Regulation of cerebellar Ins(1,4,5)P_3 receptor by interaction between Ins(1,4,5)P_3 and Ca^{2+}

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We have characterized in detail the Ca^{2+}-dependent inhibition of [^{3}H]Ins(1,4,5)P_3 ([^{3}H]InsP_3) binding to sheep cerebellar microsomes, over a short duration (3 s), with the use of a perfusion protocol. This procedure prevented artifacts previously identified in studies of this Ca^{2+} effect. In a cytosol-like medium at pH 7.1 and 20 °C, a maximal inhibition of approx. 50 % was measured. Both inhibition and its reversal were complete within 3 s. Ca^{2+} decreased the affinity of the receptor for InsP_3 by approx. 50 % (K_d 146 ± 24 nM at pCa 9 and 321 ± 56 nM at pCa 5.3), without changing the total number of binding sites. Conversely, increasing the [^{3}H]InsP_3 concentration from 30 to 400 nM tripled the IC_{50} for Ca^{2+} and decreased the maximal inhibition by 63 %. This is similar to a partial competitive inhibition between InsP_3 binding and inhibitory Ca^{2+} binding and is consistent with InsP_3 and Ca^{2+} converting InsP_3 receptor into two different states with different affinities for these ligands. Mn^{2+} and Sr^{2+} also inhibited [^{3}H]InsP_3 binding but were respectively only 1/10 and 1/200 as effective as Ca^{2+}. No inhibition was observed with Ba^{2+}. This selectivity is the same as that previously reported for the inhibitory Ca^{2+} site of InsP_3-induced Ca^{2+} flux, suggesting that the same site is used by Ca^{2+} to convert cerebellar InsP_3 receptor to a low-affinity state and to inhibit its channel activity. Our results also suggest a mechanism by which InsP_3 counteracts this Ca^{2+}-dependent inhibition.

Key words: Ca^{2+}-release channel, cellular signalling, Ins(1,4,5)P_3 binding, microsomes.

INTRODUCTION

The mobilization of Ca^{2+} from the endoplasmic reticulum by the second messenger Ins(1,4,5)P_3 (InsP_3) is a key process in the cellular response to a number of extracellular stimuli [1]. The InsP_3 receptor (InsP_3R) forms a tetrameric Ca^{2+} channel in the endoplasmic reticulum that opens after InsP_3 binding. Three types of InsP_3R, encoded by related genes, exist in mammalian cells. These receptors are believed to have the same general molecular organization: an N-terminal InsP_3-binding domain and a C-terminal Ca^{2+} channel domain linked by an intermediate domain containing sites for regulatory agents [2]. Most cells contain at least two subtypes of InsP_3R but the relative amounts of the subtypes differ [3–5]. These different subunits of InsP_3R are known to assemble to form heterotetramers [6,7]. In the cerebellum, however, owing to the very high expression of the type 1 isoform (InsP_3R1) in Purkinje cells, most InsP_3Rs are homotetramers.

Cytosolic Ca^{2+} signals generated by sustained cell activation frequently display a complex pattern involving repetitive spikes. For InsP_3-dependent Ca^{2+} signals, regulation at the level of InsP_3R is thought to have a major role. Current evidence supports a mechanism in which the spikes in cytosolic Ca^{2+} concentration reflect a positive feedback effect on InsP_3R quickly followed by its inactivation [8]. In cerebellum, as in most cells and tissues studied so far, InsP_3 and Ca^{2+} seem to be the most important determinants of channel activity. Increasing the cytosolic Ca^{2+} concentration results in a bell-shaped dose–response curve for InsP_3-induced Ca^{2+} release (IICR) or channel activity [9]. The activating and inhibiting effects of Ca^{2+} are rapid processes, developing within 1 s [10,11]. In several tissues, including the cerebellum, InsP_3, in addition to opening the Ca^{2+} channel, triggers a slower effect leading to the conversion of its receptor into a less active state with a higher affinity for InsP_3 [12–16]. High concentrations of InsP_3 have also been found to counteract the inhibition of cerebellar InsP_3R activity by Ca^{2+}, so that inhibition is shifted to higher Ca^{2+} concentrations [17–19]. All these processes are potentially important but their precise involvement in the general regulation of InsP_3R activity remains unclear. The precise characterization of all the effects of InsP_3 and Ca^{2+} is essential in addressing this question.

The mechanisms underlying the Ca^{2+}-dependence of InsP_3R channel function are poorly known. The selectivities of stimulation and inhibition of IICR for bivalent cations differ [20–22], suggesting that different sites are involved. Ca^{2+}-binding regions have been identified in type 1 [23–25] and type 2 InsP_3Rs [23] but their function is unknown. The molecular basis of the Ca^{2+}-dependence might differ for each receptor. Ca^{2+} inhibits InsP_3 binding to InsP_3R1 [26–29] but it stimulates InsP_3 binding to InsP_3R2 [29,30] and InsP_3R3 [27,28]. The inhibitory effect of InsP_3R1 has been reported to be mediated by calmedin, a membranous Ca^{2+}-binding protein first detected in cerebellum, which is readily separated from InsP_3R [31]. However, we have recently obtained evidence for a direct effect of Ca^{2+} on InsP_3R1 or on a tightly associated protein in the sheep cerebellum [32].

Given the major physiological importance of the control of InsP_3R by Ca^{2+}, we studied in detail the Ca^{2+}-dependent inhibition of InsP_3 binding to sheep cerebellar membranes. To characterize the cation-binding site involved, we examined its selectivity by also investigating the influence of Mn^{2+}, Sr^{2+} and Ba^{2+} on InsP_3 binding. Several experimental problems can be encountered in studies of the inhibitory effect of Ca^{2+} on InsP_3 binding to membranes, including Ca^{2+}-stimulated InsP_3 formation [23], alteration of the affinity of InsP_3R for InsP_3 during prolonged exposure of membranes to this ligand [14] and the ability of Ca^{2+} chelators to compete with InsP_3 [32]. We avoided

Abbreviations used: IICR, Ins(1,4,5)P_3-induced Ca^{2+} release; InsP_3, Ins(1,4,5)P_3; InsP_3R, Ins(1,4,5)P_3 receptor; NTA, nitrilotriacetic acid.

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these difficulties by measuring InsP_3 binding on a time scale of seconds with the use of a perfusion protocol in the presence of low concentrations of Ca^{2+} chelators. Each ligand decreased the affinity of the other for its binding site, in an apparently competitive manner. This mechanism and the selectivity of the Ca^{2+}-binding site suggest that this site is identical with that through which Ca^{2+} inhibits the channel activity of the cerebellar InsP_3R [22]. Taken together, these results provide an explanation for the ability of InsP_3 to counteract this effect of Ca^{2+} [17–19].

