Biochem. J. (1991) 277, 125–131 (Printed in Great Britain)

Involvement of the *C*-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies

Shinji NAKADE,* Nobuaki MAEDA*‡ and Katsuhiko MIKOSHIBA*†§

*Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita Osaka 565, Japan, and †Department of Biochemistry, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

We have studied the effects of monoclonal antibodies that recognize different epitopes of the cerebellar Ins(1,4,5)P_3 receptor on Ins(1,4,5)P_3-induced Ca^{2+}-release activity. Ins(1,4,5)P_3 stimulated Ca^{2+} flux from cerebellar microsomes, and half-maximal Ca^{2+} release occurred at 112 ± 8 nM-Ins(1,4,5)P_3 [concentration causing half-maximal effect (EC_{50}) = 112.8 nM]. The minimum concentration of Ins(1,4,5)P_3 necessary to initiate Ca^{2+} release (threshold concentration) was 20 ± 5 nM. A monoclonal antibody (mAb) 18A10 (50 μg/ml), which recognizes the *C*-terminal region of the Ins(1,4,5)P_3 receptor, suppressed Ins(1,4,5)P_3-induced Ca^{2+} release: the EC_{50} and threshold concentration shifted to 460 ± 56 nM and 61 ± 6 nM respectively. On the other hand, the antibody at the same concentration raised the affinity of the receptor for binding to Ins(1,4,5)P_3, and the K_d value decreased from 43 ± 12 nM to 25 ± 4 nM without a change in the number of Ins(1,4,5)P_3-binding sites. However, mAbs that recognize the *N*-terminal domain affected neither Ca^{2+} release nor Ins(1,4,5)P_3 binding. Among the various synthetic peptides, only the 12-residue-long peptide from the most *C*-terminal portion of the receptor (amino acid residues 2736–2747) reacted strongly with mAb18A10. From these findings, combined with the Immunogold localization of the cerebellar Ins(1,4,5)P_3 receptor [Otsu, Yamamoto, Maeda, Mikoshiba & Tashiro (1990) Cell Struct. Funct. **15**, 163–173], we concluded that the *C*-terminus of the Ins(1,4,5)P_3 receptor is exposed to the cytoplasmic side of the smooth endoplasmic reticulum and plays an important role in the regulation of both Ins(1,4,5)P_3-binding affinity and channel gating.

INTRODUCTION

The intracellular Ca^{2+} concentration is controlled by both Ca^{2+} influx from the outside of the cell and Ca^{2+} release from the intracellular Ca^{2+} storage sites [1,2]. The voltage-sensitive Ca^{2+} channels [3] and ligand-gated cation channels [4,5] located in the plasma membrane have roles in the influx of Ca^{2+} from outside the cell. Recently, the importance of Ins(1,4,5)P_3 generated from polyphosphoinositides in response to receptor stimulation by various kinds of hormones and neurotransmitters has been stressed. Ins(1,4,5)P_3 is believed to be a second mediator of Ca^{2+} release from the Ca^{2+} storage sites [6–8]. Ins(1,4,5)P_3-induced Ca^{2+} release is modulated by various mediators such as ATP [9,10], GTP [11], fatty acids [12] and Ca^{2+} [13], and the Ins(1,4,5)P_3 receptor is involved in various intracellular phenomena such as Ca^{2+} 'waves' [14–16] and Ca^{2+} oscillations [16–18]. However, the detailed molecular mechanism of Ca^{2+} release is unknown because of the paucity of knowledge about the structure of the receptor. Recently we succeeded in purifying the mouse cerebellar Ins(1,4,5)P_3 receptor [19] and in cloning its cDNA [20], and we predicted the primary structure of the receptor. Experiments on transfection with the cerebellar Ins(1,4,5)P_3 receptor cDNA revealed that the membrane fraction obtained from the transfected cells has enhanced Ins(1,4,5)P_3-binding activity [20] and enhanced Ins(1,4,5)P_3-induced Ca^{2+} release [21]. Furthermore, reconstitution of the purified cerebellar Ins(1,4,5)P_3 receptor into planar lipid bilayers [22] demonstrated that the receptor constitutes the cation-selective channel that is opened by Ins(1,4,5)P_3 and conducts Na^+ and Ca^{2+}. These findings indicate that the cerebellar Ins(1,4,5)P_3 receptor is a Ca^{2+}-permeable cation-selective channel having Ins(1,4,5)P_3-binding sites. To study the structure–function relationship of the receptor, we used monoclonal antibodies (mAbs) that recognize different epitopes [19,20,23,24] and examined their effects upon Ca^{2+} release and Ins(1,4,5)P_3 binding. We found that the antibodies that recognize the *N*-terminus did not affect either Ins(1,4,5)P_3 binding or Ca^{2+} release, but an antibody that recognizes the *C*-terminus (mAb18A10) inhibited Ca^{2+} release, accompanied by reproducibly increased Ins(1,4,5)P_3 binding without changing the number of Ins(1,4,5)P_3-binding sites. From these findings, together with the precise epitope mapping of mAb18A10, we concluded that the *C*-terminal portion of the receptor plays a crucial role in the regulation of the channel activity of the Ins(1,4,5)P_3 receptor.

