KEY WORDS: liver • Purkinje neurons • calcium • $InsP_3$ • flash photolysis

INTRODUCTION

Calcium release from intracellular stores by $InsP_3$ mediates the activation of liver metabolism by several hormones, resulting, for example, in glycogenolysis and bile secretion. In guinea pig and rabbit liver, the rise of $[Ca^{2+}]_i$ produces a large increase in K^+ and Cl^- permeability through Ca^{2+}-activated conductances in the plasma membrane. The increase of $[Ca^{2+}]_i$ typically occurs as a sequence of periodic spikes of high concentration lasting several seconds (Woods et al., 1987; Field and Jenkinson, 1987) with frequency dependent on the degree of stimulation. The mechanisms generating this pattern of Ca^{2+} release appear to depend to some degree on local regulation by $InsP_3$ receptor activation itself, probably via local feedback through Ca^{2+} released into the cytosol. Evidence supporting this idea is the generation of periodic spiking of the Ca^{2+} concentration in single hepatocytes by $InsP_3$ alone perfused directly into the cytosol from a patch pipette (Capiod et al., 1987; Ogden et al., 1990) or by stable 5-thio-$InsP_3$ released by photolysis (Wootton et al., 1995), although there are additional factors secondary to receptor activation that modify this process during hormone action (Cobbold et al., 1991).

The regulation of the $InsP_3$ receptor by cytosolic Ca^{2+} in several tissues has been shown to be facilitatory at low concentrations close to resting levels (Iino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Iino and Endo, 1992) and inhibitory at high concentrations of 1 μM or greater (Berridge, 1988; Payne et al., 1988; Iino, 1990; Parker and Ivorra, 1990; Ogden et al., 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Iino and Endo, 1992; Combettes et al., 1993; Khodakhah and Ogden, 1995). The facilitatory action is less pronounced or apparently absent in some studies (see

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Combettes et al., 1994, Khodakhah and Ogden, 1995), but the inhibitory action of high $[Ca^{2+}]_i$ is clearly demonstrated in all studies cited. In hepatocytes, there is one report of facilitation by cytosolic free $[Ca^{2+}]$ (Marshall and Taylor, 1993) and several studies showing inhibition of Ca^{2+} release (Ogden et al., 1990; Combettes et al.,1993) and Ca^{2+}-induced desensitization of $InsP_3$ receptors (Pietri et al., 1990). Additionally, inactivation processes that do not depend on $[Ca^{2+}]_i$ have been described in *Xenopus* oocytes (Ilyin and Parker, 1994) and in rat hepatocytes (Hajnozcky and Thomas, 1994).

To study the mechanisms of activation and local regulation of $InsP_3$ receptors in single hepatocytes, it is necessary to gain access to the receptors in the cytosol and release $InsP_3$ in a controlled fashion and to measure the kinetics of the resulting Ca^{2+} release. Generally, kinetic studies of $InsP_3$-activated Ca^{2+} fluxes have been made with fast perfusion of cell suspensions after permeabilizing the surface membrane, but endogenous regulatory processes and differences between cells would not be detected with these methods. In a previous single cell study, $InsP_3$ was released by flash photolysis of caged $InsP_3$ in the cytosol of single hepatocytes, and the activation by Ca^{2+} of the endogenous apamin-sensitive Ca^{2+}-activated K^+ conductance was used as a monitor of free $[Ca^{2+}]$ (Ogden et al., 1990). This showed that $InsP_3$ produced activation at concentrations of 0.4 μM or higher, that there is delay of up to several hundred milliseconds at low $InsP_3$ concentrations, and that there is a subsequent prolonged inactivation of the Ca^{2+} release process, requiring ~1 min to recover, produced by the elevation of cytosolic free $[Ca^{2+}]$. However, the Ca^{2+}-activated K^+ conductance of guinea pig hepatocytes has a strongly cooperative dependence on $[Ca^{2+}]$ (Capiod and Ogden, 1989*a*) and is unsuitable for quantitative kinetic investigation of Ca^{2+} flux, although it provides a useful indicator of free $[Ca^{2+}]$ changes. To overcome this limitation, the experiments described here make use of fluorescent Ca^{2+} indicators to measure particularly the kinetics of activation and inactivation of Ca^{2+} release into the cytosol soon after $InsP_3$ is liberated by photolysis in single guinea pig hepatocytes. The delays are shorter than those found with the K^+ conductance, the Ca^{2+} efflux is quantified as $d[Ca^{2+}]_i/dt$ and found to be small relative to other tissues, particularly in comparison with cerebellar Purkinje neurons, and the rate and mechanism of termination of Ca^{2+} efflux from stores is investigated and shown to depend on $[Ca^{2+}]_i$.

Evidence from cell to cell and tissue to tissue variation in the kinetics of Ca^{2+} release supports the idea that $InsP_3$ receptor density is an important factor determining the duration as well as the magnitude of Ca^{2+} flux. Large Ca^{2+} flux which results from high channel density produces a rapid termination of release by inactivating closed $InsP_3$ channels via local high free $[Ca^{2+}]$. Hepatocytes have a low density of $InsP_3$ receptor/channels, producing small Ca^{2+} flux and slow termination of release. Comparison is made with similar data from cerebellar Purkinje neurons, where the receptor density is very high and which have high flux (Khodakhah and Ogden, 1995) and rapid rates of termination. The results suggest that $InsP_3$ receptor density can account for most of the differences in $InsP_3$ action between hepatocytes and Purkinje neurons and supports the idea that the density of open $InsP_3$ receptor/channels determines the time course of the rise and fall of free $[Ca^{2+}]$ during hormonal signalling in hepatocytes.

METHODS

Cell Preparation and Solutions

Guinea-pig hepatocytes were isolated by perfusion with collagenase followed by mechanical dispersion (Capiod and Ogden, 1989*a*, *b*). Cells were plated from suspension onto 40-mm collagen-coated quartz coverslips in Williams medium E and kept at 37°C in a hydrated atmosphere of CO_2/5% in air. Recordings were made after 2–6 h in Cl^--free solution with gluconate as the major anion. External solution contained (in mM): Na gluconate, 145; K gluconate, 5.6; $CaSO_4$, 5; $MgSO_4$, 1.2; HEPES, 8. For experiments in absence of external Ca^{2+}, solutions contained (in mM): Na gluconate, 149; K gluconate, 5.6; $MgSO_4$, 3; EGTA, 0.04; HEPES, 8. Internal Cl^--free solutions contained (in mM): K gluconate 153; $ATPNa_2$, 3; $MgSO_4$, 3; HEPES, 8; 200 μM fluo-3 or 500 μM furaptra and caged $InsP_3$ or caged 5-thio-$InsP_3$. Fura-2–buffered internal solutions contained (in mM): K gluconate, 40; $ATPNa_2$, 3; $MgSO_4$, 3; fura-2–K_5, 10; $CaCl_2$, 4 or 7.5; HEPES, 100; KOH, 40. All the solutions were buffered at pH 7.3 and 0.2-μm filtered. Experiments were made at room temperature (~27°C).

Somata of rat cerebellar Purkinje neurons were isolated from slices of cerebellar cortex from 12–16-d-old Wistar rats by mild proteolysis (Sigma Type 23, 3 mg · ml^{-1} for 7 min at 37°C; Sigma Chemical Co., St. Louis, MO) and gentle dissociation (see Khodakhah and Ogden, 1995).

Whole-cell Recordings

Standard tight-seal whole-cell recording techniques were used (Hamill et al., 1981). Patch pipettes were made from pyrex glass and coated with a wax comprising parafilm (40%) and mineral oil (60%).

Fluorescence Measurements and Flash Photolysis

The fluorescent indicator used to detect changes in Ca^{2+} concentration was chosen according to the type of measurement made. Fluo-3 has a dissociation constant of $K = 0.7$ μM in situ, close to the resting $[Ca^{2+}]_i$, has a large fluorescence increase on binding Ca^{2+}, and was used to measure delays in the activation of Ca^{2+} release.

Estimates of the time course of $[Ca^{2+}]_i$ changes from changes in fluo-3 fluorescence were found to be distorted when compared with data obtained with lower affinity indicators (see Ogden et al., 1995). This results presumably from saturation of the fluo-3 at high local $[Ca^{2+}]$ near release sites, averaging fluorescence changes across the cell producing underestimates of the

$[Ca^{2+}]$ changes at early times after $InsP_3$ release. Therefore experiments to measure the time course of the rise and decline of $[Ca^{2+}]_i$ were made with the low affinity indicator furaptra, (K = 44 μM, Konishi et al., 1991; K = 48 μM, Ogden et al., 1995; K estimated as 100 μM in frog muscle myoplasm, Zhao et al., 1996).

Finally, in some experiments it was desired to buffer cytosolic free $[Ca^{2+}]$ with high concentrations of fura-2/calcium mixtures. To obtain precise values of free $[Ca^{2+}]$, the concentration of fura-2 was determined from the absorbance of the solution and extinction coefficient e_{362} = 28,700 M^{-1} cm^{-1} (Baylor and Hollingworth, 1988), and Ca^{2+} was added to give the desired free $[Ca^{2+}]$ assuming K = 0.2 μM (see, e.g., Zhou and Neher, 1993).

Fluo-3 (200 μM), furaptra (500 μM), or fura-2 (10 mM) free acids were introduced into the cell together with caged compounds via the patch pipette, and diffusional equilibration between pipette solution and cell was determined from the fluorescence record; recordings were made at least 5 min after membrane rupture. Microspectrofluorimetry was on a Nikon TMD microscope with 40× 1.3 NA objective. Excitation light (fluo-3, 450–490 nm; furaptra or fura-2, 400–440 nm) was from a xenon arc lamp, and light emitted from a single cell was viewed with long pass filters at 520 nm (fluo-3) or 470 nm (furaptra, fura-2). The area viewed was restricted to a single cell by an adjustable rectangular diaphragm, and emitted light was detected by a photomultiplier operated in photon counting mode. Pulse output was counted by a Tecmar interface, corrected for missed pulses, and stored via software (Photon Technology International, South Brunswick, NJ). Photon counts were converted in parallel to an analogue signal by an integrating amplifier with correction for missed counts (Cairn Research) and stored on FM tape.

Fluorescence changes in fluo-3 were expressed as a ratio with respect to the initial resting fluorescence. Furaptra was used at a single excitation wavelength of 420 nm (in most experiments 400–440 nm) conditions in which the fluorescence is quenched close to zero on binding Ca^{2+}. The method for and background to the calculation of free Ca^{2+} from the fluorescence has been described previously (Ogden et al., 1995; see Konishi et al., 1991). Briefly, the intrinsic fluorescence recorded before whole cell recording was taken as the fluorescence at saturating $[Ca^{2+}]_i$, F_{Camax}, where furaptra fluorescence is quenched. The resting $[Ca^{2+}]_i$ in guinea pig hepatocytes has been estimated as 0.2 μM (Burgess et al., 1984) and therefore the fluorescence of furaptra under resting conditions can be taken as an estimate of F_{Camin}. The free Ca^{2+}, Ca_f,was calculated from the fluorescence F by means of the relation:

$$Ca_f = K \cdot (F_{Camin} - F)/(F - F_{Camax}). \quad (1)$$

Fura-2 was used at high free concentration, 2.5 or 5 mM, to buffer Ca_f to levels near resting $[Ca^{2+}]$, i.e., conditions in which it is the predominant source of intracellular Ca^{2+} binding. Fura-2 was used at a single excitation wavelength of 420 nm, and emission was measured at >470 nm, conditions in which the fluorescence is quenched by high $[Ca^{2+}]$ to zero. As for furaptra, the intrinsic fluorescence recorded before fluophore loading was taken as the fluorescence at saturating free $[Ca^{2+}]$, F_{Camax}. The dissociation constant K, the free and bound indicator concentrations, D_f, D_{Ca}, and the Ca_f are related by:

$$K = D_f \cdot Ca_f / D_{Ca}.$$

Total indicator and Ca^{2+} concentrations are:

$$Ca_T = Ca_f + D_{Ca} \text{ and } D_T = D_f + D_{Ca},$$

which on substitution gives:

$$Ca_f / K = (Ca_T - Ca_f)/\{D_T - (Ca_T - Ca_f)\}.$$

The dissociation constant for fura-2 binding Ca^{2+} was assumed to be K = 0.2 μM (see Zhou and Neher, 1993), and D_T and Ca_T have values such that Ca_f is << Ca_T, so

$$Ca_f / K = Ca_T/(D_T - Ca_T). \quad (2)$$

Ca^{2+} released into the cytosol binds predominantly to fura-2 and is measured as the change of D_{Ca}. To measure changes of D_{Ca} an estimate of F_{Camin} is needed. If Ca_T and D_T added to the cytosol have fluorescence $F = F_0$, and Ca_f << Ca_T as above, rearranging Eqs. 1 and 2 gives:

$$(D_T F_0 - Ca_T F_{camax})/(D_T - Ca_T) = F_{Camin},$$

permits calculation of F_{Camin} from the initial fluorescence, F_0, the composition of the fura-2/Ca mixture, and F_{Camax}, which in these measurements was close to zero. Thus at high fura-2 concentration, Ca^{2+} released onto fura-2 appears as an increase of D_{Ca} and was calculated from the fura-2 fluorescence, F, as the proportion of F_{Camin} quenched, by:

$$D_{Ca} = D_T \cdot (F_{Camin} - F)/(F_{Camin} - F_{camax})$$

$$= D_T \cdot \{1 - F \cdot (D_T - C_T)/F_0 \cdot D_T\}.$$

The Ca^{2+}-dependent K^+ conductance has a range of activation by cytosolic Ca^{2+} ion concentration of 0.3–1.5 μM and a maximum open probability of 0.9 (Capiod and Ogden, 1989*a*). It is not detectably activated at $[Ca^{2+}]_i$ less than 0.3 μM and provides a good index of low resting $[Ca^{2+}]_i$. Cells with any degree of activation of Ca^{2+}-dependent K^+ conductance before $InsP_3$ stimulation were not used. The Ca^{2+}-dependent K^+ conductance was also used as an index of changes of $[Ca^{2+}]_i$ during experiments.

