The effect of external calcium and pH on inositol trisphosphate-mediated calcium release from cerebellum microsomal fractions

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Binding of D-*myo*-inositol 1,4,5-trisphosphate (InsP₃) to rat cerebellum membranes has previously been shown to be stimulated by alkaline pH and inhibited by low concentrations of Ca²⁺ [Worley, Baraban, Suppatopone, Wilson & Snyder (1987) *J. Biol. Chem.* **262**, 12132–12136]. In the present study, Scatchard analysis of InsP₃ binding to cerebellum microsomes indicates that the effects of Ca²⁺ and pH are exerted through changes in the apparent affinity of the receptor without effects on maximal binding. The influence of extravesicular Ca²⁺ and pH on InsP₃-mediated ⁴⁵Ca²⁺ release was investigated. Extravesicular Ca²⁺ inhibited InsP₃-mediated Ca²⁺ release. The inhibitory effect of Ca²⁺ was most marked when a sub-optimal concentration of InsP₃ was used. An increase in extravesicular pH produced a decrease in the concentration of InsP₃ that yielded half-maximal Ca²⁺ release. Regulation of the affinity of the InsP₃ receptor by Ca²⁺ and pH can qualitatively account for the observed effects of these factors on InsP₃-mediated Ca²⁺ release. Feedback inhibition of InsP₃ binding by Ca²⁺ could provide a mechanism to generate Ca²⁺ oscillations, particularly under hormonal conditions that produce sub-optimal elevations of InsP₃ concentration.

INTRODUCTION

An increase in cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) is an intrinsic part of the mechanism by which certain hormones regulate cellular processes. Recently, technical advances have made it possible to measure [Ca²⁺]_i in single cells, and several of these studies have documented the interesting phenomena that agonist-induced changes in [Ca²⁺]_i sometimes occur as a complex series of oscillations [1–7]. For example, in aequorin-microinjected hepatocytes, low concentrations of the agonists phenylephrine, angiotensin II and vasopressin can produce oscillations in [Ca²⁺]_i, and the frequency of these oscillations increases with agonist concentration. High agonist concentrations, however, produced only a single transient of [Ca²⁺]_i, whose duration resembled that obtained under similar conditions in bulk cell suspensions [1].

It has been firmly established that an increased turnover of the inositol-containing phospholipids represents an early step in the signal-transduction mechanism induced by Ca²⁺-mobilizing hormones [8]. Despite our knowledge of this process and of cellular Ca²⁺-transport mechanisms, the nature of the biochemical mechanisms generating [Ca²⁺]_i oscillations has not been identified. One possible mechanism, suggested by Cobbold and colleagues [9], relies on feedback inhibition of cell surface receptors (or associated GTP-binding proteins) by protein kinase C, which is activated as a result of the accumulation of 1,2-diacylglycerol. More recently, Worley *et al.* [10] have demonstrated that the binding of InsP₃ to its putative receptor in crude cerebellum membranes is itself inhibited by low concentrations of Ca²⁺. In the present paper we have further characterized this

effect of Ca²⁺ on the InsP₃-binding site and have attempted to relate this to the regulation of InsP₃-mediated Ca²⁺ release by extravesicular Ca²⁺. The data are compatible with an alternative model in which the inhibition of InsP₃ binding by Ca²⁺ could, under certain conditions, be involved in the generation of [Ca²⁺]_i oscillations.

