Characterization of a membrane protein from brain mediating the inhibition of inositol 1,4,5-trisphosphate receptor binding by calcium

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Inositol 1,4,5-trisphosphate (InsP_3) is a component of the phosphoinositide second-messenger system which mobilizes Ca^{2+} from intracellular stores. Recently, an InsP_3 receptor binding protein from rat cerebellar membranes was solubilized and purified to homogeneity. The potent inhibition by Ca^{2+} of $[\text{}^3\text{H}]\text{InsP}_3$ binding to the InsP_3 receptor in cellular membranes is not apparent in the purified receptor. The Ca^{2+} -dependent inhibition of $[\text{}^3\text{H}]\text{InsP}_3$ binding in the crude homogenate (concn. giving 50% inhibition = 300 nM) can be restored by addition of solubilized cerebellar membranes to the purified receptor. In the present study, we further characterize the protein in solubilized membranes which confers Ca^{2+} -sensitivity to the receptor, and which we term 'calmedin'. Calmedin appears to be a neutral membrane protein with an estimated M_r of 300 000 by gel filtration in the presence of Triton X-100. Calmedin confers a Ca^{2+} -sensitivity to InsP_3 receptor binding, which can be completely reversed by 10 min incubation with EDTA and therefore does not represent Ca^{2+} -dependent proteinase action. Calmedin effects on the purified InsP_3 receptor depend on Ca^{2+} binding to the calmedin, although Ca^{2+} also binds directly to the InsP_3 receptor. The regional distribution of calmedin differs from that of the InsP_3 receptor in the brain, suggesting that it also mediates other Ca^{2+} -dependent functions. Calmedin activity in peripheral tissues is much lower than in brain.

INTRODUCTION

Ca^{2+} is a major mediator of the effects of the phosphoinositide system which serves as a second messenger for numerous neurotransmitters, hormones and growth factors [1–5]. In the phosphoinositide cycle, phosphatidylinositol 4,5-bisphosphate is converted into diacylglycerol and inositol 1,4,5-trisphosphate (InsP_3) after receptor activation [1,6]. InsP_3 appears to bind to receptors on intracellular membranes to release Ca^{2+} [1,7]. Specific binding sites for InsP_3 have been demonstrated in peripheral tissues [8–11] and in the brain, where sites are highly concentrated in the cerebellum [12,13]. The InsP_3 -binding site in the brain is potently inhibited by Ca^{2+} with 50% inhibition at about 300 nM [13]. We have purified the InsP_3 -binding site from brain to homogeneity [14]. To date, we have not shown direct evidence for involvement of this binding site in Ca^{2+} release. The purified InsP_3 -binding site solubilized in Triton X-100 has a native M_r of about 10^6 , whereas on SDS/polyacrylamide-gel electrophoresis its apparent M_r is 260 000 [14]. In contrast with the potent inhibition of InsP_3 binding to brain membranes by Ca^{2+} , InsP_3 binding to the purified receptor is unaffected by Ca^{2+} [14]. We have shown that, in the course of purification of the receptor protein, a detergent-solubilized membrane protein which conveys the inhibitory effects of Ca^{2+} was separated from the InsP_3 receptor [14]. Inhibition of receptor binding by Ca^{2+} could be restored by adding detergent-solubilized membrane fractions back to the purified receptor [14]. In the present study we characterize several properties of the protein which mediates the

inhibitory effect of Ca^{2+} on the InsP_3 receptor, and which we term 'calmedin'.

MATERIALS AND METHODS

Materials

$[\text{}^3\text{H}]\text{InsP}_3$ (3.6 Ci/mmol) was obtained from NEN/DuPont. InsP_3 was obtained from Amersham. Other reagents were from Sigma or Bio-Rad.