Photolysis of caged $InsP_3$, the P-4 or the P-5 1-(2-nitrophenyl)ethyl esters of $InsP_3$ (Walker et al., 1989), was produced by a 1-ms pulse from a short arc xenon flashlamp (Rapp and Guth, 1988) focused to produce an image 2–3 mm across at the cell as described previously (Ogden et al., 1990). The output of the lamp was adapted for most experiments to be adjustable in the range producing 6–14% photolysis of caged $InsP_3$. The coefficient of variation of photolysis of caged ATP was 10%, indicating the degree of reproducibility of photolysis of $InsP_3$ in each cell. In some experiments a caged stable analogue of $InsP_3$, caged 5-thio-$InsP_3$, the S-1 (2-nitrophenyl)ethyl ester of 1-D-myo-inositol 1,4 bisphosphate 5-phosphothiorate. This has a smaller quantum yield than caged $InsP_3$, 0.57 compared to 0.65, and a slower conversion, 87 against 220 s^{-1} (Wootton et al., 1995).

The optical artefact, mainly phosphorescence in the objective, arising from the UV pulse was minimized (4–8 ms) by use of quartz coverslips and UV block (suntan) oil between the cell and objective (Carter and Ogden, 1992; Ogden et al., 1993). This artefact constituted the main limitation to resolving the latency of responses at high $InsP_3$ concentration, the fluorescence changes of fluo-3 and furaptra following photolysis of DM-Nitrophen being complete in less than 4 ms. Photolysis of caged $InsP_3$ by fluorescence excitation was minimized by shuttering the xenon lamp when not recording.

Data were recorded on FM tape at 200 Hz (fluorescence) or 1 kHz (membrane current) bandwidth, and in software fluorescence was integrated over 10 or 20 ms.

Materials

Chemicals were Analar grade from BDH Chemicals Ltd. (Poole, UK) or Sigma Chemical Co. Collagenase CLS 2 was from Wor-

thington Biochemical Corp., (Freehold, NJ). Fluo-3, furaptra and fura-2 were from Molecular Probes (Eugene, OR). The concentration of fura-2 stock solutions was determined spectroscopically with extinction coefficient at 362 nm of $2.8 \times 10^4\ M^{-1}\ cm^{-1}$ (Baylor and Hollingworth, 1988).

RESULTS

Rate of Change of $[Ca^{2+}]_i$ as a Measure of $InsP_3$-evoked Ca^{2+} Flux

Activation of $InsP_3$ receptors results in flux of Ca^{2+} from stores into the cytosol which appears as an increase of free $[Ca^{2+}]$. The rate of change of free $[Ca^{2+}]$, $d[Ca^{2+}]_i/dt$ (units moles $\cdot\ s^{-1} \cdot$ liters^{-1}), is proportional to the net flux of Ca^{2+} into unit volume of cytosol (Ogden et al., 1990), the proportionality depending on the fraction of Ca^{2+} bound to endogenous buffers and to the Ca^{2+} indicator itself. Measurement of $d[Ca^{2+}]_i/dt$ with spatially averaged fluorescence of Ca^{2+} indicators is susceptible to distortion arising from local Ca^{2+} accumulation and the saturating relation between fluorescence and $[Ca^{2+}]_i$. Precise measurement depends on K_{Ca} of the indicator being several fold higher than the peak local free Ca^{2+} so that spatial summation occurs on the linear part of the fluorescence-$[Ca^{2+}]$ relation. Furaptra used here has $K_{Ca} = 48$ μM, and at 500 μM binds approximately 10 Ca^{2+} ions for each free Ca^{2+} ion, producing minimal exogenous Ca^{2+} buffering. Peak $d[Ca^{2+}]_i/dt$ of each response was estimated from the slope of a straight line fitted to the rising phase.

The characteristic time course of the $[Ca^{2+}]_i$ and Ca^{2+}-dependent K^+ conductance of guinea pig hepatocytes after flash photolysis of caged $InsP_3$ in the cytosol is illustrated by the records shown in Fig 1. After the flash the $[Ca^{2+}]_i$ increases after a delay, rises quickly due to net Ca^{2+} flux into the cytosol, then declines slowly due to net loss of Ca^{2+} from the cytosol. The rate of rise of $[Ca^{2+}]_i$ due to $InsP_3$ was up to 52 μM $\cdot\ s^{-1}$ and the rate of decline following the peak was −0.2 to −1.3 μM s^{-1} (range, $n = 9$). In Purkinje neurons peak flux was much higher (<1,400 μM $\cdot\ s^{-1}$), and rates of decline were similar to hepatocytes (Khodakhah and Ogden, 1995). At the peak $[Ca^{2+}]_i$, where $d[Ca^{2+}]_i/dt = 0$, the net flux is zero, and the $InsP_3$-evoked efflux at this point is similar in magnitude to the flux of Ca^{2+} from the cytosol during the decline, small when compared to the maximum $d[Ca^{2+}]_i/dt$. The flux, $d[Ca^{2+}]_i/dt$, was estimated as the slope of a straight line fitted to the maximum rate of rise. The analysis presented below is of (*a*) the delay and its dependence on $InsP_3$ concentration, (*b*) the rate of rise of $[Ca^{2+}]_i$ as a measure of activation of $InsP_3$ gated Ca^{2+} efflux, (*c*) the mechanism that terminates $InsP_3$-gated flux at the peak of the response, and therefore determines the period of high efflux, and (*d*) the effect of $InsP_3$ concentration on the duration of Ca^{2+} release.

The lower record in Fig. 1 shows the K^+ current recorded at 0 mV membrane potential and measures the activation of Ca^{2+}-dependent K^+ conductance of the plasma membrane by cytosolic Ca^{2+}. This conductance has been characterized (activation range 0.3–1.5 μM free $[Ca^{2+}]$, Capiod and Ogden, 1989*a*) and serves to show that the resting $[Ca^{2+}]_i$ is low, and the Ca^{2+} indicator does not affect the response. Because of the steep dependence on free Ca^{2+} (Capiod and Ogden, 1989*a*) and saturation at relatively low free $[Ca^{2+}]$, the Ca^{2+}-dependent K^+ conductance cannot be used to measure absolute values of $d[Ca^{2+}]_i/dt$ for kinetic studies.

Initial Delay of $InsP_3$-evoked Ca^{2+} Release

Previous work (Ogden et al., 1990) had shown a delay of up to 1 s between releasing low concentrations, <0.5

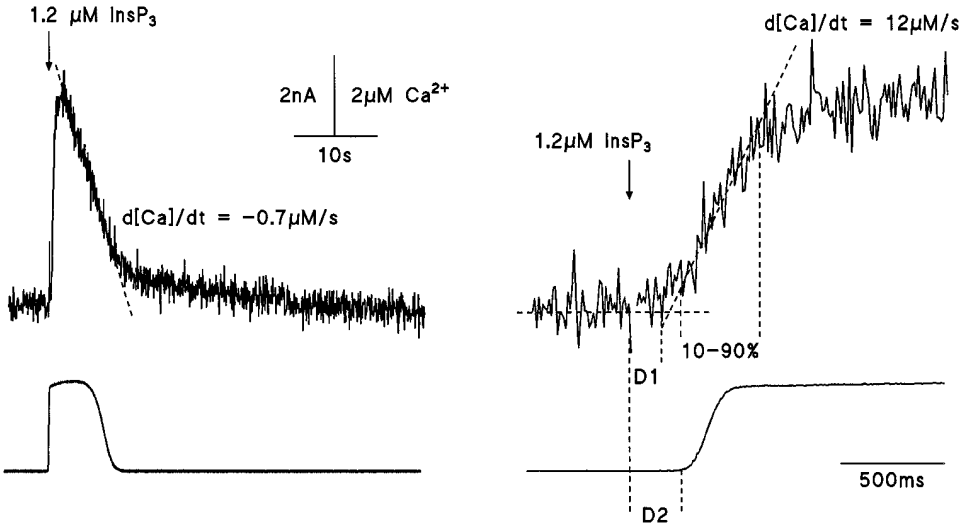

FIGURE 1. $InsP_3$-evoked increase of $[Ca^{2+}]_i$ and Ca^{2+}-activated K^+ current. Single hepatocyte whole cell voltage clamp at 0 mV with pipette solution containing 500 μM furaptra and 10 μM caged $InsP_3$. 1.2 μM $InsP_3$ released by 1-ms UV pulse at time indicated by the arrow. Ca^{2+}-activated K^+ current at 0 mV (*lower trace*) and $[Ca^{2+}]_i$ calculated from fluorescence (*upper trace*) after release of $InsP_3$. Rate of decline in $[Ca^{2+}]_i$ illustrated by the dashed line (*left panel*). (*Right panel*) Expanded records illustrating the delays (*D1*, fluorescence; *D2*, Ca^{2+}-activated K^+ current), rate of rise ($d[Ca^{2+}]/dt$) and duration (*10–90%*) of the increase in $[Ca^{2+}]_i$.

μM $InsP_3$, and activation of the plasmalemmal Ca^{2+}-dependent K^+ conductance, and that this delay was reduced to a minimum of ~120 ms at high (>2 μM) $InsP_3$ concentration. To measure the delays in $[Ca^{2+}]_i$ changes, increases in cytosolic $[Ca^{2+}]$ close to the resting level were detected with the high affinity Ca^{2+} indicator fluo-3 to reduce noise near resting $[Ca^{2+}]_i$ ($K_{Ca} \sim$ 0.4 μM). The delay was estimated as the time from the flash until the fluorescence deviates from the baseline (Fig. 2 *A*). Delays in the rise of fluo-3 fluorescence declined from a mean of 290 ± 90 ms (mean ± SEM, n = 6) at 0.4 μM $InsP_3$ to <30 ms at >5 μM $InsP_3$ (data summarized in Fig. 2 *B*). The delays in the rise of the Ca^{2+}-dependent K^+ conductance were longer than those of the Ca^{2+} increase detected by fluo-3, from 1,000 ± 170 ms at 0.4 μM $InsP_3$ declining to 104 ± 16 ms at 10 μM $InsP_3$ (Ogden et al., 1990). The difference can be explained by the need to raise $[Ca^{2+}]_i$ to 0.3 μM to activate the Ca^{2+}-dependent K^+ conductance, higher than resting $[Ca^{2+}]_i$, and the steep activation of the Ca^{2+}-dependent K^+ conductance by free Ca^{2+}. The minimum delay in fluo-3 fluorescence at high $InsP_3$ concentration was often obscured by an optical artifact lasting up to 20 ms due to flashlamp discharge. Even at very high $InsP_3$ concentrations of 25–100 μM, the delays were >20 ms.

