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Regulation of inositol 1,4,5-trisphosphate receptors in rat basophilic leukemia cells. II. Modulation of the receptor in permeabilized cells by the cytosolic compartment

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Engagement of the IP_3 receptor by its ligand releases Ca^{2+} from intracellular stores of the rat basophilic leukemia (RBL) cell. The IP_3 receptor in washed permeabilized cells has high affinity ($K_d = 1.2 \pm 0.3$ nM) for [^{3}H]IP_3 and is not sensitive to physiological concentrations of Ca^{2+}. Moreover, washed permeabilized cells only release small amounts of Ca^{2+} when stimulated with IP_3. When [^{3}H]IP_3 binding to permeabilized cells is performed in the presence of cytosolic constituents (unwashed cells), the IP_3 receptor has a lower affinity for [^{3}H]IP_3 (K_d from 20 to 100 nM) and has enhanced Ca^{2+} release. Cytosolic supernatant, prepared by centrifugation of permeabilized cells and added back to washed permeabilized cells, decreases [^{3}H]IP_3 binding in a dose-dependent manner and increases the amount of Ca^{2+} released by IP_3. Depletion of either MgATP or IP_3 in the cytosolic supernatant does not affect the supernatant's ability to decrease [^{3}H]IP_3 binding. Though MgATP competitively inhibits [^{3}H]IP_3 binding, it cannot fully account for the shift in K_d or the modulation of IP_3-stimulated Ca^{2+} release in the presence of cytosol. These findings suggest that components present in the cytosolic supernatant modulate the function of the IP_3 receptor by maintaining it in a low affinity state capable of promoting Ca^{2+} release.

Introduction

The inositol 1,4,5-trisphosphate (IP_3) receptor-Ca^{2+} release channel complex (IP_3 receptor) has a major role in the regulation of the cytoplasmic free ionized calcium (Ca^{2+}) concentration during agonist stimulation. In the accompanying paper, we demonstrate that a microsomal preparation obtained from solid tumors of rat basophilic leukemia (RBL) cells, a cell model for nonexcitable cells, can be used to study the properties of the IP_3 receptor [1]. Biochemical characterization of the receptor shows both specificity and selectivity for IP_3. In addition, kinetic binding studies demonstrate the existence of at least two conformational states of the receptor that interact in a complex manner. The suggestion of complex binding behavior and the description of the binding of IP_3 to more than one class of binding sites on the IP_3 receptor has also been described in other nonexcitable cells [2–5].

Experiments examining the functional aspects of the IP_3 receptor from permeabilized RBL cells show that concentrations of IP_3 from 60 to 100 nM are required to induce half-maximal release rates (EC_{50}) of stored intracellular Ca^{2+}, a concentration of IP_3 that is much greater than the binding affinities for IP_3 measured in microsomal preparations [1]. Thus, the results from these two types of experiments show the same discrepancy that is observed in other cell types: higher concentrations of IP_3 are needed to cause half-maximal Ca^{2+} release than are needed to half-saturate the IP_3 binding sites [6–8]. However, the Ca^{2+} release studies are performed with streptolysin O-permeabilized RBL cells, whereas IP_3 binding studies are performed with RBL cell microsomal membranes.

This paper characterizes the binding properties of the IP_3 receptor in permeabilized RBL cells under conditions more comparable to those used in the Ca^{2+} release studies. The major finding is that when the cells are not washed free of cytosolic components, the

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Abbreviations: ATPγS, adenosine 5′-[γ-thio]triphosphate; B_{max}, maximum number of binding sites; EC_{50}, concentration resulting in 50% of the maximal effect; IC_{50}, concentration resulting in 50% inhibition; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_6, inositol hexakisphosphate; K_d, dissociation constant; RBL, rat basophilic leukemia.

affinity of the IP_3 receptor is shifted into a range of IP_3 concentrations that is close to that required for half-maximal Ca^{2+} release rates. Only when the receptor is maintained in this low-affinity state is IP_3 then able to induce Ca^{2+} release from intracellular stores.

Materials and Methods

Reagents. D-[1-^{3}H]Inositol 1,4,5-trisphosphate (17 Ci/mmol, 99% purity) was purchased from Du Pont-New England Nuclear (Wilmington, DE). Inositol 1,4,5-trisphosphate and inositol hexakisphosphate were purchased from Calbiochem (San Diego, CA), and inositol 1,3,4,5-tetrakisphosphate was purchased from Cayman Chemical (Ann Arbor, MI). ATP, GTP, ATPγS were purchased from Sigma (St. Louis, MO). Reduced streptolysin O was purchased from Wellcome Reagents (Greenville, NC), and fluo-3 was from Molecular Probes (Eugene, OR).

Cells. Rat basophilic leukemia (RBL) cells, subline 2H3, were used either as a permeabilized cell preparation or as a microsomal membrane preparation derived from solid tumors. The details for the preparation of the microsomal membranes from solid tumors are outlined in the accompanying paper [1].

Preparation of permeabilized cells. [^{3}H]IP_3 binding was performed with two different permeabilized cell preparations: one in which the permeabilized cells were washed free of cytosolic components prior to assessing radioligand binding (washed cells) and one in which the cytosolic components were allowed to remain during the binding reaction (unwashed cells). In both cases, the cells were prepared for permeabilization in the same manner. Cells in monolayer culture were exposed briefly to trypsin and then dislodged. The cells were washed twice in a saline solution containing 135 mM NaCl, 5 mM KCl, 10 mM Hepes adjusted to pH 7.2 with NaOH. The cells were then resuspended in a saline solution consisting of 140 mM KCl, 30 mM Hepes (pH 7.4) adjusted with KOH (we refer to this throughout the text as K^+ saline), and streptolysin O (0.2 U/ml) was added to permeabilize the cells. The suspension was shaken at 37°C for 30 min, and permeabilization was monitored by uptake of the dye Trypan blue.

