Two Inositol 1,4,5-Trisphosphate Binding Sites in Rat Basophilic Leukemia Cells: Relationship between Receptor Occupancy and Calcium Release†

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ABSTRACT: Quantal calcium release is a novel paradigm for second messenger signal transduction which provides spatial and temporal control of calcium release from intracellular stores by inositol 1,4,5-trisphosphate ($InsP_3$). We have proposed a mechanism to account for this phenomenon [Kindman, L. A., & Meyer, T. (1993) *Biochemistry 32*, 1270−1277], which hypothesized the existence of five channels, each with a different affinity for $InsP_3$. As a direct test of this hypothesis, $InsP_3$ binding to microsomes from RBL cells was examined under conditions similar to those used for calcium release. Scatchard analyses performed under a variety of conditions indicates the presence of high affinity ($K_D = 0.9 \pm 0.3$ nM) and low affinity ($K_D = 47 \pm 5$ nM) $InsP_3$ binding sites. The low affinity sites are more prevalent, constituting 82 ± 5% of the total. Both sites are identified in the presence and absence of MgATP. Moreover, both sites are selective for $InsP_3$ over $InsP_4$, though high concentrations of $InsP_4$ displace $InsP_3$ from each site (with inhibition constants of 16 and 267 nM $InsP_4$, respectively). The relative abundance of the two $InsP_3$ binding sites is Ca^{2+} dependent. An increase in Ca^{2+} from 0.1 to 0.5 μM results in the apparent conversion of a portion of the low affinity sites into high affinity sites. Ca^{2+} (0.5 μM) also increased the K_D of the low affinity $InsP_3$ binding site. Given the presence of both high and low affinity $InsP_3$ binding sites, two simple mathematical models describing both the kinetics of calcium release and quantal calcium release from RBL cells were developed. Each model assumes that the two types of $InsP_3$ receptors interact randomly to form five different calcium channels (i.e., two homotetramers and three heterotetramers), with a distribution reflective of the relative abundance of the two binding sites. In the first model, binding of three or four molecules of $InsP_3$ to any of the five channel types is sufficient to open the channel. In the second model, $InsP_3$ binding to two or three low affinity binding sites only will open the channel. This latter model predicts that channels composed of three or four high affinity $InsP_3$ binding sites do not contribute to Ca^{2+} release. Given the Ca^{2+} dependence of the conversion between low affinity and high affinity $InsP_3$ binding sites, the latter model predicts the desensitization of some of the channels following elevation of cytosolic Ca^{2+}. Neither model requires cooperativity, consistent with the lack of cooperativity in the $InsP_3$ binding assays.

Signaling by inositol 1,4,5-trisphosphate ($InsP_3$)[1] is highly regulated spatially, temporally, and mechanistically (Meyer, 1991). A novel feature of $InsP_3$-gated Ca^{2+} release from permeabilized cells and from isolated Ca^{2+} stores is that it occurs in a quantal fashion (Muallem et al., 1989; Meyer & Stryer, 1990; Kindman & Meyer, 1993). Additions of small amounts of $InsP_3$ results in the release of linearly proportional amounts of Ca^{2+}, over a defined physiologic range. Recently, we proposed a model to account for the mechanism of quantal Ca^{2+} release (Kindman & Meyer, 1993). We suggested that this apparently complex phenomenon could be readily explained by the interaction of several well described properties of the system. It is known, for example, that the $InsP_3$-gated Ca^{2+} channels are associated with structurally and functionally heterogeneous Ca^{2+} stores (Volpe et al., 1991; Takei et al., 1992; Kasai et al., 1993; Renard-Rooney et al., 1993) and that the binding of more than one molecule of $InsP_3$ may be required to open the channel (Meyer et al., 1990). Several isoforms of the $InsP_3$ receptor have been cloned (Furuichi et al., 1989; Mignery et al., 1989; Südhoff et al., 1991; Ross et al., 1992; Blondel et al., 1993), and multiple receptor isoforms may be found in a given cell type (Ross et al., 1992). The isoform(s) of the $InsP_3$ receptor in RBL cells have not been identified, although preliminary evidence suggests the presence of both type 1 and type 3 isoforms (A. R. Maranto, personal communication).

With our earlier model for quantal calcium release, we used iterative computation to identify a satisfactory fit to the experimental data. In this report, we now demonstrate the presence of one high and one low affinity $InsP_3$ binding site in RBL cells. Given the tetrameric structure of the $InsP_3$-

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperizine-*N′*-2-ethanesulfonic acid; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; RBL, rat basophilic leukemia cell line.

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gated calcium channel (Chadwick et al., 1990), we developed refined mathematical models to describe calcium release. The models are based on open probability distributions. Two models yield excellent fits to both the kinetics of calcium release (Meyer et al., 1990), and the quantal calcium release data (Kindman and Meyer, 1993) and assume that both high and low affinity receptors combine randomly to form five types of channels (each containing four $InsP_3$ receptors). Consistent with all data obtained from ligand binding studies (Worley et al., 1987; Supattapone et al., 1988; Mohr et al., 1993), neither model of calcium release invokes cooperativity, though multiple $InsP_3$ binding events are required. One of the models includes a mechanism for desensitizing the $InsP_3$ gated Ca^{2+} channel and thus may more accurately predict the complex behavior of this channel *in vivo* (Hajnóczky & Thomas, 1994).

MATERIALS AND METHODS

Rat basophilic leukemia cells for the preparation of microsomes were grown by the Cell Culture Center, Minneapolis, MN, in roller bottles using 10% calf serum in Dulbecco's modified minimal essential media. At confluence, they were harvested in phosphate-buffered saline supplemented with 5 mM EDTA, pH 7.4. The cells were washed once with this media. Ten gram wet weight aliquots were frozen in liquid nitrogen and stored at −80 °C. Cells grown under these conditions were demonstrated to bear IgE F_c receptor using antibodies provided by Dr. David Holowka of Cornell University. Fresh RBL cells were grown in our laboratory under conditions described previously (Kindman & Meyer, 1993).

Microsomes were prepared by a modification of our previously described method (Watras & Benevolensky, 1987). Ten gram wet weight of frozen RBL cell pellet was suspended in 30 mL of 250 mM sucrose, 25 mM HEPES, pH 7.0, and homogenized with a Brinkmann Polytron. The suspension was centrifuged at 100000*g* for 30 min, and the pellet was resuspended in 250 mM sucrose, 25 mM HEPES, and 0.6 M KCl, pH 7.0. This suspension was centrifuged at 5000*g* for 20 min, and the supernatant was centrifuged at 100000*g* for 30 min. The resulting supernatant was discarded, and the tubes were inverted for 5 min to allow drainage of any remaining supernatant. The pellet was then suspended in 2 mL of 10 mM MOPS and 10% sucrose, pH 7.0, aliquoted, frozen in liquid N_2, and stored at −80 °C. The final protein concentration was 3 mg/mL, as determined by the method of Bradford (1976). Preparations of microsomes used in these studies typically stored 5 nmol of Ca^{2+}/mg of protein and released 1.5 nmol of Ca^{2+}/mg of protein in response to $InsP_3$.