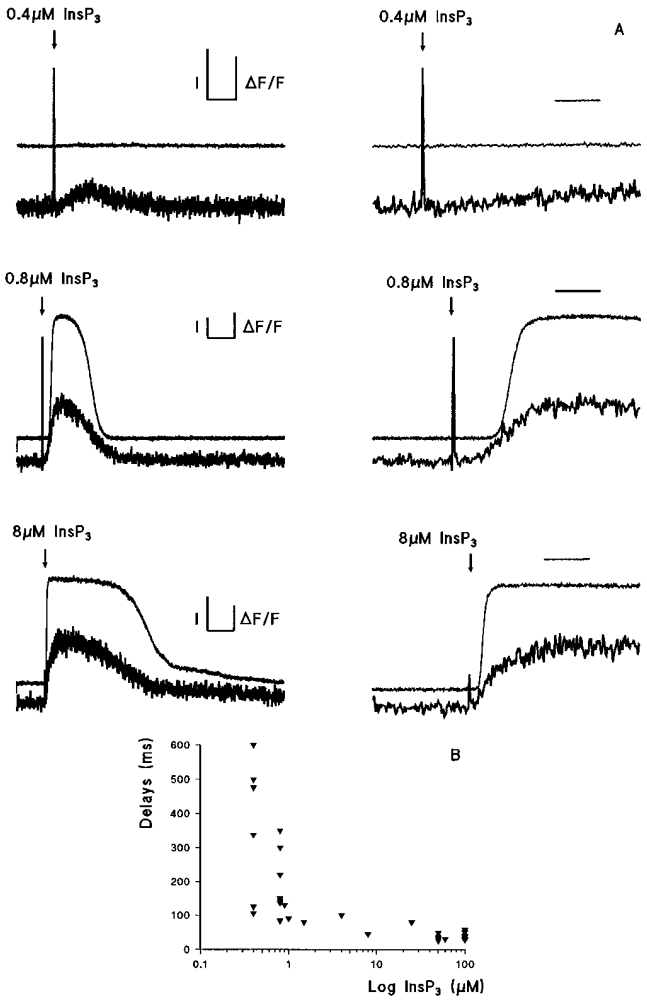

FIGURE 2. Initial delays in time course of $InsP_3$-evoked $[Ca^{2+}]_i$ and Ca^{2+}-activated K^+ current increases. (*A*) Whole cell voltage clamp with 200 μM fluo-3 at 0 mV. The Ca^{2+}-induced fluorescence change (ΔF) is expressed as a fraction of fluorescence (F) in the unstimulated cell. (*Left*) Whole cell Ca^{2+}-activated K^+ current at 0 mV (*upper traces*) and fluo-3 fluorescence (*lower traces*) after release of 0.4, 0.8, and 8 μM $InsP_3$ at the times indicated by the arrows in three different hepatocytes. (*Right*) Expanded records illustrating the delay and initial rate of rise. Horizontal bar, 2 s (*left*), 500 ms (*right*); left vertical bar, I = 200 pA; right vertical bar, $\Delta F/F$ = 0.5. (*B*) Delays between $InsP_3$ release and initiation of Ca^{2+} flux plotted against $InsP_3$ concentration (log scale).

Two explanations for the delay in Ca^{2+} release may be cooperativity in the binding of $InsP_3$ to produce activation, data in RBL cells suggesting n = 4 (Meyer et al., 1988), or a cooperative effect of Ca^{2+} ions at concentrations around the resting level, shown by Iino (1990), Bezprozvanny et al. (1991), and Finch et al. (1991). In the present experiments, when cells were loaded with 500 μM furaptra, the delay to a pulse of $InsP_3$ was substantially reduced when $[Ca^{2+}]_i$ was elevated by Ca^{2+} influx, as illustrated by the records in Fig. 3, which show Ca^{2+} release by 1.2 μM $InsP_3$ in the same hepatocyte in the presence (*middle trace*) and absence (upper and lower pre and post controls) of elevated $[Ca^{2+}]_i$ evoked by hyperpolarization. In five experiments of this kind, no delay was discernible with prior elevation of $[Ca^{2+}]_i$, whereas control release of the same $InsP_3$ concentration in the same cells had delays of mean 380 ± 62 ms (mean ± SEM, n = 5). These results agree with the idea that the initial efflux of Ca^{2+} ions produces a localized increase of $[Ca^{2+}]_i$ and accelerates activation of the $InsP_3$ receptor. It should be noted for later reference that although the delays were reduced, the subsequent Ca^{2+} flux induced by $InsP_3$, measured by $d[Ca^{2+}]_i/dt$, was also reduced in the same cells by the rise in $[Ca^{2+}]_i$ (discussed below).

Mean delays at similar $InsP_3$ concentrations were not significantly shorter with fluo-3 (200 ± 40 ms at 0.8 μM $InsP_3$, range 80–350 ms, n = 7) than with furaptra (310 ± 40 ms at 1.2 μM $InsP_3$, range 90–560 ms, n = 11). However, the low affinity Ca^{2+} dye furaptra is less likely to detect small changes in $[Ca^{2+}]_i$ than fluo-3, making it more difficult to estimate the deviation of the trace from the baseline.

The cooperativity and narrow range of $[Ca^{2+}]_i$ producing Ca^{2+} activation of the K^+ conductance in guinea pig liver cells (Capiod and Ogden, 1989*a*) is most likely responsible for the additional delay, saturation at submaximal $InsP_3$ concentrations (>0.6 μM), and steep rise of the conductance increase compared to the rise of $[Ca^{2+}]_i$ (see Fig. 1).

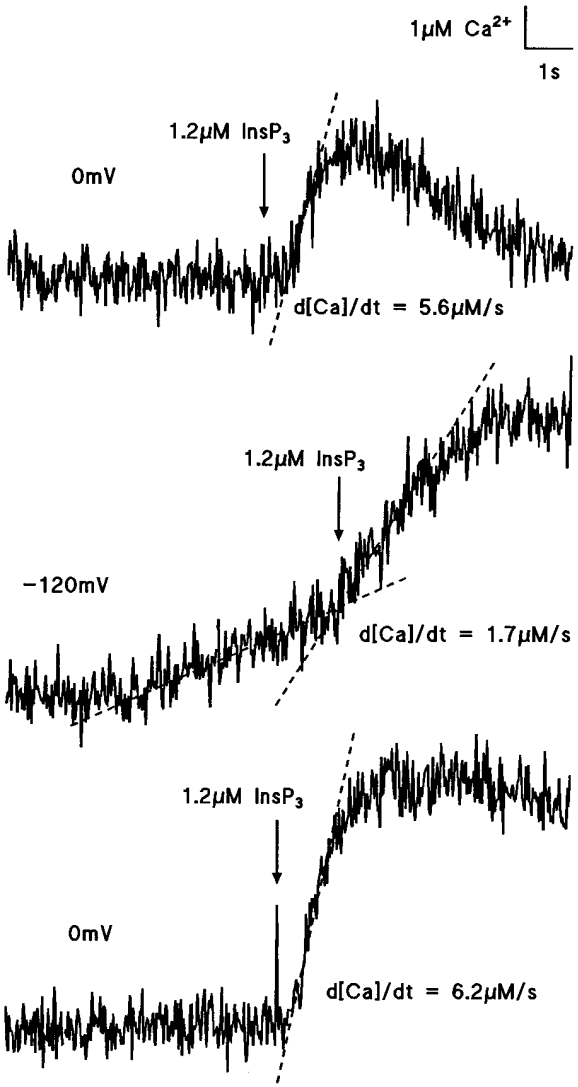

FIGURE 3. Effect of raised $[Ca^{2+}]_i$ on $InsP_3$-evoked Ca^{2+} flux and delay. The cell was loaded with 500 μM furaptra, and 1.2 μM $InsP_3$ was released as indicated by the arrows. The top and bottom traces show the control responses at 0 mV with rates of rises in $[Ca^{2+}]_i$ ($d[Ca^{2+}]_i/dt$) of, respectively, 5.6 and 6.2 $\mu M \cdot s^{-1}$. An elevation in $[Ca^{2+}]_i$ of ~2 μM obtained by hyperpolarizing the cell to −120 mV (*middle trace*) resulted in a slower rate of Ca^{2+} release, $d[Ca^{2+}]/dt$ of 1.7 $\mu M \cdot s^{-1}$, after the release of $InsP_3$. Flash release of $InsP_3$ evoked an increase in $[Ca^{2+}]_i$ without detectable delay when $[Ca^{2+}]_i$ was elevated (*middle trace*) and with a delay of ~500 ms in control conditions at 0 mV in the same cell (*upper trace*). Each traces were separated by a interval of 2 min.

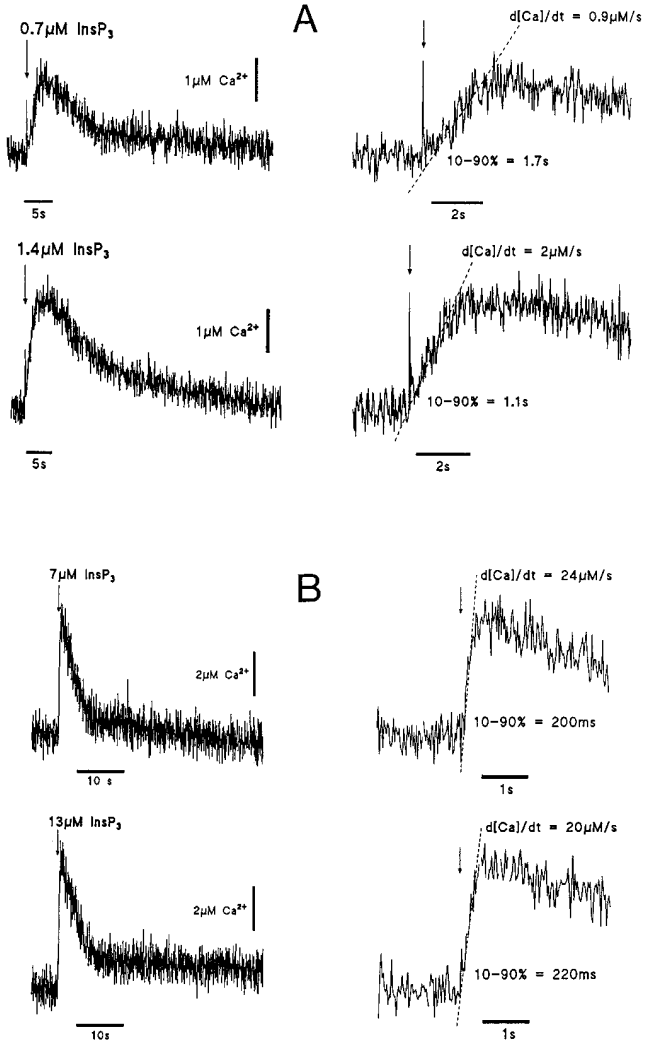

FIGURE 4. Concentration dependence of the $InsP_3$-evoked $[Ca^{2+}]_i$ increase. Increase in $[Ca^{2+}]_i$ evoked by $InsP_3$ in two different hepatocytes loaded with 500 μM furaptra at 0 mV. The photolytic releases indicated by the arrows of 0.7 and 1.4 μM $InsP_3$ in the first hepatocyte are shown in the top four traces (*A*) and of 7 and 13 μM in the second in the four bottom traces (*B*). Expanded records illustrating the delays, rates of rise ($d[Ca^{2+}]_i/dt$) and durations (*10–90%*) of the increase in $[Ca^{2+}]_i$ are shown on the right sides of the figure.

Amplitude and Rate of Rise of $[Ca^{2+}]_i$

Experiments with fluo-3 as the Ca^{2+} indicator showed evidence of distortion of the kinetics and amplitude of the $[Ca^{2+}]_i$ increase averaged over the cell as measured by the fluorescence change (Ogden et al., 1995). The problem was overcome by use of the lower affinity indicator furaptra to extend the range of linear summation of $[Ca^{2+}]_i$ (Raju et al., 1989, Konishi et al., 1991) (see METHODS). The effect of increasing the $InsP_3$ concentration released by a flash in cells loaded with 500 μM furaptra was to increase the rate of rise and peak amplitude of $[Ca^{2+}]_i$ in the range of $InsP_3$ concentrations 0.2 μM to ~5 μM. The maximum rate of rise, $d[Ca^{2+}]_i/dt$, provides a measure of the $InsP_3$-evoked Ca^{2+} flux into unit cytosolic volume and can be used to compare different $InsP_3$ concentrations in the same cell, and the variation from cell to cell at the same $InsP_3$ concentration. The effect of increasing concentration within sin-

gle hepatocytes is shown by the records of $[Ca^{2+}]_i$ in Fig. 4. Fig. 4 *A* shows an increase of peak and rate of rise of $[Ca^{2+}]_i$ between pulses of 0.7 and 1.4 μM $InsP_3$ in the same cell. The $d[Ca^{2+}]_i/dt$ (slopes of the fitted lines ± SD) were significantly different ($P < 0.01$). Fig. 4 *B* shows $[Ca^{2+}]_i$ in response to 7 and 13 μM in another cell with no increase in either parameter at the higher concentration, showing saturation of the response. The pooled data show saturation of the $InsP_3$-evoked flux, measured as the maximum rate of rise in $[Ca^{2+}]_i$ at 5–10 μM $InsP_3$. The data normalized to 1.2 μM $InsP_3$ in each of 10 hepatocytes is shown for peak $[Ca^{2+}]_i$ in Fig. 5 *A* and for $d[Ca^{2+}]_i/dt$ in Fig. 5 *B*. Both parameters increased in each cell with $InsP_3$ concentrations from 0.6 to 2.4 μM, showing that the $InsP_3$-evoked flux increased in this range of concentrations. An estimate of the Hill coefficient for $InsP_3$-evoked flux measured by $d[Ca^{2+}]_i/dt$ in this range was 1.5, but was poorly defined.

