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

Jonathan S. Marchant and Colin W. Taylor*

*Department of Pharmacology, Tennis Court Road, University of Cambridge, Cambridge CB2 1QJ, U.K.*

*Received April 10, 1998; Revised Manuscript Received June 16, 1998*

ABSTRACT: During superfusion of permeabilized hepatocytes, submaximal concentrations of inositol 1,4,5-trisphosphate (Ins$P_3$) 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 Ins$P_3$-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 Ins$P_3$ under conditions that prevented $Ca^{2+}$ mobilization. This change was accompanied by a decrease in the Ins$P_3$ dissociation rate: the response declined more quickly when Ins$P_3$ was removed during the initial stages of a response than later. We suggest that Ins$P_3$ directly causes its receptor to rapidly switch ($t_{1/2}$ = 250 ms) between a low-affinity ($K_d \sim 1$ $\mu$M) 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 Ins$P_3$ receptors are more stringent (Ins$P_3$ and then $Ca^{2+}$ binding) than those for partial inactivation (Ins$P_3$ 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 (Ins$P_3$)[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 Ins$P_3$ (*1*, *2*). The complex changes in cytosolic [$Ca^{2+}$] that follow (*3*) have focused attention on the characteristics of Ins$P_3$ 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 Ins$P_3$-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*), Ins$P_3$ receptors are biphasically regulated by increases in cytosolic [$Ca^{2+}$]: a rapid sensitization of Ins$P_3$-evoked $Ca^{2+}$ release is followed by a slower inhibition (*5*, *8*, *9*). The molecular mechanisms underlying the effects of cytosolic $Ca^{2+}$ on Ins$P_3$ receptors are not yet resolved and probably differ between receptor isoforms (*10*−*12*). Second, during prolonged exposure to Ins$P_3$ the rate of $Ca^{2+}$ mobilization decreases, consistent with a form of receptor desensitization (*8*, *13*) that may result directly from binding of Ins$P_3$ (*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 Ins$P_3$ rapidly release only a fraction of the Ins$P_3$-sensitive $Ca^{2+}$ stores (*19*, *20*) without preventing responses to subsequent challenges with higher concentrations of Ins$P_3$ (*21*, *22*). Such "quantal" (*19*, *23*) responses may allow Ins$P_3$ 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 Ins$P_3$ (*19*, *22*, *25*) or various forms of receptor desensitization (*14*−*16*). The relationships between these three important properties of Ins$P_3$ receptors—$Ca^{2+}$ regulation, desensitization, and quantal $Ca^{2+}$ mobilization—are unclear, although each could contribute to the complex physiological responses to Ins$P_3$.

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 Ins$P_3$ 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 Ins$P_3$ concentrations. Rapid superfusion overcomes these limitations and by stringently controlling the [$Ca^{2+}$]

† J.S.M. was supported by a Wellcome Trust Fellowship (018484). This work was supported by grants from the Wellcome Trust (039662) and the Biotechnology and Biological Sciences Research Council of the U.K.

* To whom correspondence should be addressed at Department of Pharmacology, Tennis Court Road, Cambridge CB2 1QJ. U.K. Tel: (+) 1223 334058. Fax: (+) 1223 334040. E-mail: cwt1000@cam.ac.uk

[1] Abbreviations: CLM, cytosol-like medium; CKF, $Ca^{2+}$- and $K^+$-free CLM; $EC_{50}$, half-maximally effective concentration; Ins$P_3$, inositol 1,4,5-trisphosphate; $t_{1/2}$, half-time; $t_{slow}$ and $t_{fast}$, half-times of slow and fast components, respectively.

S0006-2960(98)00808-3 CCC: $15.00 
Published on Web 07/28/1998

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 Ins$P_3$ receptors during the decaying phase of the response in order to understand the relationship between the initial activation of Ins$P_3$ receptors (*26*) and their subsequent behavior.