Fluorometric calcium release assays using freshly isolated saponin-permeabilized cells and microsomes were performed as previously described (Kindman & Meyer, 1993). Cells (2×10^6) were suspended in 2.0 mL of buffer containing 20 mM HEPES-KOH, pH 7.40, 2 mM $MgCl_2$, 0.4 mM ATP, 0.1 mg/mL saponin, and the Ca^{2+} indicator fluo-3 (1 μM) at 37 °C to allow permeabilization and complete loading of Ca^{2+} stores. For efflux experiments performed at 22 or 11 °C, cells were rapidly chilled following loading by briefly dipping the cuvette in liquid nitrogen, as described previously (Kindman & Meyer, 1993).

$InsP_3$ binding was determined by a centrifugation technique (Benevolensky et al., 1994). RBL membranes were first diluted 1:10 in buffer A (20 mM HEPES, 5 mM NaCl, 130 mM KCl, 0.25 mM EGTA, pH 7.4) at 4 °C and centrifuged for 15 min at 16000*g*. The pellet (containing 90% of the protein) was resuspended in buffer A to give a final protein concentration of 5 mg/mL. $InsP_3$ binding was initiated by addition of membranes to buffer A containing 0.6−1200 nM [^{3}H]$InsP_3$, with or without ATP, $MgCl_2$, and 2,3-diphosphoglycerate, and $CaCl_2$ as specified. After an 8-min incubation at the specified temperature, samples were centrifuged at the incubation temperature at 16000*g* for 15 min. The supernatant was removed by aspiration. The pellets were dissolved in Soluene 350 and then assayed for radioactivity by liquid scintillation counting. Nonspecific binding was determined in the presence of 40−160 μM nonradioactive $InsP_3$.

Calculations of the total Ca^{2+} needed to obtain a given free Ca^{2+} concentration were based on previously published association constants (Fabiato, 1981), adjusted for pH 7.4 at 4 °C. The apparent association constants (M^{-1}) used for these calculations were 1.37×10^7 for Ca-EGTA, 7.619×10^3 for Ca-ATP, 5.57 for K-ATP, and 7.2 for Na-ATP. Mg^{2+} and 2,3-DPG were not included in the Ca^{2+} dependence studies.

Hydrolysis of $InsP_3$ during the binding reaction was assayed by anion exchange chromatography. Binding reactions were conducted as above. Reactions were terminated by addition of 7% trichloroacetic acid, followed by centrifugation at 16000*g* for 2 min. Trichloroacetic acid was extracted from the supernatant with hydrated diethyl ether (4 vol; 3×). The aqueous phase was then applied to a 2-mL Dowex-1 formate column, which was then washed with 12 mL of each of the following: (1) water (to elute inositol), (2) 0.1 M formic acid/0.2 M ammonium formate (to elute inositol monophosphate), (3) 0.1 M formic acid/0.4 M ammonium formate (to elute inositol bisphosphate), and (4) 0.1 M formic acid/1 M ammonium formate (to elute $InsP_3$). Two-milliliter fractions were collected and assayed for radioactivity by liquid scintillation counting.

Scatchard analysis of $InsP_3$ binding was performed using an iterative curve-fitting routine for two binding sites (SigmaPlot, Jandel Scientific). Curve-fitting for the $InsP_3$/$InsP_4$ competition analysis (Figure 3) was also performed using SigmaPlot (as described under Results). Curve-fitting of calcium release kinetics using SigmaPlot (Figure 5) involved the construction of several models (see Results). Each model was compared for its ability to fit both the calcium release kinetics (Figure 5) and quantal calcium release (Figure 6).

RESULTS

Identification of Two Distinct $InsP_3$ Binding Sites. Our previous work (Kindman & Meyer, 1993) predicted that the RBL cell should contain five distinct ligand binding sites for $InsP_3$. Under physiologic conditions, these sites were expected to have dissociation constants for $InsP_3$ ranging from nanomolar to micromolar. The presence of low affinity sites had not been sought in the RBL previously (Hershey et al., 1993; Mohr et al., 1993), and the dissociation constant of the high affinity site had not been determined under physiologic conditions. In Figure 1A, the $InsP_3$ binding media is comparable to that used to monitor calcium release (Kindman & Meyer, 1993), except that magnesium is omitted. Scatchard plots of $InsP_3$ binding are nonlinear but

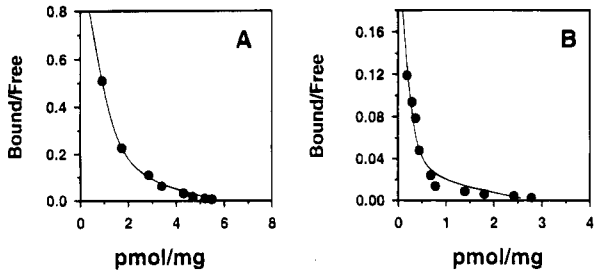

FIGURE 1: Scatchard analysis of $InsP_3$ binding to RBL microsomes. Binding of [^{3}H]$InsP_3$ was assayed in media containing 0.4 mM ATP with (panel B) or without (panel A) 2 mM $MgCl_2$ and 1.5 mM 2,3-diphosphoglyceric acid. Reactions were carried out as described under Materials and Methods (4 °C). These data were fit using a two-site model (see Table 1 for calculated values of dissociation constants).

Table 1: Calculated Dissociation Constants for $InsP_3$ Binding to RBL Membranes[a]

condition[b]	K_{D1}	K_{D2}	% B_2[c]
buffer (4 °C) ($n = 3$)	0.9 ± 0.3 nM (0.5−1.6 nM)	47 ± 5 nM (39−56 nM)	78 ± 2% (75−83)
buffer (22 °C) ($n = 1$)	4 nM	120 nM	80%
Mg^{2+} and DPG (4 °C) ($n = 3$)	1.6 ± 0.09 nM	146 ± 21 nM	87 ± 1%

[a] The numbers in parentheses in column 1 represent the number of experiments. Numbers in parentheses in columns 2−4 represent range of values. [b] All assays included buffer A [20 mM HEPES, 5 mM NaCl, 130 mM KCl, 0.25 mM EGTA, 0.4 mM ATP, pH 7.4 (4 or 22 °C; as specified)], with or without 2 mM $MgCl_2$ and 1.5 mM DPG. [c] $\%B_2$ was calculated at the ratio of $B_2/(B_1 + B_2)$, where B_1 and B_2 represent the densities of high affinity and low affinity $InsP_3$ binding sites, respectively.

can be fit assuming the presence of two binding sites. The calculated dissociation constants for these sites is 0.9 ± 0.3 nM and 47 ± 5 nM (Table 1; 4 °C). When the binding and centrifugation is performed at room temperature (22 °C), the dissociation constants are higher (4 nM and 120 nM; Table 1). From these data we calculate a Q_{10} of 1.68 for the dissociation constants of the two $InsP_3$ binding sites.