Pooled data from 48 hepatocytes showed considerable variability in the amplitude and rate of rise of the response from cell to cell. This is shown by the data plots in Fig. 5, *C* and *D*, which show a large variation in both parameters at each $InsP_3$. The differences in $d[Ca^{2+}]_i/dt$ at each $InsP_3$ concentration from cell to cell may be due to real differences in Ca^{2+} flux because of differing $InsP_3$ receptor densities, from differing single $InsP_3$ receptor-channel properties, or the Ca^{2+} flux may be similar but with differences in Ca^{2+} buffering, resulting in lower $d[Ca^{2+}]_i/dt$ when buffering is high. The variations in free $d[Ca^{2+}]_i/dt$ from cell to cell are analyzed further below.

Termination of $InsP_3$-evoked Ca^{2+} Efflux

The time course of the $InsP_3$-evoked rise of $[Ca^{2+}]_i$ shown in Figs. 1 and 3 has a well-defined peak where the net flux of Ca^{2+} ions into the cytosol, measured as $d[Ca^{2+}]_i/dt$, is zero. The mechanism terminating Ca^{2+} efflux was investigated previously with twin pulse protocols in hepatocytes (Ogden et al., 1990) and showed that Ca^{2+} release evoked by a second pulse of $InsP_3$ is inhibited for a period of ~1 min after a response to the initial pulse, an effect that recovers with a half-time of 10–20 s for the pulse interval. The Ca^{2+}-activated K^+ conductance was used to monitor Ca^{2+} flux by $InsP_3$ in the earlier study but could not show the time course of the onset of this effect because of the distortion produced by cooperative activation of the K^+ conductance by Ca^{2+}. The onset close to peak $[Ca^{2+}]_i$ was tested here with twin pulse experiments monitoring $[Ca^{2+}]_i$ with furaptra, and results from three cells are shown in Fig. 6 to illustrate the onset of inhibition. (*1*) On the left-hand side (Fig. 6 *A*), the upper record shows Ca^{2+} release evoked by 0.4 μM $InsP_3$, the peak flux $d[Ca^{2+}]_i/dt$ = 25 $\mu M \cdot s^{-1}$ is indicated by the line fitted to the fast rise. The middle trace shows that a second pulse of 0.4 μM $InsP_3$ 500 ms later, before $[Ca^{2+}]_i$ had peaked, produced no further activation of Ca^{2+} flux, i.e., no further increase of $d[Ca^{2+}]_i/dt$ above 24 $\mu M \cdot s^{-1}$ produced by

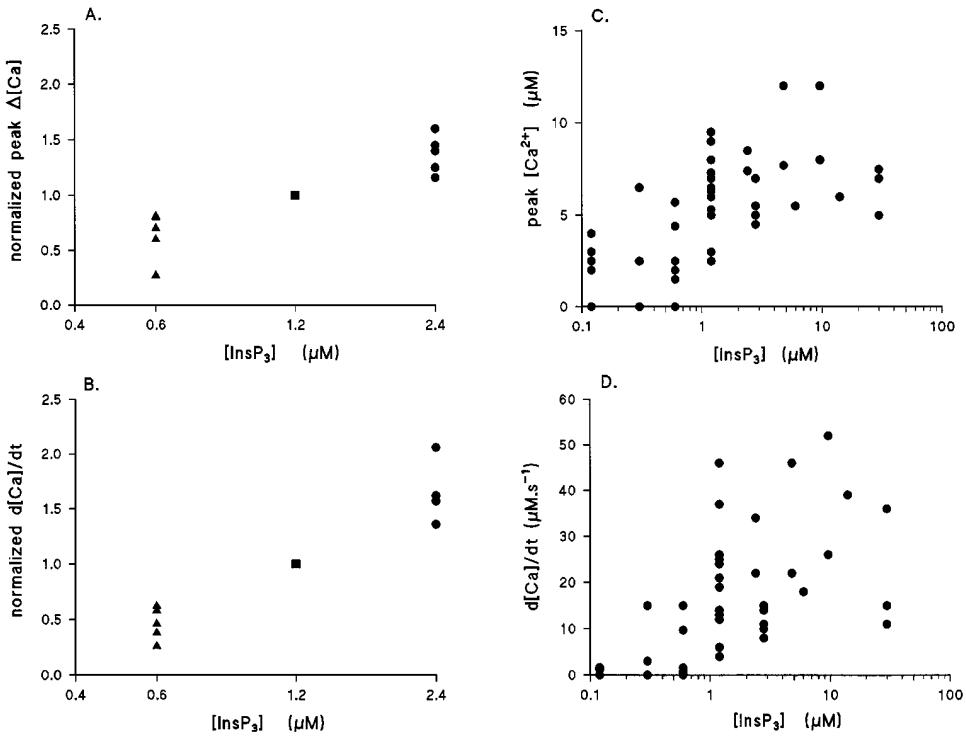

FIGURE 5. Dependence of Ca^{2+} flux and peak $[Ca^{2+}]_i$ on $InsP_3$ concentration. (*A*) Peak $[Ca^{2+}]_i$ (*Δ[Ca]*) and (*B*) rates of rise of free $[Ca^{2+}]_i$ (*$d[Ca]_i/dt$*) normalized to value at 1.2 μM $InsP_3$ in each cell and plotted against $InsP_3$ concentration (log scale). Data from 10 hepatocytes whole cell voltage clamped with 500 μM furaptra at 0 mV. (*C*) Peak $[Ca^{2+}]_i$ and (*D*) $d[Ca^{2+}]_i/dt$ plotted against $InsP_3$ concentration released by photolysis of caged $InsP_3$ in 48 guinea-pig hepatocytes. 500 μM furaptra, 0 mV.

the first pulse. The effect was not due to receptor saturation as 1.4 μM $InsP_3$ subsequently produced a larger flux ($d[Ca^{2+}]_i/dt$ = 46 μM · s^{-1}) in the same cell shown in the lower trace. (*2*) This effect is shown in another cell where a second photolytic release of 0.6 μM $InsP_3$ at about 50% of the rising phase of $[Ca^{2+}]_i$ showed no change in the slope (Fig. 6 *B*, *upper trace*), and a single release of 0.6 μM $InsP_3$ in the same cell evoked a response of similar amplitude and rate of rise of $[Ca^{2+}]_i$ as that established by the first flash with submaximal $InsP_3$ concentration (Fig. 6 *B*, *bottom trace*). It may be noted that $InsP_3$ sensitivity was suppressed at the time of the second pulse in both Fig. 6, *A* and *B*, although receptor/channels were still open as judged by the high steady flux occurring at that time. This suggests that closed receptor/channels were inhibited at the time of the second pulse (see DISCUSSION). (*3*) Finally, in the third example illustrated, twin pulses of 0.6 μM in a cell with low flux showed a significant increase of $d[Ca^{2+}]_i/dt$ from 1 μM · s^{-1} to 3.4 μM · s^{-1} (slopes of the fitted lines ± SD, $P < 0.01$) after the second flash with a 1.6-s interval (Fig. 6 *C*, *upper trace*). The delay after the first pulse of $InsP_3$ was determined with the Ca^{2+}-dependent K^+ conductance (not shown). The rate of rise in $[Ca^{2+}]_i$ after the second flash can be compared to that evoked by a single release of 1.2 μM $InsP_3$ (Fig. 6 *C*, *bottom trace*), which produced a flux of 3.7 μM · s^{-1} later in the same cell showing that in this case the effect of two pulses of 0.6 μM were similar to a single pulse of 1.2 μM. The data of Fig. 6, *A* and *B*, show that the onset of the inhibition can occur early in the response even though $InsP_3$ receptor/channels are still activated; a difference in the cell of Fig. 6 *C*, where no clear inhibition was seen, was the smaller magnitude of the Ca^{2+} flux.

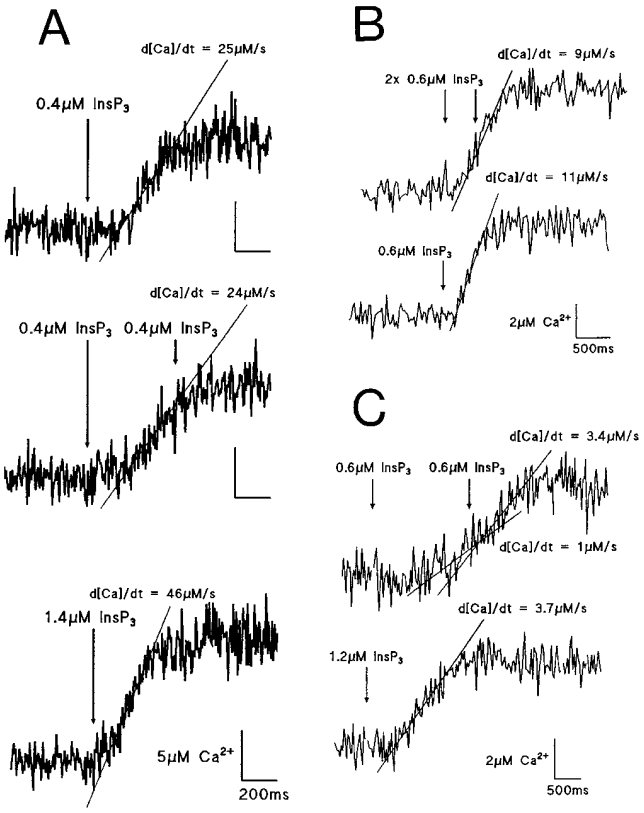

FIGURE 6. Time course of onset of loss of $InsP_3$ sensitivity. $[Ca^{2+}]_i$ after photolytic release of $InsP_3$ at times indicated by the arrows in three different guinea pig hepatocytes loaded with 500 μM furaptra at 0 mV. Traces separated by ~2 min. Rates of rise of $[Ca^{2+}]_i$ ($d[Ca]_i/dt$) are indicated by lines fitted to data. (*A*) Top trace shows the control to 0.4 μM $InsP_3$, middle trace two successive releases of 0.4 μM $InsP_3$ with a interval of 500 ms showing no change in the rate in $[Ca^{2+}]$ release ($d[Ca]_i/dt$) after the second flash, and the bottom trace the response to photolytic release of 1.4 μM $InsP_3$. (*B*) Two successive pulses of 0.6 μM $InsP_3$ (*top trace*) show no change in the rate of rise of $[Ca^{2+}]_i$ after the second flash (600-ms interval) when compared to a single pulse of 0.6 μM $InsP_3$ later in the same cell (*bottom trace*). (*C*) Two successive pulses of 0.6 μM $InsP_3$ (*top trace*) show an increase in the rate of rise of $[Ca^{2+}]_i$ after the second flash (1.6-s interval). Bottom trace shows a single release of 1.2 μM $InsP_3$ in the same cell.

