Rapid Activation and Partial Inactivation of Inositol Trisphosphate Receptors by Inositol Trisphosphate †

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Rapid Activation and Partial Inactivation of Inositol Trisphosphate Receptors by Inositol Trisphosphate†

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ABSTRACT: During superfusion of permeabilized hepatocytes, submaximal concentrations of inositol 1,4,5-trisphosphate (InsP_3) evoked quantal Ca^{2+} mobilization: a rapid acceleration in the rate of $^{45}Ca^{2+}$ release abruptly followed by a biphasic decline to the basal rate before the InsP_3-sensitive stores had fully emptied. During the fast component of the decay, the Ca^{2+} permeability of the stores fell rapidly by 40% ($t_{1/2}$ = 250 ms) to a state indistinguishable from that evoked by preincubation with InsP_3 under conditions that prevented Ca^{2+} mobilization. This change was accompanied by a decrease in the InsP_3 dissociation rate: the response declined more quickly when InsP_3 was removed during the initial stages of a response than later. We suggest that InsP_3 directly causes its receptor to rapidly switch ($t_{1/2}$ = 250 ms) between a low-affinity ($K_d \sim 1$ μM) active, and a higher-affinity ($K_d \sim 100$ nM) less active, conformation, and that this transition underlies the fast component of the decaying phase of Ca^{2+} release. Ca^{2+} continues to leak through the unchanging less active state of the receptor until those stores that responded initially are completely empty, accounting for the slow phase of the response. The requirements for activation of InsP_3 receptors are more stringent (InsP_3 and then Ca^{2+} binding) than those for partial inactivation (InsP_3 binding); rapid inactivation is therefore likely to determine whether the cytosolic [Ca^{2+}] reaches the threshold for regenerative Ca^{2+} signals.

Inositol 1,4,5-trisphosphate (InsP_3)[1] receptors are large conductance Ca^{2+} channels that release Ca^{2+} from intracellular stores in response to activation of the many plasma membrane receptors that stimulate formation of InsP_3 (*1*, *2*). The complex changes in cytosolic [Ca^{2+}] that follow (*3*) have focused attention on the characteristics of InsP_3 receptors that might both account for elementary Ca^{2+} release events and contribute to the cycles of Ca^{2+} release and reuptake responsible for Ca^{2+} spikes and Ca^{2+} waves (*3*, *4*). Several important properties of InsP_3-evoked Ca^{2+} mobilization are particularly relevant in attempting to understand the mechanisms underlying the spatio-temporal organization of intracellular Ca^{2+} signals. First, most (*5*), though perhaps not all (*6*, *7*), InsP_3 receptors are biphasically regulated by increases in cytosolic [Ca^{2+}]: a rapid sensitization of InsP_3-evoked Ca^{2+} release is followed by a slower inhibition (*5*, *8*, *9*). The molecular mechanisms underlying the effects of cytosolic Ca^{2+} on InsP_3 receptors are not yet resolved and probably differ between receptor isoforms (*10*−*12*). Second, during prolonged exposure to InsP_3 the rate of Ca^{2+} mobilization decreases, consistent with a form of receptor desensitization (*8*, *13*) that may result directly from binding of InsP_3 (*14*, *15*) or as a consequence of the increases in cytosolic [Ca^{2+}] (*16*) or decreases in luminal [Ca^{2+}] (*17*, *18*) that follow channel opening. Third, submaximal concentrations of InsP_3 rapidly release only a fraction of the InsP_3-sensitive Ca^{2+} stores (*19*, *20*) without preventing responses to subsequent challenges with higher concentrations of InsP_3 (*21*, *22*). Such "quantal" (*19*, *23*) responses may allow InsP_3 receptors to mediate rapid graded Ca^{2+} release from finite intracellular stores (*24*). The mechanisms underlying these quantal responses are unknown, but they have been proposed to reflect all-or-nothing emptying of stores that differ in their sensitivities to InsP_3 (*19*, *22*, *25*) or various forms of receptor desensitization (*14*−*16*). The relationships between these three important properties of InsP_3 receptors—Ca^{2+} regulation, desensitization, and quantal Ca^{2+} mobilization—are unclear, although each could contribute to the complex physiological responses to InsP_3.

To address these issues, we have used methods that allow rates of unidirectional $^{45}Ca^{2+}$ efflux from permeabilized rat hepatocytes to be measured during rapid and repeated changes of superfusion medium, the composition of which can be precisely controlled. The results obtained with this rapid superfusion approach (*8*, *9*, *26*, *27*) are complementary to those obtained by confocal imaging of intact cells. The latter allows the spatial organization of local Ca^{2+} release events mediated by small numbers of InsP_3 receptors to be resolved with high temporal resolution (*3*, *4*, *28*), but it is limited in its ability to reversibly manipulate cytosolic Ca^{2+} and InsP_3 concentrations. Rapid superfusion overcomes these limitations and by stringently controlling the [Ca^{2+}]

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[1] Abbreviations: CLM, cytosol-like medium; CKF, Ca^{2+}- and K^+-free CLM; EC_{50}, half-maximally effective concentration; InsP_3, inositol 1,4,5-trisphosphate; $t_{1/2}$, half-time; t_{slow} and t_{fast}, half-times of slow and fast components, respectively.

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eliminates regenerative behavior. The responses therefore arise from individual Ca^{2+} release events (*28*), but because they are the average of many such responses from many cells, the heterogeneous behavior of individual events can no longer be resolved. In the present study, we focus on defining the kinetic properties of InsP_3 receptors during the decaying phase of the response in order to understand the relationship between the initial activation of InsP_3 receptors (*26*) and their subsequent behavior.

EXPERIMENTAL PROCEDURES

Materials. Radiochemicals were from NEN DuPont (Stevenage, U.K.). InsP_3 was from American Radiolabeled Chemicals (St. Louis, MO), and 2,3-dideoxy InsP_3 and 3-deoxy-InsP_3 were from Calbiochem (Nottingham, U.K.). All other reagents were from suppliers reported previously (*26*, *27*).

Rapid Superfusion Apparatus. A superfusion apparatus based on a design originally developed by Turner et al. (*29*) was used to effect rapid changes of the medium bathing permeabilized rat hepatocytes immobilized within a sandwich of filters (Schleicher and Schuell, AE99, GF51, GF52) (*26*, *27*). The immobilized cells were mounted in a chamber linked by solenoid valves to pressurized stainless steel vessels containing superfusion media, and the entire apparatus was enclosed in a chamber maintained at 20 °C. The sequence and duration of the superfusion periods were controlled from a Viglen III/LS computer fitted with an Amplicon PC36AT digital input/output board linked to an 8-channel valve driver (General Valves, 9031100) which gated the solenoid valves within 2 ms of activation. Each gating event was recorded as an input signal from the valve driver. Initiation of each superfusion protocol was synchronized with the position of the fraction collector by means of a reference signal from the turntable. Inclusion of ^{3}H-inulin in appropriate media allowed the timing of medium changes to be precisely related to changes in $^{45}Ca^{2+}$ efflux. The half-time ($t_{1/2}$) for solution exchange was 46 ± 6 ms (n = 10) at 20 psi and 30 ± 3 ms (n = 6) at 30 psi.

The flow of medium from the superfusion chamber was collected into 100 samples during a single rotation of a turntable, the circumference of which was fitted with 100 tightly juxtaposed cuvettes. To maximize both temporal resolution and the opportunity to record over protracted time courses, we designed a turntable capable of very rapidly switching between speeds. The turntable was driven with a stepper motor (Microdrives, HY200 3424 310 A8) and integral gear box controlled from a programmable 5A ministep driver (Digiplan, PDX15). By minimizing turntable torque, the turntable speed could be rapidly stepped (±999 rev/s^2) to any velocity between 67 and 0.2 rpm giving collection bins of between 9 and 3000 ms. Rotation speeds were verified electronically and with an optical tachometer. Closure of a reed switch by a magnet mounted on the turntable allowed the superfusion and rotation protocols to be synchronized. The fluid collected into each cuvette measures the amount of $^{45}Ca^{2+}$ released during a defined collection interval (80 ms for most experiments reported herein) and is therefore a direct measurement of the average rate of unidirectional Ca^{2+} release during that interval.

