Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3841–3845, May 1990
Biochemistry

Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate

(signal transduction/"phosphoinositide" cascade/calcium channels/increment detection/oscillations)

TOBIAS MEYER AND LUBERT STRYER

Department of Cell Biology, Sherman Fairchild Center, Stanford University School of Medicine, Stanford, CA 94305

Contributed by Lubert Stryer, February 20, 1990

ABSTRACT **Many hormonal, neurotransmitter, and sensory stimuli trigger the formation of inositol 1,4,5-trisphosphate, which in turn releases calcium from intracellular stores. We report here that inositol 1,4,5-trisphosphate-induced calcium release from saponin-permeabilized rat basophilic leukemia cells at 37°C is markedly biphasic, in contrast with nearly monophasic release kinetics at 11°C. Hepatoma, PC-12 neuronal cells, and several other cell types exhibit similar biphasic release at 37°C. The biphasic kinetics are not due to degradation of inositol 1,4,5-trisphosphate or to increased Ca^{2+}-ATPase pump activity. Biphasic calcium release was also seen when ATP was quenched to <0.4 μM by adding hexokinase and glucose, suggesting that phosphorylation is not involved. External calcium (100 nM–600 nM) range had little influence on the biphasic kinetics. Rapid-mixing experiments revealed that rapid efflux of calcium is followed in ≈0.5 s by a 30-fold slower efflux. Most striking, successive additions of the same amount of inositol 1,4,5-trisphosphate induced short bursts of calcium release of similar size. This retention of responsiveness, which we term increment detection, may be a distinct mode of signal transduction. Like inactivation and adaptation, increment detection gives rise to transient responses to sustained stimuli. Systems exhibiting inactivation, adaptation, and increment detection differ in their responsiveness (none, partial, and full, respectively) to stepwise increases in stimulus intensity. Increment detection could be advantageous in generating receptor-triggered calcium oscillations.**

Many signal-transduction processes are mediated by increases in cytosolic calcium levels produced by the action of the "phosphoinositide" cascade (1, 2). The receptor-triggered hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C results in the formation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ then releases calcium from an endoplasmic reticulum store by directly opening channels (3–5). In many cell types, the receptor-mediated increases in cytosolic calcium levels are transient and repetitive (6, 7). The generation of calcium oscillation requires a tight control of the calcium-release process.

In permeabilized cells or microsomal cell preparations, submaximal concentrations of $InsP_3$ do not release all of the stored calcium (8). Partial calcium release has been attributed to rapid degradation of $InsP_3$ or to the attainment of a steady state between calcium efflux and sequestration. Muallem *et al.* (9) reported that net $InsP_3$-induced calcium release in permeabilized pancreatic acinar cells at 37°C stops in <30 s. They proposed that the internal calcium stores are extensively compartmentalized and that a submaximal dose of $InsP_3$ completely releases calcium from only some compartments. Champeil *et al.* (10) recently reported stopped-flow kinetic studies of $InsP_3$-induced calcium release in permeabilized rat hepatocytes at 20°C and 37°C. They observed biphasic release kinetics with a fast component in the subsecond time range and proposed that $InsP_3$ receptors undergo spontaneous inactivation after ligand binding. We report here kinetic studies of $InsP_3$-induced calcium release in permeabilized rat basophilic leukemia cells at 37°C that were motivated by two questions: (*i*) What is the mechanism of partial calcium release induced by submaximal concentrations of $InsP_3$? (*ii*) What is the response of the calcium-release system to incremental additions of $InsP_3$?

MATERIALS AND METHODS

$InsP_3$ was purchased from Calbiochem, and fluo-3 was from Molecular Probes. The standard buffer for flux measurements contained 20 mM Hepes (pH 7.4), 145 mM KCl, 5 mM NaCl, 2 mM Mg^{2+}, and 0.7 mM ATP. High-sensitivity measurement of released calcium depended on the removal of contaminating metal ions by a heptadentate chelating resin (calcium sponge, ref. 5). Rat basophilic leukemia cells (RBL cells) were grown and prepared as described (3, 5). The cells were permeabilized with saponin at 85 μg/ml for 10 min at room temperature. Similar release kinetics were observed with cells permeabilized with streptolysin-O or with concentrations of saponin ranging from 40 to 300 μg/ml. External calcium concentrations were measured fluorimetrically (5) by using the calcium indicator fluo-3 (11). An apparent dissociation constant of 600 nM (5) was used to determine free calcium concentrations. Rapid kinetic studies were carried out by using a stopped-flow apparatus with an integration time of 22 ms (5).