These results and experiments reported earlier with twin pulses of $InsP_3$ (Ogden et al., 1990) and stable 5-thio-$InsP_3$ (Wootton et al., 1995) rule out the possibility that the Ca^{2+} release is terminated by $InsP_3$ breakdown. There is evidence in guinea pig (Ogden et al., 1990) and rat hepatocytes (Combettes et al., 1993) that elevation of $[Ca^{2+}]_i$ inhibits $InsP_3$-evoked Ca^{2+} release. It is well documented that high $[Ca^{2+}]_i$ inhibits the $InsP_3$ receptor of several other cell types (Payne et al., 1988; Iino 1990; Parker and Ivorra, 1990; Bezprozvanny et al., 1991; Finch et al., 1991). Ca^{2+} appears to act on both closed and open $InsP_3$ channels (Bezprozvanny et al., 1991; Finch et al., 1991; Bezprozvanny and Ehrlich, 1994). One possible mechanism for the abrupt termination is inhibition of $InsP_3$ channels by the locally elevated $[Ca^{2+}]$ released by $InsP_3$. This hypothesis was tested in two ways, first by clamping the $[Ca^{2+}]_i$ with a high concentration of fura-2 and recording the Ca^{2+} flux as the increase in Ca^{2+}-bound indicator concentration, second by producing an elevation of $[Ca^{2+}]_i$ independently of $InsP_3$ and testing inhibition of Ca^{2+} release measured by free $d[Ca^{2+}]_i/dt$.

High Ca^{2+} Buffering with Fura-2

An experiment in which $[Ca^{2+}]_i$ was controlled is shown in Fig. 7. The stable 5-thio analogue of $InsP_3$ was released photolytically from caged 5-thio-$InsP_3$ to avoid complications arising from metabolism of $InsP_3$. 5-thio-$InsP_3$ is about 5 times less potent than $InsP_3$ in guinea pig hepatocytes (Wootton et al., 1995). The cell was perfused from the pipette with 36 μM caged 5-thio $InsP_3$, 10 mM fura-2, and 7.5 mM total Ca^{2+}. There was a small activation of the Ca^{2+}-dependent K^+ conduc-

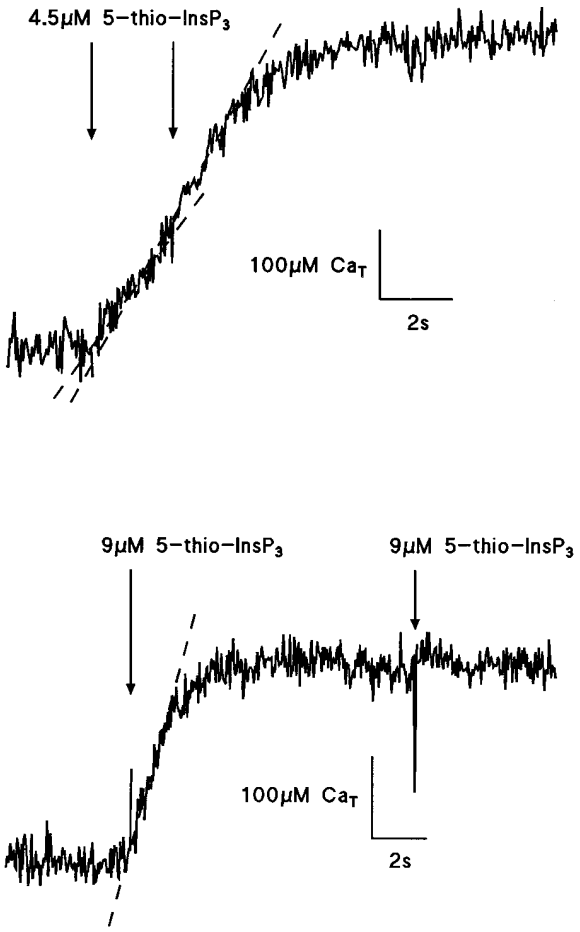

FIGURE 7. Total Ca^{2+} increases evoked by photolytic release of 5-thio-$InsP_3$ in $[Ca^{2+}]_i$ clamped cells. The two traces show the effect of photolytic release of 4.5 and 9 μM 5-thio-$InsP_3$ in two different guinea pig hepatocytes loaded with 10 mM fura-2 and 7.5 mM Ca^{2+}. The top trace shows the response to successive release of 4.5 μM 5-thio-$InsP_3$ with an interval of 2 s. An increase in the rate of total Ca^{2+} release from 60 to 90 μM · s^{-1} was observed after the second release. A third release of 4.5 μM 5-thio-$InsP_3$ at the plateau had no effect (not shown). The release of 9 μM 5-thio-$InsP_3$ raised total Ca^{2+} by 200 μM at a rate of 100 μM · s^{-1} (*bottom trace*). A second release of 9 μM 5-thio-$InsP_3$ at the plateau had no effect. Holding potential 0 mV.

tance suggesting that the $[Ca^{2+}]_i$ was elevated above the normal resting level as expected for this mixture. When the fura-2 fluorescence was steady, release of 5-thio $InsP_3$ produced Ca^{2+} flux into the cytosol, measured as the increase of Ca^{2+} bound to fura-2, at a rate similar to the generation of free Ca^{2+} seen with furaptra as the Ca^{2+} indicator, but with much prolonged duration. Data from two cells are shown in Fig. 7. The upper trace shows release evoked by 2 pulses of 4.5 μM 5-thio-$InsP_3$ separated by 2 s. Ca^{2+} flux in response to the first pulse is 60 μM · s^{-1}, is increased by release of a further 4.5 μM 5-thio-$InsP_3$ to 90 μM · s^{-1} 2 s later, and the total duration of the response is 4 s. The lower record shows in another cell the Ca^{2+} flux produced by a single release of 9 μM 5-thio-$InsP_3$, with flux also of ~100 μM · s^{-1} and duration 2 s. Once the flux had declined to zero at the peak, further release of 5-thio-$InsP_3$ evoked no further flux. In these experiments no additional activation of the Ca^{2+}-dependent K^+ conductance was seen, showing that $[Ca^{2+}]_i$ was buffered. These data show first that the Ca^{2+} flux is much prolonged when $[Ca^{2+}]_i$ is prevented from rising by high fura-2 concentrations, that the inhibition of a second pulse of $InsP_3$, which normally occurs within 0.5 s, is not operating even at 2 s when $[Ca^{2+}]_i$ is buffered to near resting levels, and, finally, that further release does not occur at the peak of the response even though $[Ca^{2+}]_i$ has not changed and is near resting levels, suggesting that another inhibitory mechanism, possibly simply store depletion, has occurred.

Prior Elevation of $[Ca^{2+}]_i$

Cytosolic free $[Ca^{2+}]$ was raised by polarizing the membrane to −120 mV to increase Ca^{2+} leak from the external solution. As discussed above in connection with Fig. 3, the effects on $InsP_3$-evoked release were to greatly reduce the latency before Ca^{2+} efflux, and also to decrease the magnitude of the $InsP_3$-evoked efflux. In Fig. 3 the upper record shows the Ca^{2+} efflux of 5.6 μM · s^{-1} evoked by 1.2 μM $InsP_3$ at 0 mV, the middle trace an $InsP_3$-evoked efflux of 1.7 μM · s^{-1} 2 min later in the same cell, after $[Ca^{2+}]_i$ had been elevated to ~2 μM by Ca^{2+} from the external solution at V = −120 mV, and the lower record shows a further control producing a flux of 6.2 μM · s^{-1} at 0 mV 2 min later. The main effect of prior elevation of $[Ca^{2+}]_i$ to ~2 μM was to suppress the $InsP_3$-evoked efflux to 33% (±6% SD, $n = 6$) of the control flux in the same cell. This confirms more rigorously data reported previously (Ogden et al., 1990) in which elevation of $[Ca^{2+}]_i$ by a bile acid was shown to inhibit $InsP_3$-evoked release as judged by the rate of change of the Ca^{2+}-dependent K^+ conductance in guinea pig hepatocytes. Other effects noted in these experiments were a reduction of the delay in $InsP_3$ activation of efflux (discussed above) and also a larger second response amplitude, possibly as a result of additional Ca^{2+} loading of the stores by the prior elevation of $[Ca^{2+}]_i$.

Rate of Termination of Ca^{2+} Flux Correlates with $d[Ca^{2+}]_i/dt$

Inspection of many $[Ca^{2+}]_i$ records from hepatocytes, and comparison with data from other tissues such as Purkinje neurons, showed that the duration of the period of $InsP_3$-evoked Ca^{2+} flux was brief when the flux was high, suggesting a mechanistic link between duration and Ca^{2+} flux. Evidence was presented above that $InsP_3$-evoked flux was inhibited at the peak $[Ca^{2+}]_i$, indicating that the rate of the process terminating Ca^{2+}

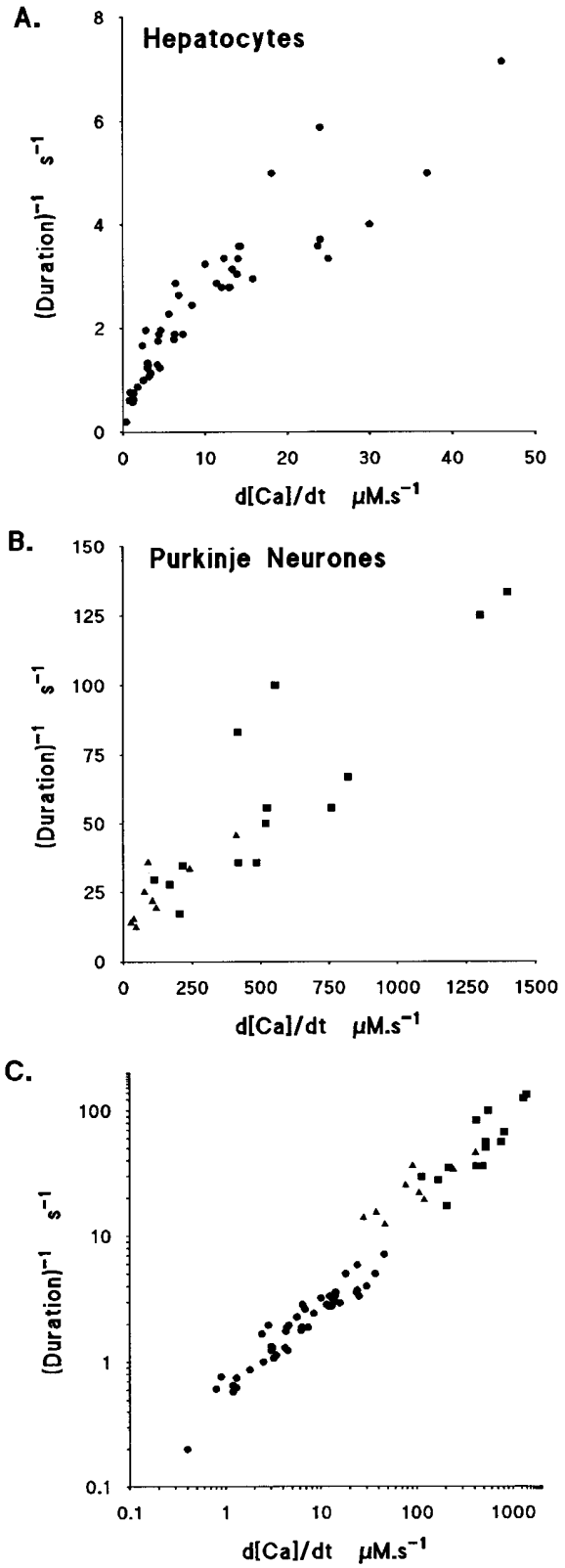

FIGURE 8. Relation between rate of termination of Ca-flux and $d[Ca^{2+}]_i/dt$ in hepatocytes and Purkinje neurons. The duration of the $InsP_3$-evoked Ca^{2+} flux estimated as the reciprocal of the 10–90% risetime (as indicated in Fig. 1) plotted against $d[Ca^{2+}]_i/dt$. (*A*) Rate of termination (duration^{-1}) of the rise of $[Ca^{2+}]_i$ plotted against $d[Ca^{2+}]_i/dt$ on linear scales data from 48 hepatocytes. (*B*) Data from 14 isolated Purkinje neuronal soma, linear scales. Squares represent control cells, triangles data where $[Ca^{2+}]_i$ was elevated via voltage-dependent Ca^{2+} channels before $InsP_3$ release, inhibiting $d[Ca^{2+}]_i/dt$ to 10–20% of control. (*C*) Data from hepatocytes and Purkinje neurons plotted on log scales.

release determines the duration of the Ca^{2+} flux. The relation between duration and magnitude of flux was examined by making use of cell to cell variation of the flux, $d[Ca^{2+}]_i/dt$, shown in Fig. 5 *D*. The rate of termination was measured as the reciprocal of the 10–90% risetime of the $[Ca^{2+}]_i$ and is plotted in Fig. 8 *A* against the flux measured as $d[Ca^{2+}]_i/dt$ (moles $\cdot$ s^{-1} $\cdot$ liters^{-1} cytosol) for each response. A good linear correlation was found between the rate of termination and the magnitude of flux ($r = 0.91$). Further, the large cell–cell variation when Ca^{2+} flux or peak $[Ca^{2+}]_i$ was plotted against $InsP_3$ concentration (see Fig. 5) is not apparent in the dependence of duration on flux shown in Fig. 8.