MATERIALS AND METHODS

Preparation of a cerebellar microsomal fraction

Adult *ddY* mice were anaesthetized and killed by decapitation and the cerebella were dissected. Fresh cerebella (10 g) was mixed with 9 vol. of a homogenization buffer containing 0.32 M-sucrose, 1 mM-EGTA, 0.1 mM-phenylmethanesulphonyl fluoride (PMSF), 10 μM-leupeptin, 10 μM-pepstatin A, 1 mM-dithiothreitol (DTT) and 5 mM-Tris/HCl, pH 7.4, and was homogenized using a

Abbreviations used: mAb, monoclonal antibody; EC_{50}, concentration causing half-maximal effect; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PBS, phosphate-buffered saline; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; SER, smooth endoplasmic reticulum; *p*CMB, *p*-chloromercuribenzoic acid; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; ABC, avidin/biotin/peroxidase complex.

‡ Present address: Institute for Developmental Research, Aichi Prefectural Colony, Kamiya-cho, Kasugai, Aichi 480-03, Japan.

§ To whom correspondence should be addressed, at: Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565, Japan.

Teflon–glass Potter homogenizer (10 strokes at 850 rev./min). The homogenate was centrifuged at 10500 *g* for 15 min at 2 °C, and then the supernatant was collected and the pellet was mixed with 9 vol. of homogenization buffer and homogenized again under the same conditions. The homogenate was centrifuged at 10500 *g* for 15 min at 2 °C, and then the supernatant was collected. The combined supernatants were centrifuged at 105000 *g* for 60 min at 2 °C to precipitate the microsomal fraction. That fraction was resuspended in a solution containing 0.1 M-sucrose, 1 mM-EGTA, 0.1 mM-PMSF, 10 μM-leupeptin, 10 μM-pepstatin A, 1 mM-DTT and 10 mM-Tris/HCl, pH 7.4, and frozen with liquid N_2 in small amounts. The microsomal fraction could be stored at −80 °C for at least 1 month without significant loss of Ins(1,4,5)P_3-binding activity and Ca^{2+}-release activity.

Measurement of Ins(1,4,5)P_3-induced Ca^{2+} release

The microsomal fraction was centrifuged at 105000 *g* for 15 min and the pellet was mixed with a Ca^{2+} mobilization buffer containing 110 mM-KCl, 1 mM-$MgCl_2$, 1 mM-DTT, 10 mM-NaCl, 5 mM-KH_2PO_4 and 10 mM-Hepes, pH 7.2, and washed three times under the same conditions and then resuspended in the same solution. Ca^{2+} uptake was initiated by adding 200 μl of microsomal suspension (560–600 μg of protein) into a quartz cuvette containing 1.8 ml of a medium composed of (final concentrations, mM): KCl 110, Hepes 10, $MgCl_2$ 1, DTT 1, NaCl 10, KH_2PO_4 5, disodium ATP 1.25 and phosphocreatine 1.25, plus creatine kinase (10 units/ml), oligomycin (2.5 μg/ml) and fura-2 free acid (2 μM), pH 7.2. mAbs were added, and after 15 min of incubation various concentrations of Ins(1,4,5)P_3 were tested. The free Ca^{2+} concentration of the solution was monitored with fura-2 under constant agitation at 30 °C in a dual-excitation wavelength RF-5000 spectrofluorimeter (Shimazu Factory, Tokyo, Japan). Changes in fluorescence were monitored at the emission wavelength of 510 nm, with the excitation wavelength alternating between 340 and 380 nm, so that a ratio R of the fluorescence intensities of the dye at both excitation wavelengths could be obtained 30 times/min. The ratio signal was found to be proportional to the free Ca^{2+} concentration in the cuvette by using Ca^{2+}/EGTA buffers [25]. The actual Ca^{2+} concentration of the medium was calculated from the maximal fluorescence ratio ($R_{max.}$) and autofluorescence ratio ($R_{min.}$) values obtained on addition of excess Ca^{2+} and Mn^{2+} respectively, after treatment with ionomycin (5 μM). The equation used was $[Ca^{2+}] = K_d$ (224 nM) $[(R-R_{min.})/(R_{max.}-R)]\cdot(S_{f2}/S_{b2})$; the K_d is 224 nM, a value appropriate for the medium. S_{f2} is the fluorescence intensity for free dye at 380 nm and S_{b2} is that for Ca^{2+}-bound dye at 380 nm [25].