EXPERIMENTAL

Preparation of the microsomal fraction

The membrane fraction was prepared from sheep cerebellum (Institut National de la Recherche Agronomique, Jouy en Josas or Nouzilly, France) as described previously [14]. In brief, cerebellum was homogenized in 90 ml of an ice-cold medium containing 5 mM Hepes, pH 7.4, 250 mM sucrose, 10 mM KCl, 1 mM 2-mercaptoethanol, 10 μg/ml leupeptin and 0.2 mM PMSF. Nuclear and mitochondrial fractions were sedimented by successive centrifugations at 1000 and 9000 ***g***. The supernatant obtained was centrifuged at 100000 ***g*** for 75 min. The pellet was resuspended in the initial medium at 20–30 mg/ml protein, homogenized, frozen in liquid nitrogen and stored at −80 °C.

Equilibrium Ins(1,4,5)P_3-binding studies

Microsomal membranes were thawed and diluted in an ice-cold cytosol-like medium (buffer A) containing 110 mM KCl, 20 mM NaCl, 1 mM NaH_2PO_4, 25 mM Hepes/KOH, pH 7.1, and 10 μg/ml leupeptin. In some experiments the free Ca^{2+} concentration of this medium was decreased to a nanomolar level; in others it was increased to 10 μM, as indicated in the text. [^{3}H]InsP_3 binding was measured at 20 °C by using a perfusion protocol similar to that described before [14,33]. A 120 μl aliquot of membrane preparation was diluted 1:10 in buffer A at 20 °C; 1 ml of the resulting suspension, containing 200–500 μg of protein, was layered on a glass-fibre filter (GF/C). The adsorbed membranes were then manually perfused for 3 s at 20 °C with 1.2 ml of binding medium, consisting of buffer A supplemented with 0.5 nM [^{3}H]InsP_3, various concentrations of unlabelled InsP_3 and 10 μg/ml leupeptin. Taking into account the rate of perfusion (0.4 ml/s) and the wet volume of the filter (40 μl), the complete replacement of the solution of membrane suspension by binding medium in the filter should have taken place within 100 ms. The free Ca^{2+} concentration was adjusted as described below. Non-specific binding was determined in the presence of 5 μM unlabelled InsP_3. Unless indicated otherwise, most of the free [^{3}H]InsP_3 in the filter was removed immediately by a short, fast (less than 1 s) rinse at 4 °C with 1 ml of a medium composed of 50 mM Tris/HCl, pH 8.3, 250 mM sucrose and 0.2 mM EDTA. The filter was then transferred to a counting vial and radioactivity was determined in a scintillation counter. The accurate measurement of [^{3}H]InsP_3 binding under our experimental conditions (pH 7.1; 20 °C; presence of an inhibitory cation) required a large quantity of membranes to be layered on the GF/C filter (more than 0.2 mg of protein). We therefore checked the dependence of [^{3}H]InsP_3 binding on the quantity of membranes. At both pCa 9 and pCa 5, [^{3}H]InsP_3 binding was linear with respect to the amount of membrane, at least up to 0.85 mg of protein per filter. Total binding and non-specific binding were determined at least in triplicate; the results are expressed as means ± S.E.M. Inplot software (GraphPad) was used to fit curves to data points by non-linear regression analysis.

Bivalent cation solutions

In most experiments, the free Ca^{2+} concentration in the perfusion medium was buffered, with EGTA and EDTA as chelators. At pH 7.1, the dissociation constants for the EGTA-Ca and EDTA-Ca complexes were taken to be 250 and 30 nM respectively. Possible inhibitory effects of chelators on [^{3}H]InsP_3 binding [32] were avoided by limiting their final concentration to 0.3 mM. Media with appropriate free Ca^{2+} concentrations were prepared by mixing, in buffer A, suitable volumes of stock solutions of chelators with or without equimolar Ca^{2+}, buffered at pH 7.1. pCa values were checked by titration with quin-2.

In the experiments in which we compared the effect of several bivalent cations including Mn^{2+} on [^{3}H]InsP_3 binding, buffer A was depleted of its contaminating Ca^{2+}. This was necessary because the usual chelators exhibit a much higher affinity for Mn^{2+} than for Ca^{2+} and the concentration of residual Ca^{2+} in buffer A (3 μM, as measured with quin-2) is sufficient to cause maximal inhibition of [^{3}H]InsP_3 binding. Contaminating Ca^{2+} was removed by passing buffer A through two columns, the first being Chelex 100 and the second Calcium Sponge S. The free Mn^{2+} concentration in Ca^{2+}-free buffer A was adjusted with 0.3 mM nitrilotriacetic acid (NTA); free Sr^{2+} and Ca^{2+} concentrations were adjusted with 0.3 mM EGTA. The dissociation constants for the NTA-Mn^{2+} and EGTA-Sr^{2+} complexes in buffer A at pH 7.1 were taken to be 19 and 70 μM respectively, on the basis of the constants in [34]. Ba^{2+} solutions were prepared in Ca^{2+}-free buffer A without chelators. All four bivalent cations were added as chloride salts.

Western blotting analysis

The microsomal fraction was analysed by conventional Western blot analysis by using antibodies specific for the three different subtypes of InsP_3R, as described previously [35]. SDS/PAGE was performed on 4–10 % (w/v) polyacrylamide gradient gels; the separated proteins were electrotransferred to a Hybond C-Super nitrocellulose membrane (Amersham), as described in [36]. The blots were saturated with 5 % (w/v) non-fat dried milk and 0.1 % (v/v) Tween 20 in PBS for 1 h at 37 °C. The blots were then incubated overnight at 4 °C with the appropriate antibodies diluted in PBS containing 2.5 % (w/v) non-fat dried milk and 0.1 % Tween 20 (v/v). After five washes the nitrocellulose membranes were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (1:2000) (Diagnostic Pasteur, Marnes-la-Coquette, France). Blots were then washed 5 times and developed with the enhanced chemiluminescence (ECL) detection system, with Hyperfilm (Amersham).

Materials

[^{3}H]InsP_3 (21 Ci/mmol) was obtained from Du Pont–New England Nuclear, InsP_3 from Calbiochem and other reagents were from Sigma. Chelex 100 was from Bio-Rad; Calcium Sponge S was from Molecular Probes.