RESULTS

Biphasic Calcium Release Induced by $InsP_3$. The kinetics of $InsP_3$-induced calcium release from internal stores of saponin-permeabilized RBL cells at 37°C were measured. Before $InsP_3$ addition, calcium efflux from the endoplasmic reticulum store is balanced by ATP-driven calcium uptake. As shown in Fig. 1*a*, the change in external calcium level elicited by addition of $InsP_3$ is clearly biphasic, in contrast with monophasic kinetics seen earlier at 11°C (3, 5). The rate of calcium efflux is much higher immediately after $InsP_3$ addition than several seconds later (Fig. 1*b*). Degradation of $InsP_3$ cannot account for these biphasic kinetics because the half-time for $InsP_3$ destruction under these conditions was 500 s, as evidenced by HPLC analysis of $[^3H_3]InsP_3$ added to an identical cell suspension (12). When $InsP_3$ was replaced by a 3-fold higher concentration of the hydrolysis-resistant thio analog of $InsP_3$ ($InsPS_3$, gift from B. Potter, University of Leicester, Leicester, U.K.), the same biphasic calcium-release kinetics was seen. The amounts of $InsP_3$ added are >100 times the number of $InsP_3$-binding sites in this suspension (≈0.1 nM; ref. 3), indicating that the free $InsP_3$ concentration also remains essentially constant. The response to a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RBL cells, rat basophilic leukemia cells; $InsP_3$, inositol 1,4,5-trisphosphate.