## EXPERIMENTAL PROCEDURES

*Materials.* Radiochemicals were from NEN DuPont (Stevenage, U.K.). Ins$P_3$ was from American Radiolabeled Chemicals (St. Louis, MO), and 2,3-dideoxy Ins$P_3$ and 3-deoxy-Ins$P_3$ 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 ($2 \times 10^6$ 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 Ins$P_3$, 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 Ins$P_3$. 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 Ins$P_3$ 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 Ins$P_3$. Each concentration of Ins$P_3$ 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

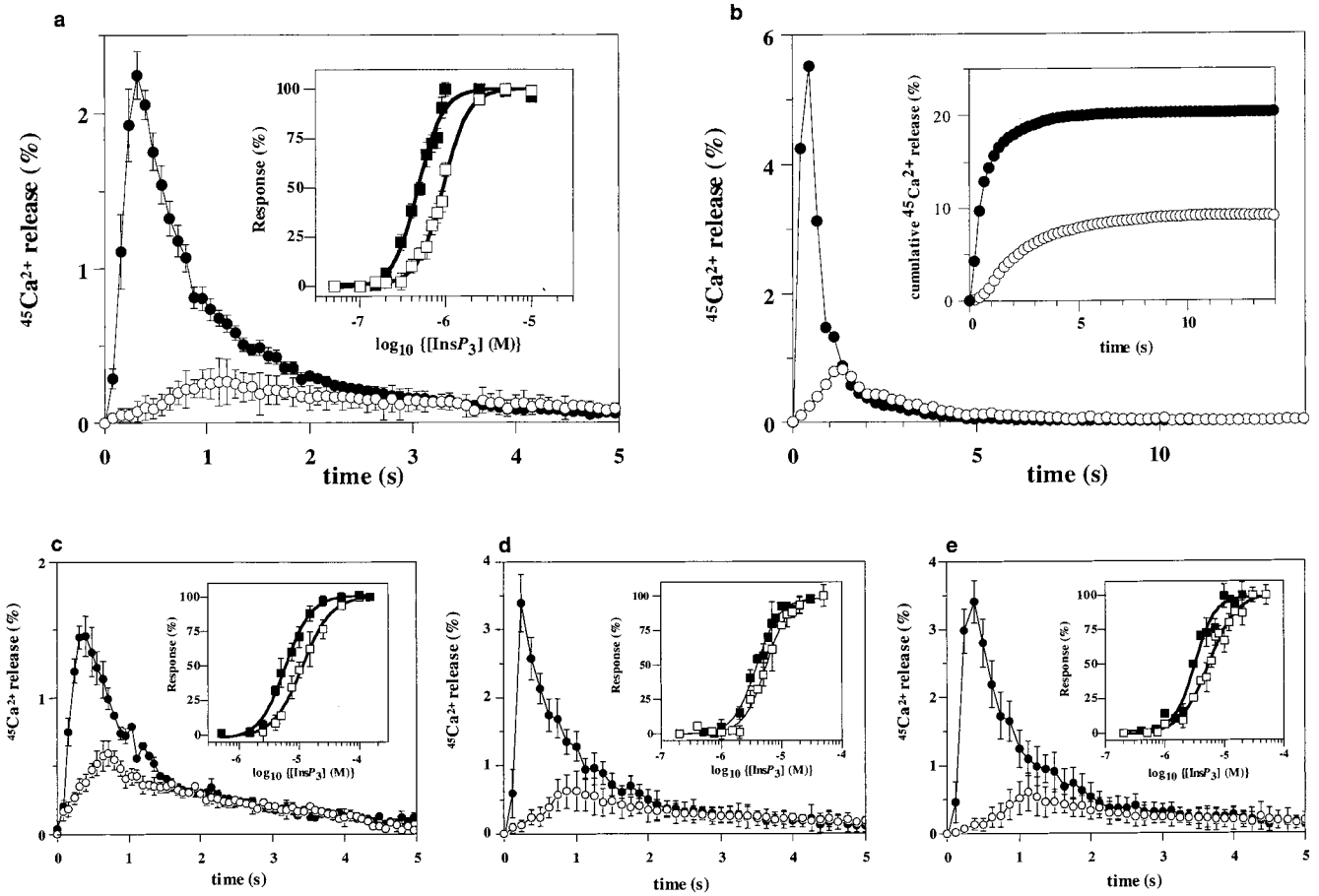


FIGURE 1: Kinetics of $^{45}Ca^{2+}$ mobilization by Ins$P_3$ and its analogues. Permeabilized cells were superfused for 5 s with CLM containing the following: a, Ins$P_3$ (10 $\mu$M, ●; 400 nM, ○); c, Ins(2,4,5)$P_3$ (100 $\mu$M, ●; 8 $\mu$M, ○); d, 3-deoxy-Ins$P_3$ (50 $\mu$M, ●; 3 $\mu$M, ○); e, 2,3-dideoxy-Ins$P_3$ (50 $\mu$M, ●; 2.5 $\mu$M, ○). 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 $\mu$M, ●) and submaximal (350 nM, ○) concentrations of Ins$P_3$ 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 Ins$P_3$.