$^{45}Ca^{2+}$ Efflux from Permeabilized Hepatocytes. Isolated hepatocytes were prepared by collagenase digestion of the livers of male Wistar rats (200−300 g) as previously described (*26*, *27*) and stored at 4 °C in Eagle's minimal essential medium buffered with $NaHCO_3$ (26 mM) for up to 24 h. Hepatocytes (2×10^6 cells/mL) were permeabilized by incubation with saponin (10 μg/mL) in a Ca^{2+}-free cytosol-like medium (CLM) at 37 °C (140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM piperazine-*N*,*N*′-bisethanesulfonic acid (Pipes), pH 7.0). After permeabilization, the cells were washed and resuspended (10^7 cells/mL) in CLM supplemented with $CaCl_2$ (300 μM, free [Ca^{2+}] = 200 nM). The free [Ca^{2+}] values of the superfusion media were determined as previously described (*26*, *27*). The intracellular Ca^{2+} stores were loaded with $^{45}Ca^{2+}$ by incubating the cells (5 min, 37 °C) with $^{45}CaCl_2$ (15 μCi/mL), ATP (7.5 mM), phosphocreatine (15 mM), creatine kinase (15 units/mL) and carbonyl cyanide *p*-trifluormethoxyphenylhydrazone (FCCP, 10 μM). They were then immobilized by filtration onto a prerinsed filter assembly and rapidly secured into the superfusion chamber. The superfusion fluid flow rate was adjusted to 2 mL/s during two prewashes (5 s), and the superfusion protocol was initiated. In the interval between transferring the cells to the filter and initiating the experimental protocol, the Ca^{2+} contents of the intracellular stores declined by no more than 8% from their steady-state level of 1−2 nmol/10^6 cells.

At the end of each experiment, the cells were superfused with medium containing Triton X-100 (0.05%) to release all $^{45}Ca^{2+}$ remaining within the intracellular stores. Similar results, though with a slower time course, were obtained with ionomycin (10 μM). The radioactivity (^{3}H and $^{45}Ca^{2+}$) of each sample was determined by liquid scintillation counting in EcoScint A scintillation cocktail.

Analysis of Results. To allow comparison with previous publications (*8*, *9*), the amount of $^{45}Ca^{2+}$ detected in each sample was initially expressed as a fraction of the total $^{45}Ca^{2+}$ content of the intracellular stores. In subsequent analyses (Figure 3), responses were expressed as fractional release rates. These were computed by first summing the total amount of Ca^{2+} released over a protracted time course by that concentration of InsP_3, and then expressing the $^{45}Ca^{2+}$ released into each time bin as a fraction of the $^{45}Ca^{2+}$ remaining to be released by that concentration of InsP_3. The justification for, and assumptions underlying, this form of analysis are discussed below. Concentration−response relationships were fitted to four-parameter logistic equations using a nonlinear curve-fitting program (*26*, *30*). Computer-assisted curve-fitting with >200 iterations using both Kaleidagraph and Microsoft Excel was used to fit exponential equations. The statistical significance of mono- and multi-exponential fits was assessed according to the "extra sum of squares" principle (*31*); $p < 0.05$ was considered significant. All results are presented as the mean ± sem.

RESULTS AND DISCUSSION

Quantal Ca^{2+} Mobilization by InsP_3 and its Analogues. Figure 1a illustrates the kinetics of $^{45}Ca^{2+}$ release from the intracellular stores of permeabilized rat hepatocytes in response to maximal (10 μM) and submaximal (400 nM) concentrations of InsP_3. Each concentration of InsP_3 evoked an acceleration in the rate of $^{45}Ca^{2+}$ release to a peak, which was abruptly followed by a protracted decline in the rate of

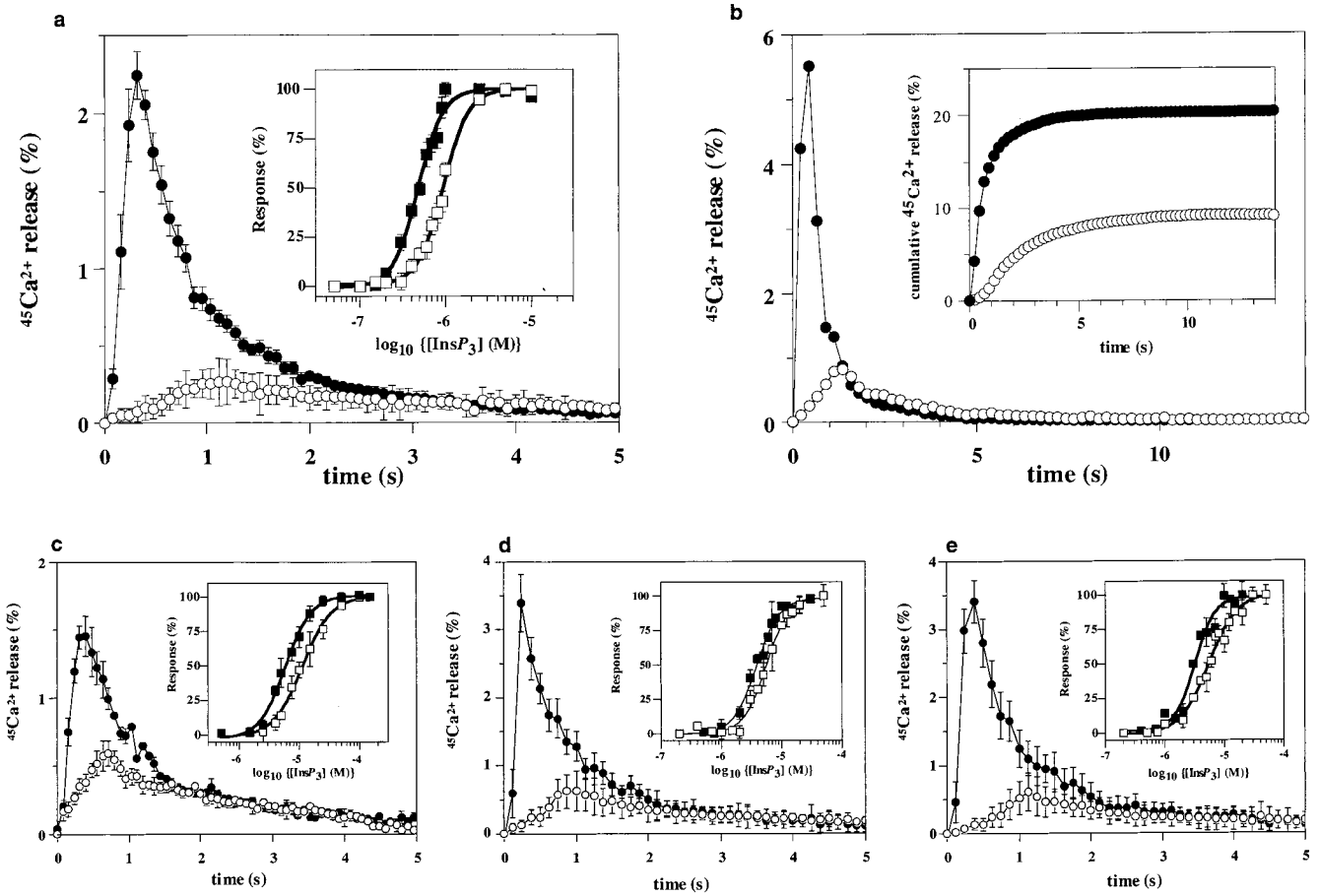

FIGURE 1: Kinetics of $^{45}Ca^{2+}$ mobilization by InsP_3 and its analogues. Permeabilized cells were superfused for 5 s with CLM containing the following: a, InsP_3 (10 μM, ●; 400 nM, ○); c, Ins(2,4,5)P_3 (100 μM, ●; 8 μM, ○); d, 3-deoxy-InsP_3 (50 μM, ●; 3 μM, ○); e, 2,3-dideoxy-InsP_3 (50 μM, ●; 2.5 μM, ○). The $^{45}Ca^{2+}$ released was collected into time bins of either 80 ms (a,c) or 125 ms (d,e), and the rates of $^{45}Ca^{2+}$ release are represented as percentages of the total initial $^{45}Ca^{2+}$ content of the intracellular stores. The insets show the concentration-dependent effects of each analogue on the peak rate (□) and extent (■) of $^{45}Ca^{2+}$ release, each of which are expressed as percentages of the maximal response evoked by that analogue. Results are the means ± sem of ≥3 independent experiments. The responses to maximal (5 μM, ●) and submaximal (350 nM, ○) concentrations of InsP_3 are shown over a more protracted time course (b) with samples collected at intervals of 225 ms. The inset shows the cumulative extent of the Ca^{2+} release evoked by each concentration of InsP_3.

$^{45}Ca^{2+}$ release. With 400 nM InsP_3, the rate of $^{45}Ca^{2+}$ release accelerated more slowly, the peak rate of $^{45}Ca^{2+}$ release was lower, and the rate of $^{45}Ca^{2+}$ mobilization returned to its unstimulated level before all the Ca^{2+} stores had completely emptied (Figures 1a,b). The protracted time course shown in Figure 1b more clearly illustrates how the $^{45}Ca^{2+}$ release evoked by a submaximal concentration of InsP_3 (350 nM) terminates without having fully emptied the InsP_3-sensitive stores. Therefore both the peak rate and the eventual extent of $^{45}Ca^{2+}$ release increased with InsP_3 concentration (Figure 1a, inset). The inability of submaximal concentrations of InsP_3 to completely empty the InsP_3-sensitive Ca^{2+} stores, quantal Ca^{2+} mobilization (*19*), has been observed in many cell types (*20*, *23*).