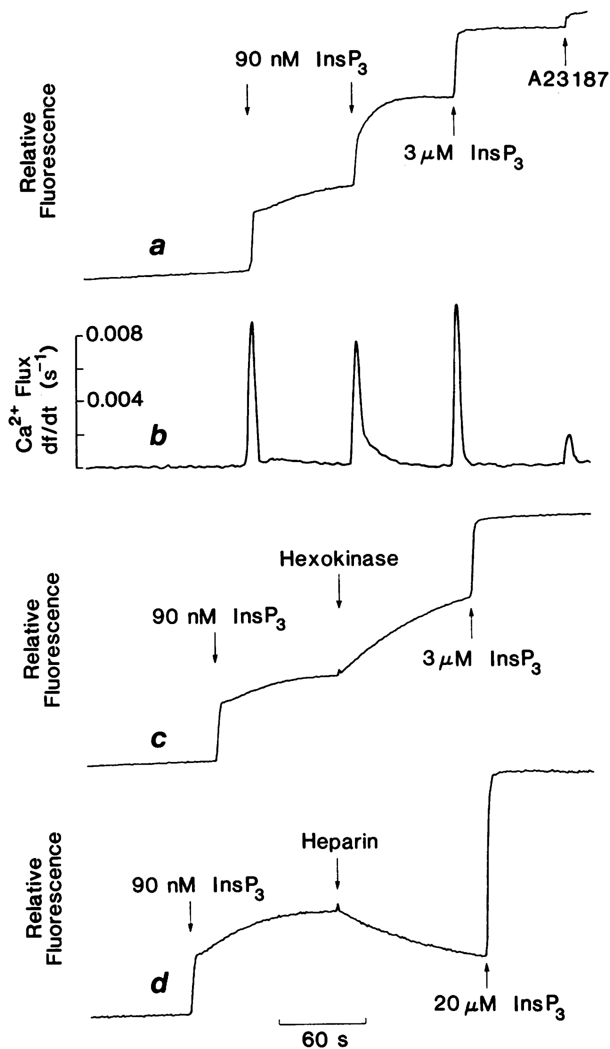

FIG. 1. Biphasic elevation in external calcium in response to the addition of InsP_3 to permeabilized RBL cells. A first addition of 90 nM InsP_3 rapidly released 20% of the stored calcium, followed by a slow release (*a*). A second addition of 90 nM InsP_3 rapidly released a larger fraction of the stored calcium. Addition of 3 μM InsP_3 then rapidly released almost all of the calcium mobilizable by the calcium ionophore A23187. The free calcium concentration in this assay increased from ≈250 nM to 450 nM. The fluorescence intensity is approximately linear with calcium concentration throughout this study. The maximal deviation from linearity is ≈35%. Diffusion of InsP_3 and released calcium occur in <65 ms (5). (*b*) Net calcium efflux rate as a function of time, calculated as the first derivative of the data in *a*. The parameter *f* represents the fraction of fluo-3 bound with calcium (5). Time resolution is ≈6 s, comparable to the 4-s mixing time of the cuvette. (*c*) Experiment in which ATP was quenched in <4 s by adding hexokinase (4 units/ml) to stop the calcium pump (10 mM glucose was present). The induced efflux of calcium then corresponds to the sum of the InsP_3-mediated efflux plus a calcium leak. (*d*) Experiment in which heparin (8 μg/ml) was added after preincubation with 90 nM InsP_3. The initial calcium uptake rate after heparin addition corresponds to the calcium flux through the InsP_3-gated channel at steady state. Competitive inhibition by heparin was reversed by adding 20 μM InsP_3.

second aliquot of InsP_3 (Fig. 1 *a* and *b*) shows that the biphasic kinetics are also not due to depletion of the internal calcium store. Inhomogeneous mixing of InsP_3 is unlikely to contribute to the biphasic release kinetics because the two phases are not observed at 11°C (3, 5). Also, two phases are seen in rapid-mixing experiments, as discussed below.

Is calcium efflux biphasic or do the biphasic kinetics reflect a delayed acceleration of calcium pump activity? These alternatives were distinguished by adding hexokinase to a cell suspension containing glucose (Fig. 1*c*) to rapidly lower the ATP level (13) and stop the Ca^{2+}-ATPase pump. The slope of the fluorescence change in the absence of pump activity is proportional to the rate of calcium efflux. The slope after hexokinase addition is much less than the slope immediately after InsP_3 addition (first arrow in Fig. 1*c*). The complementary experiment involved blocking the InsP_3-gated channel by adding heparin (Fig. 1*d*), a competitive inhibitor (14). The downward slope after heparin addition is comparable to the upward slope after hexokinase addition. These two results show that the biphasic character of the change in calcium level results primarily from biphasic calcium release rather than from activation of the calcium pump.

Calcium Pump and Leak Kinetics Are Monotonic Functions of Store Loading. A detailed analysis of the biphasic calcium-release kinetics requires knowledge of the calcium pump activity and of the InsP_3-independent release rate. The fluo-3 fluorescence of a suspension of permeabilized cells was measured after the addition of the same amount of ATP as was used in the release experiments (Fig. 2*a*). The calcium-uptake rate (given by the downward slope) decreased as the internal store became filled (0.7 fmol maximum per cell). After hexokinase addition, the fluorescence increased due to an InsP_3-independent efflux of calcium, which we term the "leak." In Fig. 2*b*, the calcium uptake and leak rates are plotted as a function of the fractional loading of the internal store. The ATP-driven calcium pump rate is the difference between the uptake and the leak rates. These data show that the leak rate and the pump rate are monotonic functions of the fractional loading of the calcium store. The release rate immediately after InsP_3 addition is much greater than the pump rate. In contrast, the pump rate is comparable to the efflux rate during the slow phase of InsP_3-induced calcium release. The dependence of the pump rate (the uptake rate minus the leak rate) on the fractional loading of the store shows that the pump slows as the store becomes filled. The calcium leak increases with the amount of calcium loaded into the store and ultimately limits the amount of calcium that can be stored. As shown in Fig. 2*c*, the pump rate was half-maximal when the external calcium concentration was ≈100 nM. Thus, the calcium pump rate was near saturation under the conditions of this study.

Biphasic Calcium Release Occurs in the Absence of ATP. A cAMP-dependent phosphorylation of the InsP_3 receptor in neuronal cells has been shown to diminish InsP_3 binding and calcium flux (15). To determine whether protein phosphorylation is required for the termination of calcium release, ATP levels were quenched by adding hexokinase and glucose 20 s before InsP_3 addition. Phosphate transfer from ATP to glucose lowered the ATP concentration to <0.4 μM within 20 s (75 nM at steady state), as determined with a lucifererase assay. Fig. 3 shows that rapid termination of InsP_3-induced calcium release is not affected by markedly lowering ATP levels, suggesting that phosphorylation is not essential. It also shows that calcium release in the slow phase increases with increasing concentration of InsP_3. In the absence of ATP, nearly all of the calcium can be released by submaximal doses of InsP_3 in times faster than can be accounted for by the leak rate. In the presence of ATP, a submaximal dose of InsP_3, such as 90 nM (Fig. 1*a*), induces only fractional release because InsP_3-induced calcium efflux during the slow phase is balanced by ATP-driven calcium uptake.