$^{45}Ca^{2+}$ release. With 400 nM Ins$P_3$, 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 Ins$P_3$ (350 nM) terminates without having fully emptied the Ins$P_3$-sensitive stores. Therefore both the peak rate and the eventual extent of $^{45}Ca^{2+}$ release increased with Ins$P_3$ concentration (Figure 1a, inset). The inability of submaximal concentrations of Ins$P_3$ to completely empty the Ins$P_3$-sensitive $Ca^{2+}$ stores, quantal $Ca^{2+}$ mobilization (*19*), has been observed in many cell types (*20*, *23*).

Other active analogues of Ins$P_3$, Ins(2,4,5)$P_3$, 3-deoxy-Ins$P_3$, and 2,3-dideoxy-Ins$P_3$, also evoked quantal $^{45}Ca^{2+}$ release, and as with Ins$P_3$, 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 Ins$P_3$ 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 Ins$P_3$-Evoked $Ca^{2+}$ Mobilization.* The kinetics of the declining phase of the response evoked by 10 $\mu$M Ins$P_3$ (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 Ins$P_3$ (≥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 Ins$P_3$ (<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 Ins$P_3$ 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-Ins$P_3$ | 2,3-dideoxy-Ins$P_3$ |
|---|---|---|---|---|
| rate | | | | |
| $EC_{50}$ | 941 ± 21 nM | 11.3 ± 2.3 $\mu$M | 5.51 ± 0.26 $\mu$M | 5.6 ± 0.44 $\mu$M |
| $n_H$ | 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 $\mu$M | 3.2 ± 0.31 $\mu$M | 3.75 ± 0.18 $\mu$M |
| $n_H$ | 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.