Cerebellar Purkinje neurons have large $InsP_3$-evoked Ca^{2+} fluxes, measured by $d[Ca^{2+}]_i/dt$, and brief duration of $[Ca^{2+}]_i$ rise. This difference between tissues was used to see if a similar relation holds more generally over a wide range of Ca^{2+} flux. A plot of data obtained with the same protocol from Purkinje neurons, shown in Fig. 8 *B*, also has a good correlation between rate of termination and $d[Ca^{2+}]_i/dt$. The data from hepatocytes and Purkinje neurons are shown plotted together on log scales in Fig. 8 *C*. If termination of Ca^{2+} flux is produced by Ca^{2+} binding to and inactivating channels then the rate of termination should be proportional to local Ca^{2+} concentration and hence the flux produced locally by open channels. The Ca^{2+} concentration in the cytosol close to $InsP_3$-gated channels due to Ca^{2+} flux through open channels will reach a high level quickly after activation, in <10 ms at distances up to 0.5 μm (see, e.g., Stern, 1992). In this model the rate of termination should be proportional to free $[Ca^{2+}]$ and hence to $d[Ca^{2+}]_i/dt$. This mechanism and an alternative, store depletion, are discussed below.

Prolongation of $[Ca^{2+}]_i$ Increase by High $InsP_3$ Concentration

Evidence was obtained of prolongation of $[Ca^{2+}]_i$ as the $InsP_3$ concentration released by photolysis was increased and occurred even in conditions where the initial flux was the same. This effect was seen by integrating the $[Ca^{2+}]_i$ records in twin pulse experiments and is illustrated by the experiment in Fig 9. Initially (*upper trace*), a single pulse of 0.4 μM $InsP_3$ produced Ca^{2+} flux of $d[Ca^{2+}]_i/dt = 21$ μM $\cdot$ s^{-1}, 10–90% risetime of high $d[Ca^{2+}]_i/dt$ of 240 ms, and area 34 μM $\cdot$ s. In the middle record the $InsP_3$ concentration was doubled by

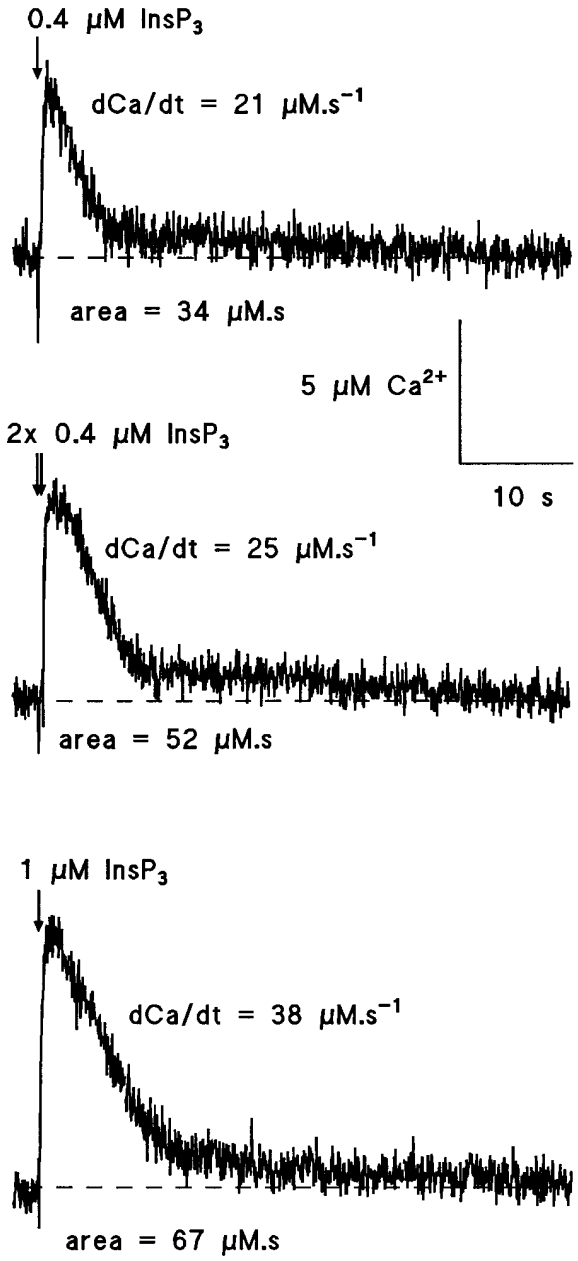

FIGURE 9. Dependence of the duration of $[Ca^{2+}]_i$ increase on $InsP_3$ concentration in hepatocytes. Photolytic release of $InsP_3$ indicated by the arrows in a single hepatocyte. Areas under the $[Ca^{2+}]_i$ increase compared between records following release of $InsP_3$. Records separated by 2 min. 500 μM furaptra, 0 mV. (*Top record*) Single pulse of 0.4 μM $InsP_3$ produced flux 21 μM · s^{-1}, peak $[Ca^{2+}]_i$ 6 μM and area 34 μM · s. (*Middle record*) Twin pulses of 0.4 μM $InsP_3$ separated by 500 ms. Flux 25 μM · s^{-1}, peak $[Ca^{2+}]_i$ 7 μM and area 52 μM · s. (*Lower record*) Single pulse of 1 μM $InsP_3$. Flux 38 μM · s^{-1}, peak $[Ca^{2+}]_i$ 9 μM, area 67 μM · s.

applying 2 pulses of 0.4 μM separated by 400 ms, producing no additional flux with the second pulse. The effect was to increase the area under the $[Ca^{2+}]_i$ trace to 52 μM · s without substantially increasing the flux (25 μM · s^{-1}), the duration of high $d[Ca^{2+}]_i/dt$ (10–90% rise 280 ms), or peak $[Ca^{2+}]_i$, thus indicating that the effect was to prolong the declining phase of $[Ca^{2+}]_i$ by prolonging release or inhibiting sequestration of free Ca^{2+}. Subsequent photolytic release of 1 μM $InsP_3$ in the same cell produced a Ca^{2+} flux of $d[Ca^{2+}]_i/dt$ = 38 μM · s^{-1}, 10–90% risetime of 180 ms and area 67 μM · s, showing that responses to 0.4 μM and 2× 0.4 μM were submaximal. In five experiments of this kind the areas resulting from a single pulse releasing 1.2 μM $InsP_3$ were compared with the areas for 2 pulses of 1.2 μM $InsP_3$ in quick succession. The twin pulse areas were increased to 190 ± 42% (mean ± SEM, $n = 5$) relative to single pulse with no increase of peak flux. This can be compared with the area evoked by a single pulse releasing 2.4 μM $InsP_3$ which produced 201 ± 30% (mean ± SEM, $n = 5$) of the area evoked by a single pulse of 1.2 μM $InsP_3$ in the same cell. Comparison of the 10–90% risetimes in twin pulse experiments with those in controls shows that risetimes are prolonged by 116 ± 7% (mean ± SEM, $n = 5$) and that the 190% prolongation occurs predominantly after the peak $[Ca^{2+}]_i$. Experiments in Ca^{2+}-free medium showed no effect of Ca^{2+} removal on duration and that the prolonged $[Ca^{2+}]_i$ increase at high $InsP_3$ concentration is not a result of enhanced Ca^{2+} entry from the extracellular fluid. These data suggest that the $InsP_3$-evoked flux is prolonged at high $InsP_3$ concentration even when the peak flux is the same as at low concentration. The simplest explanation would be repeated activation of receptors recovering from Ca^{2+} inactivation, an effect that would be prolonged by the higher concentrations. Alternatively, a weaker inhibitory effect of elevated free Ca^{2+} at higher $InsP_3$ concentrations (Combettes et al., 1994) or a reduced rate of Ca^{2+} pumping into stores or out of the cell may account for the prolonged responses in double flash experiments.

DISCUSSION

The hormonal activation of guinea pig hepatocytes, for example by norepinephrine, results in release of Ca^{2+} from intracellular stores as a series of spikes of high $[Ca^{2+}]_i$ relative to resting $[Ca^{2+}]_i$, with fast rise and decline and a period of ~20 s (Field and Jenkinson, 1987; rat hepatocytes, Woods et al., 1987). Spikes of $[Ca^{2+}]_i$ of similar waveform and period can be generated by perfusing $InsP_3$ directly into the cytosol from a patch pipette in whole cell patch clamp (Capiod et al., 1987; Ogden et al., 1990). This observation suggests that the mechanisms important in forming the spikes involve properties inherent in the $InsP_3$ receptor and its interaction with $InsP_3$ and free Ca^{2+}. The aim of the experiments described above was to analyze in situ in single cells the kinetics of activation and inactivation of Ca^{2+}

release, following $InsP_3$ release as a well defined pulse, to see how receptor properties shape the Ca^{2+} spike. The time course of $[Ca^{2+}]_i$ change after $InsP_3$ release comprises a latency, a period of rapid Ca^{2+} efflux into the cytosol which terminates abruptly at the peak where $d[Ca^{2+}]_i/dt$ is zero, and a slow recovery as Ca^{2+} is removed from the cytosol. $InsP_3$-evoked Ca^{2+} flux into the cytosol was measured as the rate of change of free cytosolic $[Ca^{2+}]$, $d[Ca^{2+}]_i/dt$, and the rate of termination of the Ca^{2+} flux as the reciprocal of the 10–90% risetime to the peak $[Ca^{2+}]_i$.

$InsP_3$ Concentrations

$InsP_3$ reproducibly produced Ca^{2+} release in hepatocytes in whole cell patch clamp at concentrations of 0.1–0.2 μM or greater, approximately the same as that reported in permeabilized guinea pig hepatocytes (Burgess et al., 1984; Combettes et al., 1989) and similar to those in whole cell clamp endothelial cells (Carter and Ogden, 1992) and astrocytes, but much less than the concentrations needed in cerebellar Purkinje neurons (Khodakhah and Ogden, 1993). As judged by shortening of delays and the saturation of $d[Ca^{2+}]_i/dt$, maximal $InsP_3$ concentrations were between 2 and 10 μM.

At low $InsP_3$ concentration (0.4 μM), a delay of mean 290 ms was found between release and the start of Ca^{2+} flux. In a previous study with photorelease of metabolically stable 5-thio-$InsP_3$ in hepatocytes, it was found that delays of many seconds duration occurred at low concentrations of 5-thio $InsP_3$ (Wootton et al., 1995), suggesting that in the absence of phosphatase activity lower concentrations of $InsP_3$ might be effective and produce longer delays. Further, this observation suggests that the accumulation of $InsP_3$ is important during hormone action, requiring $InsP_3$ generation locally at a rate that exceeds breakdown in order to produce a response and resulting in gradients of $InsP_3$ concentration (see Marty et al., 1989, for discussion).

It was found that the $[Ca^{2+}]_i$ increase was prolonged as $InsP_3$ concentration was increased and was due to an action additional to the increase in peak Ca^{2+} flux. This effect was demonstrated in closely spaced twin pulse experiments when, although the $InsP_3$ concentration was doubled, the peak flux activated was not increased and the duration of elevated $[Ca^{2+}]_i$ increased by a factor of 1.9. Comparison of 10–90% risetimes between twin pulse and control responses shows that the prolongation occurs after the peak $[Ca^{2+}]_i$ when the efflux from stores has fallen to a low level. If $InsP_3$ remaining after the peak $[Ca^{2+}]_i$ produces a low level of Ca^{2+} release, possibly due to the recycling of inactivated $InsP_3$ channels on a seconds timescale, then this result is expected simply because the higher concentration takes longer to metabolize and the low level activation of receptors persists for longer. The possibility that a second pool of activatable receptors is recruited to produce the prolongation is not supported by the observation that the second pulse of $InsP_3$ produces no immediate increase in Ca^{2+} flux into the cytosol, requiring simultaneous activation of a new pool of receptors as the initial pool is inhibited to sustain this explanation. The importance of breakdown of $InsP_3$ in the total duration of action is also apparent from data with 5-thio-$InsP_3$, which induces a small, steady, persistent component of Ca^{2+} release in hepatocytes (Wootton et al., 1995) and Purkinje neurons (Khodakhah and Ogden, 1995).