[^{3}H]IP_3 binding to permeabilized RBL cells. Kinetic and equilibrium binding experiments were performed with $(2–3) \cdot 10^6$ permeabilized cells in a final volume of 1 ml. For binding experiments utilizing washed permeabilized cells, the permeabilized cells were centrifuged ($1500 \times g$ for 10 min) and resuspended in either a saline solution containing 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.1% BSA, 25 mM Na_2HPO_4 (pH 7.4) (referred to as Binding assay solution) or K^+ saline. Experiments performed in K^+ saline had 1 mM EDTA added directly to the assay tubes. Every binding experiment contained 1 mM EDTA except for that shown in Fig. 5. In both solutions (Binding assay solution and K^+ saline with EDTA), the free Ca^{2+} and Mg^{2+} concentrations were less than 1 nM. Experiments which examined the binding of [^{3}H]IP_3 to unwashed cells utilized the cells immediately following permeabilization in K^+ saline. [^{3}H]IP_3 (0.5 nM) was added, and binding reactions were performed at 4°C, unless otherwise indicated. Non-specific binding was determined in the presence of 100-fold excess unlabeled IP_3 (50 nM). Variations in the above assay conditions are described in the appropriate figure legends. Equilibrium binding reactions were terminated after 15–30 min by rapid, single manifold filtration through Whatman GF/C filters pre-soaked in the respective ice-cold saline solution. The filters were immediately washed with 2.5 ml ice-cold binding buffer; the entire process required fewer than 5 s per sample. Specific [^{3}H]IP_3 binding was proportional to the number of cells in the assay in the range of $(0.25–3) \cdot 10^6$ cells. In a typical experiment, specific binding was approx. 2000 dpm, while non-specific binding was approx. 10% of total binding. Binding of [^{3}H]IP_3 to whole non-permeabilized cells was low (total: 161 ± 9 dpm, specific: 65 ± 9 dpm). [^{3}H]IP_3 binding to permeabilized RBL cytoplasts, which are plasma membrane ghosts containing cytosol but no organelles or nucleus [9] was also insignificant (total: 132 ± 4 dpm, specific: 62 ± 4 dpm).

The length of the incubation time for equilibrium binding reactions was determined by kinetic studies which examined the association of [^{3}H]IP_3 (0.5 nM) with $3 \cdot 10^6$ permeabilized RBL cells. Maximal binding was attained within 10 min and remained constant for at least 45 min.

IP_3-stimulated release of Ca^{2+} from intracellular stores of permeabilized RBL cells. Cells were permeabilized with streptolysin O as described in the section on preparation of permeabilized cells except that the procedure was performed at 18°C. Following permeabilization, the cells were centrifuged ($300 \times g$ for 3 min) twice and resuspended to a final concentration of $2 \cdot 10^6$ cells/ml in either K^+ saline or in the original cytosolic supernatant. Ca^{2+} chelators were not added to the bathing saline solution for these experiments. The cells were placed in a polystyrene cuvette which was maintained at 18°C with constant stirring. Ca^{2+} levels were monitored in a Perkin-Elmer LS-5B fluorescence spectrophotometer using the Ca^{2+}-sensitive fluorescent dye fluo-3. The excitation and emission wavelengths used and the determination of the concentration of free ionized Ca^{2+} were described in the accompanying paper [1].

ATP assay. Total ATP was measured in pellets of permeabilized cells or in supernatants using a modification of the firefly luciferase assay (Sigma). Luminescence was measured with a Turner TD-20E Lumi-

nometer. ATP associated with the permeabilized cells was expressed as a concentration assuming that the volume of 10^6 RBL cells was 1 μl [10].

Analysis of data. Data obtained from equilibrium and kinetic binding experiments with permeabilized cells were analyzed with the computer programs LIGAND and ENZFITTER. Each experiment was performed two or more times, and within each experiment, each data point was performed in duplicate. The figures shown are from a representative experiment.

Results and Discussion

Equilibrium binding to washed permeabilized cells

Binding of [^{3}H]IP_3 to washed permeabilized cells ($3 \cdot 10^6$) in binding assay solution is saturable in the range of 0.5–10.5 nM (Fig. 1A). Scatchard analysis (Fig. 1A, inset) of the data in this range of IP_3 concentrations indicates a single class of binding sites having an apparent K_d of 1.0 nM and a binding capacity of 0.2 pmol/$3 \cdot 10^6$ cells. (mean values $\pm$ S.D. are 1.2 ± 0.3 nM and 0.24 ± 0.05 pmol/$3 \cdot 10^6$ cells, $n = 4$). This binding capacity is equivalent to $4.7 \cdot 10^4$ binding sites/cell. Equilibrium binding experiments are also performed with washed permeabilized RBL cells ($3 \cdot 10^6$ cells) utilizing a lower range of [^{3}H]IP_3 concentrations (0.025 to 3.4 nM) as shown in Fig. 1B. Scatchard analysis of the data results in a convex curve (Fig. 1B, inset). When the data are transformed according to Hill [11], the calculated Hill number is 1.2.

Control experiments detailed in Methods demonstrate that IP_3 binding is to an intracellular site that is not the plasma membrane. Binding is rapid, stable, and proportional to the number of cells in the assay. The apparent K_d (1 nM) measured in the permeabilized cells (Fig. 1A) is consistent to the 1.5 nM value measured in RBL cell microsomal membranes by rapid filtration methods over the same range of [^{3}H]IP_3 concentrations [1]. Also, the convex Scatchard from saturation experiments measured in the 0.025–4 nM range of [^{3}H]IP_3 suggests that the complex interactions observed in the microsomes are also present in the permeabilized cell system. These results indicate that the washed permeabilized RBL cell system closely resembles the RBL microsomal system in its [^{3}H]IP_3-binding properties.