Preparation of mAbs

The mAbs against the Ins(1,4,5)P_3 receptor were prepared by injecting partially purified mouse Ins(1,4,5)P_3 receptor protein into a rat and fusing the spleen cells with mouse Sp2 myeloma cells, as described previously [23]. The hybridomas were cultured in chemically defined Nissui SFM 101 medium. The culture supernatant was concentrated with an Amicon YM10 membrane and the IgG was purified by h.p.l.c. on a Bio-Gel HTP column (Bio-Rad). The purified mAbs were dialysed against phosphate-buffered saline (PBS; pH 7.3) and stored at −80 °C. The mAbs used for assay were more than 98% pure IgG.

Measurement of [^{3}H]Ins(1,4,5)P_3 binding

[^{3}H]Ins(1,4,5)P_3 binding was measured by the centrifugation method. The microsomal fraction was washed three times with a buffer containing 110 mM-KCl, 1 mM-EGTA, 1 mM-DTT, 10 mM-NaCl, 5 mM-KH_2PO_4 and 10 mM-Hepes, pH 7.2, and then resuspended in the same buffer at a protein concentration of 280 μg/ml. mAbs were added to the samples, which were then incubated for 2 h on ice. [^{3}H]Ins(1,4,5)P_3 was added to 50 μl quantities of the samples at a concentration of 11 nM. The samples were incubated for 10 min on ice. Non-specific binding was measured in the presence of 2 μM unlabelled Ins(1,4,5)P_3. After centrifugation at 6500 *g* for 5 min at 2 °C, the precipitate was dissolved in 500 μl of Protosol (NEN) and mixed with 6 ml of Aquasol (Du Pont), and the radioactivity was measured with a liquid scintillation counter.

E.l.i.s.a.

The wells of a 96-well microtitre plate (Nunc) were coated with the purified cerebellar Ins(1,4,5)P_3 receptor (1 μg of protein/ml in sodium carbonate buffer, pH 9.5; 100 μl/well) and the plate was stored in a humid atmosphere at 4 °C overnight. Then unattached antigen was washed out with PBS (pH 7.3) containing 0.05% Tween 20 (PBS/Tween), various concentrations of each mAb were added and the plate was incubated at room temperature for 30 min. Then the plate was washed with PBS/Tween and 100 μl of biotinylated anti-(rat IgG) antibody (diluted 1:500) was added. After incubation at room temperature for 30 min, the plate was washed with PBS/Tween, and 100 μl of avidin/biotin/peroxidase complex (ABC) solution diluted 1:500 was added. The plate was incubated and then washed as above. Finally, 200 μl of the substrate solution (6 mM-H_2O_2 and 40 mM-*o*-phenylenediamine dihydrochloride in PBS/Tween) was added. A PBS/Tween wash is sufficient to prevent non-specific binding. The reaction was stopped by the addition of 50 μl of 8 M-H_2SO_4, and the absorbance was read at 480 nm with a Micro Plate Reader (Toyosoda, Tokyo, Japan). The biotinylated anti-(rat IgG) and ABC solution were from an ABC kit (Vector Laboratories). For the competition experiments, the competing peptides were incubated with mAb18A10 for 30 min at room temperature before addition to microtitre plates coated with the purified Ins(1,4,5)P_3 receptor.