In an effort to closely approximate the conditions used for calcium release, we have also assayed $InsP_3$ binding in the presence of 2 mM $MgCl_2$. Magnesium is critical for the calcium flux experiments, but it can activate an endogenous phosphatase (Watras & Benevolensky, 1987). As shown in Figure 2A, in the time required to complete the assay, 2 mM magnesium results in a substantial (20%) hydrolysis of $InsP_3$. We have previously shown that diphosphoglycerate (DPG; 1.5 mM) inhibits $InsP_3$ hydrolysis (Watras & Benevolensky, 1987), and, as shown in Figure 2B, 1.5 mM DPG reduces $InsP_3$ hydrolysis substantially. Over the $InsP_3$ concentration range 3−150 nM, less than 5% of the added $InsP_3$ is hydrolyzed during the binding assay when 1.5 mM DPG is included with 2 mM Mg^{2+} (Figure 2C,D). For analysis of $InsP_3$ binding in the presence of 2 mM Mg^{2+}, 1.5 mM DPG was therefore included in the binding assay.

As shown in Figure 1B, a Scatchard plot of $InsP_3$ binding to RBL microsomes in the presence of Mg^{2+} and DPG is also nonlinear. The data can be fit assuming the presence of both high affinity and low affinity binding sites (K_D = 1.6 and 146 nM; Table 1). These dissociation constants are higher than those calculated in the absence of Mg^{2+} and

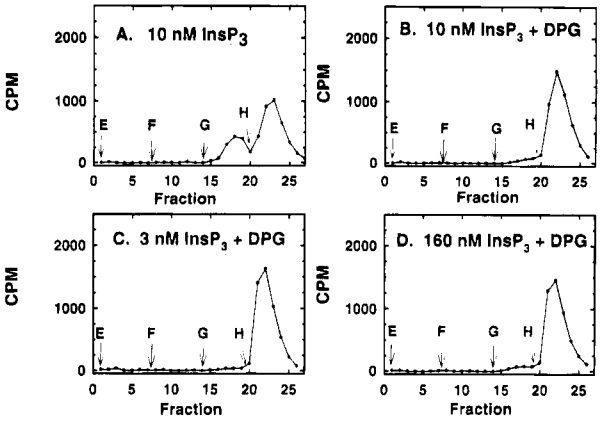

FIGURE 2: Hydrolysis of $InsP_3$ is prevented by the addition of 1.5 mM 2,3-diphosphoglyceric acid. RBL microsomes were incubated for 8 min in media containing 2 mM $MgCl_2$, and 3−160 nM [^{3}H]-$InsP_3$ ± 1.5 mM 2,3-diphosphoglyceric acid (DPG; as specified), and then the extent of $InsP_3$ hydrolysis was determined by anion exchange chromatography (as described under Materials and Methods). Symbols E, F, G, H represent washes with water or various concentrations of ammonium formate (0.2, 0.4, 1 M) to elute inositol, inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, respectively.

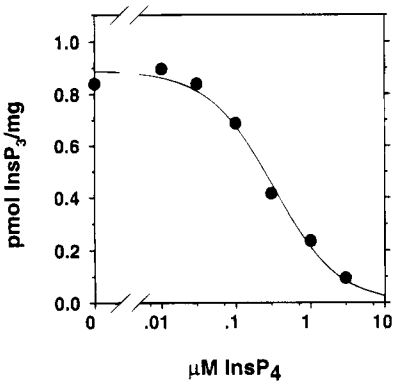

FIGURE 3: Competition of $InsP_3$ binding by $InsP_4$. Binding media included 2 mM $MgCl_2$, 0.4 mM ATP, 1.5 mM 2,3-diphosphoglyceratic acid, 30 nM [^{3}H]$InsP_3$, and 0−3.0 μM $InsP_4$. A curve-fitting routine was used to calculate the dissociation constant for the low affinity $InsP_3$ binding site (151 nM) and the inhibition constants for $InsP_4$ (16 and 267 nM) as indicated in the text.

DPG. The discrepancy may reflect an inhibition of $InsP_3$ binding by the high concentration of DPG used in this assay, as addition of 1.5 mM DPG alone also results in a similar shift in Ca^{2+} release by $InsP_3$ (not shown).

Both High and Low Affinity $InsP_3$ Binding Sites also Bind $InsP_4$. To further characterize the pharmacological properties of the two $InsP_3$ binding sites, we examined the influence of inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) at these sites. $InsP_3$ binding to RBL microsomes was measured at 4 °C in the presence of 30 nM $InsP_3$ and 0−3.0 μM $InsP_4$, with 2 mM $MgCl_2$ plus 1.5 mM DPG (as in Figure 1B). The $InsP_3$ concentration (30 nM) was chosen on the basis of the $InsP_3$ dissociation constants (1.6 and 146 nM) and the relative abundance of the two $InsP_3$ binding sites (viz., 87% low affinity sites; Table 1) so that approximately half (54%) of the [^{3}H]$InsP_3$ binding was to low affinity sites in the absence of $InsP_4$. As shown in Figure 3, $InsP_4$ (3 μM) reduces [^{3}H]-$InsP_3$ binding over 90%, indicating that $InsP_3$ binding to both the high affinity and low affinity sites is inhibited. A curve-fitting algorithm was then applied to the data to determine

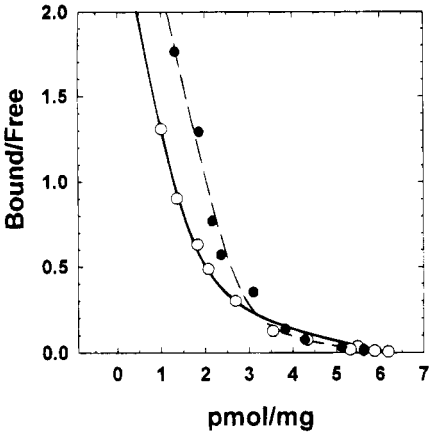

FIGURE 4: Submicromolar calcium converts low affinity $InsP_3$ binding sites into high affinity sites. $InsP_3$ binding was performed as in Figure 1A, with either 0.1 μM (open circles) or 0.5 μM (filled circles) free Ca^{2+}. Lines represent the best fit assuming a two-site model as described in the text.