Preparation of the InsP_3 receptor and heparin flow-through

The InsP_3 receptor protein was purified as in our earlier study [14], with minor modifications. Cerebella from male Sprague–Dawley rats (200–300 g) or bovine cerebella obtained from a local slaughterhouse were homogenized (Polytron setting 9, 10 s) in 50 vol. of ice-cold buffer A (50 mM-Tris/HCl, pH 7.7, 1 mM-EDTA, 1 mM- β -mercaptoethanol) and pelleted by centrifugation (35 000 g, 15 min). The supernatants were decanted and the pellets were twice resuspended in 50 vol. of buffer A and centrifuged (35 000 g, 15 min). The pellets were resuspended in a final volume of buffer A to give 50 mg original wet wt./ml. Triton X-100 was added to a final concentration of 1% (v/v) and the membranes were incubated for 30 min at 4 °C. The membranes were centrifuged for 1 h at 48 000 g and the supernatant was collected. The solubilized fraction was made 0.25 M in NaCl and then passed over a heparin–agarose column (3 cm $\times$ 3 cm) equilibrated with buffer A containing 0.25 M-NaCl and 0.1% Triton X-100. The membrane

Abbreviation used: InsP_3 , inositol 1,4,5-trisphosphate.

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component responsible for Ca^{2+} inhibition flowed through the column completely (subsequently designated as 'heparin flow-through'), whereas all InsP_3 -binding activity was retained on the column. The column was washed with 5 column vol. of buffer A with 0.25 M-NaCl and 0.1 % Triton X-100, and eluted with buffer A with 0.5 M-NaCl and 0.1 % Triton X-100. The eluate was then passed through a concanavalin A column (3 cm $\times$ 3 cm), washed with 100 mM-NaCl/50 mM-Tris (pH 7.7)/0.1 % Triton X-100/1 mM- β -mercaptoethanol (buffer B), and the purified receptor was eluted with buffer B with 1 M- α -methyl mannopyranoside.

$[^3\text{H}]\text{InsP}_3$ binding to the purified receptor

Purified InsP_3 receptor (0.1 μg) solubilized in buffer A containing detergent was incubated with 12.5 nM- $[^3\text{H}]\text{InsP}_3$ and either buffer A or heparin flow-through (containing calmodin activity) in a total volume of 200 μl . In experiments with Ca^{2+} , 1.7 mM- CaCl_2 (final free $[\text{Ca}^{2+}] = 700 \mu\text{M}$) was added and the mixture incubated for 1 min (or for a specified time) at 4 $^\circ\text{C}$, and then loaded on a 1 ml column packed with 70 g of Bio-Gel P-10 resin/l and centrifuged at 1000 g for 6 min. Liquid-scintillation cocktail (5 ml) was added to the void volume of these columns, and radioactivity was determined. $[^3\text{H}]\text{InsP}_3$ binding was linear with purified InsP_3 -receptor concentration, and not more than 10 % of the radioactivity added was bound. All of the radioactivity could be displaced by 1 μM - InsP_3 .

Assessment of calmodin activity

$[^3\text{H}]\text{InsP}_3$ binding was assayed as described above. Parallel determinations were made (a) in the absence of Ca^{2+} , (b) in the presence of 500 μM - Ca^{2+} and (c) in the presence of 500 μM - Ca^{2+} followed by 15 min treatment with 1 mM-EDTA. Calmodin activity is defined as the EDTA-reversible Ca^{2+} -dependent inhibition of $[^3\text{H}]\text{InsP}_3$ binding.

$^{45}\text{Ca}^{2+}$ binding

Samples were filtered in Centriprep 30 units and washed in 10 vol. of buffer A to remove EDTA. Retentate (1 ml) was incubated with 1 μCi of $^{45}\text{Ca}^{2+}$ (NEN/DuPont) for 10 min and loaded on to two 500 μl Chelex columns (Bio-Rad). Scintillation cocktail (Formula 963, NEN/DuPont; 5 ml) was added to the flow-through of the Chelex column, and the sample was counted for radioactivity in a Beckman scintillation counter.

Other methods

Protein concentrations were determined by the method of Bradford [15].

RESULTS

Time course of reversal of calmodin activity by EDTA

We have previously shown that $[^3\text{H}]\text{InsP}_3$ binding to the purified InsP_3 receptor is not inhibited by Ca^{2+} alone, but that addition of detergent-solubilized membranes to the purified receptor restores fully the inhibitory effects of Ca^{2+} seen in crude cerebellar membranes [14]. The factor in detergent extracts which confers Ca^{2+} -sensitivity appears to be a protein, as its activity was destroyed by treatment with trypsin or heating [14].