MATERIALS AND METHODS

Microsomes (microsomal fractions) were prepared from rat cerebellum as described in ref. [11] by using an isolation buffer containing 0.32 M-sucrose, 10 mM-Tris/Hepes (pH 7.6) and 10 μM-EGTA. Unless otherwise stated, binding of [³H]InsP₃ (15 nM) was measured in a medium containing 120 mM-KCl, 20 mM-Tris/Hepes (pH 8.3) and 0.5 mM-nitrilotriacetate. Microsomes were incubated on ice in the above buffer at 0.5 mg of protein/ml. The amount of [³H]InsP₃ bound to the microsomes was measured by using glass-fibre filters (Gelman, GF/C). The filter was rapidly washed with 2 × 4 ml of a buffer containing 50 mM-Tris/Hepes (pH 8.3), 1 mg of bovine serum albumin/ml and 1 mM-EDTA. Non-specific binding was assessed in the presence of 10 μM unlabelled InsP₃. Under these conditions equilibrium of labelled InsP₃ with its binding site was attained within 5 min. Similar values for specific binding were obtained by using an assay in which microsomes were rapidly sedimented in a bench-top ultracentrifuge (results not shown). H.p.l.c. analyses also indicated no detectable metabolism of the added label under these incubation conditions.

⁴⁵Ca²⁺ fluxes were studied in a medium containing 120 mM-KCl, 20 mM-Tris/Hepes (pH 7.2), 0.3 mM-

Abbreviations used: InsP₃, D-*myo*-inositol 1,4,5-trisphosphate; HEDTA, N-hydroxyethylenediaminetriacetic acid; [Ca²⁺]_i, intracellular free Ca²⁺ concentration.

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MgCl₂, 10 mM-phosphocreatine, 10 units of creatine kinase (Boehringer Mannheim)/ml, 3.75 μ M-antimycin A, 3.75 μ M-Ruthenium Red, 1 mM-MgATP and 0.5 mM-EGTA. Known amounts of CaCl₂ were added to this medium to generate a range of free Ca²⁺ concentrations as measured directly with a Ca²⁺-sensitive mini-electrode and calibrated as described previously [12]. The medium was supplemented with ⁴⁵Ca²⁺ (1 μ Ci/ml; Amersham). Incubations were performed at 30 °C for 20 min with 0.5 mg of microsomal protein/ml. Intravesicular Ca²⁺ contents before and after InsP₃ additions were determined with a filtration assay (Millipore; 0.4 μ m pore size) and a wash buffer containing 120 mM-KCl and 20 mM-Tris/Hepes (pH 7.2). ⁴⁵Ca²⁺ fluxes were calculated as nmol of ⁴⁵Ca²⁺/mg of protein, by using the specific radioactivity of the individual EGTA/Ca²⁺ buffers.

[³H]InsP₃ (3.3 Ci/mmol) was obtained from New England Nuclear. Unlabelled InsP₃ was from Calbiochem.

RESULTS

Initial experiments with cerebellum microsomes confirmed the stimulatory effect of alkaline pH and inhibitory effect of Ca²⁺ on InsP₃ binding to crude membranes previously observed by Worley *et al.* [10]. The nature of these interactions was further investigated in experiments of the type shown in Fig. 1. The amount of labelled InsP₃ bound to the microsomes was measured at pH 8.3 in the presence of various concentrations of unlabelled InsP₃ and 15 nM-[³H]InsP₃. The displacement curve obtained from such experiments was shifted to the right in the presence of 1 μ M-Ca²⁺. Analysis of the data indicate that the inhibitory effect of Ca²⁺ results from an increase in the K_d of the binding site for InsP₃, with no change in the maximal number of binding sites (Table 1). Similar experiments were carried out at pH 7.2. The lower binding of InsP₃ observed at this pH was also associated with an increase in the K_d . At pH 7.2, 1 μ M-Ca²⁺ increased the K_d further without altering B_{max} . (Table 1).

The kinetics and reversibility of these effects of Ca²⁺

Fig. 1. Displacement of [³H]InsP₃ by unlabelled InsP₃

Binding of [³H]InsP₃ (15 nM) was measured as described in the Material and methods section. The amount of InsP₃ bound (B) at any given concentration of InsP₃ was expressed as a ratio to the binding observed in the absence of unlabelled ligand (B_0). The data shown are means $\pm$ S.E.M. for four experiments in which 1 μ M free Ca²⁺ inhibited B_0 by $40.3 \pm 7.9\%$.

Table 1. Summary of binding parameters

Data from experiments shown in Fig. 1 were converted into a form that related the amount bound (labelled and unlabelled) to the InsP₃ concentration. The K_d and B_{max} were estimated from the data by using the computer program LIGAND [29]. Values are means $\pm$ S.E.M.