The antibodies against the 14 C-terminal residues of InsP_3R1 have been described elsewhere [35,37]. A synthetic peptide (FLGSNTPHENHHMPPH) corresponding to the 16 C-terminal residues (2686–2701) of InsP_3R2 was prepared by Covalab (Oullins, France). The N-terminal amino acid was cross-linked to keyhole-limpet haemocyanin by glutaraldehyde; polyclonal antibodies against the peptide were produced in rabbit and have been described elsewhere [29]. Monoclonal antibodies against InsP_3R3 were purchased from Transduction Laboratories.

RESULTS

Preliminary measurements of Ca^{2+}-dependent inhibition of $InsP_3$ binding

In the present study we characterized Ca^{2+}-dependent inhibition of $InsP_3$ binding to cerebellar membranes in a cytosol-like medium (buffer A) under experimental conditions designed to avoid previously identified difficulties (see the Introduction section). The membranes were adsorbed on a filter and perfused for a few seconds only with a [^{3}H]$InsP_3$-containing binding medium. With buffer A as the binding medium at pH 7.1 and 20 °C, this is sufficient to reach equilibrium. However, the maximal level of inhibition with 0.5 nM [^{3}H]$InsP_3$ (approx. 35%) was lower than reported in previous studies (65%) [33]. This led us to investigate the influence of our experimental conditions on Ca^{2+} inhibition. We therefore found that the relatively low level of inhibition was due, at least in part, to the neutral pH at which [^{3}H]$InsP_3$ binding was measured, instead of alkaline pH as in previous studies. In the course of these investigations we also observed that inhibition was increased up to approx. 50% if binding was measured with 30–100 nM, rather than 0.5 nM, [^{3}H]$InsP_3$. This effect of the $InsP_3$ concentration will be considered further below; 30–60 nM [^{3}H]$InsP_3$ was therefore used routinely to study Ca^{2+} inhibition.

Because membranes were prepared and diluted in a buffer with a free Ca^{2+} concentration in the micromolar range, we also considered the possibility that this might affect subsequent [^{3}H]$InsP_3$ binding and/or sensitivity to Ca^{2+}. We had shown previously that prolonged incubation of sheep cerebellar microsomes with $InsP_3$ resulted in an increase in the $InsP_3$R affinity [14]. The same levels of [^{3}H]$InsP_3$ binding were measured regardless of whether membranes were pre-exposed to nanomolar

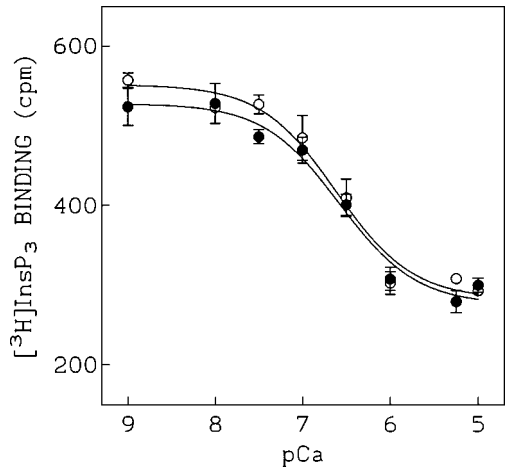


Figure 1 Presence of a micromolar concentration of free Ca^{2+} in the diluted cerebellar membrane suspension does not affect the subsequent measurement of Ca^{2+}-dependent inhibition of [^{3}H]$InsP_3$ binding

Sheep cerebellar membranes were thawed in ice-cold buffer A without Ca^{2+} chelator (●) (final free Ca^{2+} concentration 8 μM) or containing EDTA and EGTA at 0.3 mM (○) (nanomolar free Ca^{2+} concentration). A 120 μl aliquot of membrane suspension was diluted 1:10 in the same medium at 20 °C with or without Ca^{2+}; 1 ml of the dilution was immediately layered on a GF/C filter. Equilibrium [^{3}H]$InsP_3$ binding was measured by perfusing adsorbed membranes for 3 s with 1.2 ml of binding mixture. Binding mixture consisted of buffer A at 20 °C, supplemented with 60 nM [^{3}H]$InsP_3$ and adjusted for free Ca^{2+} concentration as indicated. Other experimental details are given in the Experimental section. Non-linear regression analyses gave IC_{50} values of 270 and 247 nM and maximal inhibitions of 48% and 49%, for microsomal suspensions diluted in buffer A at high and low free Ca^{2+} concentrations respectively.

Table 1 Summary of $InsP_3$-binding parameters at pCa 9 and 5.3

Free Ca^{2+} concentration (pCa)	Site	K_d (nM)	Binding sites (B_{max}) (pmol/mg of protein)
9	–	146 ± 24	29 ± 1
5.3	Low-affinity	321 ± 56	26.3 ± 2.7
	High-affinity	25 ± 6	1.1 ± 0.1