the K_i for $InsP_4$ at both $InsP_3$ binding sites:

$$B = \frac{B_1 S}{K_{D1} + S + \frac{IK_{D1}}{K_{i1}}} + \frac{B_2 S}{K_{D2} + S + \frac{IK_{D2}}{K_{i2}}}$$

where B_1 and B_2 represent the densities of the high affinity and low affinity sites, S is the $InsP_3$ concentration, and I is the $InsP_4$ concentration. K_{D1} and K_{D2} represent the $InsP_3$ dissociation constants for the high affinity and low affinity sites, respectively. K_{i1} and K_{i2} are the $InsP_4$ inhibition constants for the high affinity and low affinity sites, respectively. In this curve-fitting routine, the densities of the low affinity and high affinity sites are given (0.13 and 0.87), along with the $InsP_3$ dissociation constant for the high affinity site (1.6 nM). The dissociation constant for the low affinity $InsP_3$ binding site was then calculated, along with the two inhibition constants. The predicted dissociation constant for the low affinity $InsP_3$ binding site (151 nM) is similar to that calculated directly from the saturation isotherms in the presence of DPG (Figure 1B and Table 1; 146 nM). The predicted $InsP_4$ inhibition constant for the low affinity site (267 nM $InsP_4$) is 77% higher than the dissociation constant for $InsP_3$ binding, distinguishing it from the "polyphosphate receptor" described previously (Chadwick et al., 1992; Kijima & Fleischer, 1992). The predicted $InsP_4$ inhibition constant for the high affinity site (16 nM), on the other hand, is 10 times higher than the dissociation constant for $InsP_3$ binding (1.6 nM), indicating that the high affinity binding site shows a high degree of selectivity for $InsP_3$, as previously observed (Supattapone et al., 1988).

Effect of Ca^{2+} on $InsP_3$ Binding. Ca^{2+} (10 μM) has been shown to increase $InsP_3$ binding to RBL cell membranes (Hershey et al., 1993). Figure 4 shows that increasing the free Ca^{2+} concentration from 0.1 to 0.5 μM results in an increase in $InsP_3$ binding at low $InsP_3$ concentrations. This is not due to an increase in the total number of $InsP_3$ binding sites but instead involves an apparent redistribution of high and low affinity sites. In 0.1 μM Ca^{2+}, 27% of the total binding sites were of high affinity (K_{D1} = 0.7 nM); whereas in 0.5 μM Ca^{2+}, the relative abundance of high affinity sites is 44% (K_{D2} = 0.8 nM). The curve fitting routine also suggests that this elevation in free Ca^{2+} increases the K_D of the low affinity site (K_{D2} increases from 26 to 67 nM). Thus, the K_D of the high affinity form of the $InsP_3$ receptor is Ca^{2+} insensitive, whereas the K_D of the low affinity form is Ca^{2+} sensitive. We have also observed this for $InsP_3$ binding to the sarcoplasmic reticulum of aortic smooth muscle (Benevolensky et al., 1994). An important distinction between the aortic smooth muscle sarcoplasmic reticulum and the RBL microsomes, however, is that some of the RBL low affinity sites are converted to higher affinity binding sites.

We also compared $InsP_3$ binding in 10 μM free Ca^{2+} with that in Ca^{2+}-free media. Consistent with the data shown in Figure 4, there is an increase in $InsP_3$ binding at low $InsP_3$ concentrations, and this is attributable to a Ca^{2+}-dependent increase in the relative amount of the high affinity $InsP_3$ binding sites. There is no change in the total number of $InsP_3$ binding sites. The relative amount of high affinity sites (K_{D1} = 0.6 nM) increases form 17% in the Ca^{2+}-free media to 45% (K_{D1} = 0.7 nM) in the presence of 10 μM free Ca^{2+}. Moreover, the K_D of the low affinity $InsP_3$ binding site increases over this Ca^{2+} range (i.e., K_{D2} = 39 nM in the Ca^{2+} free media; K_{D2} = 69 nM in 10 μM free Ca^{2+}). Thus, it appears that a further increase in the free Ca^{2+}, from 0.5 to 10 μM, has little additional effect on either the relative abundance of the high affinity sites or on the K_D of the low affinity site.

Use of $InsP_3$ Binding Data To Describe Calcium Release. Calcium release from rat basophilic leukemia cells has been described as cooperative, with a Hill coefficient of ≥ 3 (Meyer et al., 1988, 1990). Results from the our ligand binding studies, however, indicate that both high and low affinity $InsP_3$ binding sites are present in this preparation. There may thus be at least two types of calcium channels, and each of these could exhibit cooperativity as one of the four subunits within the channel complex. Consequently, efforts were made to fit the calcium release data of Meyer et al. (1990) to a two-site model. In this curve-fitting routine, we assume that there is only one channel per Ca^{2+} store and that activation of the channel releases the contents of this store as a quanta of calcium. The basic equation used in this curve-fitting routine is shown below:

$$\text{rel} = \text{rel}_{max}\left[\text{HA}\frac{S^{n_1}}{K_{D1}^{n_1} + S^{n_1}} + (1 - \text{HA})\frac{S^{n_2}}{K_{D2}^{n_2} + S^{n_2}}\right]$$

where rel = rate constant for calcium release; S = $InsP_3$ concentration; n_1 and n_2 are the Hill coefficients for the opening of the high and low affinity channels, respectively; K_{D1} and K_{D2} are the dissociation constants for the high and low affinity channels; HA = relative abundance of the high affinity sites; and r_{max} is the maximal rate constant of calcium release. The calcium release experiments of Meyer et al. were conducted at 11 °C; thus we adjusted the value of k_1 from 1 to 1.5 nM (assuming a Q_{10} of 1.68). The maximal rate of calcium release (r_{max}) was set at 5 s^{-1}. Given the variability in the observed values for the relative abundance of the low affinity site and the dissociation constant for this site (cf. Table 1), the values of HA, K_{D2}, n_1, and n_2 which yielded the best fit of the calcium release data were calculated. Initially, a constraint that the high affinity site could represent 13−25% of the total binding sites was included in the curve-fitting routine (consistent with the data in Table 1), but this yielded a dramatic overestimate of the calcium release at low $InsP_3$ concentrations (not shown). When this constraint was removed, the high affinity site was