Effect of free Ca^{2+}, inositol polyphosphates, and nucleoside triphosphates on [^{3}H]IP_3 binding to permeabilized cells

The free ionized Ca^{2+}, inositol polyphosphate, and nucleoside triphosphate concentrations have been shown to modulate the binding properties of the IP_3 receptor in many tissues [4,12,13]. Between 20 nM and 100 μM Ca^{2+}, the binding of [^{3}H]IP_3 (0.5 nM) to the washed permeabilized cells is only slightly enhanced over [^{3}H]IP_3 binding to cells in the presence of < 1 nM Ca^{2+}, whereas Ca^{2+} concentrations greater than 100 μM greatly decrease binding (Fig. 2). Inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphate (IP_6) can inhibit the binding of [^{3}H]IP_3 to its receptor in washed permeabilized cells, but with less potency than inositol 1,4,5-trisphosphate (Fig. 3); the concentrations that cause 50% inhibition (IC_{50} values) for IP_4 and IP_6 are 26 nM and 37 μM, respectively. The inhibitory effects of ATP, GTP, and adenosine 5'-[γ-thio]triphosphate (ATPγS) on [^{3}H]IP_3 binding to washed permeabilized cells are also shown in Fig. 3. MgATP is the most potent nucleoside triphosphate tested, with an IC_{50} of 220 μM. An analog of ATP, ATPγS, is significantly less potent in inhibiting the binding of [^{3}H]IP_3, having an IC_{50} of 1.4 mM. Another

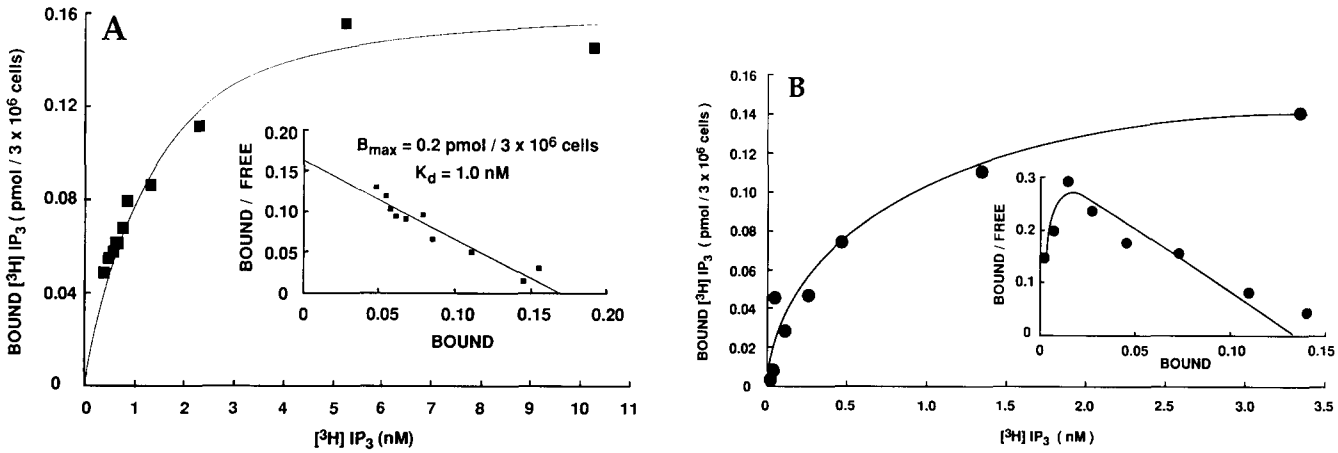

Fig. 1. Equilibrium [^{3}H]IP_3 binding and Scatchard analysis. (A) [^{3}H]IP_3 (0.5 nM) is titrated with increasing concentrations of unlabeled IP_3 for 30 min at 4°C with washed permeabilized RBL cells ($3 \cdot 10^6$) in Binding assay solution. The data are presented as pmol [^{3}H]IP_3 bound vs. free [^{3}H]IP_3. The curve shown is drawn by computer. (Inset) Scatchard analysis of the saturation curve. The plot is drawn by computer. (B) [^{3}H]IP_3 (0.025–3.4 nM) is added to washed permeabilized RBL cells ($3 \cdot 10^6$) for 30 min at 4°C in assay binding solution. (Inset) Scatchard analysis of the saturation curve. The plot is drawn by hand.

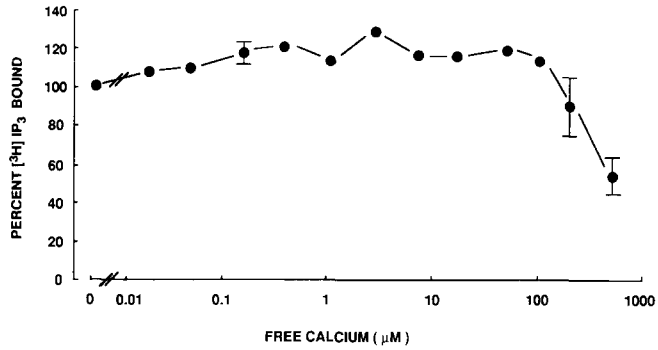

Fig. 2. Effect of free Ca^{2+} on [^{3}H]IP_3 binding to permeabilized cells. [^{3}H]IP_3 (0.5 nM) is incubated with $3 \cdot 10^6$ washed permeabilized cells for 30 min in Binding assay solution at 4°C. Ca^{2+} concentrations are between 20 nM and 500 μM, and EDTA (1 mM) was used to maintain the appropriate Ca^{2+} concentrations. For each Ca^{2+} concentration, results are expressed as a percentage of [^{3}H]IP_3 bound relative to the amount of [^{3}H]IP_3 bound in the absence of Ca^{2+} (< 1 nM) which in this experiment is 0.075 pmol. The bars represent ranges in percent and are only present when the ranges fall outside the area of the symbol.

nucleotide, GTP, inhibits IP_3 binding with low potency ($IC_{50} = 1.1$ mM).

The failure of physiological concentrations of Ca^{2+} to substantially affect IP_3 binding in permeabilized cells is consistent with what is observed in RBL cell microsomal membranes [1]. This finding contrasts with observations that physiological concentrations of Ca^{2+} inhibit IP_3 binding in cerebellar microsomal membranes [12] and enhance binding to hepatocyte membranes [4]. As we observed with the microsomal membranes, high concentrations of Ca^{2+} (> 100 μM) inhibit [^{3}H]IP_3 binding in washed permeabilized RBL cells; although, the concentration of Ca^{2+} at which inhibition begins is 10-fold higher in the permeabilized cells. IP_4 inhibits [^{3}H]IP_3 binding more strongly in the RBL cell than in brain, but IP_6 is comparably potent [13]. GTP has been shown to inhibit radiolabeled IP_3 binding at micromolar concentrations in liver and cerebellar membranes [13], but this is not the case for RBL cells. Lastly, the addition of a thiol to the terminal phosphate of ATP to form ATPγS increases the IC_{50} approx. 6.5-fold suggesting that this terminal phosphate group has a critical role in the interaction between ATP and the IP_3 receptor.