We wondered whether the effects of calmodin merely

Fig. 1. Time course of EDTA reversal of Ca^{2+} -mediated inhibition of $[^3\text{H}]\text{InsP}_3$ binding to the InsP_3 receptor

Purified InsP_3 receptor (0.1 μg), prepared as described in the Materials and methods section, was incubated with heparin flow-through (75 μl), 12.5 nM- $[^3\text{H}]\text{InsP}_3$ and 1.7 mM- CaCl_2 in a final volume of 200 μl . EDTA (1.7 mM) was added, and the mixture was assayed for $[^3\text{H}]\text{InsP}_3$ binding to the receptor (by the spun-column method) either immediately or after a period of incubation. The percentage inhibition of $[^3\text{H}]\text{InsP}_3$ binding was calculated by taking binding to the receptor in the absence of Ca^{2+} as 100 %. Data are from a typical experiment which was replicated twice.

reflected a Ca^{2+} -sensitive protease. In preliminary experiments we showed that the ability of calmodin to confer Ca^{2+} -sensitivity to the InsP_3 receptor was completely reversed by 1 mM-EDTA, ruling out proteolysis [14]. To characterize the reversibility of calmodin action, we examined the time course of the effect of EDTA (Fig. 1). Purified receptor protein was incubated with $[^3\text{H}]\text{InsP}_3$ and 500 μM - Ca^{2+} with excess EDTA added to samples at zero time, 5 min or 10 min. When EDTA was incubated together with $[^3\text{H}]\text{InsP}_3$, Ca^{2+} and receptor protein for a full 10 min, it completely reversed the inhibitory effects of Ca^{2+} , but no reversal was apparent if EDTA was present for only 5 min. Thus the ability of EDTA to reverse the effects of Ca^{2+} , presumably by chelating Ca^{2+} that has dissociated from calmodin, is time-dependent. These results suggest that Ca^{2+} binds to calmodin with substantial avidity, requiring 5–10 min to dissociate. In all subsequent experiments, to ensure that effects of Ca^{2+} do not result from Ca^{2+} -dependent proteolysis, we included control samples treated with 1 mM-EDTA for 15 min after 500 μM -calcium was added to ensure reversibility of the Ca^{2+} effect. All calmodin-mediated inhibitory effects of Ca^{2+} on InsP_3 receptor binding hereafter reflect EDTA-reversible actions.

Concentration-dependence of calmodin activity on Ca^{2+} and tissue

In the presence of calmodin, Ca^{2+} inhibited $[^3\text{H}]\text{InsP}_3$ binding in a concentration-dependent fashion (Fig. 2a); 50 % inhibition of binding was apparent at about 500 nM- Ca^{2+} , with almost complete inhibition evident at 1 μM . The potency of Ca^{2+} in these experiments is similar to its potency in inhibiting $[^3\text{H}]\text{InsP}_3$ binding to crude cerebellar membranes [13]. Confirming our earlier ob-

Fig. 2. Inhibition of $[^3\text{H}]\text{InsP}_3$ binding depends on the concentration of Ca^{2+} and heparin flow-through

Purified InsP_3 receptor and heparin flow-through were prepared and $[^3\text{H}]\text{InsP}_3$ binding was assayed by spin-column separation as described in the Materials and methods section. (a) Ca^{2+} concentrations (buffered with hydroxyl-EDTA) were varied in the presence of 0.5 mg of protein/ml of heparin flow-through. (b) Concentration of heparin flow-through containing calmedin in the final incubation was varied and Ca^{2+} concentration (500 nM) was kept constant. Data are from typical experiments replicated twice. Note that the concentration of NaCl in these experiments was 0.3 M, but no other bivalent cations were included.

servations [14] in the absence of calmedin, concentrations of Ca^{2+} as high as 500 μM failed to inhibit $[^3\text{H}]\text{InsP}_3$ binding to purified receptor protein (results not shown).

We were unable to assess unambiguously the Ca^{2+} -dependency of calmedin in the presence of Mg^{2+} because this ion activates a phosphatase which hydrolyses $[^3\text{H}]\text{InsP}_3$ under our assay conditions [13]. However, we attempted these experiments in the presence of 2 mM- MgCl_2 , assaying calmedin activity and simultaneously monitoring conversion of $[^3\text{H}]\text{InsP}_3$ into inositol mono- and bis-phosphates. Although the data varied, apparently depending on the total InsP_3 phosphatase activity of each sample, calmedin activity was the same as in the absence of MgCl_2 (results not shown). Furthermore, the time course of reversibility (see above) was identical with or without 2 mM- MgCl_2 .