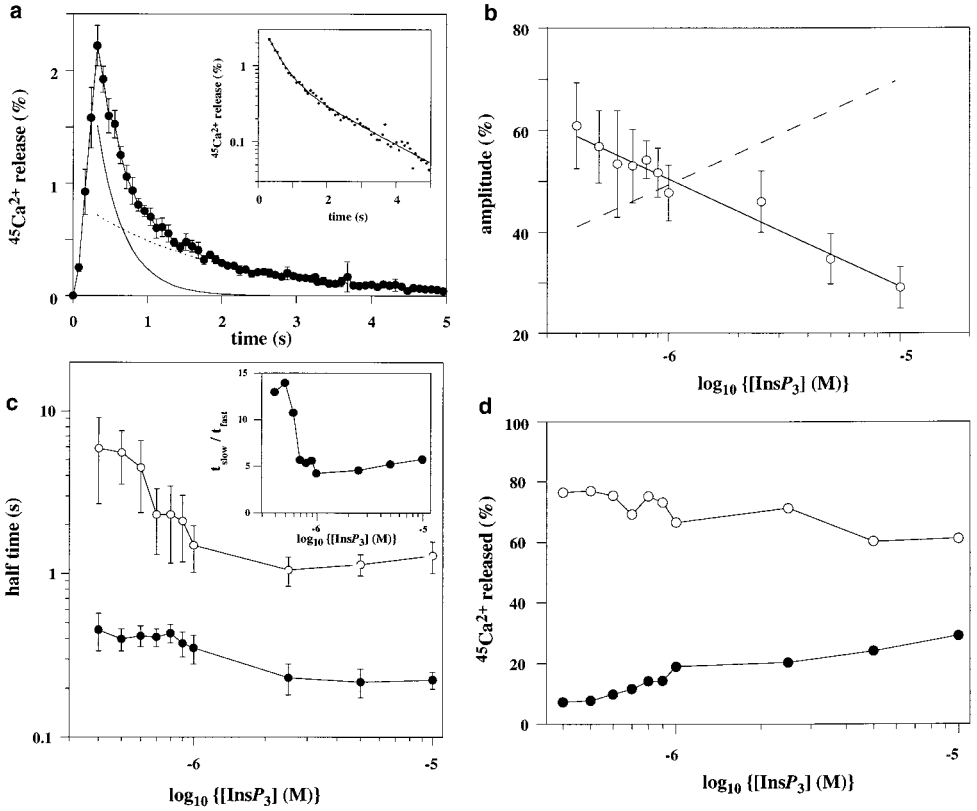


FIGURE 2: Biphasic decay in the rate of Ins$P_3$-evoked $^{45}Ca^{2+}$ release. Cells were superfused for 5 s with CLM containing 10 $\mu$M Ins$P_3$ and the rate of $^{45}Ca^{2+}$ release (●) was recorded during 80 ms intervals. (a) The kinetics of the decaying phase of the response are best fitted ($p < 0.05$) 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 Ins$P_3$. 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 Ins$P_3$ are shown (mean ± sem, $n \geq 4$). The inset shows the ratio of $t_{fast}/t_{slow}$ at various concentrations of Ins$P_3$. (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 Ins$P_3$ ($n \geq 4$ 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 Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ to 31% ± 5% ($n = 10$) for 10 $\mu$M Ins$P_3$ (Figure 2b). At 10 $\mu$M Ins$P_3$, $t_{fast}$ 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 Ins$P_3$, with the half-maximal effect ($EC_{50}$) occurring when the Ins$P_3$ concentration was 1.1 $\mu$M 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 Ins$P_3$ (Figure 2c, inset), the responses evoked by submaximal concentrations of Ins$P_3$ 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 Ins$P_3$, the long duration of the slow

Table 2: Kinetics of the Decaying Phase in the Rate of $^{45}Ca^{2+}$ Release Evoked by Ins$P_3$ and Its Analogues[a]

| | Ins(1,4,5)$P_3$ | Ins(2,4,5)$P_3$ | 3-deoxy-Ins$P_3$ | 2,3-dideoxy-Ins$P_3$ |
|---|---|---|---|---|
| $t_{fast}$ | 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 $\mu$M), Ins(2,4,5)$P_3$ (150 $\mu$M), 3-deoxy-Ins$P_3$ (80 $\mu$M), or 2,3-dideoxy-Ins$P_3$ (80 $\mu$M) 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 Ins$P_3$ 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 Ins$P_3$-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 Ins$P_3$ are consistent with previous studies of permeabilized cells (*13*, *21*), microsomes (*36*), and reconstituted Ins$P_3$ 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 Ins$P_3$-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 Ins$P_3$ receptor or from properties of the intracellular $Ca^{2+}$ stores themselves. Heterogeneity in the size, $Ca^{2+}$ content, or Ins$P_3$ 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 Ins$P_3$-evoked $Ca^{2+}$ efflux have often been equated with two distinct states of the Ins$P_3$ receptor, there is scant justification for this assumption without further evidence.

In our experiments, the biphasic kinetics of Ins$P_3$-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*). Ins$P_3$ metabolism did not contribute because the biphasic kinetics were observed during continuous superfusion with Ins$P_3$ and in response to stimulation with metabolically stable analogues of Ins$P_3$ (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 Ins$P_3$ 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 Ins$P_3$-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 Ins$P_3$ 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 Ins$P_3$ receptors (*37*) because the rates of decay of the fast and slow phases are differentially affected by Ins$P_3$ concentration (Figure 2c) (*34*).