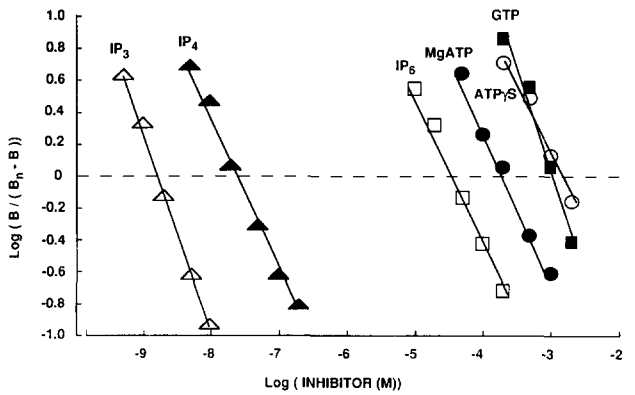

Fig. 3. Inhibition of IP_3 binding by IP_4, IP_6, MgATP, ATPγS, and GTP. [^{3}H]IP_3 (0.5 nM) is titrated against increasing concentrations of inhibitor with $3 \cdot 10^6$ washed permeabilized cells in Binding Assay Solution at 4°C. The data are presented as the Hill transformation of the inhibition experiment, and the plots are drawn by computer. The term B indicates the amount of specific binding in the presence of inhibitor; B_n indicates the amount of specific binding in the absence of inhibitor.

Effect of MgATP on apparent K_d for IP_3 binding

In the accompanying paper, IP_3-stimulated Ca^{2+} release experiments are performed with unwashed permeabilized cells without the addition of exogenous MgATP [1]. However, the measured concentration of endogenous ATP found in RBL cells after permeabilization is 50 ± 35 μM (mean $\pm$ S.D., $n = 4$), a concentration that is inhibitory to [^{3}H]IP_3 binding (Fig. 3). As a consequence, concentrations of IP_3 higher than would be predicted from the binding experiments (Fig. 1) may be required to stimulate Ca^{2+} release from IP_3-sensitive stores. To define the relationship between ATP concentration and IP_3 binding, IP_3 equilibrium experiments are performed in the presence of 0, 0.1, 0.5, and 1.5 mM MgATP. Because large quantities of receptor are required to perform this experiment and it is important to wash away any endogenous ATP, RBL cell microsomes are used. Scatchard plots for each saturation curve are shown in Fig. 4. The decreasing slopes associated with the higher MgATP concentrations indicate that MgATP is a competitive inhibitor of the IP_3 receptor in RBL cells. Apparent K_d values for each MgATP concentration are calculated (Fig. 4 inset). A similar experiment performed with washed permeabilized cells in the presence of 2 mM MgATP

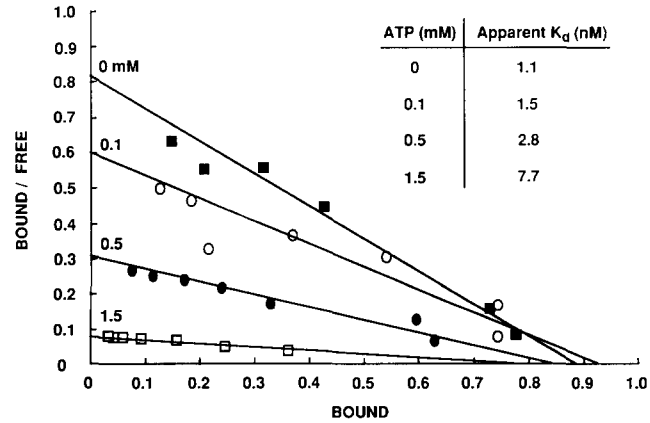

Fig. 4. Competitive inhibition of IP_3 binding by MgATP in microsomes. [^{3}H]IP_3 (0.5 nM) is incubated with 500 μg of microsomal protein in Binding assay solution, and varying amounts (0.2–10 nM) of unlabeled IP_3 are added in the presence of 0, 0.1, 0.5, or 1.5 mM MgATP. The Scatchard analysis for each saturation curve is shown, and each plot is drawn by computer. Apparent K_d values from the data are shown in the inset table.

shifted the K_d from 1 to 6 nM. In another experiment, 200 μM and 750 μM MgATP shifted the K_d to 2.5 and 4.5 nM, respectively.

It is clear that the shift in binding affinities for IP_3 (from approx. 1 to 8 nM) by MgATP in RBL microsomal membranes is not entirely responsible for the requirement of 60 to 100 nM IP_3 in generating half-maximal rates of Ca^{2+} release. The shift in apparent K_d may contribute in a small way but other factors must have a more important role.

Equilibrium binding to unwashed permeabilized cells

The conditions used for [^{3}H]IP_3 binding and for IP_3-induced Ca^{2+} release experiments [1] differ in potentially important parameters. Binding experiments, in contrast with Ca^{2+} release experiments, utilize different saline solutions and are performed at 4°C instead of 18°C. In addition, for binding assays the cells are permeabilized at 37°C whereas for Ca^{2+} release assays cells are permeabilized at 18°C. Furthermore, the permeabilized cells are washed once before binding is measured, but in Ca^{2+} release experiments the cells remain in the same solution in which permeabilization takes place. Taking these differences into account, [^{3}H]IP_3 binding experiments must be performed under the conditions that exactly mimic the conditions used to measure IP_3-stimulated Ca^{2+} release performed in the accompanying paper [1]. Cells are permeabilized at 18°C and are not subjected to centrifugation and washed prior to binding, and binding is performed at 18°C in the same saline solution (K^+ saline, no EDTA) used in Ca^{2+} release experiments. As shown in Fig. 5, when [^{3}H]IP_3 binding is tested over the range of 1.25–150 nM, binding is saturable. Scatchard analysis (inset) indicates a single class of binding sites with an apparent K_d of 40 nM and a binding capacity of 0.5 pmol/3 $\cdot 10^6$ cells. Experiments performed twice more generated apparent K_d values of 20 nM and 100 nM with B_{max} values of 0.3 and 0.9 pmol/3 $\cdot 10^6$ cells, respectively. The shift of the IP_3 receptor to a single class of low affinity binding sites in the unwashed permeabilized cells correlates better with the EC_{50} for IP_3-stimulated Ca^{2+} release (60–100 nM) determined under identical conditions.