Although 1 μM - CaCl_2 ensures complete activation of calmedin, we have utilized 500 μM - CaCl_2 in subsequent

Fig. 3. Gel-filtration profile of calmedin

Bovine cerebellar membranes were solubilized with 1% Triton as described in the Materials and methods section. The soluble fraction was passed over a heparin-agarose column and the flow-through collected and concentrated to 20% of the original volume with an Amicon filter (cut-off 15000 M_r). The retentate was diluted to the original volume with buffer A (containing no NaCl or Triton) and re-concentrated to 20% of the original volume twice. Then 3 ml of the retentate was loaded on a Sephacryl S-400 column (0.5 cm $\times$ 45 cm). Fractions (2.2 ml) eluted with buffer A containing 0.1% Triton were collected and assayed for calmedin activity as described in the Materials and methods section. The yield of calmedin activity from this column [void volume = 25.2 ml; included volume = 56.0 ml] was approx. 60%. Data depicted are from a typical experiment. Arrows indicate positions of marker proteins, with M_r values in parentheses.

experiments to ensure saturation, because we found that subtle pipetting errors could lead to large variations in free Ca^{2+} concentrations in EDTA buffer as measured by a Ca^{2+} electrode.

$[^3\text{H}]\text{InsP}_3$ binding to detergent-solubilized cerebellar membranes is inhibited potently by Ca^{2+} . The effect of Ca^{2+} is lost after purification of the receptor on a heparin-agarose column, to which the InsP_3 receptor protein adheres [14]. Calmedin activity does not adhere to the heparin column, and can be routinely assessed in portions of the heparin flow-through. The ability of calmedin to confer Ca^{2+} inhibition was concentration-dependent (Fig. 2b). Ca^{2+} inhibition of $[^3\text{H}]\text{InsP}_3$ binding was almost maximal with as little as 250 μg (final protein concentration) of the heparin flow-through preparation/ml in binding incubations, with about 50% of maximal inhibition of Ca^{2+} binding apparent at about 100–150 $\mu\text{g}/\text{ml}$ when 0.1 μg of purified InsP_3 receptor was used to assess total binding.

Characterization of calmedin

To assess the approximate M_r of calmedin, we fractionated the heparin flow-through on a Sephacryl S-

Table 1. Effects of preincubation of EDTA with InsP_3 receptor and calmedin on Ca^{2+} inhibition of $[^3\text{H}]\text{InsP}_3$ binding to purified receptor

Purified InsP_3 receptor and heparin flow-through were prepared as described in the Materials and methods section. For assays, 0.1 μg of InsP_3 receptor and 0.5 mg of protein/ml of heparin flow-through were separately incubated with 500 μM - CaCl_2 ; 1 mM-EDTA was added to some fractions (+preincubation), but not to others (–preincubation). After 15 min, InsP_3 receptor and heparin flow-through fractions were combined, and $[^3\text{H}]\text{InsP}_3$ binding was immediately assayed (< 4 min); $[^3\text{H}]\text{InsP}_3$ and the purified receptor were incubated for 1 min at 4 °C, and bound $[^3\text{H}]\text{InsP}_3$ was measured as in the Materials and methods section. This experiment was performed twice in quadruplicate. Data are mean values which varied less than 10 %.

Fractions preincubated with EDTA

Purified InsP_3 receptor	Heparin flow-through (calmedin)	$[^3\text{H}]\text{InsP}_3$ binding (c.p.m.)
–	–	25
+	+	800
–	+	850
+	–	140

400 column (Fig. 3). Calmedin activity emerged as a relatively sharp peak with an apparent M_r greater than that of alcohol dehydrogenase (80 000) but less than that of thyroglobulin (700 000). We estimate the native M_r of calmedin to be about 300 000. This value is likely to be an overestimate, owing to the well-known increase in apparent Stokes radius of membrane proteins which bind Triton X-100.