Other active analogues of InsP_3, Ins(2,4,5)P_3, 3-deoxy-InsP_3, and 2,3-dideoxy-InsP_3, also evoked quantal $^{45}Ca^{2+}$ release, and as with InsP_3, the responses were both positively cooperative (Hill coefficients, $n_H \sim 2-3$) (Figures 1c−e; Table 1) and inhibited by heparin, a competitive antagonist of InsP_3 receptors (not shown). For all these agonists, the concentration required to evoke half the maximal response (EC_{50}) was ~2-fold higher for the peak rate of $^{45}Ca^{2+}$ release than for the extent of the $^{45}Ca^{2+}$ release (EC_{50}^{rate}/ EC_{50}^{extent} ~2) (Table 1).

Biphasic Kinetics of InsP_3-Evoked Ca^{2+} Mobilization. The kinetics of the declining phase of the response evoked by 10 μM InsP_3 (Figure 2a) were best described ($p < 0.05$) by a linear combination of two exponential components:

$$\text{Rate of } ^{45}Ca^{2+} \text{ release} = A_{fast}e^{-T(0.693/t_{fast})} + A_{slow}e^{-T(0.693/t_{slow})}$$

where T is the time after attainment of the peak rate of $^{45}Ca^{2+}$ release; t_{fast} and t_{slow} are the half-times ($t_{1/2}$) of the fast and slow components and describe the rate of decline in the rate of $^{45}Ca^{2+}$ release; and A_{fast} and A_{slow} are the amplitudes of the fast and slow components at the peak of the response and reflect the maximal rate of $^{45}Ca^{2+}$ release through each component during the declining phase.

Similarly, for all other concentrations of InsP_3 (≥400 nM), the decline in the rate of $^{45}Ca^{2+}$ release was best described by a biexponential equation. Although the slower rates of $^{45}Ca^{2+}$ release recorded during the decaying phase of the response to lower concentrations of InsP_3 (<400 nM) were adequately described by monoexponential functions, the traces were too noisy to be subjected to more complex curve-fitting analyses (*9*, *32*). Table 2 summarizes the characteristics of the biphasic declining phase of the responses to

Table 1: Effects of InsP_3 and Analogues on the Peak Rate and Extent of $^{45}Ca^{2+}$ Release[a]

	Ins(1,4,5)P_3[b]	Ins(2,4,5)P_3[b]	3-deoxy-InsP_3	2,3-dideoxy-InsP_3
rate				
EC_{50}	941 ± 21 nM	11.3 ± 2.3 μM	5.51 ± 0.26 μM	5.6 ± 0.44 μM
n_H	3.0 ± 0.3	2.1 ± 0.1	2.4 ± 0.2	1.8 ± 0.3
extent				
EC_{50}	477 ± 21 nM	6.0 ± 0.3 μM	3.2 ± 0.31 μM	3.75 ± 0.18 μM
n_H	3.0 ± 0.3	2.1 ± 0.1	2.6 ± 0.6	2.2 ± 0.2
rate/extent				
EC_{50}	2.0 ± 0.1	1.9 ± 0.4	1.7 ± 0.1	1.5 ± 0.1
n_H	1.0	1.0	1.1	1.2

[a] Experiments similar to those shown in Figure 1 were used to measure maximal rates of $^{45}Ca^{2+}$ release and, by integration of areas beneath the complete time courses, extents of $^{45}Ca^{2+}$ release. Results are the mean ± sem of 3 or more independent experiments. [b] Results with Ins(1,4,5)P_3 and Ins(2,4,5)P_3 are taken from ref 27.

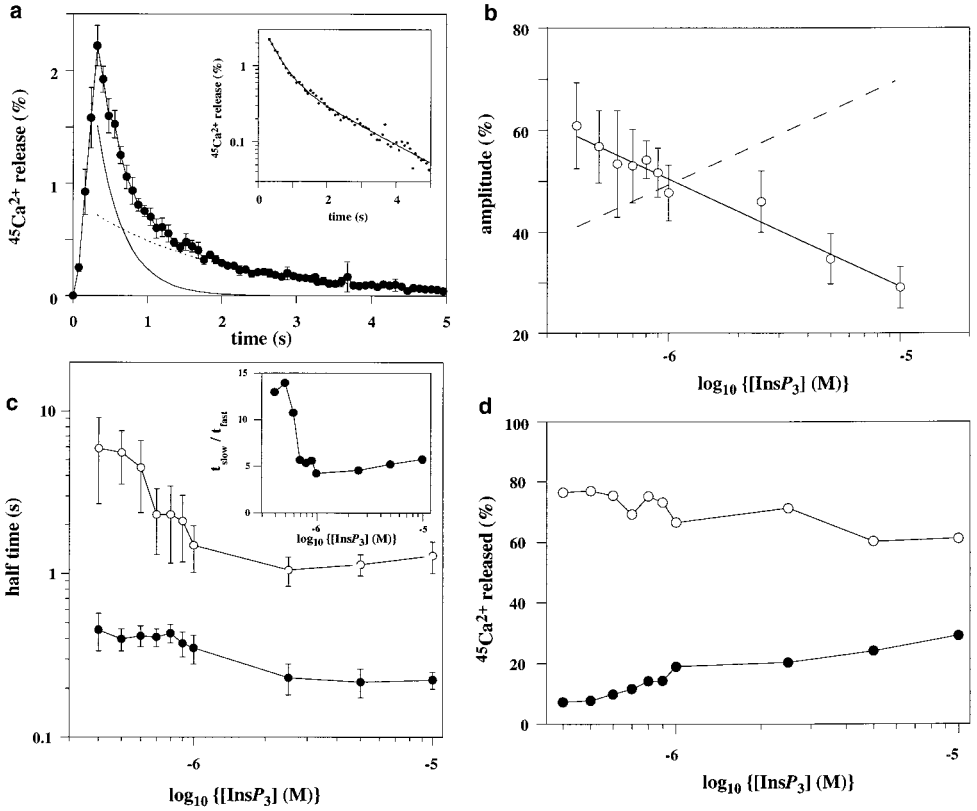

FIGURE 2: Biphasic decay in the rate of InsP_3-evoked $^{45}Ca^{2+}$ release. Cells were superfused for 5 s with CLM containing 10 μM InsP_3 and the rate of $^{45}Ca^{2+}$ release (●) was recorded during 80 ms intervals. (a) The kinetics of the decaying phase of the response are best fitted ($p < 0.05$) by the sum of a fast (solid curve) and a slow (dashed curve) exponential component. Results are the mean ± sem of 10 independent experiments. The inset shows the data plotted semilogarithmically to illustrate the biexponential nature of the decay in the rate of $^{45}Ca^{2+}$ release. (b) The relative contribution of the slow component (○) to the amplitude of the peak rate of $^{45}Ca^{2+}$ release is shown for various concentrations of InsP_3. The dashed line reports the contribution of the fast component. (c) The $t_{1/2}$ values of the fast (●) and slow (○) components of the decay in the rate of $^{45}Ca^{2+}$ release at each concentration of InsP_3 are shown (mean ± sem, $n \geq 4$). The inset shows the ratio of t_{fast}/t_{slow} at various concentrations of InsP_3. (d) The relative contribution of the fast (●) and slow (○) components to the cumulative extent of Ca^{2+} release was calculated by integration of the area beneath curve fits to the average profile of responses at each concentration of InsP_3 ($n \geq 4$ for each). Since the $^{45}Ca^{2+}$ mobilized before the maximal rate of $^{45}Ca^{2+}$ release was attained was not assigned to either component, the sums of the components are less than 100%.

maximal concentrations of InsP_3 and its analogues.