We conclude that the two components of the decay in the rate of Ins$P_3$-evoked $Ca^{2+}$ release reflect different properties of the Ins$P_3$ receptor rather than properties of the intracellular $Ca^{2+}$ stores, consistent with the biphasic pattern of Ins$P_3$-evoked $Ca^{2+}$ release observed from immunoaffinity-purified type 1 Ins$P_3$ receptors reconstituted into liposomes (*37*). Subsequently, we investigated whether these components result from distinct Ins$P_3$ receptor populations or from a change in channel behavior following Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ (*22*, *25*, *30*). The observation that during the biphasic response to submaximal concentrations of Ins$P_3$, the rate of $^{45}Ca^{2+}$ release during the slow phase invariably extrapolated to the basal rate despite the fact that the Ins$P_3$-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 Ins$P_3$ receptors, we have applied a different analysis which recognizes that intracellular $Ca^{2+}$ stores respond in this all-or-nothing manner to Ins$P_3$. In this analysis, the total amount of $Ca^{2+}$ released by each concentration of Ins$P_3$ was assumed to be the entire $Ca^{2+}$ pool to which that Ins$P_3$ concentration had access. This pool size was then used to compute fractional release rates during sustained stimulation with each concentration of Ins$P_3$ (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 Ins$P_3$-sensitive store (*45*), the revised quantal analysis acknowledges that each concentration of Ins$P_3$ has access to a different fraction of the total store and that its content declines during the response to Ins$P_3$. The advantage of this analysis is that it provides direct insight into the permeability of Ins$P_3$ receptors: the fractional release rate will be constant unless there is a change in the behavior of the Ins$P_3$ receptor.

The profile of the fractional $^{45}Ca^{2+}$ release rates (Figure 3) has three important implications. First, at maximal concentrations of Ins$P_3$ (≥2.5 $\mu$M), the fractional rate of

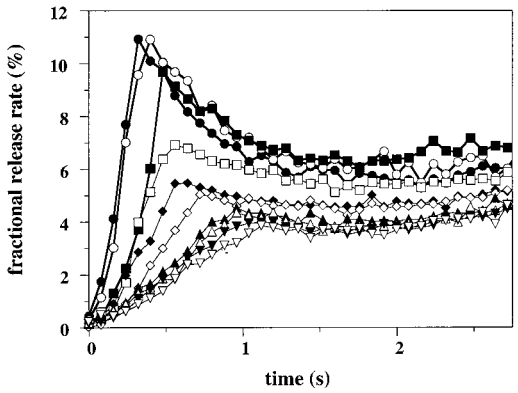


FIGURE 3: Effects of Ins$P_3$ on the fractional rate of $^{45}Ca^{2+}$ release. Data derived from experiments similar to those shown in Figure 1 were used to establish the fractional release rate of $^{45}Ca^{2+}$ (see Experimental Procedures) during each 80 ms interval. The concentrations of Ins$P_3$ were 10 $\mu$M (●), 5 $\mu$M (○), 2.5 $\mu$M (■), 1 $\mu$M (□), 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 Ins$P_3$, hepatic Ins$P_3$ receptors inactivate to a state with ~40% lesser activity. At submaximal concentrations of Ins$P_3$, 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 Ins$P_3$ (Figure 1) was less apparent because at lower concentrations of Ins$P_3$ 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 Ins$P_3$ (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 Ins$P_3$ (10 $\mu$M) releases only 9.6% of the Ins$P_3$-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 Ins$P_3$ receptor, but rather reflects the monophasic loss of $Ca^{2+}$ from the intracellular stores through a state of the Ins$P_3$ receptor that undergoes no subsequent change in activity.

We conclude that after binding of Ins$P_3$, the Ins$P_3$ 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 Ins$P_3$ 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-Ins$P_3$ and 2,3-dideoxy-Ins$P_3$ (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 $\mu$M 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 Ins$P_3$ receptor activation and inactivation.