Voltage-dependent channel closure does not appear to be involved because gramicidin A and valinomycin do not alter the biphasic kinetics of calcium release. In neuronal cells, calcium diminishes InsP_3 binding, an effect mediated by calmedin, a calcium-binding protein (16). Also in *Xenopus* oocytes, calcium inhibits InsP_3-induced calcium release (17). However, in our system, varying the initial calcium level

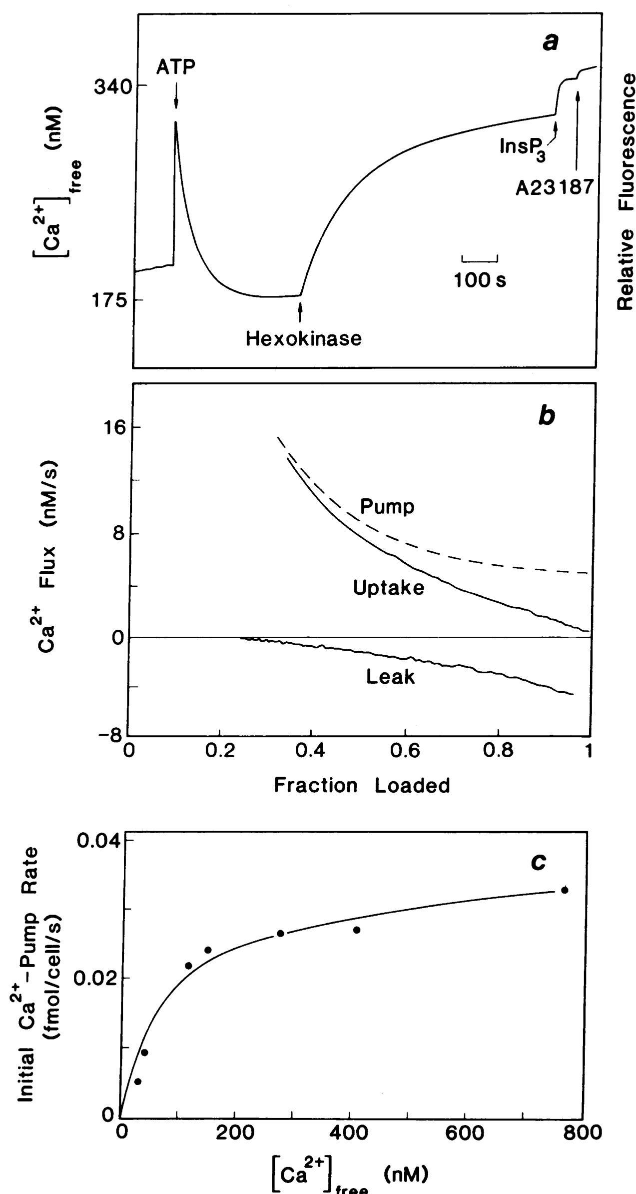

FIG. 2. Calcium pump and leak rates across the InsP_3-sensitive store without InsP_3. Permeabilized RBL cells were stirred for 10 min in the absence of added Mg^{2+}-ATP. Addition of 0.7 mM ATP/2 mM Mg^{2+}/1 μM Ca^{2+} then leads to calcium uptake at a maximal rate; complete loading occurs in 30 s (*a*). Lowering the ATP concentration by hexokinase addition causes a slow calcium efflux. Nearly all the remaining calcium can be released by saturating amounts of InsP_3 (3 μM); subsequent addition of calcium ionophore A23187 (1 μM) releases little additional calcium. (*b*) Plot of net flux versus fraction of maximal calcium loading. The flux is the first derivative of the data in Fig. 3. The uptake curve corresponds to the part in *a* after Mg^{2+}-ATP addition, and the leak curve corresponds to the part after hexokinase addition. Subtraction of the leak from the uptake curves reveals the activity of the calcium pump (---). (*c*) Dependence of initial Ca^{2+}-uptake rate on the external calcium level after ATP addition.

between 100 and 600 nM had little effect on the release kinetics. Likewise a 5-fold reduction in cell concentration to minimize the change in external calcium had little effect on the shape of calcium-release curves. Thus, termination of calcium release probably does not depend on a rise in the external calcium level. A G protein is probably not involved because guanosine[γ-thio]trisphosphate had no effect.