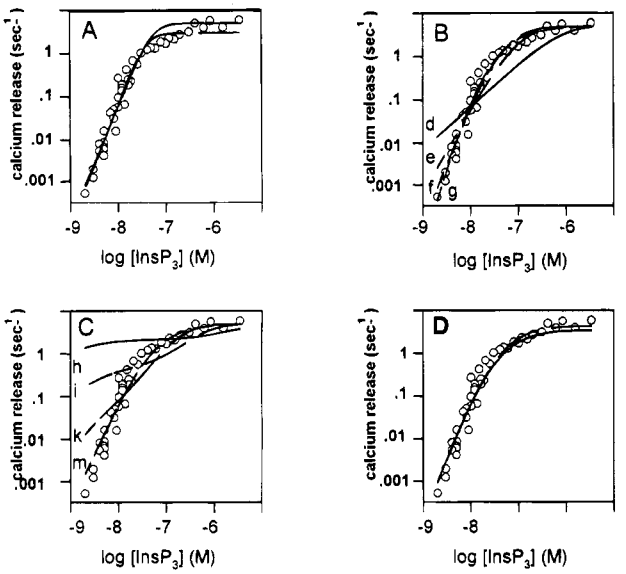

FIGURE 5: Comparison of four models of calcium release. The open circles represent data of Meyer et al. (1990) and show the rate constant of calcium release from RBL cells as a function of $InsP_3$ concentration. The model in panel A assumes that the channel is composed of four low affinity receptors and that opening is a cooperative process (calculated Hill coefficient = 2.7−2.9). The model in panel B also assumes that the channel is composed of four low affinity receptors but opening is not cooperative. Opening requires the binding of at least one (d), two (e), three (f), or four (g) molecules of $InsP_3$. Panel C shows fits obtained if the high and low affinity receptors associate randomly and if channel opening is noncooperative, again requiring the binding of at least one (h), two (i), three (k) or four (m) molecules of $InsP_3$. The model in panel D also assumes random association of the receptors but requires the binding of $InsP_3$ to at least three low affinity sites to open the channel. The top and bottom traces in panel D assume that the high affinity sites represent 16 and 27% respectively of the total $InsP_3$ binding. See the text for details.

predicted to make a negligible contribution to Ca^{2+} release (HA < 0.01%). We therefore omitted the participation of the high affinity site in this curve-fitting routine by setting HA to zero and obtained the fit in Figure 5A (top trace). The calculated affinity is K_{D2} = 52 nM, with a Hill coefficient of 2.7. The bottom trace in this figure represents the best fit when the maximal rate constant of calcium release was determined by the curve-fitting routine. In this case, the calculated affinity is 37 nM, with a Hill coefficient of 2.9.

As an alternative to the cooperativity model, we also prepared curve-fitting routines to determine if noncooperative binding of up to four molecules of $InsP_3$ to a channel could explain the kinetics of calcium release. We assumed that two types of calcium channels, high affinity and low affinity, were present. Four curve-fitting routines were then developed to determine the fit assuming that one, two, three, or four molecules of $InsP_3$ were needed to open the channel. The basic assumption in each routine was that $InsP_3$ binding is random and thus can be predicted by polynomial distributions:

$$(p_1 + q_1)^4 = 1$$

$$(p_2 + q_2)^4 = 1$$

where p_1 and p_2 represent the probabilities of $InsP_3$ binding to the high and low affinity binding sites, respectively. The terms q_1 and q_2 represent the probabilities of these receptors being unoccupied by $InsP_3$. In the expanded form of the polynomials, each term describes the relative amount of channels with the number of occupied and free sites corresponding to the exponents of p and q, that is $4p^3q_1$ gives the relative amount of high affinity tetramers with three occupied and one unoccupied sites. The values of p_1, p_2, q_1, and q_2 are calculated from the Michaelis−Menton equation as follows:

$$p_1 = \frac{S}{K_{D1} + S} \quad p_2 = \frac{S}{K_{D2} + S}$$

$$q_1 = 1 - p_1 \quad q_2 = 1 - p_2 \quad (1)$$

For a mixture of high and low affinity tetramers of relative abundances HA and LA, respectively, $InsP_3$ binding is described by expansion of:

$$\mathrm{HA}(p_1 + q_1)^4 + (1 - \mathrm{HA})(p_2 + q_2)^4 = 1$$

since HA + LA = 1. The curve-fitting routines included the value of K_{D1} (1.4 nM) and a constraint that HA (the relative amount of high affinity sites) must be between 13 and 24% (as suggested from Table 1). The values of K_{D2} and HA which best fit the calcium release data were then calculated. Again, the fits were poor and always overestimated the extent of calcium release at low $InsP_3$ concentrations. When the constraint regarding HA was eliminated, the curve-fitting routine predicted a near zero level of high affinity $InsP_3$-gated calcium channels, again suggesting that the high affinity $InsP_3$ receptor does not contribute to calcium release. We therefore set HA to 0 and found that excellent fits of the calcium release were obtained when binding of three or four molecules of $InsP_3$ are needed to open the channel (Figure 5B, traces f and g) and predicted values for K_{D2} of 56 and 19 nM, respectively. Thus, both the cooperativity model (shown in Figure 5A) and the noncooperative model (shown in Figure 5B) provide excellent fits of the calcium release data, but only when the high affinity site is predicted to *not* participate in Ca^{2+} release. Approximately three molecules of $InsP_3$ appear to be needed to open the low affinity channel.

In a third model, we assumed that the high and low affinity receptors could associate randomly to form the tetrameric channel complex. The mathematical description of $InsP_3$ binding in this case is based on the assumptions that (i) formation of the tetrameric complexes is purely random, governed by the relative amounts of the two monomers (HA and LA) and (ii) binding of $InsP_3$ to each monomer is completely independent of binding to other monomers in the same complex (i.e., noncooperative). We will now derive the equations for the general case of n-mers resulting from random association of two types of monomers.

The relative amount of any of the $n + 1$ possible types of n-mers resulted from combinations of two different monomers is calculated from the expansion of the polynomial (HA + LA)^n = 1 and is

$$r^h = \binom{n}{h}\mathrm{HA}^h(1 - \mathrm{HA})^{n-h} \quad (2)$$

for a n-mer with h high affinity sites and $(n - h)$ low affinity sites. The probabilities of monomers being occupied (p) or unoccupied (q) are calculated as before from eq 1. Occupancy of sites among all types of n-mers is calculated from

the sum of the polynomials describing binding statistics in each type of n-mer:

$$\sum_{h=0}^{n} r_h (p_1 + q_1)^h (p_2 + q_2)^{n-h} = 1$$

Expansion of these polynomials will yield

$$\sum_{\alpha=0}^{h} \sum_{\beta=0}^{n-h} \binom{h}{\alpha} p_1^{\alpha} q_1^{h-\alpha} \binom{n-h}{\beta} p_2^{\beta} q_2^{(n-h)-\beta} = 1$$

where α and β are the numbers of occupied sites of high and low affinity, respectively, in each of the n-mers having h high affinity sites and $(n - h)$ low affinity sites. Grouping terms by the total number of n-mer channels having any given number b of total bound molecules:

$$o_b = \sum_{h=0}^{n} r_h \sum_{i=0}^{\min(h,b)} \binom{h}{i} p_1^i \binom{n-h}{b-i} p_2^{b-i} \qquad (3)$$

If b molecules of $InsP_3$ are needed to open a channel, then all channels with *b or more* occupied sites will be open. Assuming that there is only one channel per microsome and that opening of the channel releases the contents of that microsome, the relative rate of Ca^{2+} release will be proportional to the number of open channels:

$$\text{rel} = \text{rel}_{\max}(o_b + o_{b+1} + \ldots + o_n) \qquad (4)$$

For our particular case of tetramers ($n = 4$), five distinct types of channels could be present, composed of four high affinity receptors, three high affinity and one low affinity receptor, two high affinity and two low affinity receptors, one high affinity and three low affinity receptors or four low affinity receptors, respectively. If the low affinity sites represent 87% of the total (i.e., HA = 0.13 and LA = 0.87), then the relative amounts of tetramers calculated from (2) and expressed as percentiles are 0.03%, 0.76%, 7.68%, 34.24%, and 57.29% respectively.