Both the relative initial amplitude of the slow component of the decaying phase ($A_{slow}/(A_{fast} + A_{slow})$) and the $t_{1/2}$ for the decay of each component (t_{fast} and t_{slow}) decreased as the concentration of InsP_3 increased (Figures 2b,c). The relative contribution of the slow phase at the peak of the response decreased from 71% ± 11% ($n = 7$) for 400 nM InsP_3 to 31% ± 5% ($n = 10$) for 10 μM InsP_3 (Figure 2b). At 10 μM InsP_3, t_{fast} and t_{slow} tended to lower limits of 244 ± 27 ms and 1498 ± 280 ms ($n = 10$), respectively (Figure 2c). There was an ~2-fold difference in the sensitivity of t_{fast} and t_{slow} to InsP_3, with the half-maximal effect (EC_{50}) occurring when the InsP_3 concentration was 1.1 μM for t_{fast} and 600 nM for t_{slow} (Figure 2c), consistent with the slow phase involving a higher affinity conformation of the receptor (see below). Because the fast and slow phases differ in their sensitivity to InsP_3 (Figure 2c, inset), the responses evoked by submaximal concentrations of InsP_3 are not simply smaller scale versions of those evoked by maximal concentrations (*33*, *34*). Even though the relative contribution of the fast phase of Ca^{2+} release becomes more significant at higher concentrations of InsP_3, the long duration of the slow

Table 2: Kinetics of the Decaying Phase in the Rate of $^{45}Ca^{2+}$ Release Evoked by InsP_3 and Its Analogues[a]

	Ins(1,4,5)P_3	Ins(2,4,5)P_3	3-deoxy-InsP_3	2,3-dideoxy-InsP_3
t_{fast}	244 ± 27 ms	378 ± 42 ms	252 ± 23 ms	248 ± 61 ms
t_{slow}	1498 ± 280 ms	2667 ± 472 ms	2036 ± 558 ms	1910 ± 524 ms
A_{fast}	69% ± 5%	70% ± 4%	60% ± 5%	72% ± 7%

[a] From the results of experiments similar to those shown in Figure 2a, the decaying phase of the response to a maximally effective concentration of Ins(1,4,5)P_3 (10 μM), Ins(2,4,5)P_3 (150 μM), 3-deoxy-InsP_3 (80 μM), or 2,3-dideoxy-InsP_3 (80 μM) was fitted to the sum of two exponentials (see Experimental Procedures). The table shows the calculated $t_{1/2}$ values of the fast (t_{fast}) and slow (t_{slow}) components of the biexponential curve fit and the relative initial amplitude of the fast phase (A_{fast}). Results are the mean ± sem of 3 or more independent experiments.

phase ensures that even with a maximal concentration of InsP_3 it always contributed more than 60% of the total amount of $^{45}Ca^{2+}$ released (Figure 2d). A similar long-lasting decay in the rate of InsP_3-evoked $^{45}Ca^{2+}$ release toward the basal rate has been observed in other studies (*32*, *35*). The biexponential pattern of Ca^{2+} release, the ~10-fold difference in the $t_{1/2}$ of the two components, and the greater sensitivity of the slow phase to InsP_3 are consistent with previous studies of permeabilized cells (*13*, *21*), microsomes (*36*), and reconstituted InsP_3 receptors (*37*).

Mechanisms Underlying the Biphasic Decay in the Rate of Ca^{2+} Release. Previous measurements of the time course of the cumulative *extent* of InsP_3-evoked Ca^{2+} release were analyzed by fitting the observations to the sum of two exponentials (*9*, *13*, *21*, *36*−*38*). This form of analysis assumes the existence of two separate and independent processes and is therefore limited by its inability to account for transitions between receptor conformations (*39*). Furthermore, it is not trivial to resolve whether the biphasic pattern of $^{45}Ca^{2+}$ release results from changes in the kinetic properties of the InsP_3 receptor or from properties of the intracellular Ca^{2+} stores themselves. Heterogeneity in the size, Ca^{2+} content, or InsP_3 receptor density of the intracellular Ca^{2+} stores as well as diffusional considerations could all contribute to a multiphasic time course of Ca^{2+} release (*13*, *40*). While the two phases of InsP_3-evoked Ca^{2+} efflux have often been equated with two distinct states of the InsP_3 receptor, there is scant justification for this assumption without further evidence.

In our experiments, the biphasic kinetics of InsP_3-stimulated $^{45}Ca^{2+}$ efflux were unaffected by the monovalent cation ionophore, gramicidin D (500 nM), indicating that the behavior was not a consequence of changes in membrane potential arising from the electrogenic Ca^{2+} leak (*41*). InsP_3 metabolism did not contribute because the biphasic kinetics were observed during continuous superfusion with InsP_3 and in response to stimulation with metabolically stable analogues of InsP_3 (Table 2). The combination of EGTA in the CLM and the rapid flow of medium through the superfusion chamber (2 mL/s) minimized the likelihood of a role for feedback regulation of the InsP_3 receptor by an increase in free [Ca^{2+}] (*6*). Furthermore, our measurements of unidirectional $^{45}Ca^{2+}$ efflux excluded the possibility that resequestration of $^{45}Ca^{2+}$ contributed to the pattern of InsP_3-stimulated $^{45}Ca^{2+}$ release (*21*, *42*), and ATP was not involved (*13*) because when it was included (1 mM) in the superfusion media it had no effect on the kinetics of $^{45}Ca^{2+}$ release (*26*). The switch to the slow phase of Ca^{2+} release is not a consequence of the fast phase having exhausted the pool of free Ca^{2+} within the stores, because the relative amplitude of the slow phase, which would then reflect the kinetics of Ca^{2+} dissociation from luminal Ca^{2+} buffers, increases at low concentrations of InsP_3 when the initial rate of Ca^{2+} release is less (Figure 2c) (*9*, *13*). Finally, our results cannot be explained by proposing that stores differ solely in their density of InsP_3 receptors (*37*) because the rates of decay of the fast and slow phases are differentially affected by InsP_3 concentration (Figure 2c) (*34*).

We conclude that the two components of the decay in the rate of InsP_3-evoked Ca^{2+} release reflect different properties of the InsP_3 receptor rather than properties of the intracellular Ca^{2+} stores, consistent with the biphasic pattern of InsP_3-evoked Ca^{2+} release observed from immunoaffinity-purified type 1 InsP_3 receptors reconstituted into liposomes (*37*). Subsequently, we investigated whether these components result from distinct InsP_3 receptor populations or from a change in channel behavior following InsP_3 binding. In analyses of other ligand-gated ion channels, it has also proven difficult to resolve whether biphasically decaying currents result from different receptors or changes in receptor behavior following agonist binding (*39*, *43*, *44*).

Time-Dependent Decrease in InsP_3 Receptor Activity. We previously suggested that quantal Ca^{2+} release in permeabilized hepatocytes results from the all-or-nothing emptying of stores that differ in their sensitivities to InsP_3 (*22*, *25*, *30*). The observation that during the biphasic response to submaximal concentrations of InsP_3, the rate of $^{45}Ca^{2+}$ release during the slow phase invariably extrapolated to the basal rate despite the fact that the InsP_3-sensitive Ca^{2+} stores had not been fully depleted (Figure 1b) is entirely consistent with this interpretation. Therefore, to provide a more direct assessment of the properties of activated InsP_3 receptors, we have applied a different analysis which recognizes that intracellular Ca^{2+} stores respond in this all-or-nothing manner to InsP_3. In this analysis, the total amount of Ca^{2+} released by each concentration of InsP_3 was assumed to be the entire Ca^{2+} pool to which that InsP_3 concentration had access. This pool size was then used to compute fractional release rates during sustained stimulation with each concentration of InsP_3 (see Experimental Procedures). Whereas previous analyses expressed the rate of Ca^{2+} release either as a fraction of the total $^{45}Ca^{2+}$ store available at the start of the experiment (Figure 1) (*8*, *9*) or as a fractional release from the entire InsP_3-sensitive store (*45*), the revised quantal analysis acknowledges that each concentration of InsP_3 has access to a different fraction of the total store and that its content declines during the response to InsP_3. The advantage of this analysis is that it provides direct insight into the permeability of InsP_3 receptors: the fractional release rate will be constant unless there is a change in the behavior of the InsP_3 receptor.

The profile of the fractional $^{45}Ca^{2+}$ release rates (Figure 3) has three important implications. First, at maximal concentrations of InsP_3 (≥2.5 μM), the fractional rate of

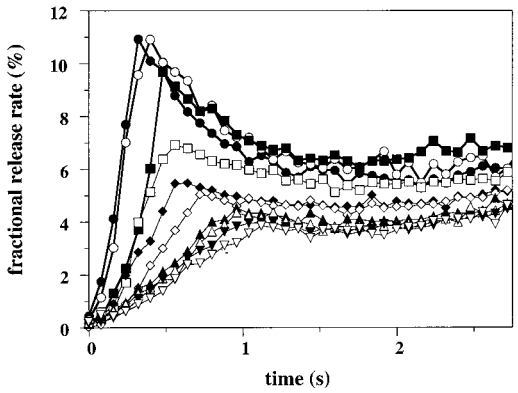

FIGURE 3: Effects of InsP_3 on the fractional rate of $^{45}Ca^{2+}$ release. Data derived from experiments similar to those shown in Figure 1 were used to establish the fractional release rate of $^{45}Ca^{2+}$ (see Experimental Procedures) during each 80 ms interval. The concentrations of InsP_3 were 10 μM (●), 5 μM (○), 2.5 μM (■), 1 μM (□), 900 nM (◆), 800 nM (◇), 700 nm (▲), 600 nM (△), 500 nM (▼), and 400 nM (▽). Each trace represents the average profile of between 4 and 23 independent experiments.