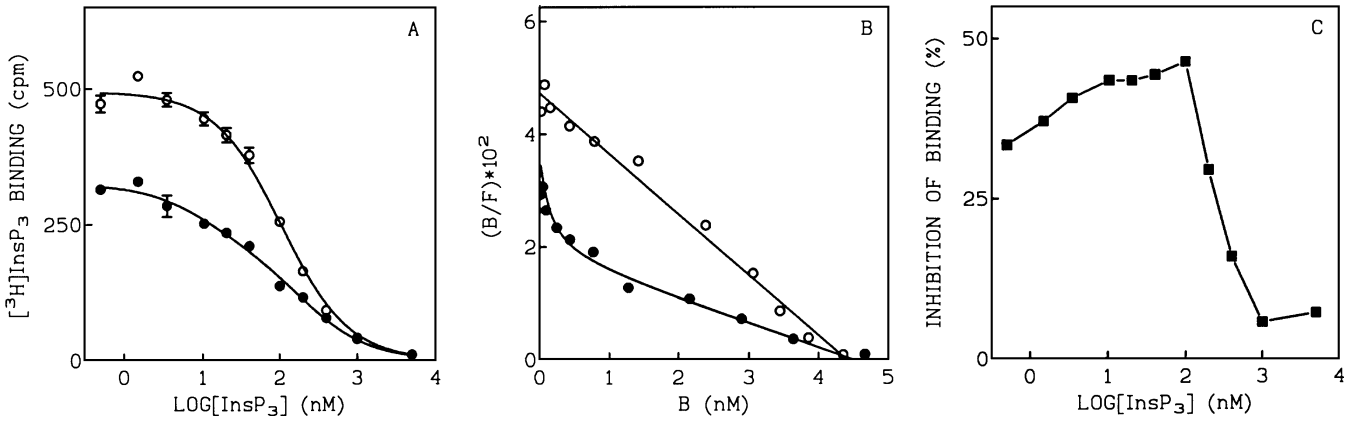


Figure 2 Effect of Ca^{2+} on $InsP_3$ binding characteristics

[^{3}H]$InsP_3$ displacement by increasing concentrations of unlabelled $InsP_3$ was measured at pCa 9 (○) or pCa 5.3 (●). Microsomes adsorbed on a GF/C filter were perfused with binding mixtures consisting of buffer A, with the free Ca^{2+} concentration adjusted and containing 0.5 nM [^{3}H]$InsP_3$ and various concentrations of unlabelled $InsP_3$. Other experimental conditions were as indicated in the legend to Figure 1 and in the Experimental section. (**A**) Specific [^{3}H]$InsP_3$ binding as a function of total $InsP_3$ concentration. Non-linear regression analysis suggested only one type of binding site for the data at pCa 9 (K_d 106 nM) but two different sites for the data at pCa 5.3 [K_d = 11 and 207 nM; variance ratio (F) = 10.8, $P < 0.008$]. The same total numbers of $InsP_3$-binding sites were measured at low and high Ca^{2+} concentration (B_{max} = 27 and 25 pmol/mg of protein respectively). The low-affinity site accounted for 95% of the total binding sites. (**B**) Scatchard plot of the same data as shown in (**A**). (**C**) Inhibition measured at pCa 5.3 in the same experiment, expressed as a function of total $InsP_3$ concentration. The results are the means of triplicates from one of three experiments.

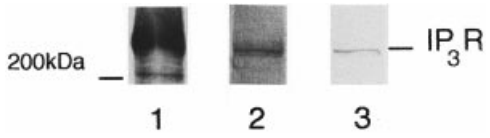


Figure 3 Immunodetection of InsP_3R subtypes in sheep cerebellar microsomes

Sheep cerebellar membranes (10 μg of protein on lane 1; 50 μg of protein on lanes 2 and 3) were loaded on a 4–10% (w/v) polyacrylamide gradient gel, subjected to electrophoresis and blotted to nitrocellulose. The blots were developed with antibodies against InsP_3R1 (lane 1), InsP_3R2 (lane 2) or InsP_3R3 (lane 3) and with peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:2000). The position of a 200 kDa size marker is indicated at the left.

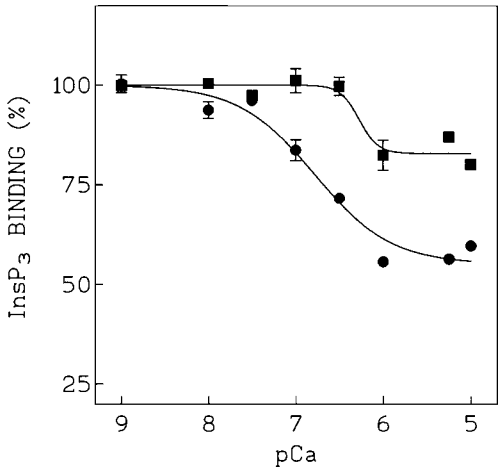


Figure 4 Comparison of the dose–response curves for Ca^{2+} inhibition at low and high InsP_3 concentrations

Cerebellar membranes adsorbed on a GF/C filter were perfused with binding mixtures containing either 30 nM (●) or 400 nM (■) [^{3}H]InsP_3, and various free Ca^{2+} concentrations. Results are expressed as percentages of maximal [^{3}H]InsP_3 binding. The data were fitted with a Hill slope of 1 and an IC_{50} of 165 nM for 30 nM [^{3}H]InsP_3, and with a Hill slope of 4 and an IC_{50} of 533 nM for 400 nM [^{3}H]InsP_3. Other experimental conditions were as described in the legend to Figure 1 and in the Experimental section. Results are means for two experiments.

or to micromolar concentrations of free Ca^{2+} (Figure 1). Data in Figure 1 provided important additional information: both the onset and the reversal of inhibition of [^{3}H]InsP_3 binding by Ca^{2+} took place within 3 s i.e. the duration of perfusion with the binding medium. Unless indicated otherwise, membranes were therefore routinely diluted in standard buffer A, containing contaminating Ca^{2+}.

Determination of InsP_3-binding parameters affected by Ca^{2+}

We performed competitive binding experiments at pCa 9 or 5.3 with 0.5 nM [^{3}H]InsP_3 and various concentrations of unlabelled ligand. As in previous studies [14,33], non-linear regression analysis of the data obtained at pCa 9 suggested that there was one type of binding site (Hill slope 1.05 ± 0.06; $n = 3$). Assuming a Hill slope of 1, a one-site model provided a K_d of 146 ± 24 nM. In contrast, a non-linear regression analysis of measurements performed at pCa 5.3 suggested heterogeneity in InsP_3-binding sites: the fit calculated with a two-site model was significantly better than that with a single-site model. In three experiments, mean K_d values of 25 ± 6 and 321 ± 56 nM were determined for these sites. However, almost all (96 ± 1 %) of the maximal binding capacity was due to the low-affinity site. The same total number of InsP_3-binding sites was measured in low and high Ca^{2+} medium (B_{max} 29 ± 1 and 27 ± 3 pmol/mg of protein respectively). Figure 2(A) presents the results of one of these experiments. The corresponding Scatchard plots (Figure 2B) illustrate the conclusions derived from the analyses of untransformed results in Figure 2(A). Consistent with these conclusions derived from equilibrium binding experiments was an examination of kinetics studies performed by Hannaert-Merah et al. (Figure 6C in [33]) on sheep cerebellar microsomes, which also suggested the presence of a high-affinity site at high Ca^{2+} concentration but not at low Ca^{2+} concentration. Table 1 summarizes the characteristics of InsP_3 binding determined at pCa 9 and 5.3.

Figure 2(C), constructed from the same data as Figure 2(A), shows changes in Ca^{2+}-induced inhibition as a function of total InsP_3 concentration. Increasing the [^{3}H]InsP_3 concentration to above 0.5 nM increased the inhibition to a maximum at 30–100 nM [^{3}H]InsP_3. Further increasing the [^{3}H]InsP_3 concentration resulted in a gradual decrease in Ca^{2+} inhibition. This unusual relationship between the extent of inhibition and ligand