Synthesis of peptides and coupling to carrier protein

Peptides corresponding to the *C*-terminal region of the Ins(1,4,5)P_3 receptor were custom-synthesized on an Applied Biosystems synthesizer, model 430A. The peptides were conjugated to BSA via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as described by Richardson *et al.* [26]. Briefly, 20 mg of peptide and 20 mg of BSA were dissolved in 5 ml of PBS, pH 7.3, and 20 mg of EDC was added at 4 °C with constant stirring. The mixture was stirred overnight. The remaining unreacted EDC and peptides were separated by gel filtration on a Sephadex G-50 column equilibrated in 50 mM-ammonium acetate. The fractions containing peptide–BSA were collected and lyophilized, and then dissolved in PBS.

Immunoblot analysis

SDS/PAGE (7.5% gels) was carried out by the method of Laemmli [27]. The proteins were then transferred to a nitrocellulose paper as described by Towbin *et al.* [28]. The nitrocellulose paper was soaked in 0.5% skim milk in PBS for 30 min at room temperature and then incubated in the mAb18A10 solution for 30 min at room temperature. The paper was processed with a Vectastain ABC rat IgG kit according to the manufacturer's instructions. Finally, the paper was treated with 0.1% diaminobenzidine/0.02% H_2O_2/PBS.

Other methods

Protein concentration was measured with a Bio-Rad protein assay kit, with BSA as standard. Ins(1,4,5)P_3 receptor protein was purified as described previously [19].

Materials

[^{3}H]Ins(1,4,5)P_3 was purchased from NEN. Synthetic Ins(1,4,5)P_3, fura-2, EGTA and Hepes were obtained from Dojindo Laboratories (Kumamoto, Japan). Bio-Gel HTP columns were obtained from Bio-Rad. Creatine kinase, phosphocreatine, oligomycin and disodium ATP were obtained from Sigma. All of the other chemicals were of the highest purity commercially available.

RESULTS

Effects of mAbs against the Ins(1,4,5)P_3 receptor on Ca^{2+} release

We examined Ins(1,4,5)P_3-induced Ca^{2+}-release activity by using the mouse cerebellar microsomal fraction, in which the Ins(1,4,5)P_3 receptor is highly concentrated. The microsomal fraction showed ATP-dependent Ca^{2+}-sequestering activity, and the surrounding Ca^{2+} concentration decreased to 300 nM when ATP was added (Fig. 1*a*). Addition of low concentrations of Ins(1,4,5)P_3 resulted in an immediate efflux of Ca^{2+} followed by a rapid re-uptake (Fig. 1*a*). As the Ins(1,4,5)P_3 concentration increased, restoration to the basal Ca^{2+} concentration was delayed. The re-accumulation of Ca^{2+} in the microsomes seems to be correlated with the degradation of Ins(1,4,5)P_3 [29]. Desensitization of Ca^{2+}-release activity by Ins(1,4,5)P_3 was not observed (Fig. 1*a*), and repeated addition of the same concentration of Ins(1,4,5)P_3 did not change the magnitude of the response (results not shown). The concentration of released Ca^{2+} increased from zero to 300 nM when the Ins(1,4,5)P_3 concentration was increased from 10 nM to 1 μM. Addition of 5 μM-ionomycin immediately released all of the accumulated Ca^{2+}. After addition of ionomycin, the Ca^{2+} concentration increased to 3 μM (results not shown).

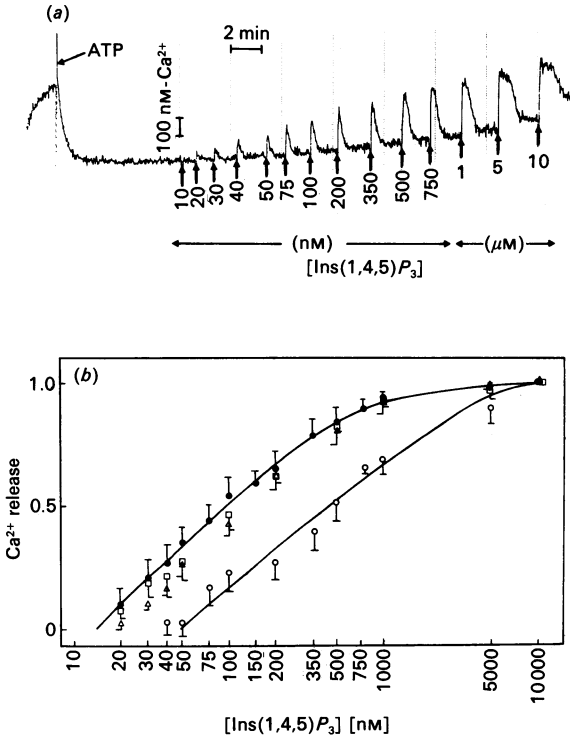

Fig. 1. Ca^{2+} release induced by different concentrations of Ins(1,4,5)P_3 from mouse cerebellar microsomes in the presence or absence of mAbs