Origin of the Delay in Activation of Ca^{2+} Efflux at Low $InsP_3$ Concentrations

The delay between $InsP_3$ release in the cytosol and the rise of fluorescence of fluo-3 from baseline levels at resting $[Ca^{2+}]_i$ decreased from mean 290 ms at 0.4 μM $InsP_3$ to 30 ms or less at high concentration, close to the kinetic limit imposed by photolytic release of $InsP_3$. Similar delays were reported in fast perfusion experiments with permeabilized guinea pig hepatocytes (Champeil et al., 1989). Delays have also been reported in $InsP_3$ activation of Ca^{2+} release in smooth muscle (Walker et al., 1987; Somlyo et al., 1992), RBL cells (Meyer et al., 1990), oocytes (Parker and Ivorra, 1990), and Purkinje neurons (Khodakhah and Ogden, 1993, 1995). Evidence that Ca^{2+} ions act cooperatively with $InsP_3$, thus generating the delay at low $InsP_3$ concentration, was obtained in experiments in which the $[Ca^{2+}]_i$ was elevated before $InsP_3$ release, resulting in delays reduced or abolished when compared with controls in the same cell. In previous work (Ogden et al., 1990), elevation of $[Ca^{2+}]_i$ by a bile acid was shown to reduce the delay in the activation of the Ca^{2+}-dependent K^+ conductance. Reduction of the latency has also has been reported by Iino and Endo (1992) in smooth muscle and by Parker et al. (1996) in *Xenopus* oocytes. The whole cell averaged $[Ca^{2+}]_i$ required to produce this effect here was ~2 μM, but the local concentration could be different because influx is via the plasma membrane. In the same recordings, it was found that $[Ca^{2+}]_i$ was high enough to have produced inhibition of the $InsP_3$-evoked Ca^{2+} efflux.

A minimal delay at high $InsP_3$ concentration has been reported in 2 studies, by Parker et al. (1996) in *Xenopus* oocytes with a protocol similar to that used here, who found a minimal delay of about 30 ms at resting $[Ca^{2+}]_i$, and by Marchant and Taylor (1996) who found a minimal delay of about 30 ms in a perfused microsomal preparation of hepatocytes. Champeil et al. (1989) found a minimum resolvable delay of 20 ms in perfused permeabilized hepatocytes, which is, however, attributable to the resolution of rapid mixing studies. In the experiments reported here, the mean latency at 5–10

μM $InsP_3$ was 30 ms with a minimum of 25 ms. The photolysis of caged $InsP_3$ has a half-time of 3 ms, so it might be expected that a 5–10-fold supramaximal concentration would act with no detectable delay. Responses at 25–100 μM $InsP_3$ showed delays that could be distinguished from the optical artifact, supporting the idea that there is a minimal delay. The effect of raising $[Ca^{2+}]_i$ in reducing the delay to zero supports the interpretation of Parker et al. (1996) that Ca^{2+} priming of the receptor occurs before activation by $InsP_3$.

It is not clear from the present experiments, as in many other studies, whether there is cooperativity produced by multiple $InsP_3$ binding at constant $[Ca^{2+}]_i$, as reported at low $InsP_3$ concentrations in RBL cells (Meyer et al., 1988). The concentrations of $InsP_3$ are higher here than those required in RBL and may be in the range where the "low concentration" Hill coefficient is substantially underestimated.

The delay in activation of the Ca^{2+}-dependent K^+ conductance is 100 ms longer than the delay of fluo-3 fluorescence at all but very high $InsP_3$ concentrations. This can be explained by the activation of the Ca^{2+}-dependent K^+ conductance at free $[Ca^{2+}]$ greater than resting levels and by the strongly cooperative activation by Ca^{2+} (Ogden et al., 1990; Capiod and Ogden, 1989*a*). There is no evidence that the additional delay in activation of the conductance results from slow diffusion of Ca^{2+} to the membrane.

$InsP_3$-evoked Ca^{2+} Flux

After the latency, free $[Ca^{2+}]$ rises quite linearly with a rate, $d[Ca^{2+}]_i/dt$, that depends on the flux of Ca^{2+} into unit cytosolic volume (moles · s^{-1} · $liter^{-1}$) and on the proportion of Ca^{2+} bound to endogenous and exogenous buffers. If the unitary flux through $InsP_3$-gated channels is similar from one to another, then $d[Ca^{2+}]_i/dt$ measures in each cell the density of channels open in unit volume. This quantity $d[Ca^{2+}]_i/dt$ was found to increase with $InsP_3$ concentration up to 2.4 μM in each cell without showing evidence of cooperative $InsP_3$ binding and was maximal by about 7 μM. In guinea pig hepatocytes the peak $[Ca^{2+}]_i$ also increased with $InsP_3$ concentration in this range in each cell but with lower slope. The peak $[Ca^{2+}]_i$ at high $InsP_3$ concentration were 5–9.5 μM and rates of change $d[Ca^{2+}]_i/dt$ up to 52 μM · s^{-1}. These maximum values are small compared with cerebellar Purkinje neurons (Khodakhah and Ogden, 1995) and aortic endothelial cells (Carter and Ogden, manuscript in preparation) examined with the same methods, but are generally high compared with $[Ca^{2+}]_i$ reported for hormonal stimulation of hepatocytes in studies with fluorescent Ca^{2+} indicators (for review, Rooney and Thomas, 1991). In *Xenopus* oocytes (Parker et al., 1996), an increase in the flux, measured as rate of change of calcium green fluorescence, was found as $InsP_3$ concentration was increased, although the $InsP_3$ and Ca^{2+} concentrations were not measured.

A striking finding in these experiments was a large cell to cell variation in $d[Ca^{2+}]_i/dt$ measured at each $InsP_3$ concentration. This may be due to a large variation in density of $InsP_3$ receptors from cell to cell, a variation in the driving potential for Ca^{2+} efflux due to different degrees of store loading, or different extents of Ca^{2+} binding to cytosolic buffers. A large variation of peak $[Ca^{2+}]_i$ from cell to cell was also found here and in cerebellar Purkinje neurons (Khodakhah and Ogden, 1995).

Termination of $InsP_3$-evoked Ca^{2+} Release: Comparison with Cerebellar Purkinje Neurons

The net flux into the cytosol at the peak $[Ca^{2+}]_i$ is zero, indicating that the Ca^{2+} flux due to $InsP_3$ has declined to a low level at this time. It was noted that the duration of the period of high, fairly constant $d[Ca^{2+}]_i/dt$ during the rise of $[Ca^{2+}]_i$ was shortened when flux, $d[Ca^{2+}]_i/dt$, was high. The rate of the process underlying the termination of flux, whatever the mechanism, can be represented by the reciprocal of the 10–90% rise time of $[Ca^{2+}]_i$. A good linear correlation was found between this rate and $d[Ca^{2+}]_i/dt$ in liver cells, shown in Fig. 8 *A*, suggesting a simple one-step mechanism. The $d[Ca^{2+}]_i/dt$ represents the flux of free Ca^{2+} into unit cytosolic volume and will depend on the density of $InsP_3$ receptors. To test this idea, data from Purkinje neurons, which have a very high receptor density and high Ca^{2+} flux into the cytosol (Khodakhah and Ogden, 1995), were analyzed and presented in the same way in Fig. 8 *B*. A linear correlation with similar slope was found but over a much higher range of flux, suggesting that the same mechanism operates to terminate the Ca^{2+} flux in Purkinje neurons. The data from hepatocytes and Purkinje neurons are plotted together in Fig. 8 *C* on log–log scales, illustrating a similar correlation over a wide range, four log units, in both tissues. The large cell–cell variability seen when the flux or free $[Ca^{2+}]$ were plotted against $InsP_3$ concentration, was not present in the correlation between duration and flux, supporting a mechanistic link between rate of termination and the flux. Empirically, this correlation indicates that high Ca^{2+} flux into the cytosol via $InsP_3$ channels, produced, for instance, by a high channel density as in Purkinje neurons, results in a more rapid termination of Ca^{2+} release, producing a fast rising but brief pulse of free $[Ca^{2+}]$. Local differences in $InsP_3$ receptor density might be expected to produce localized fast rising, brief free $[Ca^{2+}]$ changes, as well as producing localized high $InsP_3$ sensitivity as proposed by Hirose and Iino (1994).

Mechanism of Termination

The rate of termination correlates with the rate of appearance of free [Ca^{2+}] in the cytosol of hepatocytes and Purkinje neurons over a wide range of flux. Purkinje neurons have a very large endogenous Ca^{2+} buffering, estimated as 2,000 Ca^{2+} ions bound for each free, due to high density of Ca^{2+}-binding proteins (Fierro and Llano, 1996), compared with estimates of 50–100:1 in other cells (Neher, 1995), supporting the idea that free Ca^{2+} is important rather than total Ca^{2+} flux. The inhibition produced by cytosolic free [Ca^{2+}] of the $InsP_3$ receptor has been shown in many tissues (Payne et al., 1988; Iino, 1990; Ogden et al., 1990; Ivorra and Parker, 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Combettes et al., 1993). Strong inhibition of $InsP_3$-evoked Ca^{2+} release by Ca^{2+} influx through voltage-gated channels has been shown in Purkinje neurons (Khodakhah and Ogden, 1995) and through nicotinic channels in *Xenopus* oocytes (Parker et al., 1996). Similar experiments were less straightforward in hepatocytes. Previously (Ogden et al., 1990), a bile acid had been used to release Ca^{2+} from $InsP_3$-independent stores and shown to reduce the rate of rise of the Ca^{2+}-dependent K^+ conductance after $InsP_3$ release. In the present study, the $[Ca^{2+}]_i$ was raised more reproducibly by polarizing the membrane to −120 mV to increase the leak of Ca^{2+}, and $[Ca^{2+}]_i$ was measured here with a fluorescent indicator. $InsP_3$-evoked flux was inhibited to ~33% by prior elevation of $[Ca^{2+}]_i$ to ~2 μM. Controlling the $[Ca^{2+}]_i$ close to resting levels with a high concentration of free fura-2 during $InsP_3$-evoked release prolonged the period of flux and prevented inactivation of the response normally seen to a second pulse of $InsP_3$ (5-thio-$InsP_3$ in this case). These experiments support the idea that free Ca^{2+} adjacent to the release sites inactivates $InsP_3$ receptor/channels. The free Ca^{2+} close to open channels is proportional to the flux through the channels and is established very quickly, in ~10 ms at 0.5 μm calculated on the basis of Ca^{2+} diffusion from a discrete source (see Stern, 1992; Roberts, 1994). The linear correlation between rate of termination and free $d[Ca^{2+}]_i/dt$, measuring flux into unit cytosolic volume, can therefore be explained by accumulation of free [Ca^{2+}] close to the release sites and a first order inactivation by Ca^{2+} binding to channels. Open channels have a high and invariant [Ca^{2+}] within the channel because of high constant flux density in the pore, and fast inactivation by pore [Ca^{2+}] has been reported to determine the channel open lifetime of 2–3 ms (Bezprozvanny and Ehrlich, 1994). The whole cell flux $d[Ca^{2+}]_i/dt$ measured here depends on the open probability of $InsP_3$ channels and operates on a slower timescale of 10–1,000 ms. Peak open probability of $InsP_3$-gated channels is low (<0.15; Bezprozvanny et al., 1991) so a large proportion of channels are closed even with high $InsP_3$ concentrations. Because open channels have a high, constant rate of inhibition due to high pore [Ca^{2+}], the dependence of termination rate on whole cell flux indicates an inhibition of closed channels by Ca^{2+} accumulating in the cytosol within a few hundred nm adjacent to open channels, an effect that would increase at high density of open channels (observed here as high $d[Ca^{2+}]_i/dt$). This is supported by the observations discussed in connection with Fig. 6, that a second pulse of $InsP_3$ is ineffective even when applied during the period of high flux when open probability is high but submaximal, indicating that the process terminating flux acts on the closed channels. The first order dependence on $[Ca^{2+}]_i$ implied in the linear correlation between rate of termination and flux in both hepatocytes and Purkinje neurons differs from the conclusions of Oancea and Meyer (1996) from Ca^{2+} injection experiments, who found a relation suggesting 3–4 Ca^{2+} ions bind to produce inhibition of $InsP_3$-evoked Ca^{2+} transients in RBL cells.