$^{45}Ca^{2+}$ release rose to a peak (~10%/80 ms) and then decayed monoexponentially toward a stable lower rate (~6%/80 ms), which was maintained for as long as the Ca^{2+} content of the stores remained high enough to allow meaningful analysis (Figure 3). Therefore, in the sustained presence of a maximal concentration of InsP_3, hepatic InsP_3 receptors inactivate to a state with ~40% lesser activity. At submaximal concentrations of InsP_3, the fractional rate of $^{45}Ca^{2+}$ release also stabilized after ~1 s. However, the abrupt transition in the rate of Ca^{2+} release observed at high concentrations of InsP_3 (Figure 1) was less apparent because at lower concentrations of InsP_3 the receptors activate over a more protracted time course (*26*) and individual receptors therefore complete the sequence of activation and inactivation reactions asynchronously.

Second, the $t_{1/2}$ for the decay in the fractional rate of $^{45}Ca^{2+}$ release at maximal concentrations of InsP_3 (249 ± 13 ms) was very similar to the lower limit of the $t_{1/2}$ (244 ± 27 ms) governing the transition from the fast to slow phase of $^{45}Ca^{2+}$ release (Figure 2c). The close agreement between these values, together with the observation that even a maximal concentration of InsP_3 (10 μM) releases only 9.6% of the InsP_3-sensitive stores before the rate of release declines, suggests that it is the transition between the active and less active conformations of the receptor, rather than depletion of the intracellular Ca^{2+} stores, that underlies the rapid phase of the decay (t_{fast}) in the rate of $^{45}Ca^{2+}$ release.

Third, since the fractional $^{45}Ca^{2+}$ release rate stabilized after 1 s, the slow component of the decay in the rate of $^{45}Ca^{2+}$ release (t_{slow}) does not reflect a further change in the behavior of the InsP_3 receptor, but rather reflects the monophasic loss of Ca^{2+} from the intracellular stores through a state of the InsP_3 receptor that undergoes no subsequent change in activity.

We conclude that after binding of InsP_3, the InsP_3 receptor initially opens to an active state that rapidly ($t_{1/2}$ ~ 250 ms) switches to a less active conformation which then mediates Ca^{2+} release for as long as Ca^{2+} remains within the responsive stores. The fast component of the decay in the rate of $^{45}Ca^{2+}$ release (t_{fast}) (Table 2) therefore appears largely to report the time course of the changing behavior of the InsP_3 receptor, whereas the slow component (t_{slow}) reflects depletion of the Ca^{2+} stores.

Similar results were obtained from analysis of the kinetics of $^{45}Ca^{2+}$ release evoked by 3-deoxy-InsP_3 and 2,3-dideoxy-InsP_3 (data not shown). However, with Ins(2,4,5)P_3, the transition between the active and less active conformations occurred more slowly ($t_{1/2}$ = 391 ms at 300 μM Ins(2,4,5)P_3). Our previous kinetic analysis revealed that Ins(2,4,5)P_3 was a partial agonist, with maximal concentrations initiating $^{45}Ca^{2+}$ release over the same time course as Ins(1,4,5)P_3, but to a peak rate that was only ~65% of that evoked by Ins(1,4,5)P_3 (*27*). Since the rate at which Ins(1,4,5)P_3 causes the receptor to switch from the active to the less active conformation is also ~65% faster (249 ms/391 ms) than that evoked by Ins(2,4,5)P_3, our results suggest that the lesser efficacy of Ins(2,4,5)P_3 is manifest equally through the slower kinetics of InsP_3 receptor activation and inactivation.

Role of InsP_3 in Receptor Inactivation. InsP_3-stimulated Ca^{2+} efflux from intracellular stores is electrically compensated by an inward movement of K^+. We and others (*46*−*48*) previously demonstrated that removal of monovalent counterions effectively prevented InsP_3-evoked Ca^{2+} efflux without preventing InsP_3 from binding to its receptor (*46*). We have adapted this protocol to the rapid superfusion apparatus to assess whether InsP_3 binding in the absence of both cytosolic Ca^{2+} and Ca^{2+} flux through the InsP_3 receptor affects the rate of $^{45}Ca^{2+}$ efflux when the essential counterions are subsequently restored. Permeabilized cells loaded with $^{45}Ca^{2+}$ in normal CLM were immobilized within the superfusion chamber and superfused (5 s) with Ca^{2+}-free and K^+-free medium (CKF: 160 mM *N*-methyl-D-glucamine, 14 mM EGTA, 20 mM Pipes, pH 7.0). The release of $^{45}Ca^{2+}$ was recorded during superfusion with CKF with or without 20 μM InsP_3, and then during the subsequent restoration of counterions together with 20 μM InsP_3. The results demonstrate that InsP_3 in the absence of K^+ had no direct effect on the rate of $^{45}Ca^{2+}$ release (Figure 4). However, a 3.85 s preincubation with InsP_3 in CKF reduced the peak rate of $^{45}Ca^{2+}$ release when K^+ was restored by 20% ± 1.4% (n = 3) without affecting the eventual extent of the Ca^{2+} release (95% ± 1.0% of control).

Analysis of these data as fractional release rates revealed that preincubation with InsP_3 caused the difference between the peak and sustained fractional Ca^{2+} release rates to be reduced by ~55%, consistent with InsP_3 having driven more than half the receptors to their less active conformations during the preincubation. However, preincubation with InsP_3 did not alter the $t_{1/2}$ of the transition of the remaining active receptors to the less active state (220 versus 240 ms, for control and pretreated) or the activity of the less active receptor state (~6.2%/80 ms versus 5.6%/80 ms, n = 3). Therefore, the population of InsP_3 receptors that remain in the most active state after pretreatment with InsP_3 subsequently inactivate to the less active state with the same kinetics as naive receptors. This observation suggests that a single inactivation mechanism is responsible for the fast component of the decaying response and that, under our experimental conditions, preincubation with InsP_3 alone, in the absence of both cytosolic Ca^{2+} and a flux of Ca^{2+} through the receptor, is sufficient to drive the InsP_3 receptor into the conformation that mediates the slow phase of Ca^{2+} release. It is, however, noteworthy that only ~55% of the receptors

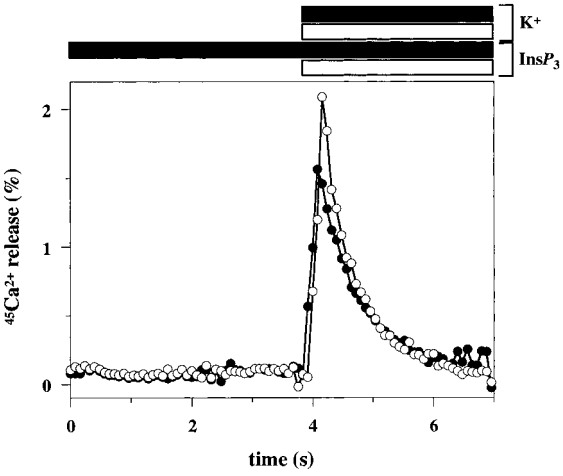

FIGURE 4: Effect of InsP_3 preincubation on the kinetics of $^{45}Ca^{2+}$ mobilization. Permeabilized cells were superfused for 5 s with Ca^{2+}- and K^+-free medium (CKF) and then (at $t = 0$) for 3.85 s in either CKF alone (○) or CKF supplemented with 20 μM InsP_3 (●). After 3.85 s, both K^+ (140 mM) and InsP_3 (20 μM) were included in the Ca^{2+}-free superfusion medium. Rates of $^{45}Ca^{2+}$ efflux, collected into 80 ms bins, are expressed as percentages of the $^{45}Ca^{2+}$ contents of the entire intracellular Ca^{2+} stores. The results are typical of 3 independent experiments. The kinetics of solution exchange were monitored using ^{3}H-inulin and are depicted by the bars (solid for ● and open for ○) denoting the intervals during which K^+ and InsP_3 were included in the superfusion media.