It is interesting to note that as the apparent K_d increases, the B_{max} increases as well, suggesting that modulation of the IP_3 binding site to a lower affinity conformation also unmasks stable binding sites. The presence of a single class of binding sites for [^{3}H]IP_3 when binding is performed with unwashed permeabilized cells is in contrast to the two conformational states identified when binding is performed with RBL cell microsomal membranes [1] or with washed cells (Fig. 1B). Even at lower concentrations of [^{3}H]IP_3 (0.1–10 nM) the binding to unwashed permeabilized cells is still to a single site with an estimated apparent

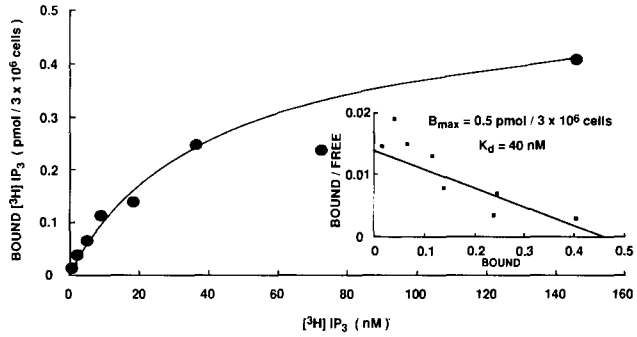

Fig. 5. Saturation curve of [^{3}H]IP_3 binding performed with unwashed permeabilized cells. Varying amounts of [^{3}H]IP_3 (0.1–150 nM) are incubated with $3 \cdot 10^6$ unwashed permeabilized cells for 30 min. The cells are used in the binding assay immediately following permeabilization. Permeabilization and binding are performed at 18°C in K^+ saline and in the absence of EDTA. (Inset) Scatchard analysis of the data is shown. The line is drawn by computer.

K_d of 40 nM, ruling out the possibility of a second class of conformational states in this preparation (unpublished data). In fact an attempt to fit the data into a two-site model using the computer program LIGAND failed.

Cytosolic supernatant modulation of [^{3}H]IP_3 binding

The experiments described above show a marked difference in the binding properties of the IP_3 receptor between washed and unwashed cells. A number of factors may be responsible for these differences. Table I compares the binding of [^{3}H]IP_3 (0.5 nM) to washed and unwashed permeabilized cells both in K^+ saline with 1 mM EDTA but with different binding and permeabilization temperatures. The most striking difference in the amount of [^{3}H]IP_3 bound is found between permeabilized cells that have either been centrifuged and resuspended in K^+ saline (washed cells) or have not been centrifuged and allowed to remain in

TABLE I

Comparison of the effects of washing, permeabilization temperature, and binding temperature on [^{3}H]IP_3 binding to permeabilized cells

RBL cells ($3 \cdot 10^6$) are permeabilized at the indicated temperatures for 30 min and are centrifuged and resuspended in fresh K^+ saline (i.e., washed) or are unwashed as indicated. Binding is performed at the indicated temperature in the presence of [^{3}H]IP_3 (0.5 nM) and EDTA (1 mM) for 15 min. The amount of bound [^{3}H]IP_3 is expressed as the mean of duplicate samples with a range between duplicates never varying by more than 14%.

Reaction No.	Permeabilization temp. (°C)	Binding temp. (°C)	Washing	fmol [^{3}H]IP_3 bound
1	18	4	–	11
2	18	18	–	11
3	18	4	+	64
4	18	18	+	49
5	37	18	+	58

the same solution that is used for permeabilization (unwashed cells), e.g., compare pmol bound of reactions 1 vs. 3 and 2 vs. 4. It is apparent that the temperature at which the permeabilization takes place does not affect binding as significantly as washing (compare reaction 4 vs. 5). Similarly, the temperature of the binding reactions is not contributing to any difference in binding (compare reaction 1 vs. 2 and 3 vs. 4). Neither the physical process of centrifugation and resuspension of the cells nor the presence of EDTA (1 mM), streptolysin O (0.2 units/ml), or fluo-3 (1 μM) in the binding assay is found to have an effect on [^{3}H]IP_3 binding (unpublished data). These findings suggest that cytosolic constituents in the supernatant, removed or reduced in concentration during the washing step, are responsible for maintaining the RBL cell IP_3 receptor as a single class of binding sites with a low binding affinity.

Cytosolic supernatants obtained from centrifugation of permeabilized RBL cells modulate IP_3 binding when added to another set of washed permeabilized cells. As increasing amounts of supernatant are added to $3 \cdot 10^6$ washed permeabilized cells, the amount of [^{3}H]IP_3 (0.5 nM) binding decreases (Table II), indicating that the effect of the supernatant is concentration-dependent. When cytosolic supernatant equivalent to the number of cells in the assay is added, the average inhibition is $73 \pm 26\%$ (mean $\pm$ S.D., $n = 7$).

Properties of the active component in the cytosolic supernatant

Neither heat (100°C, 1 h) nor trypsin treatment (100 μg/ml) of the cytosolic supernatant removes its modulatory activity on [^{3}H]IP_3 binding in unwashed permeabilized cells. This suggests that a globular protein is not responsible for modulating IP_3 binding. Furthermore, treatment of the cytosolic supernatant with hexokinase (1 unit/ml) and glucose (1 mg/ml) to deplete endogenous ATP before the supernatant is added to the washed permeabilized cells does not affect its ability to inhibit [^{3}H]IP_3 binding (Table III). Also, freezing the supernatant, which reduces its ATP concentration by 73%, does not affect the ability of the supernatant to inhibit [^{3}H)IP_3 binding (unpublished data). These findings support the previous observation that ATP is not responsible for substantially lowering the affinity of the IP_3 receptor for its ligand (Fig. 4).