Mouse cerebellar microsomal fractions were incubated at 30 °C in a buffer containing 1.25 mM-ATP and an ATP-regeneration system, and their Ca^{2+}-uptake and -release activities were monitored with fura-2 (free acid). The arrows indicate the addition of increasing amounts for Ins(1,4,5)P_3 (between 10 nM and 10 μM). The left-hand ordinate scale shows the free Ca^{2+} concentration. The steady-state free Ca^{2+} concentration was 200 nM. The results shown are from a typical experiment (*a*). (*b*) Dose-dependent effects of Ins(1,4,5)P_3 on Ca^{2+} release in the presence or absence of 50 μg of mAbs/ml. The amount of Ca^{2+} released is expressed relative to the maximal response (= 1.0). The maximal response of Ca^{2+} release in each experiment was almost the same in the presence and absence of mAbs. Each plotted value is the mean ± S.D. of 5–10 independent experiments. Plotted are dose-dependent curves of Ca^{2+} release in the absence of mAb (●), or in the presence of 50 μg of mAb18A10/ml (○), 50 μg of mAb4C11/ml (△) or 50 μg of mAb10A6/ml (□).

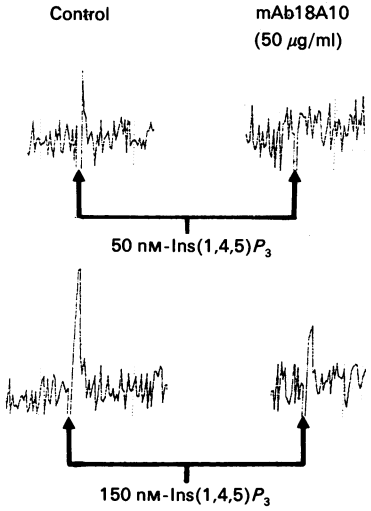

Fig. 2. Inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release by mAb18A10 at submaximal Ins(1,4,5)P_3 concentrations

Ins(1,4,5)P_3-induced Ca^{2+} release was measured as described in the Materials and methods section and the legend to Fig. 1. Shown is a comparison of Ca^{2+} release from a microsomal fraction preincubated for 15 min with or without (control) 50 μg of mAb18A10/ml. The ATP-dependent Ca^{2+} uptake phase is not shown. The arrows indicate the additions of 50 nM- and 150 nM-Ins(1,4,5)P_3.

mAbs against the Ins(1,4,5)P_3 receptor, 4C11 (IgG_{2a}), 10A6 (IgG_{2b}) and 18A10 (IgG_{2a}), each exhibiting unique epitope specificity [19,20,23,24], were used to determine their effects on Ins(1,4,5)P_3-induced Ca^{2+} release. The response to various doses of Ins(1,4,5)P_3 (20 nM–10 μM) in the presence or absence of mAbs was plotted (Fig. 1*b*). In the absence of mAb the threshold response occurred at 20 ± 5 nM for Ins(1,4,5)P_3; this value was constant at various concentrations of both the microsomal protein (from 100 μg/ml to 400 μg/ml) and ambient Ca^{2+} (from 100 nM to 500 nM, results not shown). Half-maximal Ca^{2+} release occurred at 112 ± 8 nM for Ins(1,4,5)P_3 (EC_{50} = 112 ± 8 nM). This value was of the same order as the k_d value for Ins(1,4,5)P_3 binding described below. These results were in agreement with earlier reports [29]. In the presence of 50 μg of mAb18A10/ml, there were apparent shifts in the EC_{50} and the threshold value (Fig. 1*b*). At the maximal concentration of Ins(1,4,5)P_3 tested, the presence of mAb18A10 did not alter the absolute amount of Ca^{2+} released from the microsomal fraction (Fig. 1*b*). However, mAb18A10 decreased the responses at all submaximal Ins(1,4,5)P_3 concentrations. Fig. 2 shows a comparison of Ca^{2+} release at 50 and 150 nM-Ins(1,4,5)P_3 in the presence or the absence of 50 μg of mAb18A10/ml. mAb18A10 apparently inhibited Ca^{2+} release. On the other hand, mAb4C11 and