Kinetics of the Rapid and Slow Phase of Calcium Release. The kinetics of the rapid phase of calcium release were resolved by rapid-mixing experiments, which had a time resolution of 22 ms (Fig. 4*a*). The fraction of stored calcium that was released depended on the InsP_3 concentration. The initial rate of calcium release increased with InsP_3 concentration until a limiting rate ($\approx$14 s^{-1}) was reached at $\approx$1 μM InsP_3. The rapid phase ended at $\approx$1 s at 125 nM InsP_3 and at 500 ms at 350 nM InsP_3. The plateaus seen at longer times correspond to a steady state between slow calcium efflux (due to the leak and the InsP_3-induced release) and ATP-driven calcium uptake. InsP_3-induced release during the slow phase was blocked with heparin to provide a measure of its rate. The initial rate of calcium uptake after heparin addition is equal to the InsP_3-induced calcium release. The InsP_3-dependence of the rate constant during the rapid and slow phases are shown in Fig. 4*b*. Between 100 nM and 500 nM InsP_3, the efflux rate constant during the slow phase is 30-fold less than in the fast phase.

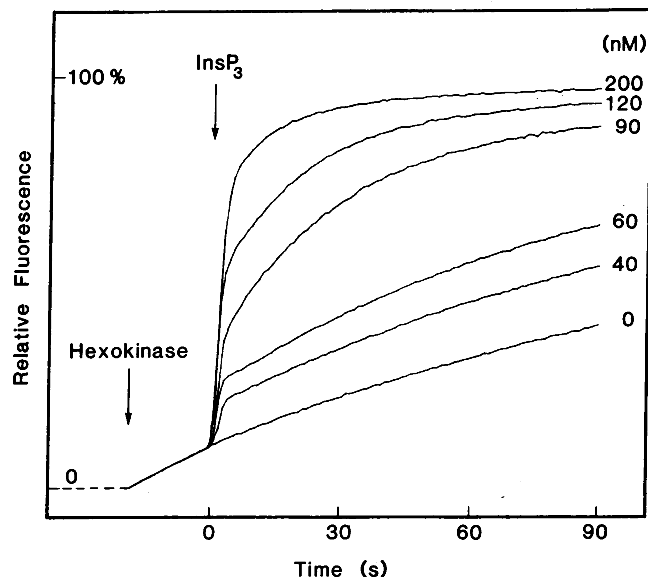

FIG. 3. Biphasic release kinetics in the absence of ATP. ATP levels were rapidly quenched by adding hexokinase. After 20 s, different amounts of InsP_3 were added, and calcium efflux was measured for 3 min. Saturating amounts of InsP_3 were then added to determine the total amount of InsP_3-releasable calcium.

Successive Additions of InsP_3 Lead to Short Pulses of Calcium Release. The calcium-release system in the slow-efflux phase is highly responsive to incremental additions of InsP_3. As shown in Fig. 1 *a* and *b*, the addition of a second dose of 90 nM InsP_3 elicits nearly as large an efflux as the first 90 nM dose. Multiple calcium bursts elicited by incremental additions of the same dose of InsP_3 are shown in Fig. 5. Nearly the same amount of calcium is released by a single addition of 200 nM InsP_3, two additions of 100 nM InsP_3, or four additions of 50 nM InsP_3. Although the calcium release mechanism nearly turns off after each addition of 50 nM or 100 nM InsP_3, it remains fully responsive to the next addition.

Sequential bursts of calcium release induced by successive additions of InsP_3 were also seen in PC-12 neuronal cells and hepatoma (human G2) cells (data not shown). PC-12 cells exhibit a less pronounced rapid phase and require higher levels of InsP_3 to elicit calcium release than do RBL cells. Hepatoma cells display strikingly biphasic calcium release. Indeed, an overshoot is observed. Epithelial cells (T84 and $9HTEO^-$) also gave a rectangular release profile. A more rounded but clearly biphasic calcium-release profile was exhibited by fibroblasts (L cells), smooth muscle (R22), and lymphocytes (Jurkat, transformed B cells). Thus, transient InsP_3-induced calcium release with full retention of responsiveness occurs in diverse cell types.