Condition	K_d (nM)	B_{max} (pmol/mg)	n
pH 8.3, no Ca ²⁺	64.2 ± 7.7	25.2 ± 1.7	6
pH 8.3, 1 μ M-Ca ²⁺	220.0 ± 83.0	27.4 ± 1.8	4
pH 7.2, no Ca ²⁺	145.2 ± 22.8	31.3 ± 4.4	3
pH 7.2, 1 μ M-Ca ²⁺	542	36.9	2

are shown in Figs. 2 and 3. With low concentrations of InsP₃ (15 nM; Fig. 2), the addition of a pulse of Ca²⁺ to raise the free concentration from 0.1 μ M to 1.0 μ M produced a rapid fall in bound InsP₃, which was complete within 5 s (results not shown). Decreasing the free Ca²⁺ concentration back to 0.1 μ M by adding a Ca²⁺ chelator restored binding to the original value. The amount of InsP₃ bound to the receptor could be increased or decreased by repeatedly changing Ca²⁺ between 0.1 and 1.0 μ M, a range of concentrations over which Ca²⁺ has been observed to oscillate [1–7]. This was not the case,

Fig. 2. Ca²⁺-mediated dissociation of bound InsP₃ measured at low InsP₃ concentrations

Binding of [³H]InsP₃ (15 nM) was measured at pH 8.3 as described in the Materials and methods section. The upper record shows changes in free Ca²⁺ of the incubation mixture measured with a Ca²⁺ electrode by using a medium from which microsomes and labelled InsP₃ had been omitted. The final concentrations of the additions indicated were: 0.24 mM (Ca²⁺), 0.2 mM (HEDTA), 0.81 mM (Ca²⁺) and 2.2 mM (HEDTA). Results shown are representative of two experiments. The dotted line indicates non-specific binding measured in the presence of 10 μ M-InsP₃.

Fig. 3. Ca^{2+} -mediated dissociation of bound InsP_3 measured at high InsP_3 concentrations

The experiment was carried out as described for Fig. 2, except for the use of 150 nM $[\text{}^3\text{H}]\text{InsP}_3$. The final concentrations of the additions were: 0.24 mM (Ca^{2+}), 0.59 mM (HEDTA). Similar results were obtained when the experiment was repeated with the concentration of Tris/Hepes buffer increased to 100 mM .

however, when higher concentrations of InsP_3 were used (Fig. 3). At 150 nM InsP_3 the amount of InsP_3 bound was 4-fold higher. An increase in Ca^{2+} from 0.1 and $1.0 \mu\text{M}$ had no significant effect on InsP_3 binding; concentrations as high as $30 \mu\text{M}$ were required to obtain a 50% inhibition. Attempts were made to repeat these experiments and assess the Ca^{2+} -sensitivity of $[\text{}^3\text{H}]\text{InsP}_3$ binding by using 0.3 mM MgCl_2 in the incubation medium. However, interpretation of these experiments was complicated, since $[\text{}^3\text{H}]\text{InsP}_3$ was metabolized in the presence of MgCl_2 and metabolism was further stimulated by Ca^{2+} addition (results not shown).

The data (Table 1 and Fig. 3) suggest that the efficacy of Ca^{2+} as an inhibitor of InsP_3 binding is dependent on both Ca^{2+} and InsP_3 concentration. For this reason the effect of extravesicular Ca^{2+} on InsP_3 -mediated Ca^{2+} release was measured at saturating and sub-saturating amounts of InsP_3 (Fig. 4a). These experiments were carried out at pH 7.2. The Ca^{2+} released by a saturating dose of InsP_3 ($10 \mu\text{M}$) showed a biphasic relationship with respect to extravesicular free Ca^{2+} . With Ca^{2+} concentrations up to $2 \mu\text{M}$, the amount of Ca^{2+} released by InsP_3 increased in proportion to the total intravesicular Ca^{2+} content of the microsomes. Further increases in extravesicular Ca^{2+} inhibited InsP_3 -mediated Ca^{2+} release, although the microsomal Ca^{2+} content remained elevated (Fig. 4b). When a lower concentration of InsP_3 was used ($0.2 \mu\text{M}$), the extravesicular free Ca^{2+} concentration required to produce an inhibition of InsP_3 -mediated Ca^{2+} release was shifted to sub-micromolar values, and complete inhibition of the release was obtained above $10 \mu\text{M}$ Ca^{2+} .