Table 1. Effects of mAb18A10 on EC_{50} and threshold values for Ins(1,4,5)P_3-induced Ca^{2+} release

EC_{50} values and threshold concentrations for Ins(1,4,5)P_3-induced Ca^{2+} release were determined with various mAb18A10 concentrations. Experiments were performed as described in the legend to Fig. 1. Each value is the mean ± S.D. of at least five experiments, except for the 100 μg of mAb18A10/ml series, where the value is the mean of the two experiments.

mAb18A10 concentration (μg/ml)	EC_{50} (nM)	Threshold (nM)
0	112± 8	20±5
5	143±26	33±5
10	174±28	40±2
20	248±36	46±2
50	460±56	61±6
100	500	60

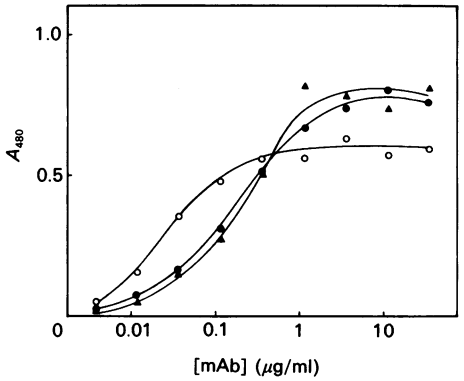

Fig. 3. Comparison of the binding affinities of the mAbs to the purified Ins(1,4,5)P_3 receptor

The binding affinities of the mAbs to the Ins(1,4,5)P_3 receptor were compared by the e.l.i.s.a. method. Shown are the results for mAb18A10 (▲), mAb4C11 (●) and mAb10A6 (○).

mAb10A6, which recognize the *N*-terminal domain of the Ins(1,4,5)P_3 receptor, did not suppress Ins(1,4,5)P_3-induced Ca^{2+} release even in the presence of 50 μg of mAbs/ml (Fig. 1*b*). The inhibition of the Ins(1,4,5)P_3-induced Ca^{2+} release by mAb18A10 was dependent on the concentration of the antibody (Table 1). mAb18A10 (50 μg/ml) raised the EC_{50} from 112±8 nM to 460±56 nM and the higher concentrations of the antibody did not further inhibit Ca^{2+} release. Table 1 also shows the effect of the dose of mAb18A10 on the threshold concentration, which increased with increasing antibody concentration, similar to the case of the EC_{50}.

Binding of the mAbs to the Ins(1,4,5)P_3 receptor was examined by e.l.i.s.a. at various antibody concentrations in order to determine whether the inhibitory effect of mAb18A10 on Ca^{2+} release was due to its stronger affinity with the Ins(1,4,5)P_3 receptor compared with the other mAbs (Fig. 3). From these experiments, the relative affinity of each antibody to its epitope could be determined. The order of the affinities of these antibodies with the receptor was 10A6 > 4C11 = 18A10, indicating that the affinity of mAb18A10 was not greater than that of the others. Thus the inhibitory effect of mAb18A10 on Ins(1,4,5)P_3-induced Ca^{2+} release is presumably due to its binding to an important site for channel activity.

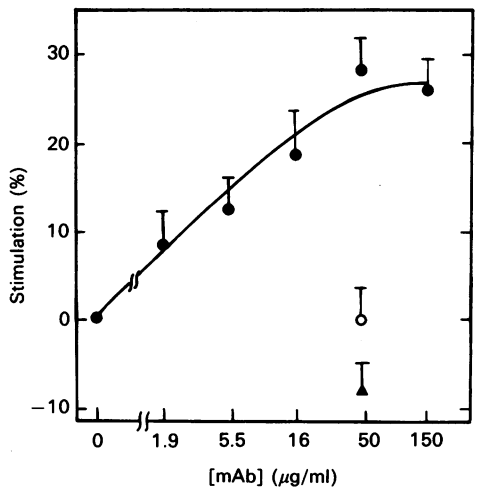

Fig. 4. Stimulation of [^{3}H]Ins(1,4,5)P_3 binding by mAb18A10

Microsomal fractions were preincubated with the various concentrations of mAb18A10 (●) or with 50 μg of mAb4C11/ml (○) or 50 μg of mAb10A6/ml (▲) for 2 h on ice. In the control experiments an equal volume of PBS was added instead of the mAb. [^{3}H]Ins(1,4,5)P_3 (11 nM) was allowed to bind to the microsomal fractions for 10 min on ice. The specific binding of [^{3}H]Ins(1,4,5)P_3 for both conditions was measured and the percentage stimulation of binding by mAbs over the value of the control experiments was calculated and plotted. Each value is the means ± S.D. of three experiments, each in triplicate.