We assayed the ability of each of the fractions from the S-400 column to bind $^{45}\text{Ca}^{2+}$, using Chelex columns to separate free $^{45}\text{Ca}^{2+}$ from bound radioactivity. $^{45}\text{Ca}^{2+}$ binding was low throughout most of the column and highest in the included fraction. There was no correlation between $^{45}\text{Ca}^{2+}$ binding and calmedin activity (results not shown). Calmedin does not appear to be identical with either calmodulin or calcineurin, well-known Ca^{2+} -binding proteins, because these proteins partition predominantly into the soluble fraction, unlike calmedin, which is membranous.

We also examined the chromatographic properties of calmedin with ion-exchange columns (results not shown). Calmedin was not retained by a DEAE-cellulose column at pH 7.7, in 0.1 M-NaCl. Calmedin was also not retained by a CM-cellulose column at pH 7.7 in the absence of NaCl. This suggests that calmedin is a neutral protein.

Calmedin activity was stable when frozen at –70 °C for at least 4 weeks. However, when maintained at 4 °C calmedin activity was stable for only about 2 h. After 2 h, calmedin preparations could inhibit $[^3\text{H}]\text{InsP}_3$ binding, but such effects were not reversed by EDTA. This non-reversible inhibitory activity could be separated from the InsP_3 receptor by passing the mixture through a heparin-agarose column.

Interaction of Ca^{2+} with calmedin

In preliminary experiments we observed saturable binding of $^{45}\text{Ca}^{2+}$ to purified InsP_3 receptor protein, with

Table 2. Distribution of calmedin activity in rat brain regions and peripheral tissues

Various tissues and brain regions were homogenized (Brinkmann Polytron) in 50 mM-Tris (pH 7.7)/1 mM-EDTA/1 mM- β -mercaptoethanol. Homogenates were centrifuged at 20 000 g for 15 min. Pellets were resuspended in the same buffer and re-centrifuged at 10 000 g for 15 min. This procedure was repeated three times. The final pellet was then solubilized in 50 mM-Tris (pH 7.7)/1 mM-EDTA/1 mM- β -mercaptoethanol/1 % Triton for 30 min at 50 mg wet wt./ml and the suspension centrifuged at 20 000 g for 1 h. Solubilized samples (0.25 mg of protein/ml) were incubated for 4 min at 4 °C with 0.5 μg of InsP_3 receptor, 12.5 nM- $[^3\text{H}]\text{InsP}_3$, and either 1.5 mM added CaCl_2 (final free Ca^{2+} concn. 500 μM) or no addition. To determine reversibility of Ca^{2+} -dependent inhibition, 1 mM-EDTA was added to the mixture, which was then maintained for 15 min at 4 °C. $[^3\text{H}]\text{InsP}_3$ binding was then assayed by spun-column separation (see the Materials and methods section). Brain stem, spleen, skeletal muscle and colon all displayed non- Ca^{2+} -dependent inhibition of $[^3\text{H}]\text{InsP}_3$ binding. Data are mean values from four experiments each in triplicate, with results varying less than 20 %.

Region	Inhibition of InsP_3 binding (%)	
	With 500 μM - CaCl_2	With 500 μM - CaCl_2 followed by 1 mM-EDTA
Cerebellum	100	0
Thalamus	100	0
Cerebral cortex	100	0
Spinal cord	100	48
Testis	20	0
Lung	18	0
Liver	15	0
Kidney	15	0
Heart	0	0

a K_D of approx. 50 μM (S. Supattapone, unpublished work). We wondered if calmedin conveyed the inhibitory effects of Ca^{2+} on the InsP_3 receptor by influencing the binding of Ca^{2+} to the receptor protein or by the binding of Ca^{2+} to calmedin itself. To examine this question, we took advantage of the fact that a 10 min incubation with EDTA is required to reverse the effects of Ca^{2+} (Fig. 1). We incubated heparin flow-through (containing calmedin) and the InsP_3 receptor protein separately with Ca^{2+} . EDTA was added to calmedin alone, to the InsP_3 receptor alone, to both calmedin and the InsP_3 receptor, or to neither (Table 1). After 15 min incubation with EDTA the InsP_3 receptor and calmedin samples were mixed, $[^3\text{H}]\text{InsP}_3$ was added, and the preparation was immediately assayed for $[^3\text{H}]\text{InsP}_3$ binding to the InsP_3 receptor. The short incubation time employed for $[^3\text{H}]\text{InsP}_3$ binding (1 min) is much less than the 10 min required for EDTA to reverse the inhibitory effect of Ca^{2+} (Fig. 1). Thus, in samples not pretreated for 15 min with 1 mM-EDTA, tightly bound Ca^{2+} which inhibits InsP_3 binding should still be present during the $[^3\text{H}]\text{InsP}_3$ binding assay, despite the removal of free Ca^{2+} by excess EDTA introduced at the initiation of $[^3\text{H}]\text{InsP}_3$ binding. As in previous experiments, in the absence of EDTA, Ca^{2+} (500 μM) completely inhibited