Fig. 5 shows the dose/response relationships for InsP_3 -mediated Ca^{2+} release measured with a Ca^{2+} -sensitive electrode at pH 7.2 and 8.3. The concentration of InsP_3 required for half-maximal release was found to

Fig. 4. Effect of external Ca^{2+} on InsP_3 -mediated Ca^{2+} release

$^{45}\text{Ca}^{2+}$ release was measured over a range of free Ca^{2+} concentrations as described in the Materials and methods section. Triplicate $75 \mu\text{l}$ samples were removed after 20 min incubation, before addition of InsP_3 . A further three samples were taken 20–30 s after addition of the InsP_3 . Ca^{2+} release was measured as the difference in $^{45}\text{Ca}^{2+}$ content before and after InsP_3 addition (a). Then $2 \mu\text{M}$ A-23187 was added and additional samples were taken at 25 min for measurement of the total $^{45}\text{Ca}^{2+}$ content of the vesicles (b). The data shown are representative of two experiments that gave similar results.

decrease from 170 nM at pH 7.2 to 80 nM at pH 8.3. These values are in agreement with the observed effect of pH on the affinity of the InsP_3 -binding site (Table 1).

DISCUSSION

The diverse pattern of $[\text{Ca}^{2+}]_i$ oscillations seen in different cell types makes it unlikely that a single mechanism is responsible for their generation. In insulin-secreting cells [3], peritoneal macrophages [5] and BC3H-1 muscle cells [7] it has been demonstrated that $[\text{Ca}^{2+}]_i$ oscillations persist in the absence of external Ca^{2+} . InsP_3 microinjected directly into guinea-pig hepatocytes has been reported to trigger oscillations in Ca^{2+} -dependent K^+ currents [13]. These data suggest that some aspect of the regulation of intracellular Ca^{2+} mobilization can participate in generating oscillations. A plausible model suggested by the data is that Ca^{2+} discharged into the cytoplasm as a result of InsP_3 generation rapidly decreases the affinity of the InsP_3 receptor for its ligand and causes displacement of bound InsP_3 . This would have the effect of terminating Ca^{2+} release. Removal of Ca^{2+} from the cytoplasm by resequestration into intra-

Fig. 5. Effect of pH on the InsP_3 dose/response for Ca^{2+} release

Microsomes were incubated at 1 mg/ml in the medium described in the Materials and methods section but containing no EGTA/ Ca^{2+} buffer and no $^{45}\text{Ca}^{2+}$. The medium was adjusted to pH 8.3 with Tris/HCl or to pH 7.2 with Tris/Hepes. Incubations were carried out in a final volume of 200 μl in a thermostatically controlled chamber, and changes were registered with a Ca^{2+} -sensitive electrode. The amounts of Ca^{2+} released in response to increasing concentrations of InsP_3 were calibrated by addition of known amounts of Ca^{2+} . The sensitivity of the electrode was not altered by pH. Maximal Ca^{2+} release measured at 10 μM - InsP_3 was 3.6 ± 0.7 nmol/mg of protein at pH 7.2 and 2.6 ± 0.2 nmol/mg of protein at pH 8.3 (means $\pm$ S.E.M. of three preparations).

cellular compartments or into the extracellular space would return the InsP_3 receptor to its high-affinity form and allow the initiation of further Ca^{2+} release. An important constraint arising from the data is that such a mechanism can be involved only when the InsP_3 concentration is sub-saturating. In this regard, it is noteworthy that $[\text{Ca}^{2+}]_i$ oscillations in aequorin-loaded hepatocytes are observed only under conditions of weak hormonal stimulation [1]. Exceptions to this rule include α_1 -adrenergic stimulation of hepatocytes [1], α_1 -adrenergic and H1-histaminergic stimulation of BC3H-1 cells [7] and carbachol stimulation of insulin-secreting cells [3]. In all three cases, however, the particular agonists have been shown to be relatively impotent at increasing InsP_3 ([14,15]; M. Prentki & M. C. Glennon, personal communication).