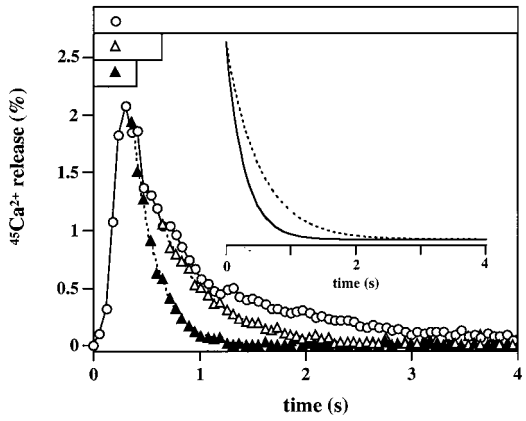

FIGURE 5: Kinetics of channel closure after removal of InsP_3. Cells were exposed to InsP_3 for the periods indicated, and the rate of decay in the rate of $^{45}Ca^{2+}$ release was measured in response to a 380 ms pulse (▲), a 640 ms pulse (△), and a 4 s challenge with 10 μM InsP_3 (○). For clarity, only the latter parts of the traces that employed short pulses of InsP_3 are shown. Results are typical of at least 4 independent experiments. The inset shows normalized monophasic curve fits to the kinetics of the decay in the rate of $^{45}Ca^{2+}$ release following the removal of InsP_3 after 380 ms (solid curve) and 640 ms (dashed curve).

were driven to their less active conformation during a preincubation (3.85 s) with InsP_3 (Figure 4) that was substantially longer than the $t_{1/2}$ (250 ms) for the transition under more physiological conditions (Figure 3). A possible explanation for this incomplete effect of preincubation with InsP_3 is discussed below.

By measuring the retrograde flux of Mn^{2+} into the lumen of the fura-2-loaded Ca^{2+} stores of hepatocytes, Hajnóczky and Thomas (*14*, *15*) concluded that InsP_3 binding caused InsP_3 receptors to slowly ($t_{1/2} = 15$ s) adopt a less conducting conformation, but only in the presence of cytosolic Ca^{2+}. Our results concur in demonstrating that InsP_3 binding causes partial inactivation of its receptor but differ in two respects. Under our conditions, this effect occurs much more rapidly ($t_{1/2} \sim 250$ ms, see also ref *9*) and furthermore can proceed in the absence of cytosolic Ca^{2+} (free $[Ca^{2+}] < 2$ nM). It is noteworthy that in both bilayer recordings from hepatic InsP_3 receptors (*49*) and patch clamp recordings from nuclei of *Xenopus* oocytes (*50*), channel activity declines, albeit over seconds, during prolonged exposure to InsP_3. Practical difficulties have so far prevented us from further examining the effects of cytosolic Ca^{2+} on InsP_3-evoked inactivation, but our observation that only about half of the receptor population was driven to the less active state during prolonged preincubation (3.85 s) with InsP_3 alone could be explained if removal of cytosolic Ca^{2+} slowed the rate of the InsP_3-driven inactivation process by ~15-fold. Previous observations (*14*) support this suggestion. From analyses of Mn^{2+}-evoked quench of fura-2 trapped within the endoplasmic reticulum, we previously concluded that prolonged incubation with InsP_3 did not cause receptor inactivation (*51*). However, with the temporal resolution of those experiments (~2 s), it is likely that we failed to detect the initial maximally active receptor conformation (half-life ~ 250 ms) and recorded the activity of only the less active conformation, which, in keeping with our present results (Figure 3), maintained this level of activity indefinitely.

These observations have important implications. First, the time course of InsP_3-evoked inactivation under physiological conditions (free $[Ca^{2+}] \sim 200$ nM) is sufficiently rapid ($t_{1/2} \sim 250$ ms) relative to that of Ca^{2+} mobilization (Figure 1) to significantly influence the pattern of Ca^{2+} release. Indeed, the subcellular Ca^{2+} release events thought to initiate global Ca^{2+} signals (3, 4) persist over time courses comparable to that of InsP_3-evoked inactivation. Second, the observation that InsP_3 alone in the absence of a Ca^{2+} flux through the channel can inactivate the receptor suggests, as we previously speculated (*26*), that InsP_3 binding initiates a race between receptor activation and inactivation. This competition between the stringent requirements for activation (InsP_3 and Ca^{2+} binding to the receptor) and the lesser requirements for inactivation (which may require only InsP_3 binding) may serve to reduce the likelihood of spontaneous initiation of regenerative Ca^{2+} signals (*26*).

The Less Active Receptor Conformation Has Higher Affinity for InsP_3. By rapidly ($t_{1/2} = 45 \pm 3$ ms) removing InsP_3 at different phases of the response, the superfusion apparatus allowed the rate of termination of $^{45}Ca^{2+}$ release, reflecting the rate of dissociation of InsP_3 from its receptor, to be measured for the first time. Figure 5 shows that the kinetics of the decay in the rate of $^{45}Ca^{2+}$ release were faster when InsP_3 (10 μM) was removed during the response, indicating that the slow phase of $^{45}Ca^{2+}$ release, and therefore the less active conformation of the InsP_3 receptor, was dependent on the maintained binding of InsP_3. More importantly, the rate of decay of the response was faster when InsP_3 (10 μM) was removed after 380 ms ($t_{1/2} = 195 \pm 20$ ms, $n = 10$) than when it was removed after 640 ms ($t_{1/2} = 360 \pm 35$ ms, $n = 4$). A similar pattern was observed during brief challenges with 1 μM InsP_3 (not shown). These results demonstrate that InsP_3 dissociates rapidly from the conformation of the InsP_3 receptor that mediates a large Ca^{2+} flux and more slowly from the conformation that mediates a lesser Ca^{2+} flux. Previous kinetics analyses have suggested that

the rate of InsP_3 dissociation from its receptor under physiological conditions occurs with a $t_{1/2}$ of between 125 and 300 ms (*52*, *53*).

Our evidence is consistent with the suggestion that following InsP_3 binding, the InsP_3 receptor switches from a low-affinity active conformation to a higher-affinity less active conformation. This interpretation is also consistent with the slow phase of Ca^{2+} release being more sensitive to InsP_3 than the fast phase (Figure 2c), and with the response to a submaximal concentration of InsP_3 (500 nM) becoming progressively less sensitive to inhibition by heparin as the duration of the exposure to InsP_3 is increased (not shown). The results of other studies are also consistent with our conclusion. InsP_3 binding in the absence of Ca^{2+} causes a time-dependent (albeit slow, $t_{1/2} \sim 20$ s) increase in the affinity of cerebellar InsP_3 receptors for InsP_3 (*54*). In hepatocytes, where two states of the receptor differing by 10−20-fold in their affinities for InsP_3 have been detected in Ca^{2+}-free medium, hormones that stimulate InsP_3 formation increase the fraction of receptors in the high-affinity state (*55*, *56*). Since increases in [Ca^{2+}] also increase the affinity of hepatic InsP_3 receptors (*15*, *56*, *57*), the effect of hormones has been attributed to their ability to increase the cytosolic [Ca^{2+}] (*56*), but it may also reflect a direct effect of InsP_3.

From the $t_{1/2}$ (∼250 ms) of the transition between the two receptor conformations (Figure 3), we can estimate that in the 260 ms interval separating the two measurements of channel closure after stimulation with 10 μM InsP_3, ∼52% of the receptor that was in its most active conformation at 380 ms will have transformed into the less active state by 640 ms. Our previous measurements indicate that at the peak of the response (380 ms for 10 μM InsP_3), the fast component accounted for 70% of the $^{45}Ca^{2+}$ release (Table 2) and that would therefore have decreased to 34% by 640 ms (Figure 5). By assuming that during our InsP_3 removal experiments, 70% of the response at 380 ms was mediated by the most active conformation of the receptor and 34% after 640 ms, we can estimate that InsP_3 dissociates ∼9-fold more rapidly from the most active receptor conformation. If the rate of association of InsP_3 with the two receptor conformations is similar, then the most active conformation would be expected to bind InsP_3 with ∼9-fold lower affinity than the less active conformation. This is again consistent with radioligand binding experiments suggesting that the two states of the hepatic InsP_3 receptor that are interconverted by hormones differ by ∼10−20-fold in their affinities for InsP_3 (*55*, *56*). Both our measurements of peak rates of $^{45}Ca^{2+}$ release (Figure 1a, Table 1) and previous measures of initial rates of Ca^{2+} release from permeabilized hepatocytes using fluorescent indicators (*13*) concur in concluding that the initial active conformation of the receptor is likely to have an affinity (K_d) for InsP_3 of ∼1 μM, suggesting that the less active state might have a K_d of ∼100 nM.

Quantal Ca^{2+} Release Is Not Caused by InsP_3 Receptor Inactivation. Although the biphasic kinetics of InsP_3-stimulated Ca^{2+} mobilization, regulation of the InsP_3 receptor by changes in cytosolic [Ca^{2+}], and the quantal pattern of Ca^{2+} release have often been assumed to be closely related aspects of the same phenomenon (*16*), there are many problems with this assumption. InsP_3 causes a biphasic leak of Mn^{2+} into the intracellular stores of hepatocytes in the absence of any change in cytosolic [Ca^{2+}] (*14*). Purified cerebellar InsP_3 receptors are not Ca^{2+}-sensitive (*37*, *58*), and yet they mediate both biphasic (*37*) and quantal responses to InsP_3 (*37*, *59*). Both quantal responses to InsP_3 in permeabilized hepatocytes and biphasic regulation of the hepatic InsP_3 receptor by cytosolic Ca^{2+} persist at 2 °C (*22*), while inactivation of the receptor after InsP_3 binding is prevented (*14*, *38*).