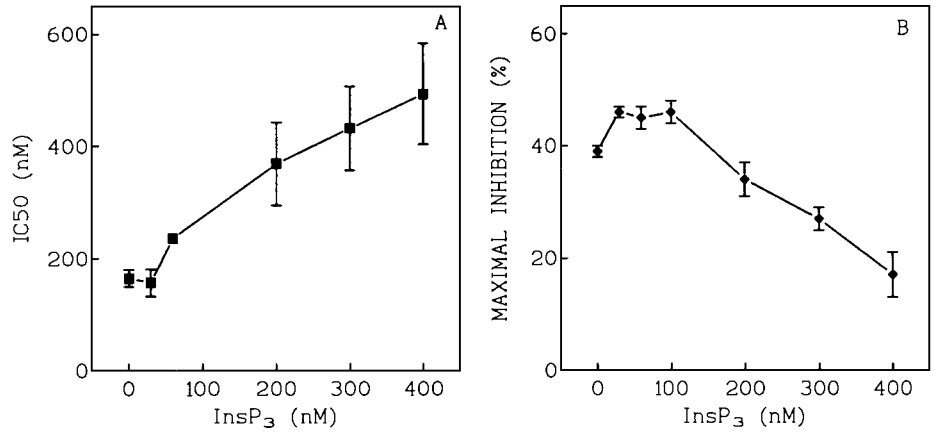


Figure 5 Effect of InsP_3 concentration on characteristics of Ca^{2+}-dependent inhibition

The characteristics of inhibition by Ca^{2+} were determined at several [^{3}H]InsP_3 concentrations from 0.5 to 400 nM. Experimental conditions were as described in the legends to Figures 1 and 4. Results are means for at least three experiments. Changes in IC_{50} (**A**) and maximal inhibition (**B**) with InsP_3 concentration are shown.

concentration was certainly due to the presence of the minor high-affinity site detected in the presence of Ca^{2+} in addition to the low-affinity site. Although the minor site accounted for only 4% of total binding sites, non-linear regression analysis indicated that at the lowest [^{3}H]InsP_3 concentration (0.5 nM) it accounted for as much as 40% of specific binding (Figures 2A and 2B). Increasing the [^{3}H]InsP_3 concentration above this low level enhanced the relative contribution of the low-affinity site to InsP_3 binding, resulting in an apparently stronger inhibition.

The detection of a minor binding site for InsP_3 at pCa 5.3 but not at pCa 9 suggests that two sites exist that do not have the same sensitivity to Ca^{2+}. Indeed, in the cerebellum of several species, InsP_3R2 and InsP_3R3 have both been detected at low concentrations compared with InsP_3R1 [4,5]. Unlike type 1 receptors, type 2 [29,30] and type 3 receptors [27] have been reported to exhibit a higher affinity for InsP_3 in the presence of Ca^{2+}. A Western blot analysis demonstrated the presence of the type 2 and 3 isoforms in microsomal fraction from sheep cerebellum (Figure 3), although at a much lower concentration than type 1 receptors.

Effect of InsP_3 on characteristics of Ca^{2+}-dependent inhibition

We then investigated the influence of InsP_3 concentration on the parameters of Ca^{2+}-induced inhibition of [^{3}H]InsP_3 binding. The dependence on Ca^{2+} concentration was assessed at several InsP_3 concentrations. Figure 4 presents inhibition curves obtained with 30 or 400 nM total InsP_3. To illustrate differences between these curves better, results were expressed as a percentage of maximal [^{3}H]InsP_3 binding. A comparison of the two curves shows that the decrease in Ca^{2+} inhibition described above at 400 nM [^{3}H]InsP_3 (Figure 2) was again observed in this experiment (from 45% inhibition to 17%). In addition, increasing [^{3}H]InsP_3 from 30 to 400 nM increased the IC_{50} for Ca^{2+} (from 165 to 533 nM). Figure 4 also illustrates a phenomenon observed repeatedly: inhibition by Ca^{2+} became steeper if the [^{3}H]InsP_3 concentration was increased. Results obtained with 400 nM [^{3}H]InsP_3 were fitted by using a value of 4 for the Hill slope, whereas a Hill slope of 1 was suitable for results obtained with 30 nM [^{3}H]InsP_3. Qualitatively identical results were obtained when the experiment in Figure 4 was performed at 4 °C instead of 20 °C.

To determine the relationship between the concentration of InsP_3 and the characteristics of Ca^{2+} inhibition, this experiment was repeated with several [^{3}H]InsP_3 concentrations from 0.5 to 400 nM. As shown in Figure 5(A), the IC_{50} for Ca^{2+} increased progressively as total [^{3}H]InsP_3 concentrations increased above 30 nM. Fitting the data with a rectangular hyperbola, a maximum IC_{50} of 572 nM was obtained. However, IC_{50} values could not be reliably determined at [^{3}H]InsP_3 concentrations above 400 nM, so it is not clear whether IC_{50} really tends towards an asymptote as InsP_3 increases. The dependence of maximal Ca^{2+} inhibition on InsP_3 concentration determined in the same and other experiments is shown in Figure 5(B). The curve reached a plateau at 30–100 nM [^{3}H]InsP_3, before declining progressively with increasing InsP_3 concentration. The Hill slope determined in the same experiments as in Figure 5(A) increased with increasing [^{3}H]InsP_3 concentration. However, values of the Hill slope determined at high [^{3}H]InsP_3 concentration were very variable. Mean values of 1.45 ± 0.17 and 4.26 ± 0.97 were obtained at 30 and 400 nM [^{3}H]InsP_3 respectively.

Selectivity of the Ca^{2+}-binding site for inhibition of InsP_3 binding

Ca^{2+}-induced activation and inhibition of InsP_3R activity are thought to occur through two distinct sites with close affinities for Ca^{2+}. However, these sites exhibit different affinities for Mn^{2+} and Sr^{2+} [20–22]. In the present study we determined the selectivity of the Ca^{2+}-binding site responsible for the inhibition of [^{3}H]InsP_3 binding to cerebellar membranes by comparing the effects of these cations on this binding. For reasons described above (see the Experimental section), the binding medium used in these experiments was prepared with buffer A containing a very low residual Ca^{2+} concentration. The efficiency of Ca^{2+} removal was assessed by checking for the possible inhibition of 30 nM [^{3}H]InsP_3 binding by treated buffer A, as revealed by the addition of 0.3 mM EGTA. This EGTA concentration was sufficient to abolish entirely the inhibition caused by contaminating Ca^{2+} in non-treated buffer A, as shown by the doubling of binding that it caused with 30 nM [^{3}H]InsP_3 (Figure 6A). Adjustment of the free Ca^{2+} concentration to 10 μM brought [^{3}H]InsP_3 binding back to the level measured in the absence of EGTA.