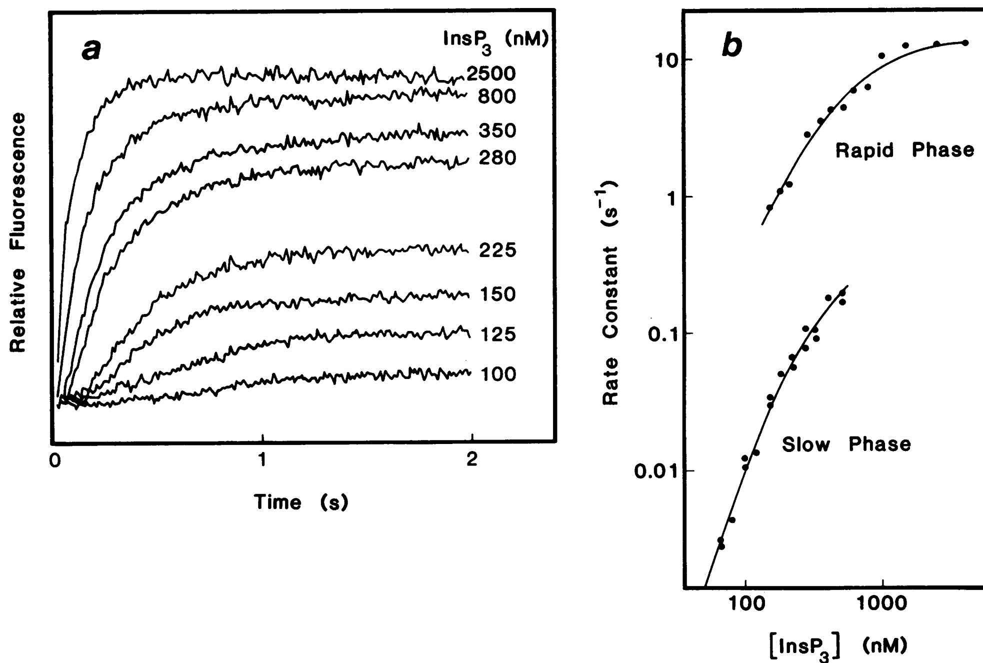

FIG. 4. Analysis of the rapid and slow phases of calcium release. ATP (0.7 mM) was present. (*a*) Rapid-mixing experiments in which the initial release kinetics after a rise in InsP_3 are resolved. (*b*) Release rate constants in the initial rapid and slow phases of the release process are compared. Rate constants for the rapid phase were determined from the initial calcium-release rate in rapid-mixing measurements, as in *a*. Rate constants for the slow phase were determined from the initial uptake rate after heparin addition to permeabilized cells preincubated with various amounts of InsP_3 (Fig. 1*d*). When heparin is added to a cell suspension at a steady state between calcium pump and calcium efflux, the block of the efflux through the InsP_3 channel leads to an initial calcium-uptake equivalent to the blocked flux.

DISCUSSION

The striking finding is that successive additions of InsP_3 induce short pulses of calcium release. After addition of InsP_3, a rapid efflux of calcium from internal stores is followed within a second by a 30-fold slower release. Changes in the Ca^{2+}-ATPase pump rate do not account for these phases. The calcium-release system during the slow phase remains highly responsive to further additions of InsP_3. The amount of calcium released by each of four additions of 50 nM InsP_3 is nearly the same. Furthermore, the total calcium released by these four additions is virtually the same as that released by a single 200 nM dose. *This full retention of responsiveness to increments of* InsP$_3$ *contrasts with previously observed inactivation or adaptation processes.* For example, the nicotinic acetylcholine receptor rapidly inactivates after the addition of ligand (18). The inactivated channel is not opened by subsequent addition of even a high dose of acetylcholine. Sensitivity is restored only after dissociation of bound ligands after a marked lowering of the acetylcholine level. The voltage-gated sodium channel is another example of a channel that becomes refractory after stimulation (19). The calcium-release system is like the acetylcholine receptor and the sodium channel in that stimulation leads to a transient response. However, the similarity ends there because subsequent additions of InsP_3 during the slow phase give nearly the same extent of calcium release as does the first dose. *Increment detection appears to be another mode of signal transduction.*