*Role of Ins$P_3$ in Receptor Inactivation.* Ins$P_3$-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 Ins$P_3$-evoked $Ca^{2+}$ efflux without preventing Ins$P_3$ from binding to its receptor (*46*). We have adapted this protocol to the rapid superfusion apparatus to assess whether Ins$P_3$ binding in the absence of both cytosolic $Ca^{2+}$ and $Ca^{2+}$ flux through the Ins$P_3$ 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 $\mu$M Ins$P_3$, and then during the subsequent restoration of counterions together with 20 $\mu$M Ins$P_3$. The results demonstrate that Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ caused the difference between the peak and sustained fractional $Ca^{2+}$ release rates to be reduced by ~55%, consistent with Ins$P_3$ having driven more than half the receptors to their less active conformations during the preincubation. However, preincubation with Ins$P_3$ 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 Ins$P_3$ receptors that remain in the most active state after pretreatment with Ins$P_3$ 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 Ins$P_3$ alone, in the absence of both cytosolic $Ca^{2+}$ and a flux of $Ca^{2+}$ through the receptor, is sufficient to drive the Ins$P_3$ receptor into the conformation that mediates the slow phase of $Ca^{2+}$ release. It is, however, noteworthy that only ~55% of the receptors

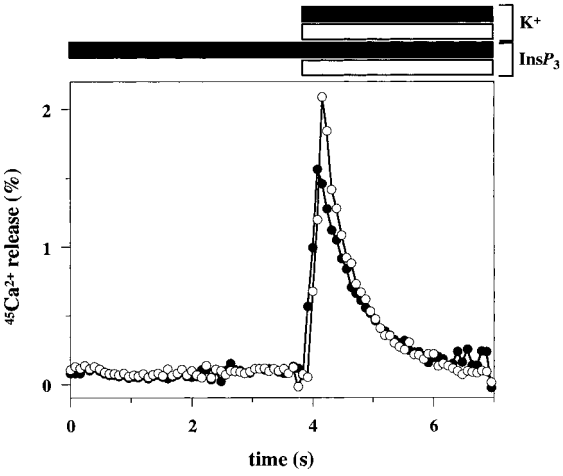


FIGURE 4: Effect of Ins$P_3$ preincubation on the kinetics of $^{45}Ca^{2+}$ mobilization. Permeabilized cells were superfused for 5 s with $Ca^{2+}$- and $K^+$-free medium (CKF) and then (at $t = 0$) for 3.85 s in either CKF alone (○) or CKF supplemented with 20 $\mu$M Ins$P_3$ (●). After 3.85 s, both $K^+$ (140 mM) and Ins$P_3$ (20 $\mu$M) 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 Ins$P_3$ were included in the superfusion media.

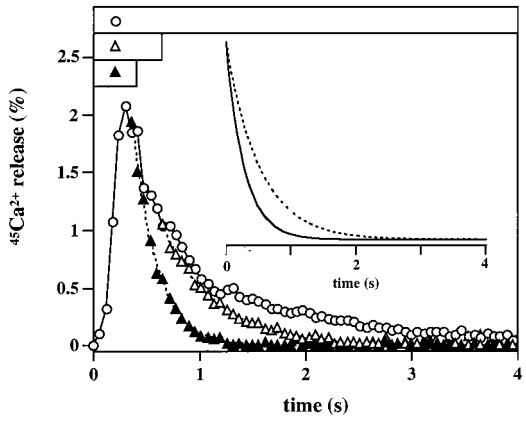


FIGURE 5: Kinetics of channel closure after removal of Ins$P_3$. Cells were exposed to Ins$P_3$ for the periods indicated, and the rate of decay in the rate of $^{45}Ca^{2+}$ release was measured in response to a 380 ms pulse (▲), a 640 ms pulse (△), and a 4 s challenge with 10 $\mu$M Ins$P_3$ (○). For clarity, only the latter parts of the traces that employed short pulses of Ins$P_3$ 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 Ins$P_3$ after 380 ms (solid curve) and 640 ms (dashed curve).