As illustrated in Figure 6(B), the addition of 0.3 mM EGTA to

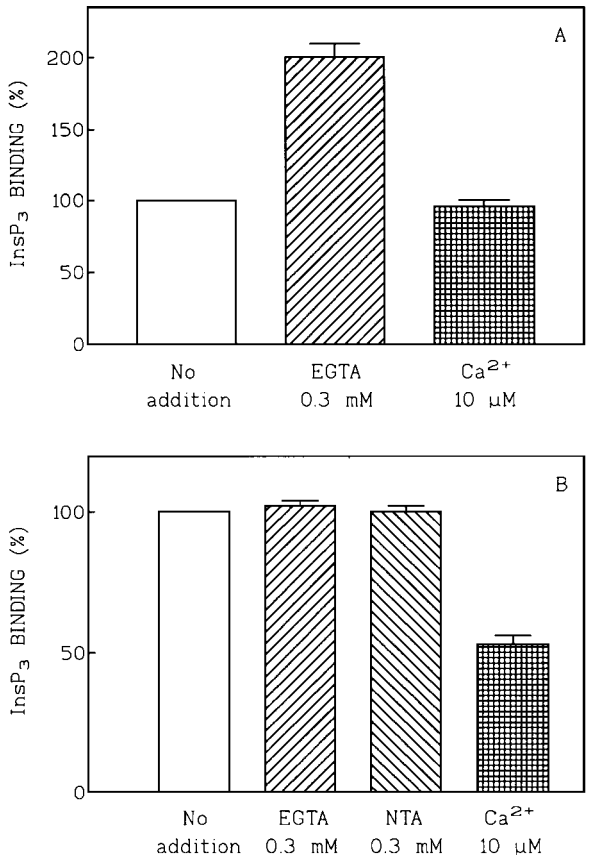


Figure 6 Lack of inhibition of [^{3}H]InsP_3 binding by buffer A purified by removal of bivalent cations

Cerebellar membranes adsorbed on a GF/C filter were perfused with a binding mixture containing 30 nM [^{3}H]InsP_3 and 10 μg/ml leupeptin. Binding mixtures were prepared either with non-purified buffer A (non-purified medium) or with buffer A passed successively through columns of Chelex 100 and Calcium Sponge S (purified medium). Binding mixtures contained EGTA, NTA and free Ca^{2+} as indicated. Other experimental conditions were as described in the legend to Figure 1 and in the Experimental section. (**A**) Effect of 0.3 mM EGTA or 10 μM free Ca^{2+} in binding mixtures prepared with non-purified medium. Results are expressed as percentages of [^{3}H]InsP_3 binding measured with non-purified medium alone, and are means for six experiments. (**B**) The addition of 0.3 mM EGTA to binding mixtures prepared with purified medium did not modify [^{3}H]InsP_3 binding. A free Ca^{2+} concentration of 10 μM was obtained by adding a small volume of a stock solution of K_2CaEGTA (final concentration of EGTA, 0.3 mM). Results are expressed as percentages of [^{3}H]InsP_3 binding measured with purified medium alone, and are means for at least six experiments.

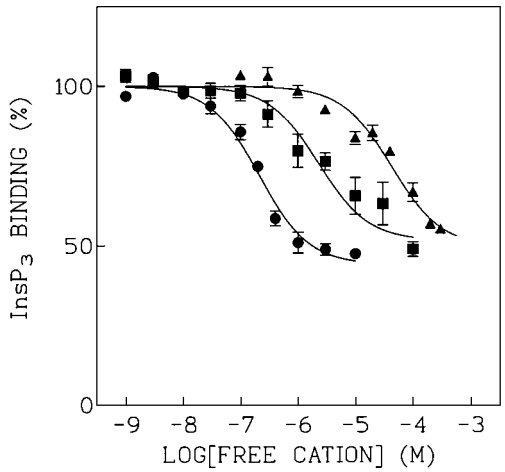


Figure 7 Comparison of dose-dependent effects of Ca^{2+}, Mn^{2+} and Sr^{2+} on [^{3}H]InsP_3 binding

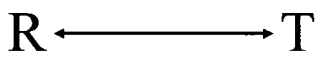
All binding mixtures were prepared with purified buffer A and contained 30 nM [^{3}H]InsP_3 and 10 μg/ml leupeptin. Concentrations of free bivalent cations were adjusted with 0.3 mM EGTA for Ca^{2+} (●) and Sr^{2+} (▲) and with 0.3 mM NTA for Mn^{2+} (■). Cerebellar membranes adsorbed on a GF/C filter were perfused for approx. 3 s with 1.2 ml of binding mixtures at 20 °C. In this experiment the filter was counted without washing of the free ligand. Non-specific binding was determined with 5 μM unlabelled InsP_3. Results are means of specific binding for three experiments.

the binding mixture prepared with purified buffer A did not affect the binding of 30 nM [^{3}H]InsP_3. The dose–response curves for Ca^{2+} inhibition at the same [^{3}H]InsP_3 level indicated that the Ca^{2+} concentration in purified buffer A was less than 30 nM, which was the minimum Ca^{2+} concentration causing inhibition in all experiments. Increasing the free Ca^{2+} concentration to 10 μM restored inhibition to approx. 50 %. NTA (0.3 mM) alone had no effect on [^{3}H]InsP_3 binding.

We compared the effect on [^{3}H]InsP_3 binding of various concentrations of different bivalent cations, with the use of solutions prepared in Ca^{2+}-depleted buffer A. Free Ca^{2+} and Sr^{2+} concentrations were fixed with 0.3 mM EGTA and free Mn^{2+} concentrations with 0.3 mM NTA. This chelator was chosen for Mn^{2+} because EGTA has too high an affinity for this cation (K_d 12 nM). Mn^{2+} and Sr^{2+} also inhibited InsP_3 binding, as determined in the presence of 30 nM [^{3}H]InsP_3, but at concentrations that were respectively 10-fold and 100-fold that of Ca^{2+} (Figure 7). A maximal inhibition of approx. 50 % was measured with the three cations. A one-site model provided IC_{50} values of 205 ± 30 nM for Ca^{2+}, 2.55 ± 0.60 μM for Mn^{2+} and 44.0 ± 0.8 μM for Sr^{2+}. We also investigated the possible effect of Ba^{2+} on [^{3}H]InsP_3 binding; no inhibition was observed, at least up to 1 mM Ba^{2+}.