Our results confirm that quantal Ca^{2+} release is not mediated by Ca^{2+} regulation of InsP_3 receptors (Figure 1) (*22*, *37*). More importantly, they establish that the biexponential pattern of InsP_3-evoked Ca^{2+} efflux that we (Figure 2) and others (*13*, *21*, *32*, *36*, *37*) have observed from the intracellular stores of many cell types is not the reason submaximal concentrations of InsP_3 fail to fully empty the InsP_3-sensitive stores. During the biphasic response to submaximal concentrations of InsP_3, the rate of $^{45}Ca^{2+}$ release during the slow phase invariably extrapolated to the basal rate despite the fact that the InsP_3-sensitive Ca^{2+} stores had not been fully emptied (Figure 1b). While the high-affinity conformation of the InsP_3 receptor is less active than the initial conformation, it is nevertheless an *open* conformation and must therefore eventually allow all of the Ca^{2+} to leak from any store in which it is present. The activity of this state of the receptor is underscored by the fact that during sustained stimulation, more Ca^{2+} is released through it than through the short-lived more active conformation (Figure 2d). The less active state of the InsP_3 receptor will therefore mediate Ca^{2+} release for as long as InsP_3 remains bound (Figure 5) and Ca^{2+} remains within the stores: the amount of Ca^{2+} released will, of course, decrease monoexponentially as the Ca^{2+} content of the stores declines, the slow phase of the response (Figure 6). We conclude that while partial inactivation of the receptor triggered by InsP_3 binding is the cause of the abrupt slowing of the rate of Ca^{2+} release in our experiments (Figures 1 and 2) and presumably those of others (*9*, *14*, *21*, *36*, *37*), it cannot, as has been widely speculated (*13*, *14*, *23*), account for quantal Ca^{2+} release. Quantal responses must result from all-or-nothing emptying of stores that differ in their sensitivities to InsP_3 (Figure 6a) (*14*, *19*, *22*, *60*), although the structural basis of the heterogeneity and extreme cooperativity required for such behavior have not yet been fully defined.

Since InsP_3 receptors retain an ability to mediate Ca^{2+} release for as long as they have InsP_3 bound, albeit at slower rates at later times, activation of a single InsP_3 receptor should ultimately be capable of draining the entire store with which it is associated. The inexorable ($t_{1/2} \sim 250$ ms) switch of the receptor from its active to its less active conformation together with the size of the Ca^{2+} store to which it has access and the number of receptors within that store will determine whether the entire Ca^{2+} store is drained while the receptors are in their most active state or whether substantial Ca^{2+} will remain after this phase and be released through the less active conformation. These factors, therefore, determine the extent of the discrepancy between the sensitivity of the peak rate of Ca^{2+} release to InsP_3 (mediated almost entirely by the active conformation of the receptor) and the extent of the release (mediated by both receptor conformations). It is not surprising that with more than one receptor present in each store, low concentrations of InsP_3, which are likely to activate only few receptors, leave more Ca^{2+} trapped within

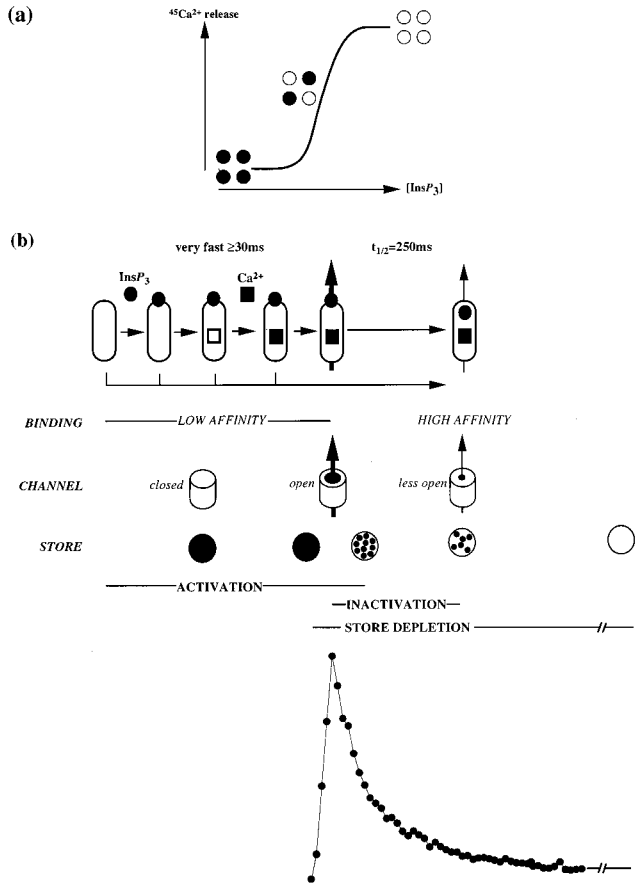

FIGURE 6: Changes in InsP_3 receptor behavior following InsP_3 binding. Changes in the Ca^{2+} contents of the stores and the underlying behavior of the InsP_3 receptors are illustrated. (a) As the InsP_3 concentration is increased, stores that differ in their sensitivities to InsP_3 respond by releasing all or none of their sequestered Ca^{2+}, at rates governed by the changing behavior of the InsP_3 receptor (b). (b) Activation. Binding of InsP_3 to its receptor unmasks a site to which Ca^{2+} can then bind, and when all four subunits of the receptor have bound both ligands, the intrinsic Ca^{2+} channel opens to its maximal activity (*26*). At normal cytosolic [Ca^{2+}] and in the presence of a maximal concentration of InsP_3, this sequence can be completed in ~30 ms; it takes longer for lower concentrations of InsP_3. Inactivation. Binding of InsP_3 also directly initiates a slower ($t_{1/2}$ = 250 ms) switch of the receptor to a higher-affinity, less active state through which the entire Ca^{2+} content of that store eventually drains. The race between activation, with its stringent need for sequential binding of InsP_3 and then Ca^{2+}, and the less demanding trigger for partial inactivation (InsP_3 binding alone) ensures that spontaneous openings of InsP_3 receptors are unlikely to cause the cytosolic [Ca^{2+}] to exceed the threshold for regenerative activity. Store depletion. Any Ca^{2+} remaining within a store after the fast phase of Ca^{2+} release will leak from it through the partially inactivated conformation of the receptor and drain that store completely.

the store at the end of the first phase of the response and therefore release proportionately more Ca^{2+} during the slow phase (Figure 2d). In our experiments, there was invariably a ~2-fold discrepancy between the sensitivities of the peak rate and the extent of the Ca^{2+} release evoked by each agonist (Table 1). By contrast, in cerebellum, where InsP_3 receptors are expressed at very much higher density, the concentration dependence of the peak rates and extents of Ca^{2+} release are similar (8). The relationship between the sensitivities of the rate and extent of InsP_3-evoked Ca^{2+} release (Table 1) is not, therefore, a fundamental property, but an emergent effect of the Ca^{2+} content and InsP_3 receptor density of the stores as well as the time-dependent changes in InsP_3 receptor behavior. Within any specific preparation, of course, the former properties would be constant and the sensitivity ratio ($EC_{50}^{rate}/EC_{50}^{extent}$) should also then be similar for different agonists unless they differ in their relative abilities to activate and inactivate the receptor. The latter is entirely consistent with our observation that while Ins(2,4,5)P_3 is less efficacious than Ins(1,4,5)P_3 in activating the receptor, it is similarly less efficacious at inactivating it and so has the same $EC_{50}^{rate}/EC_{50}^{extent}$ ratio as Ins(1,4,5)P_3 (Table 1).

Conclusions: A Model for InsP_3 Receptor Behavior. Our kinetic analysis of InsP_3-evoked Ca^{2+} mobilization leads us to propose the sequence of events shown in Figure 6. InsP_3 initially binds to a low-affinity conformation of each of the four subunits of its receptor and causes a Ca^{2+}-binding site to be exposed on each (*26*). After Ca^{2+} has bound to each of those sites, the intrinsic channel of the receptor opens (*26*) and exhibits its maximal ability to conduct Ca^{2+}. InsP_3 binding, even in the absence of cytosolic Ca^{2+}, initiates a further conformational change in the receptor, which proceeds more slowly ($t_{1/2}$ ~ 250 ms) and causes both the receptor to bind InsP_3 with ~10-fold higher affinity and the channel to become ~40% less able to mediate Ca^{2+} release. The latter may result from a decrease in conductance, a decreased probability of opening, or a shorter open time. We cannot yet define the state of the receptor from which inactivation proceeds most readily, but it must have InsP_3 bound and the rate of inactivation is probably accelerated if Ca^{2+} is also bound (*14*). It is unclear whether InsP_3 bound to a single receptor subunit is sufficient to cause inactivation or whether each subunit must have bound InsP_3. Indeed, it is possible that inactivation proceeds along several parallel paths (Figure 6); desensitization of the nicotinic acetylcholine receptor, for example, occurs when acetylcholine has bound to a single subunit, but the rate is much faster when both α subunits are occupied (*61*). Once the InsP_3 receptor has adopted its partially inactivated state, it remains within this less active conformation for as a long as InsP_3 remains bound, and Ca^{2+} continues to leak through the open channel until the store with which it is associated is drained of Ca^{2+}.