were driven to their less active conformation during a preincubation (3.85 s) with Ins$P_3$ (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 Ins$P_3$ 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 Ins$P_3$ binding caused Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ receptors (*49*) and patch clamp recordings from nuclei of *Xenopus* oocytes (*50*), channel activity declines, albeit over seconds, during prolonged exposure to Ins$P_3$. Practical difficulties have so far prevented us from further examining the effects of cytosolic $Ca^{2+}$ on Ins$P_3$-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 Ins$P_3$ alone could be explained if removal of cytosolic $Ca^{2+}$ slowed the rate of the Ins$P_3$-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 Ins$P_3$ 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 Ins$P_3$-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 Ins$P_3$-evoked inactivation. Second, the observation that Ins$P_3$ alone in the absence of a $Ca^{2+}$ flux through the channel can inactivate the receptor suggests, as we previously speculated (*26*), that Ins$P_3$ binding initiates a race between receptor activation and inactivation. This competition between the stringent requirements for activation (Ins$P_3$ and $Ca^{2+}$ binding to the receptor) and the lesser requirements for inactivation (which may require only Ins$P_3$ 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 Ins$P_3$.* By rapidly ($t_{1/2} = 45 \pm 3$ ms) removing Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ (10 $\mu$M) was removed during the response, indicating that the slow phase of $^{45}Ca^{2+}$ release, and therefore the less active conformation of the Ins$P_3$ receptor, was dependent on the maintained binding of Ins$P_3$. More importantly, the rate of decay of the response was faster when Ins$P_3$ (10 $\mu$M) 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 $\mu$M Ins$P_3$ (not shown). These results demonstrate that Ins$P_3$ dissociates rapidly from the conformation of the Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ binding, the Ins$P_3$ 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 Ins$P_3$ than the fast phase (Figure 2c), and with the response to a submaximal concentration of Ins$P_3$ (500 nM) becoming progressively less sensitive to inhibition by heparin as the duration of the exposure to Ins$P_3$ is increased (not shown). The results of other studies are also consistent with our conclusion. Ins$P_3$ 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 Ins$P_3$ receptors for Ins$P_3$ (*54*). In hepatocytes, where two states of the receptor differing by 10−20-fold in their affinities for Ins$P_3$ have been detected in $Ca^{2+}$-free medium, hormones that stimulate Ins$P_3$ formation increase the fraction of receptors in the high-affinity state (*55*, *56*). Since increases in [$Ca^{2+}$] also increase the affinity of hepatic Ins$P_3$ 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 Ins$P_3$.

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 $\mu$M Ins$P_3$, ∼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 $\mu$M Ins$P_3$), 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 Ins$P_3$ 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 Ins$P_3$ dissociates ∼9-fold more rapidly from the most active receptor conformation. If the rate of association of Ins$P_3$ with the two receptor conformations is similar, then the most active conformation would be expected to bind Ins$P_3$ 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 Ins$P_3$ receptor that are interconverted by hormones differ by ∼10−20-fold in their affinities for Ins$P_3$ (*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 Ins$P_3$ of ∼1 $\mu$M, suggesting that the less active state might have a $K_d$ of ∼100 nM.

*Quantal $Ca^{2+}$ Release Is Not Caused by Ins$P_3$ Receptor Inactivation.* Although the biphasic kinetics of Ins$P_3$-stimulated $Ca^{2+}$ mobilization, regulation of the Ins$P_3$ 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. Ins$P_3$ 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 Ins$P_3$ receptors are not $Ca^{2+}$-sensitive (*37*, *58*), and yet they mediate both biphasic (*37*) and quantal responses to Ins$P_3$ (*37*, *59*). Both quantal responses to Ins$P_3$ in permeabilized hepatocytes and biphasic regulation of the hepatic Ins$P_3$ receptor by cytosolic $Ca^{2+}$ persist at 2 °C (*22*), while inactivation of the receptor after Ins$P_3$ binding is prevented (*14*, *38*).

Our results confirm that quantal $Ca^{2+}$ release is not mediated by $Ca^{2+}$ regulation of Ins$P_3$ receptors (Figure 1) (*22*, *37*). More importantly, they establish that the biexponential pattern of Ins$P_3$-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 Ins$P_3$ fail to fully empty the Ins$P_3$-sensitive stores. During the biphasic response to submaximal concentrations of Ins$P_3$, the rate of $^{45}Ca^{2+}$ release during the slow phase invariably extrapolated to the basal rate despite the fact that the Ins$P_3$-sensitive $Ca^{2+}$ stores had not been fully emptied (Figure 1b). While the high-affinity conformation of the Ins$P_3$ 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 Ins$P_3$ receptor will therefore mediate $Ca^{2+}$ release for as long as Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ (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 Ins$P_3$ receptors retain an ability to mediate $Ca^{2+}$ release for as long as they have Ins$P_3$ bound, albeit at slower rates at later times, activation of a single Ins$P_3$ 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 Ins$P_3$ (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 Ins$P_3$, which are likely to activate only few receptors, leave more $Ca^{2+}$ trapped within