DISCUSSION

Worley et al. [26] showed that Ca^{2+} inhibits the binding of InsP_3 to its receptor in the cerebellum. More recent studies have demonstrated that this effect of Ca^{2+} is exerted on InsP_3R1, the major receptor isoform in this tissue [27–29]. In the present study we addressed the questions of the mechanism and function of this inhibition by examining in detail the inhibition by Ca^{2+} of InsP_3 binding to sheep cerebellar membranes. The perfusion protocol used to measure [^{3}H]InsP_3 binding prevented difficulties such as Ca^{2+}-stimulated InsP_3 formation [23] from occurring in this type of study. Constant perfusion with medium containing



$InsP_3$	K_d = 150 nM	K_d = 350 nM
Ca^{2+}	No binding	K_d = 150 nM

Scheme 1 Allosteric mechanism for Ca^{2+}-dependent inhibition of InsP_3 binding to cerebellar InsP_3R1

The tetrameric InsP_3R exists in two conformations, R and T, which are in equilibrium. Ca^{2+} binds exclusively to the receptor in the T state, whereas InsP_3 binds to both receptor states but its affinity for R is twice that for T. Binding of Ca^{2+} to T causes the equilibrium between the two receptor conformations to shift in favour of T. Conversely, InsP_3 favours the conformational transition to the higher-affinity state, R. These opposite effects of InsP_3 and Ca^{2+} on the affinity of InsP_3R are similar to those commonly described in competitive inhibition. Because T also binds InsP_3, even high concentrations of Ca^{2+} do not abolish InsP_3 binding; therefore only partial inhibition can be obtained. In the presence of saturating Ca^{2+} concentrations, InsP_3R is frozen in the T state, so the affinity for InsP_3 determined in this condition is that of this conformation (pCa 5.3; Figure 4). In the absence of Ca^{2+}, the affinity measured for InsP_3 is close to that of the R state (pCa 9; Figure 4).

[^{3}H]InsP_3 also prevented biases caused by InsP_3 metabolism and therefore made it possible to perform binding experiments at 20 °C in a cytosol-like medium at pH 7.1, conditions commonly used to study IICR. In addition, [^{3}H]InsP_3 binding could be measured after some seconds of contact with Ca^{2+}, so that only the short-term changes in InsP_3 binding such as those associated with Ca^{2+} effects on InsP_3R activity [10] were detected; changes developing more slowly were excluded.

With the use of the perfusion protocol, we observed partial inhibition by Ca^{2+} of InsP_3 binding to cerebellar microsomes, which could be explained by a decrease in the affinity of the InsP_3R for InsP_3. This agrees with most previous studies in the cerebellum ([17,21,29,33], but see [28]) and in other cells or tissues containing a high proportion of the InsP_3R1 isoform [27,38,39]. However, a high-affinity site was also detected in the presence of Ca^{2+}, which accounted for a small fraction (4 %) of the total number of binding sites. Although we cannot exclude the possibility that this site resulted from the conversion of a small fraction of InsP_3R1 to a high-affinity state, it more probably involves type 2 and/or type 3 InsP_3R, which were detected at low levels in sheep cerebellum (Figure 3) and in the cerebellum of other species [4,5]. The higher affinity of these two InsP_3R isoforms for InsP_3 in the presence of Ca^{2+} [27,29] might have made their detection easier in these experiments. However, the high-affinity site in cerebellar microsomes had only a minor effect on the maximal inhibition. This maximum (approx. 50 %) was consistent with the 50 % decrease in affinity for InsP_3, measured at pCa 5.3.

Conversely, we also showed that InsP_3 binding overcame Ca^{2+} inhibition by increasing the IC_{50} for Ca^{2+} and decreasing maximal inhibition. As with Ca^{2+} inhibition, its reversal was a rapid process, reaching equilibrium within seconds. This ability of each ligand to quickly decrease the affinity of the other for its binding site is similar to a competitive mechanism between InsP_3 binding and the binding of inhibitory Ca^{2+}. It could be suggested that Ca^{2+} acts as a simple competitive inhibitor for approx. 50 % of InsP_3-binding sites. However, with such a mechanism the same maximal inhibition by Ca^{2+} should be measured whatever the concentration of [^{3}H]InsP_3. In contrast, increasing the concentration of [^{3}H]InsP_3 from 30 to 400 nM gradually decreased the level of maximal inhibition. Moreover, the [^{3}H]InsP_3 dis-

placement curve obtained with unlabelled ligand at pCa 5.3 showed no binding site with a K_d equal to that determined at pCa 9. In contrast, it indicated that almost all of the binding capacity was due to the low-affinity site. Thus, except for the small fraction of high-affinity sites, all $InsP_3$-binding sites seem to be sensitive to Ca^{2+} inhibition.

Our results are consistent with an apparent partial competitive mechanism in which $InsP_3$ and cytosolic Ca^{2+} interact with distinct sites, thereby transforming the tetrameric $InsP_3R$ into one or the other of two different affinity states in equilibrium. Such a mechanism, based on the Monod–Wyman–Changeux model for allosteric proteins [40], is illustrated in Scheme 1, in which R and T are the $InsP_3R$ states favoured by $InsP_3$ and Ca^{2+} respectively. In this model, partial inhibition occurs because $InsP_3$ can also bind to the T state, although with a lower affinity than that for the R state [40]. It is consistent with the hyperbolic behaviour of the major site at pCa 5.3 (Figure 2B) if Ca^{2+} concentration is high enough to lock the $InsP_3$ receptor in the T state for all $InsP_3$ concentrations [40]. This mechanism is also compatible with the large Hill slope of the inhibitory curves at high $InsP_3$ concentrations (Figure 4). However, distinct and non-interacting inhibitory sites might also make a significant contribution to the high degree of sigmoidicity of these curves [40]. Several Ca^{2+}-binding sites have been identified in the $InsP_3R1$ subunit [25].

Several accessory proteins have been proposed to mediate the regulation of $InsP_3R$ by Ca^{2+} in cerebellum. Ca^{2+}-dependent inhibition of $InsP_3$ binding has been reported to be mediated by a membrane protein, calmedin [31]. Regulation of IICR by Ca^{2+}-sensitive phosphorylation/dephosphorylation has been shown in cerebellar microsomes [41]. Such regulation was probably not involved in our binding experiments because they were performed without ATP or Mg^{2+} and both the onset and reversal of Ca^{2+} effects were complete in a few seconds. Calmodulin, previously reported to bind to cerebellar $InsP_3R1$ [42,43], does not seem to be involved, because calmodulin antagonists had no effect on Ca^{2+} inhibition (results not shown). We have found that the Ca^{2+}-dependent inhibition of $InsP_3$ binding to cerebellar microsomes might result from a direct interaction of Ca^{2+} with $InsP_3R1$ [29]. Two of the Ca^{2+}-binding regions identified in the cytoplasmic part of $InsP_3R1$ are located in the $InsP_3$-binding domain [25], making these sites reasonable candidates for the mediation of the competitive inhibitory effects of Ca^{2+} described here. Recently, biphasic dependence on Ca^{2+} of $InsP_3R$ channel activity was found to occur with cerebellar receptor isolated and reconstituted in lipid bilayers, suggesting direct effects of Ca^{2+} on this protein [44]. However, accessory factors might also be involved in the regulation of these Ca^{2+} effects.