Another possibility to consider is the presence of endogenous IP_3 in the supernatant which could dilute the specific activity of the [^{3}H]IP_3 and give the appearance of decreased [^{3}H]IP_3 binding. Concentrations of IP_3 in nonstimulated RBL cells have been measured to be 0.4–0.9 μM [14]. If all of the IP_3 is freely diffusible upon permeabilization, the cytosolic supernatant may contain concentrations of IP_3 ranging from 1.2–2.7 nM. Mignery and co-workers have shown that high Ca^{2+} concentrations (mM range) can stimulate IP_3 formation in isolated cerebellar microsomal membrane preparations [15]. No effect is seen when free Ca^{2+} was below approx. 5 μM, a range of Ca^{2+} concentra-

TABLE II

Concentration-response effect of cell supernatant on [^{3}H]IP_3 binding to permeabilized cells

Washed permeabilized RBL cells ($3 \cdot 10^6$) are incubated with [^{3}H]IP_3 (0.5 nM) and EDTA (1 mM) in K^+ saline for 15 min at 4°C and in the presence of the indicated cell equivalent amount of cytosolic supernatant. Cytosolic supernatant is prepared 1 to 7 days before use, frozen at −70°C and thawed immediately prior to use. The values for fmol [^{3}H]IP_3 bound are expressed as the means of duplicate samples with the range between each duplicate never varying by more than 10%. The control value in the last column is derived from the fmol [^{3}H]IP_3 bound to cells in the absence of cytosolic supernatant (Row 1).

Supernatant concentration (cell equivalents) ($\times 10^{-6}$)	fmol [^{3}H]IP_3 bound	% of control
0	100	100
0.1	80	72
0.3	70	66
1	40	33
3	20	15

TABLE III

Effect of various cytosolic supernatant treatments on [^{3}H]IP_3 binding to permeabilized cells

Cytosolic supernatants (equivalent to $3 \cdot 10^6$ cells) are treated as indicated and then added to washed permeabilized RBL cells ($3 \cdot 10^6$ cells). Binding is performed in the presence of [^{3}H]IP_3 (0.5 nM) and EDTA (1 mM) for 15 min at 4°C in K^+ saline. In each treatment a control is performed to assess [^{3}H]IP_3 binding to the cells in the absence of supernatant. Results with treated and untreated supernatants and controls are expressed as the mean fmol [^{3}H]IP_3 bound of duplicate samples with a range between each duplicate never varying by more than 13%. Numbers in parentheses are % inhibition of the control values.

Treatment	fmol [^{3}H]IP_3 bound		
	control	treated	untreated
Heat (100°C, 1 h) [a]	31	15 (47%)	13 (42%)
Trypsin (100 μg/ml, 37°C, 1 h) [a,b]	85	54 (41%)	62 (61%)
ATP depletion [c]	74	26 (66%)	29 (65%)
IP_3 depletion [d]	110	10 (91%)	11 (90%)

[a] These treatments are performed with $1 \cdot 10^6$ cell equivalents of cytosolic supernatant.

[b] Following trypsin treatment, the supernatant is treated with trypsin inhibitor (0.4 mg/ml, Sigma) before addition to the binding assay.

[c] ATP depletion of the supernatant is accomplished by adding hexokinase (1 unit/ml) and glucose (1 mg/ml) for 15 min and 37°C.

[d] IP_3 depletion of the supernatant is carried out by permeabilizing the cells, that will yield the cytosolic supernatant, for 30 min at 37°C in the presence of Mg^{2+} (1 mM).

tions that permeabilized RBL cells are exposed to during the initial stages of cell permeabilization. Yet, when RBL cells are permeabilized in the presence of EGTA (1 mM), the resulting supernatant inhibits [^{3}H]IP_3 binding by only 10% compared to 74% inhibition with control (EGTA not present during permeabilization) supernatants. The inhibitory activity of the supernatant towards [^{3}H]IP_3 binding is generated during permeabilization because the activity of the supernatant in inhibiting [^{3}H]IP_3 binding is not affected if EGTA (1 mM) is added after the permeabilization step. The inhibitory component may be IP_3 although it is doubtful that large concentrations of IP_3, are produced under these experimental conditions. Nevertheless, when supernatant (containing a small amount of tracer [^{3}H]IP_3) is added to a Bio-Gel column (Bio-Rad) to size-fractionate the component responsible for modulating [^{3}H]IP_3 binding, the fraction which shows the most inhibitory activity is the one which also contains the radiolabeled tracer (unpublished data). Although these findings are consistent with endogenous IP_3 being the active component, other, yet unidentified, compounds in the fraction may be responsible for inhibiting [^{3}H]IP_3 binding. To investigate the role of endogenous IP_3 further, RBL cells are permeabilized at 37°C in the presence of Mg^{2+} (1 mM) for 30 min (conditions known to deplete endogenous IP_3 completely [16]), but the inhibitory effect of the cytosolic supernatant on [^{3}H]IP_3 binding is still present (Table III). For this reason, the modulatory activity in the supernatant cannot be due to the presence of endogenous IP_3.

Requirement of cytosolic supernatant for IP_3-stimulated Ca^{2+} release

In the accompanying paper the EC_{50} for IP_3-stimulated Ca^{2+} release is determined in permeabilized RBL cells which have not been washed [1]. Under these conditions we have determined that the IP_3 receptors are in a lower affinity state for [^{3}H]IP_3 (Fig. 5). Studies measuring Ca^{2+} release stimulated by IP_3 in washed permeabilized cells may give an indication of whether the higher affinity states of the receptor are also capable of functional responses.

Comparisons are made between permeabilized cells treated in one of three different ways: those washed free of the supernatant produced during permeabilization and resuspended in K^+ saline; those kept resuspended in the original supernatant (although the cells are still subjected to the same number of centrifugations as the washed cells, i.e., centrifugation control); or those washed once in K^+ saline and then resuspended back into the original supernatant (equivalent to the binding experiments shown in Table II). Washing the permeabilized cells reduces the amount of available ATP; for example, the ATP concentration present in permeabilized RBL cells (measured in the cell pellet of centrifuged permeabilized cells) that have been washed once is 3 ± 1 μM; the concentration of ATP in the bathing saline solution is less than 1 μM. In contrast, the ATP concentration of permeabilized cells that are maintained in the original supernatant is 50 ± 35 μM; the supernatant contains 4 ± 2 μM ATP. The quantity of ATP present can influence the amount of Ca^{2+} sequestered into intracellular stores, and therefore, the amount of Ca^{2+} that is released in response to IP_3. To control for any discrepancy among the three conditions in the concentration of Ca^{2+} stores that is available for release by agonist, the total releasable Ca^{2+} (defined as the peak free Ca^{2+} concentration after addition of 1 μM ionomycin minus the free Ca^{2+} concentration of nonstimulated cells prior to the addition of IP_3) is determined for each condition. Comparisons are made only among groups in which the total releasable Ca^{2+} in the stores varies by no more than 100 nM.