We wrote several curve fitting routines based on eqs 3 and 4 which would predict the Ca^{2+} release data of Meyer and Stryer (Meyer et al., 1990) if at least b $InsP_3$ receptors had to be occupied to open the channel (where b was varied between 1 and 4). The maximal Ca^{2+} release rate was set at 5 s^{-1} and K_{D1}(the dissociation constant of the high affinity site was set at 1.6 nM. The dissociation constant K_{D2} and the relative abundance of the low affinity site (LA) which would best fit the calcium release data were then computed. A constraint in the program required that LA must fall within the range 0.75−0.87 (consistent with the range in Table 1).

The first curve fitting routine calculated the values of K_{D2} and LA which best fit the calcium release data assuming that at least one molecule of $InsP_3$ was needed to open the channel ($b = 1$). As shown in Figure 5C, trace h, this yielded a very poor fit of the data. A good fit of the data was obtained, however, when four molecules were needed to open the channel (trace m). The predicted dissociation constant of the low affinity site in trace m was 29 nM, and the predicted LA was 0.87. Thus all three models shown in Figure 5A−C yield good fits to the calcium release data, with a predicted $InsP_3$ affinity close to the measured dissociation constant for $InsP_3$ binding. However, only the third model, which assumes random association of high and low affinity receptors, proposes that both the low *and* the high affinity receptors participate in Ca^{2+} release.

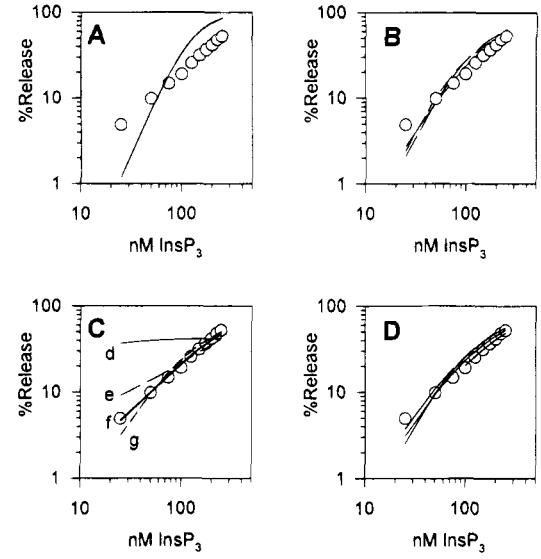

FIGURE 6: Modeling quantal calcium release data. The four models of calcium release shown in Figure 5 were applied to the quantal calcium release data of Kindman and Meyer (1993). (A) Cooperativity model (assuming Hill coefficient = 2.9); (B) noncooperativity model, assuming homotetramers of low affinity $InsP_3$ receptors with binding of at least two, three, or four molecules of $InsP_3$ needed to open the channel. (C) Noncooperative model, assuming random association of high and low affinity $InsP_3$ receptors, with binding of at least one (d), two (e), three (f), or four (g) molecules of $InsP_3$ needed to open the channel. (D) Variation of model C, where binding of $InsP_3$ to at least two (top trace) or three (bottom trace) low affinity receptors is required to open the channel. See the text for details.

To include Ca^{2+} sensitivity as a factor in the kinetics of Ca^{2+} release, we then applied the same approach as described for Figure 5C, but added the additional constraint that only binding to low affinity sites would result in channel opening. We presumed that the shift of low affinity to high affinity reflects a mechanism by which the channel could be inactivated (Pietri et al., 1990). As shown in Figure 5D, an excellent fit of the kinetics of $InsP_3$-gated Ca^{2+} release was obtained when $InsP_3$ is bound to three low affinity sites. Assuming a relative abundance of the low affinity site is 73% (as observed at 0.1 μM Ca^{2+}), the predicted K_{D2} of the low affinity site is 38 nM. In Ca^{2+}-free media, where the relative abundance of the low affinity site is 84%, the predicted K_{D2} is 45 nM.

We next determined which of these models could fit the quantal release data previously reported by Kindman and Meyer (1993). Figure 6 shows the extents of calcium release following a series of $InsP_3$ additions. Eight serial additions of 25 nM $InsP_3$ were made to a cuvette containing permeabilized RBL cells. Only the immediate release of calcium after addition of $InsP_3$ was used in these calculations. A small persistent leak of calcium following addition of $InsP_3$ was calculated to be due to dissociation of $InsP_3$ from one channel complex, followed by binding to another (not shown). All of the release data were corrected for this small leak.

The first model tested for its ability to fit the quantal calcium release data was the cooperativity model described above. The curve-fitting routine was basically as described for Figure 5A. Only channels composed exclusively of low affinity receptors were considered in this curve-fitting routine.

On the basis of the results of Figure 5A, we also included a constraint that the Hill coefficient was 2.9. The value of K_{D2} which yielded the best fit of the quantal calcium release was calculated. As shown in Figure 6A, this cooperativity model did not fit the quantal calcium release data. Thus, although this cooperativity model could accurately predict the kinetics of calcium release at 11 °C, it could not explain the quantal calcium release at 37 °C.

The second model tested was similar to that used in Figure 5B, where only homotetramers are present. This model also assumed that only the low affinity receptors were active but that $InsP_3$ bound noncooperatively to two, three, or four receptors. Curve fits assuming that $InsP_3$ must bind to at least two, three, or four receptors to open the channel are shown. None of these fits, however, provided an adequate fit of the quantal calcium release data (Figure 6B).

A model similar to that shown in Figure 5C, where high and low affinity receptors combine *randomly* to form tetrameric complexes, was also tested. This model predicts the existence of five channel types (with zero, one, two, three, or four low affinity receptors). Both high and low affinity $InsP_3$ receptors are assumed to participate in the calcium release. The curve-fitting routine included the dissociation constant of the high affinity binding site (K_{D1}), which was calculated to be 4.7 nM [assuming a Q_{10} of 1.68 (Table 1)]. The dissociation constant of the low affinity site (K_{D2}) and the relative abundance of this site which best fit the quantal calcium release data were then calculated. As shown in Figure 6C, a good fit of the calcium release data was observed if at least three or four molecules of $InsP_3$ are needed to open the channel. The predicted dissociation constants are 228 nM if three $InsP_3$ molecules are needed to open the channel and 77 nM if four $InsP_3$ molecules are needed. As the Ca^{2+} release experiments were performed in the presence of ~0.1 μM free Ca^{2+}, the $K_{D2}$of the low affinity site at 4 °C would be expected to be 26−45 nM (see Figure 4 and Table 1). Taking into account the Q_{10} of 1.68 for the K_D of this site, the K_{D2} is expected to be 144−260 nM at 37 °C. The K_D predicted from the curve-fitting routine of Figure 6C (239 nM) falls within this range. The predicted relative abundance of the low affinity sites (80%) is also what is observed experimentally (82 ± 5%; Table 1).