We conclude that InsP_3 binding directly initiates a sequence of activation and inactivation processes, each of which is rapid enough to shape the complex patterns of Ca^{2+} release recorded in intact cells. Since the requirements for activation are more stringent (InsP_3 and then Ca^{2+} binding) than those for partial inactivation (InsP_3 binding), rapid inactivation is likely to prevent openings of single channels from reaching the threshold at which regenerative Ca^{2+} signals are initiated.

REFERENCES

1. Berridge, M. J. (1993) *Nature 361*, 315−325.
2. Mikoshiba, K. (1997) *Curr. Opin. Neurobiol. 7*, 339−345.
3. Berridge, M. J. (1997) *J. Physiol. 499.2*, 291−306.
4. Parker, I., Choi, J., and Yao, Y. (1996) *Cell Calcium 20*, 105−121.
5. Taylor, C. W., and Traynor, D. (1995) *J. Membr. Biol. 145*, 109−118.
6. Combettes, L., Hannaert-Merah, Z., Coquil, J.-F., Rousseau, C., Claret, M., Swillens, S., and Champeil, P. (1994) *J. Biol. Chem. 269*, 17561−17571.
7. Horne, J. H., and Meyer, T. (1995) *Biochemistry 34*, 12738−12746.

8. Finch, E. A., Turner, T. J., and Goldin, S. M. (1991) *Science 252*, 443−446.
9. Dufour, J.-F., Arias, I. M., and Turner, T. J. (1997) *J. Biol. Chem. 272*, 2675−2681.
10. Zhang, B.-X., Zhao, H., and Muallem, S. (1993) *J. Biol. Chem. 268*, 10997−11001.
11. Cardy, T. J. A., Traynor, D., and Taylor, C. W. (1997) *Biochem. J. 328*, 785−793.
12. Yoneshima, H., Miyawaki, A., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1997) *Biochem. J. 322*, 591−596.
13. Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S., and Claret, M. (1989) *J. Biol. Chem. 264*, 17665−17673.
14. Hajnóczky, G., and Thomas, A. P. (1994) *Nature 370*, 474−477.
15. Hajnóczky, G., and Thomas, A. P. (1997) *EMBO J. 16*, 3533−3543.
16. Swillens, S., Combettes, L., and Champeil, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10074−10078.
17. Missiaen, L., De Smedt, H., Droogmans, G., and Casteels, R. (1992) *Nature 357*, 599−602.
18. Tanimura, A., and Turner, R. J. (1996) *J. Cell Biol. 132*, 607−616.
19. Muallem, S., Pandol, S. J., and Beeker, T. G. (1989) *J. Biol. Chem. 264*, 205−212.
20. Taylor, C. W. (1992) *Adv. Second Messenger Phosphoprotein Res. 26*, 109−142.
21. Meyer, T., and Stryer, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3841−3845.
22. Beecroft, M. D., and Taylor, C. W. (1997) *Biochem. J. 326*, 215−220.
23. Parys, J. B., Missiaen, L., De Smedt, H., Sienaert, I., and Casteels, R. (1996) *Pfluegers Arch. 432*, 359−367.
24. Taylor, C. W. (1995) *Biochem. Soc. Trans. 23*, 637−642.
25. Oldershaw, K. A., Nunn, D. L., and Taylor, C. W. (1991) *Biochem. J. 278*, 705−708.
26. Marchant, J. S., and Taylor, C. W. (1997) *Curr. Biol. 7*, 510−518.
27. Marchant, J. S., Chang, Y.-T., Chung, S.-K., Irvine, R. F., and Taylor, C. W. (1997) *Biochem. J. 321*, 573−576.
28. Horne, J. H., and Meyer, T. (1997) *Science 276*, 1690−1692.
29. Turner, T. J., Pearce, L. B., and Goldin, S. M. (1989) *Anal. Biochem. 178*, 8−16.
30. Patel, S., and Taylor, C. W. (1995) *Biochem. J. 312*, 789−794.
31. Rodbard, D. (1974) *Clin. Chem. 20*, 1255−1270.
32. Sugiyama, T., and Goldman, W. F. (1995) *Am. J. Physiol. 269*, C813−C818.
33. Hirose, K., and Iino, M. (1994) *Nature 372*, 791−794.
34. Mezna, M., and Michelangeli, F. (1995) *Nature 376*, 300−301.
35. Missiaen, L., Parys, J. B., De Smedt, H., Lemaire, F. X., Sienaert, I., Bootman, M. D., and Casteels, R. (1995) *Cell Calcium 18*, 100−110.
36. Mezna, M., and Michelangeli, F. (1996) *J. Biol. Chem. 271*, 31818−31823.
37. Hirota, J., Michikawa, T., Miyawaki, A., Furuichi, T., Okura, I., and Mikoshiba, K. (1995) *J. Biol. Chem. 270*, 19046−19051.
38. Hajnóczky, G., Lin, C., and Thomas, A. P. (1994) *J. Biol. Chem. 269*, 10280−10287.
39. Koch, K.-W., Cook, N. J., and Kaupp, U. B. (1987) *J. Biol. Chem. 262*, 14415−14421.
40. Bernhardt, J., and Neumann, E. (1982) *Biophys. Chem. 15*, 327−341.
41. Missiaen, L., De Smedt, H., Parys, J. B., Raeymaekers, L., Droogmans, G., Van den Bosch, L., and Casteels, R. (1996) *Biochem. J. 317*, 849−853.
42. Steenbergen, J. M., and Fay, F. S. (1996) *J. Biol. Chem. 271*, 1821−1824.
43. Rang, H. P. (1981) *J. Physiol. 311*, 23−55.
44. Cash, D. J., and Subbarao, K. (1987) *Biochemistry 26*, 7556−7562.
45. Missiaen, L., De Smedt, H., Parys, J. B., Sienaert, I., Vanlingen, S., and Casteels, R. (1996) *J. Biol. Chem. 271*, 12287−12293.
46. Oldershaw, K. A., and Taylor, C. W. (1993) *Biochem. J. 292*, 631−633.
47. Muallem, S., Schoeffield, M., Pandol, S., and Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4433−4437.
48. Mezna, M., and Michelangeli, F. (1995) *J. Biol. Chem. 270*, 28097−28102.
49. Mayrleitner, M., Chadwick, C. C., Timerman, A. P., Fleischer, S., and Schinder, H. (1991) *Cell Calcium 12*, 505−514.
50. Mak, D.-O. D., and Foskett, J. K. (1997) *J. Gen. Physiol. 109*, 571−587.
51. Combettes, L., Cheek, T. R., and Taylor, C. W. (1996) *EMBO J. 15*, 2086−2093.
52. Meyer, T., Wensel, T., and Stryer, L. (1990) *Biochemistry 29*, 32−37.
53. Hannaert-Merah, Z., Coquil, J.-F., Combettes, L., Claret, M., Mauger, J.-P., and Champeil, P. (1994) *J. Biol. Chem. 269*, 29642−29649.
54. Coquil, J.-F., Mauger, J.-P., and Claret, M. (1996) *J. Biol. Chem. 271*, 3568−3574.
55. Mauger, J.-P., Claret, M., Pietri, F., and Hilly, M. (1989) *J. Biol. Chem. 264*, 8821−8826.
56. Pietri, F., Hilly, M., and Mauger, J.-P. (1990) *J. Biol. Chem. 265*, 17478−17485.
57. Marshall, I. C. B., and Taylor, C. W. (1994) *Biochem. J. 301*, 591−598.
58. Danoff, S. K., Supattapone, S., and Snyder, S. H. (1988) *Biochem. J. 254*, 701−705.
59. Ferris, C. D., Cameron, A. M., Huganir, R. L., and Snyder, S. H. (1992) *Nature 356*, 350−352.
60. Kaplin, A. I., Ferris, C. D., Voglmaier, S. M., and Snyder, S. H. (1994) *J. Biol. Chem. 269*, 28972−28978.
61. Jackson, M. B. (1993) *Thermodynamics of Membrane Receptors and Channels*, CRC Press Inc., Boca Raton, FL.

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