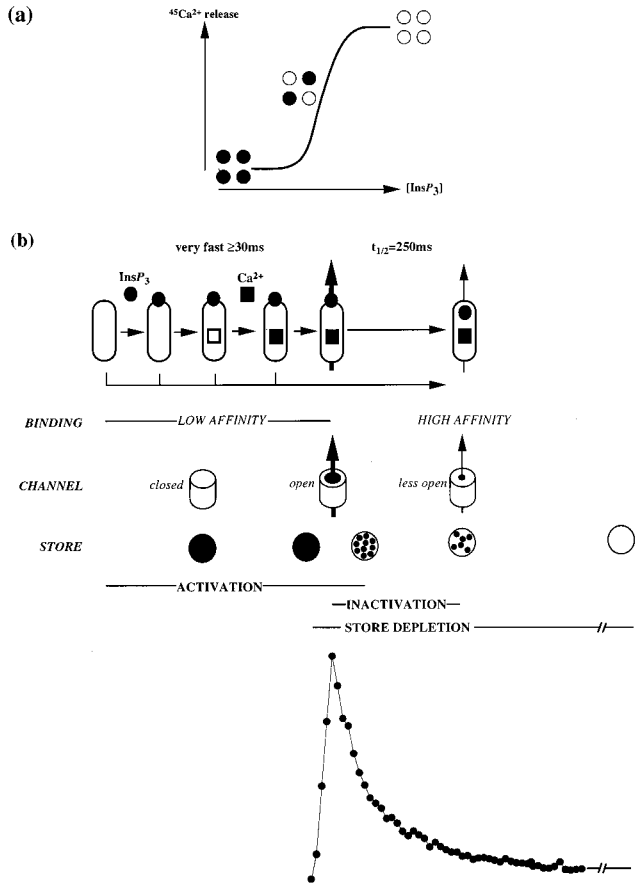


FIGURE 6: Changes in Ins$P_3$ receptor behavior following Ins$P_3$ binding. Changes in the $Ca^{2+}$ contents of the stores and the underlying behavior of the Ins$P_3$ receptors are illustrated. (a) As the Ins$P_3$ concentration is increased, stores that differ in their sensitivities to Ins$P_3$ respond by releasing all or none of their sequestered $Ca^{2+}$, at rates governed by the changing behavior of the Ins$P_3$ receptor (b). (b) Activation. Binding of Ins$P_3$ 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 Ins$P_3$, this sequence can be completed in ~30 ms; it takes longer for lower concentrations of Ins$P_3$. Inactivation. Binding of Ins$P_3$ 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 Ins$P_3$ and then $Ca^{2+}$, and the less demanding trigger for partial inactivation (Ins$P_3$ binding alone) ensures that spontaneous openings of Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$-evoked $Ca^{2+}$ release (Table 1) is not, therefore, a fundamental property, but an emergent effect of the $Ca^{2+}$ content and Ins$P_3$ receptor density of the stores as well as the time-dependent changes in Ins$P_3$ 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 Ins$P_3$ Receptor Behavior.* Our kinetic analysis of Ins$P_3$-evoked $Ca^{2+}$ mobilization leads us to propose the sequence of events shown in Figure 6. Ins$P_3$ 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 $Ca^{2+}$. Ins$P_3$ 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 Ins$P_3$ 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 Ins$P_3$ bound and the rate of inactivation is probably accelerated if $Ca^{2+}$ is also bound (*14*). It is unclear whether Ins$P_3$ bound to a single receptor subunit is sufficient to cause inactivation or whether each subunit must have bound Ins$P_3$. 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 Ins$P_3$ receptor has adopted its partially inactivated state, it remains within this less active conformation for as a long as Ins$P_3$ 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 Ins$P_3$ 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 (Ins$P_3$ and then $Ca^{2+}$ binding) than those for partial inactivation (Ins$P_3$ 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.


BI980808K