In Figure 6D, we tested the model shown in Figure 5D, where only $InsP_3$ binding to low affinity sites is required to open the channel. The quantal Ca^{2+} release data could be fit assuming that binding to at least two (top trace) or three (bottom trace) sites are needed to open the channel (Figure 6D). If binding of $InsP_3$ to three low affinity sites was needed to open the channel, the calculated K_{D2} is 101 nM. If binding to two low affinity sites is sufficient to open the channel, a K_{D2} of 224 nM is predicted. This latter prediction falls within the expected range (144−260 nM) for this temperature.

Thus, of all the models tested, two models, both of which predict random distribution of high and low affinity $InsP_3$ binding sites, fit both the kinetics of Ca^{2+} release measured at 11 °C and the quantal Ca^{2+} release observed at 37 °C. Results from the curve fitting analyses indicate that for the first of these two models binding of three or four molecules of $InsP_3$ is required to open the channel. The second model goes one step further, by taking into account the Ca^{2+} dependence of $InsP_3$ binding. It requires that binding of two or three molecules of $InsP_3$ to low affinity sites is needed to open the channel complex. This latter model may better predict the behavior of this $InsP_3$ gated Ca^{2+} channel as it provides an additional mechanism for closing (inactivating) some of the channels in the face of elevated cytosolic Ca^{2+}. This additional mechanism may contribute to quantal Ca^{2+} release under certain conditions, and may be related to the inactivation behavior observed in some studies by other investigators (Hajnóczky & Thomas, 1994).

DISCUSSION

Quantal calcium release from intracellular calcium stores represents a novel paradigm for regulation of second messenger signaling (Muallem et al., 1989; Kindman & Meyer, 1993). Small additions of $InsP_3$ lead to the release of small amounts of calcium from permeabilized cells and from isolated calcium stores (Kindman & Meyer, 1993). In this report, we describe and quantitate high and low affinity $InsP_3$ binding sites in rat basophilic leukemia cells. It is unlikely that the second binding site results from phosphorylation of the receptor since two sites were observed even in the absence of ATP and were observed at 4 °C as well as 22 °C. Similarly, we have excluded $InsP_3$ degradation as a potential artifact leading to the appearance of a second binding site. The dissociation constants for the two sites is temperature dependent having a Q_{10} of 1.6−2. Because of this temperature dependence, we were unable to accurately measure the dissociation constants at 37 °C. Assuming a Q_{10} of 1.68, the high and low affinity dissociation constants would respectively be expected to increase from 1 and 26−47 nM at 4 °C (Table 1) to 5.5 and 144−260 nM at 37 °C. The presence of two $InsP_3$ binding sites led to the development of several mathematical models, each of which was tested for its ability to predict $InsP_3$-induced calcium release from rat basophilic leukemia cells.

In our previous report (Kindman & Meyer, 1993), we hypothesized the presence of five independent $InsP_3$-gated calcium channels which were distinguished by their affinity for $InsP_3$. These unique sites would provide the range of affinities for $InsP_3$ needed to account for the observed quantal behavior of calcium release over a broad range of $InsP_3$ concentrations. In the present study, our data identify just two $InsP_3$ binding sites. We have therefore examined the possibility that two $InsP_3$ receptors could explain quantal calcium release.

An important assumption in the development of these models is that the $InsP_3$-gated calcium channel is a tetramer (Chadwick et al., 1990). Thus, if the two $InsP_3$ binding sites combine randomly, then five functionally distinct calcium channels result. The two isoenzymes of lactate dehydrogenase have been shown to combine randomly to yield five heterotetramers (Markert, 1963; Bishop et al., 1972). Ion channels also behave in this fashion. The human cardiac delayed rectifier potassium channels (Po et al., 1993) rat brain potassium channels (Ruppersberg et al., 1990) and the GluR-K1 and GluR-K3 subtypes of the rat brain glutamate receptor (Nakanishi et al., 1990) also form heterotetramers with receptor and electrophysiologic properties intermediate between their constituent subunits.

We developed several mathematical models based on random recombination of oligomers into heterotetramers to fit the kinetics of $InsP_3$-induced calcium release (Meyer et al., 1990). A model which yielded a close fit of the calcium release data required the binding of four molecules of $InsP_3$ to a given calcium channel complex in order to open the

channel. This model was consistent with the kinetics of $InsP_3$-induced calcium release for RBL cells and predicts a dissociation constant of 30 nM for the low affinity site at 11 °C. Given a Q_{10} of 1.68, the expected value for this should be 37−68 nM at 11 °C. If only three molecules of $InsP_3$ were needed to open the channel, the predicted dissociation constant for the low affinity site is 151 nM. This raises the possibility that, on average, the number of molecules needed to open the channel may be between three and four. The quantal Ca^{2+} release data was also fit best by models requiring the binding of three molecules of $InsP_3$. The dissociation constant for the low affinity site calculated from quantal calcium release data likewise is best fit if three molecules of $InsP_3$ are needed to open the channel. Moreover, the dissociation constant calculated from this curve-fitting routine (239 nM) is within the expected range of 144−260 nM. Thus, in this heterotetrameric model, we hypothesize that binding of three or four molecules of $InsP_3$ is necessary to open the channel. This model does not require a cooperative interaction between the oligomers of the tetramer. A stochastic process alone is sufficient.

A variation of this model which also fit the Ca^{2+} release data assumed that binding only to the low affinity sites would open the channel. An excellent fit of the kinetics of Ca^{2+} release was obtained when $InsP_3$ binding of three low affinity sites was required to open the channel. If 27% of the total binding sites were of high affinity, as we have found at a free Ca^{2+} of ~100 nM, a K_{D2} of 38 nM is predicted. This falls into the range of expected values at 11 °C, the temperature in which the experiments were executed.

Elevation of free Ca^{2+} from 0.1 to 0.5 μM increased the relative amount of the high affinity sites to 44% (Figure 4). A further increase in free Ca^{2+}, to 10 μM, resulted in little further change. Thus, physiologic free Ca^{2+} concentrations have a profound effect on the relative distribution of low and high affinity $InsP_3$ binding sites, without affecting the total number of $InsP_3$ binding sites. This may reflect a mechanism for inactivation of the $InsP_3$-gated Ca^{2+} channel. If channel activity only results from binding at low affinity $InsP_3$ binding sites, the model predicts that a decrease in affinity would also result in a loss of Ca^{2+} channel activity at less than saturating amounts of $InsP_3$.