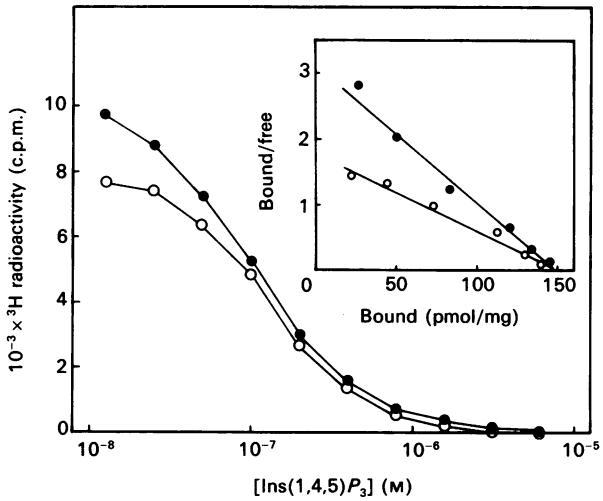

Fig. 5. Increased affinity of microsomes with [^{3}H]Ins(1,4,5)P_3 in the presence of mAb18A10

Microsomal fractions were incubated with 50 μg of mAb18A10/ml for 2 h on ice. Binding assays contained 24 μg of microsomal fraction, 23 nM-[^{3}H]Ins(1,4,5)P_3 and various concentrations of unlabelled Ins(1,4,5)P_3 in 50 μl of 110 mM-KCl/1 mM-EDTA/1 mM-DTT/10 mM-NaCl/5 mM-KH_2PO_4/10 mM-Hepes/KOH, pH 7.2, and with (●) or without (○) 50 μg of mAb18A10/ml. The samples were incubated for 10 min on ice and specific binding was measured as described in the Materials and methods section. The inset shows Scatchard analysis. Data are the means of triplicate determinations in a representative experiment. Analyses of three independent experiments revealed a K_d of 43±12 nM in the absence of mAb18A10 and a K_d of 25±4 nM in the presence of mAb18A10, with a $B_{max.}$ of 150±10 pmol/mg of protein.

Effects of the mAbs on Ins(1,4,5)P_3 binding

When we examined the effects of the mAbs on [^{3}H]Ins(1,4,5)P_3 binding, we found that only mAb18A10 resulted in 30%

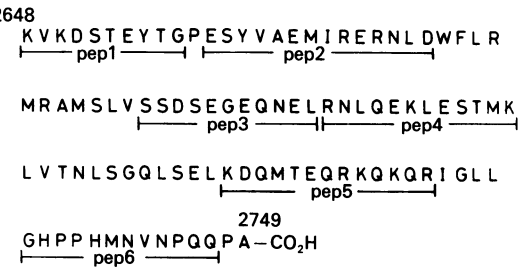

Fig. 6. Peptides used for epitope mapping

Amino acid sequences of the six synthesized peptides (pep1–pep6) tested for epitope mapping of mAb18A10 are shown.

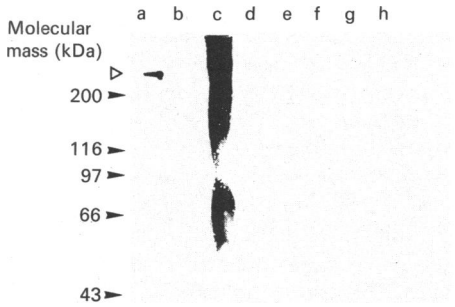

Fig. 7. Immunoreactivity of mAb18A10 with the pep6–BSA conjugate

Peptide–BSA conjugates were electrophoresed on SDS/PAGE (7.5% gels) and then processed for immunoblotting. mAb18A10 was used for immunodetection of peptide–BSA conjugates (lane b, BSA; c, pep6–BSA; d, pep1–BSA; e, pep2–BSA; f, pep3–BSA; g, pep4–BSA; h, pep5–BSA); 10 μg of peptide-BSA conjugate was applied to the each lane. Lane a was loaded with 10 μg of mouse cerebellar microsomal fraction. The smeared bands in lane c are the polymerization products between pep6 and BSA which are immunodetected by mAb18A10. The positions of molecular mass markers are shown on the left. ▷ indicates the position of intact Ins(1,4,5)P_3 receptor protein.