Alternatively, the correlation between duration and size of flux could also be explained on the basis of store depletion, the higher the flux the sooner store Ca^{2+} runs out. The observation that the peak $[Ca^{2+}]_i$ is increased by increasing the $InsP_3$ concentration released in each cell, showing that, at low $InsP_3$, stores are not depleted when the flux is terminated. Furthermore, the prolonged responses in high fura-2 concentration also argue against store depletion acting alone. However, the observation, e.g., in Fig. 3, that loading the store by increasing Ca^{2+} influx into the cytosol increases the peak $[Ca^{2+}]_i$ evoked by $InsP_3$ indicates that store loading can influence Ca^{2+} flux in hepatocytes. The strong inhibitory effect of raised $[Ca^{2+}]_i$ suggests that Ca^{2+} feedback is the primary mechanism, but store depletion or $InsP_3$-evoked desensitization of receptors (Hajnozcky and Thomas, 1994; Ilyin and Parker, 1994) may contribute to termination of the Ca^{2+} flux.

The conclusions from these experiments are (*a*) that the properties of the $InsP_3$ receptor can account for the time course of $[Ca^{2+}]_i$ spikes in guinea pig hepatocytes, the onset and rise determined by the activation and inactivation of $InsP_3$ receptor, and the minimum duration of the interspike period by the recovery of $InsP_3$ sensitivity described previously (Ogden et al., 1990), and (*b*) that the differences in kinetics of $InsP_3$-evoked Ca^{2+}-release between hepatocytes and Purkinje neurons can be explained by the differences in $InsP_3$ receptor density. The longer latencies at low concentration, slower rise of free [Ca^{2+}], and longer duration of the $InsP_3$-evoked flux into the cytosol are mostly explicable by the low density of $InsP_3$ receptors in liver, producing small flux and correspondingly long periods of Ca^{2+} release due to slow Ca^{2+}-inactivation of release channels.

These properties are consistent with prolonged, pulsatile Ca^{2+} signalling in liver cells which underlies hormonal regulation of processes such as glycogenolysis. The different receptor subtypes, mainly type 2 $InsP_3$ receptors with some type 1 in hepatocytes, and type 1 alone in Purkinje neurons (DeSmedt et al., 1994) would appear secondary to receptor density in determining the kinetics of Ca^{2+} release but may be important in determining other factors such as $InsP_3$ sensitivity or susceptibility to phosphorylation. More generally, the linear relation between Ca^{2+} flux into unit cytosolic volume and rate of termination of Ca^{2+} release, shown here for hepatocytes and Purkinje neurons over a wide range of Ca^{2+} flux, indicates that regulation of the $InsP_3$ receptor by cytosolic free $[Ca^{2+}]$ produces fast rising but brief pulses of $[Ca^{2+}]_i$ at high $InsP_3$ receptor densities.

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REFERENCES

Baylor, S.M., and Hollingworth S. 1988. Fura-2 calcium transients in frog skeletal muscle fibres. *J. Physiol. (Lond.).* 403:151–192.

Berridge, M.J. 1988. Inositol trisphosphate induced membrane potential oscillations in *Xenopus* oocytes. *J. Physiol. (Lond.).* 403:589–599.

Bezprozvanny, I., and B.E. Ehrlich. 1994. Inositol (1,4,5)-trisphosphate ($InsP_3$)-gated Ca channels from cerebellum: conduction properties for divalent cations and regulation by intraluminal calcium. *J. Gen. Physiol.* 104:821–856.

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

Burgess, G.M., R.F. Irvine, M.J. Berridge, J.S. McKinney, and J.W. Putney. 1984. Actions of inositol phosphates on Ca^{2+} pools in guinea-pig hepatocytes. *Biochem. J.* 224:741–746.

Capiod, T., A.C. Field, D.C. Ogden, and C.A. Sandford. 1987. Internal perfusion of guinea-pig hepatocytes with buffered Ca^{2+} or inositol 1,4,5-trisphosphate mimics noradrenaline activation of K^+ and Cl^- conductances. *FEBS Lett.* 217:247–252.

Capiod, T., and D.C. Ogden. 1989*a*. The properties of calcium-activated potassium ion channels in guinea-pig isolated hepatocytes. *J. Physiol. (Lond.).* 409:285–295.

Capiod, T., and D.C. Ogden. 1989*b*. Properties of membrane ion conductances evoked by hormonal stimulation of guinea-pig and rabbit isolated hepatocytes. *Proc. R. Soc. B.* 236:187–201.

Carter, T.D., and D.C. Ogden. 1992. Kinetics of intracellular calcium release by inositol 1,4,5-trisphosphate and extracellular ATP in porcine cultured aortic endothelial cells. *Proc. R. Soc. B.* 250:235–241.

Champeil, P., L. Combettes, B. Berthon, E. Doucet, S. Orlowski, and M. Claret. 1989. Fast kinetics of calcium release induced by myo-inositol trisphosphate in permeabilized rat hepatocytes. *J. Biol. Chem.* 264:17665–17673.

Cobbold, P.H., A. Sanchez-Bueno, and C.J. Dixon. 1991. The hepatocyte calcium oscillator. *Cell Calcium.* 12:87–95.

Combettes, L., B. Berthon, E. Doucet, S. Erlinger, and M. Claret. 1989. Characteristics of bile acid mediated calcium release from permeabilized liver cells and live microsomes. *J. Biol. Chem.* 264: 157–167.

Combettes, L., M. Claret, and P. Champeil. 1993. Calcium control on $InsP_3$-induced discharge of calcium from permeabilised hepatocyte pools. *Cell Calcium.* 14:279–292.

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

DeSmedt, H., L. Missiaen, J.B. Parys, M.D. Bootman, L. Mertens, L. VanDenBosch, and R. Casteels. 1994. Determination of relative amounts of inositol trisphosphate receptor mRNA isoforms by ratio polymerase chain reaction. *J. Biol. Chem.* 269:21691–21698.

Field, A.C., and D.H. Jenkinson. 1987. The effect of noradrenaline on the ion permeability of isolated mammalian hepatocytes, studied by intracellular recording. *J. Physiol. (Lond.).* 392:493–512.

Fierro, L., and I. Llano. 1996. High endogenous calcium buffering in Purkinje cells from rat cerebellar slices. *J. Physiol. (Lond.).* 496: 617–625.

Finch, E.A., T.J. Turner, and S.M. Goldin. 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate induced calcium release. *Science (Wash. DC).* 252:443–446.

Hajnoczky, G., and A.P. Thomas. 1994. The inositol trisphosphate calcium channel is inactivated by inositol trisphosphate. *Nature (Lond.).* 370:474–477.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflüg. Arch.* 391:85–100.

Hirose, K., and M. Iino. 1994. Heterogeneity of channel density in inositol 1,4,5-trisphosphate-sensitive Ca^{2+}stores. *Nature (Lond.).* 372:791–794

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

Iino, M., and M. Endo. 1992. Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. *Nature (Lond.).* 360:76–78.

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

Khodakhah, K., and D.C. Ogden. 1993. Functional heterogeneity of calcium release by inositol 1,4,5-trisphosphate in single Purkinje neurons, cultured cerebellar astrocytes and peripheral tissues. *Proc. Natl. Acad. Sci. USA.* 90: 4976–4980.

Khodakhah, K., and D.C. Ogden. 1995. Fast activation and inactiva-

tion of inositol trisphosphate evoked calcium release in rat cerebellar Purkinje neurons. *J. Physiol. (Lond.).* 487:343–358.

Konishi, M., S. Hollingworth, A.B. Hawkins, and S.M. Baylor. 1991. Myoplasmic Ca transients in intact frog skeletal muscle fibres monitored with the fluorescent indicator furaptra. *J. Gen. Physiol.* 97:271–302.

Marchant, J.S., and C.W. Taylor. 1996. Delayed opening of inositol trisphosphate receptors in permeabilized rat hepatocytes. *J. Physiol. (Lond.).* 495:85P (Abstr.).

Marshall, I.C.B., and C.W. Taylor. 1993. Biphasic effects of cytosolic Ca^{2+} on Ins(1,4,5)P_3-stimulated Ca^{2+}-mobilization in hepatocytes. *J. Biol. Chem.* 268:13214–13220.

Marty, A., R. Horn, Y.P. Tan, and J. Zimmerberg. 1989. Delay of the Ca mobilization response to muscarinic stimulation. *In* Secretion and Its Control. G. Oxford and C.M. Armstrong, editors. The Rockefeller University Press, New York. 97–110.

Meyer, T., D. Holowka, and L. Stryer. 1988. Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science (Wash. DC).* 240:653–656.

Meyer, T., T. Wensel, and L. Stryer. 1990. Kinetics of calcium channel opening by inositol 1,4,5-trisphosphate. *Biochemistry.* 29:32–37.

Neher, E. 1995. The use of Fura-2 for estimating Ca buffers and Ca fluxes. *Neuropharmacology.* 34:1423–1442.

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

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

Ogden, D.C., K. Khodakhah, T.D. Carter, M. Thomas, and T. Capiod. 1995. Mechanisms of intracellular Ca release during hormone and neurotransmitter action investigated with flash photolysis. *Pflüg. Arch.* 429:587–591.

Ogden, D.C., K. Khodakhah, P.T.A. Gray, T.D. Carter, and T. Capiod. 1993. Mechanisms of intracellular Ca release during hormone and neurotransmitter action investigated with flash photolysis. *J. Exp. Biol.* 184:105–127.

Parker, I., and I. Ivorra. 1990. Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+}. *Proc. Natl. Acad. Sci. USA.* 87:260–264.

Parker, I., Y. Yao, and V. Ilyin. 1996. Fast kinetics of calcium liberation induced in *Xenopus* oocytes by photoreleased inositol trisphosphate. *Biophys. J.* 70:222–237.

Payne, R., B. Walz, S. Levy, and A. Fein. 1988. The localization of Ca release in Limulus photoreceptors and its control by negative feedback. *Phil. Trans. R. Soc. B.* 320:359–370.

Pietri, F., M. Hilly, and J.P. Mauger. 1990. Calcium mediates the interconversion of two states of the liver inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 265:17478–17485.

Rapp, G., and K. Guth. 1988. A low cost high intensity flash device for photolysis experiments. *Pflüg. Arch.* 411:200–203.

Raju, B., E. Murphy, L.A. Levy, R.D. Hall, and R.E. London. 1989. A fluorescent indicator for measuring cytosolic free magnesium. *Am. J. Physiol.* 256:C540–C548.

Roberts, W.M. 1994. Localisation of calcium signals by a mobile calcium buffer in frog saccular hair cells. *J. Neurosci.* 14:3246–3262.

Rooney, T.A., and A.P. Thomas. 1991. Organization of intracellular calcium signals generated by inositol lipid-dependent hormones. *Pharmacol. Ther.* 49:223–237.

Somlyo, A.V., K. Horiuti, D.R. Trentham, T. Kitazawa, and A.P. Somlyo. 1992. Kinetics of $InsP_3$-induced Ca^{2+} release and contraction induced by photolysis of caged D-*myo* inositol 1,4,5-trisphosphate in smooth muscle- the effects of heparin, procaine and adenine nucleotides. *J. Biol. Chem.* 267:22316–22322

Stern, M.D. 1992. Buffering of calcium in the vicinity of a channel pore. *Cell Calcium.* 13:183–192.

Walker, J.W., J. Feeney, and D.R. Trentham. 1989. Photolabile precursors of inositol phosphates. Preparation and properties of 1-(2-nitrophenyl)ethyl esters of myo-inositol 1,4,5-trisphosphate. *Biochemistry.* 28:3272–3280.

Walker, J.W., A.V. Somlyo, Y.E. Goldman, A.P. Somlyo, and D.R. Trentham. 1987. Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5-trisphosphate. *Nature (Lond.).* 327:249–252.

Woods, N.M., K.S.R. Cuthbertson, and P.H. Cobbold. 1987. Agonist induced oscillations in cytoplasmic free calcium ion concentration. *Cell Calcium.* 8:79–100.

Wootton, J.F., J.E.T. Corrie, T. Capiod, J. Feeney, D.R. Trentham, and D.C. Ogden. 1995. Kinetics of cytosolic Ca^{2+} concentration following photolytic release of 1D-myo-inositol 1,4-bisphosphate 5-phosphorothioate (5-thio-$InsP_3$) from a caged derivative in guinea pig hepatocytes. *Biophys. J.* 68:2601–2607.

Zhao, M., S. Hollingworth, and S.M. Baylor. 1996. Properties of tri- and tetracarboxylate Ca^{2+} indicators in frog skeletal muscle fibers. *Biophys. J.* 70:896–916.

Zhou, Z., and E. Neher. 1993. Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. *J. Physiol. (Lond.).* 469: 245–273.