Extravesicular Ca^{2+} has been shown to inhibit Ca^{2+} release produced by maximal doses of InsP_3 in a number of cell types. In brain microsomes [16] or neuronal cell lines [17,18] the effective range of Ca^{2+} concentrations is between 1 and 50 μM . The data in Fig. 4 show that lower concentrations of Ca^{2+} are effective when sub-optimal doses of InsP_3 are used. Although this finding is consistent with the conclusion that external Ca^{2+} inhibits Ca^{2+} release by inhibiting the InsP_3 receptor, it should also be pointed out that external Ca^{2+} does not inhibit Ca^{2+} release promoted by saturating concentrations of InsP_3 in certain tissues (e.g. liver [19,20]) and it has not been established that the effects of Ca^{2+} on InsP_3 binding are present in all tissues. Supattopone *et al.* [21] have recently shown that the purified InsP_3 receptor from rat cerebellum is insensitive to added Ca^{2+} and requires a

separate protein to confer Ca^{2+} -sensitivity. This ' Ca^{2+} modulator protein' is present at high concentrations in cerebellum and in much lower concentrations in peripheral tissues [22]. Loss of, or selective damage to, this protein could account for the lack of effect of external Ca^{2+} on InsP_3 -mediated Ca^{2+} release or binding seen in several systems.

The affinities of InsP_3 -binding sites in isolated liver, adrenal or pituitary membranes are in the range 3–10 nM [23–25]. By comparison, the InsP_3 concentrations giving 50% of maximum Ca^{2+} release are greater than 0.3 μM [8]. There is thus a large discrepancy between the affinities for binding and Ca^{2+} release, which is usually attributed to the different experimental conditions necessary to study the two processes [24,25]. In contrast with other microsomal systems, cerebellum microsomes show a reasonable agreement between the affinities of InsP_3 binding and InsP_3 -mediated Ca^{2+} release. Alkaline pH was found to decrease the concentration of InsP_3 required to obtain half-maximal Ca^{2+} release from cerebellum microsomes (Fig. 4). A similar observation has also been made with permeabilized platelets [26]. Alkaline pH decreased the K_d of the InsP_3 -binding site (Table 1). It therefore seems likely that the stimulation of binding by alkaline pH may underlie the enhanced InsP_3 -mediated Ca^{2+} release seen at alkaline pH in disrupted systems, and possibly also the increased responsiveness of intact platelets to suboptimal doses of thrombin after cytoplasmic alkalization with monensin ([27], but see ref. [28]). N.m.r. studies of the pH-dependence of ionization of phosphate substituents on the inositol ring indicates that the profile of the appearance of the fully charged species of the molecule (bearing six negative charges) closely parallels the pH profile of InsP_3 binding (C. A. Hansen, A. McLaughlin & J. R. Williamson, unpublished work). It is therefore possible that it is this species that preferentially binds to the receptor site.

In summary, the data show that the previously observed effects of pH and Ca^{2+} on InsP_3 binding to a putative receptor site in cerebellar microsomes [10] exclusively reflects shifts in the K_d of that site. InsP_3 -mediated Ca^{2+} release from this microsomal preparation is regulated by external Ca^{2+} and pH in a manner entirely consistent with modulation of InsP_3 binding to the receptor. Furthermore, the inhibitory effect of Ca^{2+} on the receptor could contribute to the generation of repetitive spikes of $[\text{Ca}^{2+}]_i$, particularly under hormonal conditions that produce sub-optimal elevations of InsP_3 concentration.

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