Two other models which fit the kinetics of calcium release were described in this report, though they failed to adequately fit the quantal calcium release. The first model assumed cooperativity among the receptors within the channel complex, whereas the second did not. Both models assumed only the existence of homotetramers, and adequate fits of the data were obtained only when the high affinity receptors were inactive. Neither of these two models could explain quantal calcium release.

One of the hallmarks of quantal Ca^{2+} release is its disappearance at low temperatures. The data presented here account for this by showing the striking temperature dependence of the isoforms affinity for $InsP_3$ binding.

Thomas and co-workers have found that, *in vivo*, the cellular calcium store is one large continuous structure, which releases calcium in a unimolecular fashion (Renard-Rooney et al., 1993). Quantal calcium release occurs only when the calcium stores were compartmentalized during isolation either by permeabilization, or by shear (Renard-Rooney et al., 1993). Because $InsP_3$-induced calcium release *in vivo* is a highly spatially and temporally regulated process (Kasai & Augustine, 1990; Tse et al., 1993), fractionation of the endoplasmic reticulum would be expected to result in functionally distinct calcium stores with different $InsP_3$ receptors. These diverse properties yield quantal calcium release. Thus, in the present study, calcium release probably reflects the individual contributions of several functionally distinct calcium stores.

Recently, Thomas and co-workers have identified conditions under which the $InsP_3$-gated Ca^{2+} channel in hepatocytes can be inactivated by $InsP_3$ (Hajnóczky & Thomas, 1994). Hepatocytes, like RBL cells exhibit a Ca^{2+}-dependent conversion of low affinity to high affinity binding sites (Pietri et al., 1990). Local increases in free Ca^{2+} near (perhaps just within) the mouth of the channel could promote the conversion of low affinity to high affinity, inactive channels, thereby closing the channel.

Ca^{2+}-dependent interconversion of high and low affinity binding sites has not been observed for smooth muscle or cerebellum. Instead, micromolar Ca^{2+} increases the K_D of $InsP_3$ binding of a low affinity $InsP_3$ binding site (Joseph et al., 1989; Benevolensky et al., 1994). This could explain the Ca^{2+}-dependent decrease in $InsP_3$ induced Ca^{2+} release from cerebellar vesicles observed at less than saturating $InsP_3$ concentrations in these systems (Combettes et al., 1994).

We hypothesize that in RBL cells, Ca^{2+} flux through the pore of the $InsP_3$-gated channel may transiently inactivate the channel by converting the active, low affinity form of the receptor into an inactive, high affinity form. If $InsP_3$ binding to two or three low affinity sites is necessary to open the channel, as suggested by our models in Figures 5D and 6D, then this could close channels when the number of high affinity sites increases to two or three. As the calcium diffuses away, conversion back to a low affinity active form would be expected to occur. If the cytosolic Ca^{2+} concentration remains high, channel activity would be expected to remain low.

A basic feature of the two models used to describe Ca^{2+} release in this study is the presence of two unique types of $InsP_3$ binding sites. A previous study on RBL cells (Hershey et al., 1993; Mohr et al., 1993) identified only a high affinity $InsP_3$ binding site (2 nM) but observed that $InsP_3$ binding increased in the presence of 10 μM Ca^{2+}. We have confirmed this effect of Ca^{2+}, and attribute it to a Ca^{2+}-dependent interconversion of low affinity sites to high affinity sites. Both high and low affinity sites have been reported in other tissues. Microsomes isolated from rat thymus and human lymphocytes (Khan et al., 1992), bovine cerebellum (Hinogorani & Agnew, 1991), and canine aortic smooth muscle (Benevolensky et al., 1994) have all been reported to contain both high and low affinity sites. Using a broad range of $InsP_3$ concentrations, we have been able to detect two $InsP_3$ binding sites in RBL microsomes. The dissociation constant for the high affinity site (1−2 nM) is comparable to that previously reported for RBL cells (Mohr et al., 1993) and aortic smooth muscle (Benevolensky et al., 1994).

Other mechanisms for quantal Ca^{2+} release have been proposed. Luminal Ca^{2+} contributes to the regulation of $InsP_3$-gated Ca^{2+} efflux from microsomes when the stores are only partially filled (Irvine, 1990; Missiaen et al., 1992). Swillens (1992) has suggested the presence of additional modulatory proteins or other molecules regulating Ca^{2+} flux. In our previous work (Kindman & Meyer, 1993), we found that quantal release of Ca^{2+} could still be observed in isolated Ca^{2+} stores prepared in such a way as to render it unlikely

that they would contain a modulatory molecule with the proposed properties.

A low affinity site for $InsP_3$ binding has also been identified in bovine cerebellum (Chadwick et al., 1992) and canine myocardium (Kijima & Fleischer, 1992). This site is apparently the clathrin assembly protein complex, which functions as a potassium channel and whose permeability can by modulated by micromolar concentrations of $InsP_3$ and $InsP_4$ (Kijima et al., 1993). This site has some preference for $InsP_4$ over $InsP_3$. This contrasts with the present study, where the low affinity sites prefer $InsP_3$ over $InsP_4$. Other characterized $InsP_3$ binding proteins also bind $InsP_4$. $InsP_4$, for example, binds to homogeneous cloned receptor (Furuichi et al., 1989; Mignery et al., 1989; Blondel et al., 1993). In some systems, $InsP_4$ also causes the release of calcium from intracellular stores (Ely et al., 1990; Gawler et al., 1990) although this has not been observed for RBL cells (T. Meyer, personal communication). Our data are most consistent with the low affinity site being an $InsP_3$-gated calcium channel, because high concentrations of $InsP_3$ are needed to liberate calcium from the stores under the experimental conditions used here. Thus, it is likely that both the high and low affinity sites are important physiologic mediators of calcium mobilization. As $InsP_4$ does not release calcium from these RBL microsomes, metabolism of $InsP_3$ to $InsP_4$ could provide an additional means of inhibiting $InsP_3$-induced calcium release in RBL cells.

In summary, our data fit a simple, compelling hypothesis: calcium release can be accounted for by the presence of two $InsP_3$ binding sites. Two models emerged from this hypothesis. Both models provide excellent fits to the experimentally obtained kinetic and quantal Ca^{2+} release data. Both models require that the random assortment of the two distinct $InsP_3$ binding sites into five tetrameric $InsP_3$-gated Ca^{2+} channels. The first model simply predicts that the opening of a given channel requires $InsP_3$ binding to three or four subunits. The second model requires that binding to two or three low affinity sites results in opening of the Ca^{2+} channel, followed by the interconversion of low affinity active Ca^{2+} channels into high affinity inactive channels. This inactivation provides an additional mechanism by which to mediate quantal Ca^{2+} release and provides a theoretical basis for experimentally derived data (Hajnóczky & Thomas, 1994) which identifies channel inactivation as one mechanism by which $InsP_3$-gated Ca^{2+} release may be regulated.

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