The simplest model for transient calcium release (10) would be a transition from a low-affinity open state to a high-affinity closed state, as exemplified by the acetylcholine receptor channel (18). This model predicts that subsequent additions of the same amount of InsP_3 will release little calcium, in contrast with our experimental findings (Fig. 1*a*, ref. 5). Greater retention of responsiveness could be obtained if channel closure were caused by a transition from a high-affinity open state to a low-affinity open state. A time-dependent lowering of InsP_3-binding affinity requires an external energy input, which could be provided by the binding of a ligand or protein to the occupied channel. Such a model would account for the rapid turn-off of calcium release after the first addition of InsP_3. It would also explain why calcium channels can be reopened by adding more InsP_3. This model further predicts that preincubation with InsP_3 will markedly reduce the response to subsequent additions of InsP_3. However, preincubation did not appreciably lower sensitivity (see Fig. 5). Hence, increment detection is probably not solely due to a transition within a single type of InsP_3 receptor.

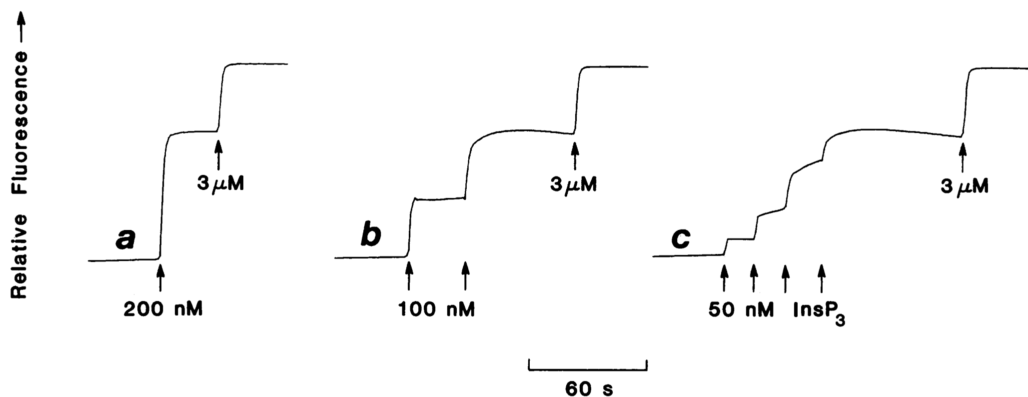

FIG. 5. Response of the InsP_3-sensitive calcium store to sequential additions of InsP_3. Most (65% of the stored calcium) is rapidly released after adding 200 nM InsP_3 (*a*). Additions of 2 × 100 nM (*b*) and 4 × 50 nM (*c*) lead to the same final fraction of calcium released. Successive additions of InsP_3 lead to bursts of calcium releases on the time scale of 1 s. For details, see Fig. 1.

It has been proposed that there are multiple calcium stores, each of which can be activated in an all-or-none fashion (9). Increment detection could occur if (*i*) the $InsP_3$ thresholds of different stores are closely spaced and span the range of responsiveness and (*ii*) each store is unloaded over a narrow range of $InsP_3$ concentration. The first criterion could be met by having stores that differ widely in the number of $InsP_3$-gated channels or in their affinity for $InsP_3$. The second criterion could be fulfilled by having a highly cooperative release process. However, the observed degree of cooperativity, corresponding to a Hill coefficient of 3–4 (3, 5), is not sufficient. All-or-none calcium release from individual stores would require an even higher degree of cooperativity or a molecular interplay that imposes a steep threshold. In short, increment detection cannot be readily explained by currently known properties of the intracellular calcium store.

Three responses—inactivation, adaptation, and increment detection—of signal transduction systems are depicted in Fig. 6. All three give transient responses to sustained stimuli. A doubling of the stimulus intensity has virtually no effect in systems that undergo inactivation. A stepwise increase in stimulus intensity leads to a partial response in systems that undergo adaptation (desensitization) as exemplified by vision (20) and bacterial chemotaxis (21). Increment detection is distinctive in that full responsiveness is retained. The three signal-transduction modes serve different biological needs. Inactivation leads to an unresponsive state that can be reset only when the stimulus intensity is markedly lowered. Inactivation is used in systems that detect large and rapid changes in stimulus intensity, such as a change in concentration of acetylcholine in the synaptic cleft from 10^{-8} M to 3×10^{-4} M. Adaptation enables a sensory system to respond effectively over a wide dynamic range of stimulus intensity. For example, amphibian retinal rod cells can detect incremental stimuli over a 10^5-fold range of background light levels (20). Likewise, bacteria sense gradients of chemoattractants over several orders of magnitude. In vision and chemotaxis, the response is approximately proportional to $\Delta S/S$, where ΔS is the increment and S is the background stimulus level. In contrast, the response in increment detection is proportional to ΔS independent of S. The dynamic range, however, is much smaller. In increment detection, the background level is ignored. This feature may be advantageous in generating receptor-triggered calcium oscillations. It will be challenging to unravel the molecular basis of increment detection and delineate its contribution to calcium spiking.