[³H]InsP₃ binding. When both receptor and calmedin were treated with EDTA, the inhibition was fully reversed. When calmedin but not the InsP₃ receptor was treated with EDTA, inhibition was also reversed. However, when the InsP₃ receptor protein but not calmedin was treated with EDTA, Ca²⁺ markedly inhibited [³H]InsP₃ binding. Accordingly, the influence of Ca²⁺ on receptor binding appears to be mediated by Ca²⁺ binding directly to calmedin rather than the InsP₃ receptor protein.

Regional and species distribution of calmedin

InsP₃ receptor binding densities vary markedly throughout the brain, with much higher densities in the cerebellum than in any other brain region, and with very low values in peripheral tissues [13,14]. To assess the regional distribution of calmedin activity, we added amounts of tissue protein from various rat brain areas equal to cerebellar protein content which was just sufficient to provide a near-maximal inhibition by Ca²⁺ of InsP₃ binding (Table 2; Fig. 2b). The calmedin activities of the three brain regions and the spinal cord were closely similar. No region appeared to possess substantially less calmedin activity than the cerebellum. From our experimental design we cannot rule out the possibility that other regions might contain more calmedin activity than the cerebellum. Calmedin activity in the cerebellum, thalamus and cerebral cortex was completely reversed by a 10 min incubation with EDTA, whereas only 50% reversal was obtained in the spinal cord.

In contrast with the robust calmedin activity of central-nervous-system areas, the five peripheral tissues assayed displayed very low calmedin activity.

DISCUSSION

The major finding of our study is that a membrane-associated protein of the brain, which we refer to as calcium-mediator protein (calmedin), enables Ca²⁺ to inhibit InsP₃ binding to purified receptor protein. Since the potency of Ca²⁺ in inhibiting receptor binding after addition of calmedin is the same as in native membranes, we believe that calmedin may be physiologically associated with the regulation of InsP₃ receptors by Ca²⁺.

The regional distribution of calmedin in the brain differs markedly from that of the InsP₃ receptor, with similar amounts of calmedin present in several areas of the central nervous system. Accordingly, we suspect that calmedin is involved in effects of Ca²⁺ on other systems in addition to the InsP₃ receptor. The evidence that we have provided that calmedin confers Ca²⁺-sensitivity to the InsP₃ receptor by the binding of Ca²⁺ to calmedin itself suggests that calmedin may be a major membrane-associated Ca²⁺-binding protein in the brain. Other high-affinity Ca²⁺-binding membrane proteins which have been previously characterized include glycerol-3-

phosphate dehydrogenase ($K_D = 150$ nM), a mitochondrial membrane protein [16], a M_r -21000 calicoprotein ($K_D = 1$ μ M) isolated from bovine brain [17], Ca²⁺-ATPase of the sarcoplasmic reticulum ($K_D = 300$ nM) [18] and Ca²⁺/phospholipid-dependent protein kinase C [K_D (in presence of diacylglycerol) = 100 nM] [3]. It is unlikely that calmedin is either glycerol-3-phosphate dehydrogenase or Ca²⁺-ATPase, since both of these are ubiquitous in peripheral tissue. The M_r -21000 brain calicoprotein and protein kinase C have different apparent M_r values from that of calmedin. InsP₃ binding to receptors in peripheral tissues is not inhibited by Ca²⁺ [8–11]. The very low amounts of calmedin detected in peripheral tissues might account for the failure of peripheral InsP₃ receptors to respond to Ca²⁺.

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