We further characterized the process by which Ca^{2+} inhibits $InsP_3$ binding to the cerebellar $InsP_3R$, by investigating the selectivity of the inhibitory site. Mn^{2+} and Sr^{2+} partly inhibited [^{3}H]$InsP_3$ binding to about the same extent as Ca^{2+}, suggesting that the inhibitory effects of Mn^{2+} and Sr^{2+} are mediated by the same site and the same mechanism as those of Ca^{2+}. However, this site had very different affinities for these cations. The order of potency for this site ($Ca^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+}$) was identical and the relative affinities very similar to those determined for the site inhibiting $InsP_3R$ activity. These characteristics were different from those of the activatory site [20–22]. The dissociation constants for Ca^{2+} and Mn^{2+} determined here are very close to those determined for $InsP_3R$ channel inhibition in electrophysiological experiments [22]. Given that the experimental conditions were different in these two studies, this might be fortuitous; however, the similarity in relative affinities is probably completely relevant. This result suggests that the site through which Ca^{2+} converts cerebellar $InsP_3R$ to a low-affinity state is identical to that through which it inhibits channel activity.

Negative interactions between $InsP_3$ and cytosolic Ca^{2+} have been described for the Ca^{2+}-dependent inhibition of $InsP_3R$ channel activity in the cerebellum [17–19] and in A7r5 smooth-muscle cells [45], which also mainly express the $InsP_3R1$ isoform [3]. In these studies, increasing the $InsP_3$ concentration overcame Ca^{2+} flux inhibition, which was restored by increasing the Ca^{2+} concentration. Investigating IICR in vascular smooth-muscle cells, Hirose et al. [46] recently suggested that $InsP_3$ and Ca^{2+} each lower the affinity of the $InsP_3R$ for the other. In this study, similar negative interactions between $InsP_3$ and Ca^{2+} were demonstrated, but for the Ca^{2+}-dependent inhibition of $InsP_3$ binding. This similarity in the mechanism and identity of the Ca^{2+} sites involved in the two inhibitory effects of Ca^{2+} indicate that the conversion of the $InsP_3R$ to the lower-affinity state by Ca^{2+} is a process intimately connected with the Ca^{2+} inhibition of channel activity. Consistent with this conclusion is the observation that the onset and reversal of $InsP_3$-binding inhibition occur rapidly, as for Ca^{2+} flux inhibition.

Whereas cytosolic Ca^{2+} might inhibit cerebellar $InsP_3R$ activity completely [10,18,19], the inhibition of $InsP_3$ binding was only partial, even at low $InsP_3$ concentrations. This suggests that the effect of Ca^{2+} on $InsP_3$ binding might be only partly responsible for flux inhibition. However, a partial inhibition of $InsP_3$ binding might result in a more complete inhibition of Ca^{2+} flux if this flux depends in a co-operative way on $InsP_3$ concentration, as has been shown in the cerebellum and other tissues [13,18,47–50]. Oancea and Meyer [51] reported that the suppression of IICR in intact RBL cells could be explained by a 2–3-fold shift in $InsP_3$ sensitivity combined with a high power dependence between $InsP_3$ and Ca^{2+} release. Thus the 50 % decrease in affinity of the cerebellar $InsP_3R$ might cause a large decrease in its channel activity. This implies that the steep inhibition of $InsP_3$ binding by Ca^{2+} at high $InsP_3$ concentrations (Figure 4) should result in a steep decrease in $InsP_3R$ activity. In accordance with this, steep curves for dose-dependent flux inhibition by cytosolic Ca^{2+} have been obtained at high $InsP_3$ concentrations in the cerebellum [17–19]. However, the co-operativity of IICR in the cerebellum remains controversial [52] and we cannot exclude the possibility that Ca^{2+} inhibits $InsP_3R$ activity via a mechanism other than the decrease of $InsP_3$ binding, by directly affecting channel activity. Nevertheless, to be effective, such a mechanism might require the conversion of $InsP_3R$ to the lower-affinity state described here. This would account for the common properties in the mechanisms of Ca^{2+} inhibition of Ca^{2+} flux and $InsP_3$ binding. It is also consistent with the observation by Thrower et al. [44] of Ca^{2+} flux inhibiton at high $InsP_3$ concentration, because the free Ca^{2+} concentrations used in this study were high enough to convert the $InsP_3R$ into this state.

The negative interaction between $InsP_3$ and Ca^{2+} described here might be specific to $InsP_3R1$. The effect of Ca^{2+} on $InsP_3$ binding to $InsP_3R$ of type 2 [29,30] or type 3 [27,28] is different from that on $InsP_3$ binding to the type 1 receptor. However, this does not exclude the possibility that the counteractive effect of $InsP_3$ on the Ca^{2+}-dependent inhibition of $InsP_3R1$ activity also occurs with other $InsP_3R$ isoforms. Oancea and Meyer [51] found that, in RBL cells containing mostly the $InsP_3R2$ isoform [3], the Ca^{2+}-induced desensitization of IICR in intact cells is overcome by increasing the $InsP_3$ concentration.

Evidence has been obtained in various tissues, including the cerebellum, that $InsP_3R$ undergoes spontaneous inactivation in the presence of $InsP_3$ [12–16,49,53]. It has been suggested that this process is the predominant mechanism by which the ac-

tivation of InsP_3R is terminated in hepatocytes [53,54]. In some studies, this inactivation has been shown to develop slowly, with a time constant of approx. 0.5 min [12,16]. The counteractive effect of InsP_3 on the Ca^{2+}-induced inhibition of InsP_3R1, the onset of which is much more rapid [18], would therefore be operative, supporting Ca^{2+} release, before the inactivation step triggered by InsP_3. Alternatively, if InsP_3-dependent inactivation also occurred in a matter of seconds [49,53] but were only partial, as has been reported [15,49,53], this InsP_3 effect, by preventing excessive inhibition by Ca^{2+}, would help to maintain InsP_3R in a partly active state. The level of InsP_3R activity would therefore depend on the relative concentrations of InsP_3 and cytosolic Ca^{2+}. The mechanism put forward for Ca^{2+} inhibition of InsP_3R1, i.e. the conversion of the receptor to a state with a lower affinity, maintains the possibility of InsP_3 binding to the transformed InsP_3R1 and is therefore compatible with the InsP_3-dependent inactivation of channel activity.

In conclusion, we have shown here that the two major determinants, InsP_3 and Ca^{2+}, in sheep cerebellar InsP_3R each decrease the binding of the other. These interactions are probably important elements in the fine regulation of the InsP_3R, which is known to have a key role in Ca^{2+} signal organization [8].

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