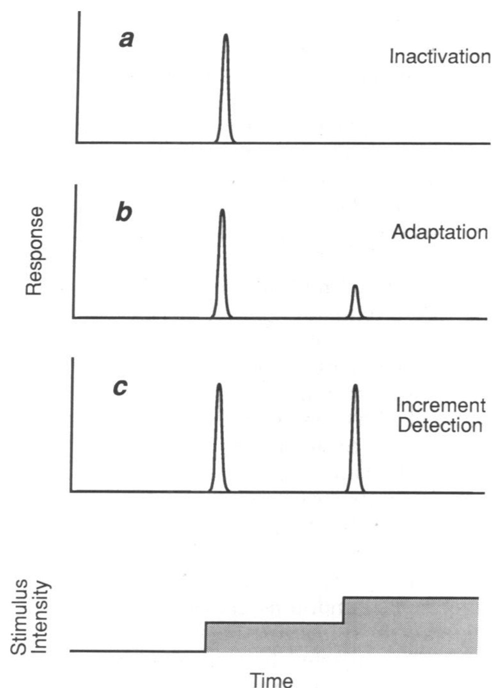

FIG. 6. Schematic diagram of different response patterns to stepwise increases in stimulus intensity. (*a*) Inactivation. (*b*) Adaptation. (*c*) Increment detection.

We thank Drs. D. A. Baylor, R. L. Brown, W. Catterall, J. Karpen, A. Kindman, R. D. Kornberg, R. W. Tsien, N. Unwin, J. Wagner, and T. Wensel for stimulating discussions. This work was supported by grants from the National Institute of General Medical Sciences (GM24032 and GM30387), the National Institute of Mental Health (MH 45324), and the Cystic Fibrosis Foundation. T.M. is a Swiss National Science Foundation Fellow.

1. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193.
2. Putney, J. W., Jr. (1987) *Am. J. Physiol.* **252**, G149–G157.
3. Meyer, T., Holowka, D. & Stryer, L. (1988) *Science* **240**, 653–656.
4. Ferris, C. D., Huganir, R. L., Supattapone, S. & Snyder, S. (1989) *Nature (London)* **342**, 87–89.
5. Meyer, T., Wensel, T. & Stryer, L. (1990) *Biochemistry* **29**, 32–37.
6. Woods, N. M., Cuthbertson, K. S. & Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602.
7. Jacob, R., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1988) *Nature (London)* **355**, 40–45.
8. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69.
9. Muallem, S., Pandol, S. J. & Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212.
10. Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S. & Claret, M. (1989) *J. Biol. Chem.* **264**, 17665–17673.
11. Minka, A., Kao, J. P. & Tsien, R. Y. (1989) *J. Biol. Chem.* **264**, 8171–8178.
12. Meyer, T. & Stryer, L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5051–5055.
13. Pollard, T. D. (1984) *J. Cell Biol.* **99**, 769–777.
14. Hill, T. D., Berggren, P. O. & Boynton, A. L. (1987) *Biochem. Biophys. Res. Commun.* **149**, 897–901.
15. Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, J. & Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8747–8750.
16. Danoff, S. K., Supattapone, S. & Snyder, S. H. (1988) *Biochem. J.* **254**, 701–705.
17. Parker, I. & Ivorra, I. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 260–264.
18. Colquhoun, D. & Sakmann, B. (1985) *J. Physiol. (London)* **369**, 501–557.
19. Aldrich, R. W., Corey, D. P. & Stevens, C. F. (1983) *Nature (London)* **306**, 436–441.
20. Dowling, J. E. (1987) in *The Retina* (Belknap, Cambridge, MA), pp. 187–223.
21. Dahlquist, F. W., Lovely, P. & Koshland, D. E., Jr. (1972) *Nature (London) New Biol.* **236**, 120–123.