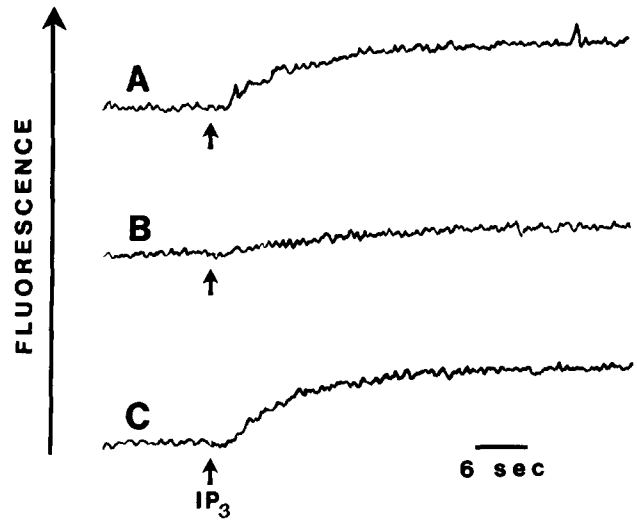

Fig. 6. Release of Ca^{2+} from intracellular stores of washed and unwashed permeabilized cells. The permeabilized cells ($2 \cdot 10^6$/ml) are suspended in K^+ saline at 18°C and are stimulated with IP_3 (1 μM) as indicated by the arrows. See Results for a detailed description of each condition. (A) Unwashed permeabilized cells. The ambient Ca^{2+} concentration prior to IP_3 addition is 455 nM. (B) Washed permeabilized cells. Pre-stimulatory Ca^{2+} concentration is 505 nM. (C) Washed permeabilized cells with re-addition of cytosolic supernatant. Pre-stimulatory Ca^{2+} concentration is 395 nM. The addition of ionomycin (1 μM) following IP_3-stimulated Ca^{2+} release resulted in final free Ca^{2+} concentrations of 660 nM, 680 nM, and 670 nM, for A, B, and C, respectively.

The addition of IP_3 (1 μM) to permeabilized RBL cells that have remained continuously suspended in the original supernatant (centrifugation control) induces a significant increase in fluo-3 fluorescence corresponding to the liberation of 64% of the total releasable Ca^{2+} from internal stores (Fig. 6A). On the other hand, if the permeabilized cells are washed and resuspended into fresh K^+ saline, IP_3 (1 μM) induces a smaller increase in fluo-3 floresence that corresponds

to 34% of the total releasable Ca^{2+} (Fig. 6B). Also, the initial release of IP_3-stimulated Ca^{2+} release is slower compared to the cells allowed to remain in the original supernatant. Furthermore, if the original supernatant is added back to washed permeabilized cells, the subsequent stimulation with IP_3 (1 μM) results in the increase of fluo-3 fluoresence to levels comparable to that observed with the centrifugation control and a release of 55% of total releasable Ca^{2+} (Fig. 6C). The initial rate of release is also faster compared to the washed cells. Thus, while IP_3 is able to induce the efflux of Ca^{2+} from stores in washed cells, the extent of Ca^{2+} release and the initial rate of release is greater in the presence of the cytosolic supernatant.

The component(s) found in the cytosolic supernatant that influence IP_3-induced Ca^{2+} release may or may not be the same factors that have a modulatory effect on [^{3}H]IP_3 binding. In previous sections the possibility that low concentrations of endogenous IP_3 may influence [^{3}H]IP_3 binding are considered. It is possible that low nM concentrations of IP_3 found in the cytosolic supernatant are maintaining the IP_3 receptor in a conformation that is more responsive to larger, stimulatory concentrations of IP_3. However, when permeabilized RBL cells are washed, and then pretreated with 5 nM IP_3, the subsequent addition of IP_3 (1 μM) 2 min later does not result in an increased efflux of Ca^{2+} from intracellular stores (unpublished data).

The possibility that endogenous ATP may be enhancing IP_3-stimulated release of Ca^{2+} from stores in RBL cells suspended in the original supernatant must also be considered. Ferris and co-workers found that low concentrations of ATP can modulate the activity of the IP_3-receptor Ca^{2+} release channel in smooth muscle microsomal membranes [17]. We found that the average concentration of ATP present in cytosolic supernatants is 4 μM. Therefore, the addition of this low concentration of ATP to washed permeabilized RBL cells should enhance IP_3-induced Ca^{2+} release. However, when MgATP (5 μM) is added 5 min before IP_3 (1 μM) there is no significant increase in the percentage of total amount of releasable Ca^{2+} that leaves the Ca^{2+} stores upon addition of IP_3 compared to control cells without additional MgATP (42% vs. 41% and 49% vs. 45%, respectively, in two separate experiments). Although it appears that low concentrations of ATP do not influence the IP_3-stimulated Ca^{2+} release channel of RBL cells, this is not the case when higher concentrations of MgATP are used. Pretreatment of washed permeabilized RBL cells with 30 μM ATP for 5 min results in a 58% and 88% increase in the concentration of calcium released after addition of IP_3 (1 μM) compared to washed cells not pretreated with MgATP (two separate experiments). Similar experiments performed with 100 μM MgATP show a 51% and 116% increase in IP_3-stimulated Ca^{2+} release over washed cells not pretreated with MgATP. While these findings support the idea that ATP can modulate the activity of the IP_3 receptor-Ca^{2+} release channel in RBL cells, the finding that 5 μM MgATP has no influence on IP_3-stimulated Ca^{2+} release in washed permeabilized RBL cells suggests that components other than ATP in the cytosolic supernatant are enhancing IP_3-stimulated efflux of Ca^{2+} from intracellular stores.

Conclusions

The major finding from this study is that in unwashed permeabilized RBL cells the IP_3 receptor is maintained in a state characterized by the binding of IP_3 to a single site with low affinity and increased binding capacity. The low-affinity state of the receptor may be coupled to Ca^{2+} release because IP_3-induced Ca^{2+} release is enhanced when the permeabilized cells are maintained under conditions that promote the low affinity state of the IP_3 receptor. This finding also correlates well with our previous finding that the EC_{50} for IP_3-stimulated Ca^{2+} release in unwashed permeabilized RBL cells ranges between 60 and 100 nM [1]. Washing the cells after permeabilization increases the binding affinity of the receptor for IP_3 and also transforms the receptor into one with complex binding reactions with IP_3 to more than one site. In addition this conversion to a high-affinity state of the IP_3 receptor is associated with a reduced sensitivity to IP_3-stimulated Ca^{2+} release.

Other studies have been performed that measure [^{3}H]IP_3 binding to permeabilized cells [3,4,13]. Mauger and co-workers show that the IP_3 receptor of permeabilized hepatocytes exists in two states when permeabilization and binding are performed at 4°C: one of high affinity (mean $K_d = 1.5 \pm 0.6$ nM) and another of lower affinity (mean $K_d = 80 \pm 30$ nM) [3]. Preincubation of the permeabilized cells at 37°C completely prevents the high-affinity state from being detected. The authors demonstrate that only the low-affinity state of the receptor is coupled to Ca^{2+} release, a finding similar to what we have found with RBL cells. However, the reversible transitions between these two states is mediated by the Ca^{2+} concentration, providing a negative feedback on IP_3-receptor binding as the Ca^{2+} concentration increases during agonist stimulation [4]. Our findings indicate that Ca^{2+} at concentrations found in agonist-stimulated RBL cells does not significantly affect [^{3}H]IP_3 binding to the IP_3 receptor. Nunn and Taylor also measured [^{3}H]IP_3 binding in permeabilized hepatocytes [13]. In this study a single binding site for [^{3}H]IP_3 was demonstrated with an apparent K_d of approx. 27 nM in the permeabilized hepatocytes whereas simultaneous studies with hepatic

membranes revealed apparent binding affinities of the IP_3 receptor for IP_3 in the range of 4 to 6 nM. However, these studies were performed with washed hepatocytes; therefore the reason for the discrepancy in that system may not be linked to the presence of a cytosolic modulator.

Cytosolic supernatant added to washed permeabilized RBL cells decreases [^{3}H]IP_3 binding in a dose-dependent manner and increases IP_3-stimulated Ca^{2+} release. The identity of the active component(s) which modulates the binding and functional properties of the IP_3 receptor is not known. Furthermore, the cytosolic factor which modifies the binding characteristics of the IP_3 receptor may not be the same one which enhances Ca^{2+} release. MgATP is ruled out because it cannot substantially increase the apparent K_d of the IP_3 receptor for [^{3}H]IP_3 in microsomal membranes or washed permeabilized cells to those observed in unwashed permeabilized cells. Ferris and co-workers have previously shown that concentrations of ATP between 1 and 100 μM are able to enhance IP_3-stimulated Ca^{2+} release [17]. We have determined that in Ca^{2+} release experiments, the unwashed permeabilized cells are exposed to approx. 5 μM free ATP in the supernatant. Adding 5 μM ATP to the washed permeabilized cells is not able to increase IP_3-stimulated Ca^{2+} release to levels greater than washed cells without added MgATP. This is further evidence that the factor in the supernatant must be something other than ATP. However, it is interesting to note that if MgATP at 30 μM or greater concentrations is added to washed permeabilized cells, then IP_3-stimulated Ca^{2+} release is enhanced.

IP_3 is also ruled out on the basis that the cytosolic supernatant remains active in inhibiting [^{3}H]IP_3 binding even though endogenous IP_3 has been removed by activation of phosphatases (Mg^{2+} pretreatment at 37°C) and that pre-incubation of washed permeabilized cells with IP_3 (5 nM) does not restore the ability of the washed cells to release Ca^{2+} in response to higher concentrations of IP_3. The active component separated into the same fraction as IP_3 did when the supernatant was size-fractionated, suggesting that it is comparable in molecular weight to IP_3 and is possibly another inositol polyphosphate.

A recent report by Kanematsu et al. identified two putative IP_3 binding proteins in rat brain cytosol that are distinct from the IP_3 receptor and IP_3 metabolizing enzymes [18]. One protein has been identified as the δ-isozyme of phospholipase C, and the other, a 130 kDa protein, has an unknown identity and function. The activity of these proteins in binding [^{3}H]IP_3 in rat brain cytosol is low; therefore, the role of these proteins in reducing the cytoplasmic concentration of free IP_3 is uncertain. It is unlikely that similar IP_3-binding proteins are responsible for reducing the affinity of the IP_3 receptor for [^{3}H]IP_3 in unwashed permeabilized RBL cells because treatment of the supernatant with either heat or trypsin (Table III) does not eliminate the supernatant's effect on [^{3}H]IP_3 binding. Nevertheless, the findings by Kanematsu et al. demonstrate that compounds may exist in the cell's cytoplasm that affect the interaction between IP_3 and its receptor.

Oscillations in the cytoplasmic Ca^{2+} concentration have been described in RBL cells, [19] and the release of Ca^{2+} from intracellular stores is an important component of this process. Therefore, Ca^{2+} oscillations may involve an interaction between IP_3 concentration and Ca^{2+}-releasing stores. However, Ca^{2+} oscillations have been induced in cells under experimental conditions that prevent changes in the IP_3 concentration, which suggests that fluctuations in the IP_3 concentration may not be a key component in generating the oscillations [20]. This implies that the IP_3 receptor itself may have a direct role in mediating Ca^{2+} oscillations. An important role for the cytosolic activity described in this paper may be to modulate the transition between low- and high-affinity conformational states of the IP_3 receptor that are either coupled or uncoupled to IP_3 receptor-mediated Ca^{2+} release. It is this interaction that may regulate the oscillatory behavior of agonist-stimulated Ca^{2+} movements.

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