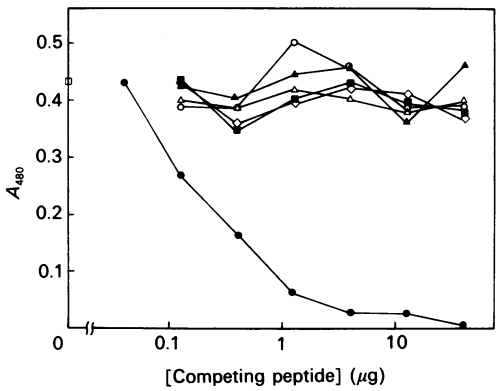

Fig. 8. Two-step competitive e.l.i.s.a.: inhibition of the binding between mAb18A10 and the purified Ins(1,4,5)P_3 receptor by pep6

Each well of a 96-well microtitre plate was coated with 100 μl of purified Ins(1,4,5)P_3 receptor (1 μg/ml). Various amounts of peptides (●, pep6; ○, pep1; ■, pep2; ◇, pep3; △, pep4; ▲, pep5; □, no peptide) were mixed with 100 μl of mAb18A10 (0.4 μg/ml) and the mixtures were added to the wells. After incubation for 30 min at room temperature, biotinylated anti-(rat IgG) antibody was introduced to each well. Avidin and biotinylated horseradish-peroxidase complex were sequentially added, and *o*-phenylenediamine dihydrochloride was used as the substrate. The colour intensity given by the product was monitored at 480 nm with a Micro Plate Reader.

stimulation of [^{3}H]Ins(1,4,5)P_3 binding at a concentration of 50 μg/ml (Fig. 4). mAbs 4C11 and 10A6 had little effect on [^{3}H]Ins(1,4,5)P_3 binding (Fig. 4), suggesting that these antibodies recognize epitopes that are different from the one necessary for Ins(1,4,5)P_3 binding.

Scatchard analysis of Ins(1,4,5)P_3 binding to the microsomal fraction with and without 50 μg of mAb18A10/ml showed that the K_d value was changed from 43 ± 12 nM to 25 ± 4 nM by addition of the mAb (Fig. 5). The $B_{max.}$ value was 150 ± 10 pmol/mg in both cases. mAb18A10 actually increased the [^{3}H]Ins(1,4,5)P_3-binding affinity without changing the number of Ins(1,4,5)P_3-binding sites.

Epitope mapping for mAb18A10

From the sequence analysis of the immunopositive clones of the Ins(1,4,5)P_3 receptor, we previously predicted that the epitope of mAb18A10 was located within amino acid residues 2648–2749 (*C*-terminal end) of the Ins(1,4,5)P_3 receptor ([20], Fig. 6). To obtain further information about the precise sequence that mAb18A10 recognizes, we carried out epitope mapping by using synthetic short peptides within residues 2648–2749. The six synthetic peptides shown in Fig. 6 were each conjugated with BSA. The six peptide–BSA conjugates were examined for binding to mAb18A10 by Western blotting. A positive reaction was observed only with pep6–BSA (covering residues 2736–2747, lane c in Fig. 7), and not with the other five peptide–BSA conjugates or with BSA itself. A competitive e.l.i.s.a. clearly showed that pep6 inhibited the binding of mAb18A10 to the purified Ins(1,4,5)P_3 receptor (Fig. 8). However, the other peptides did not compete with the purified Ins(1,4,5)P_3 receptor for binding to mAb18A10. These results indicate that the epitope of mAb18A10 is located within pep6, a 12-residue-long peptide with the sequence Gly-His-Pro-Pro-His-Met-Asn-Val-Asn-Pro-Gln-Gln (residues 2736–2747 of the receptor).

DISCUSSION

The cerebellar Ins(1,4,5)P_3 receptor is a large membrane-bound glycoprotein of 320 kDa (313 kDa, as deduced from its amino acid sequence [20]; SDS/PAGE estimate of 250 kDa [19], agarose/PAGE estimate of 320 kDa [22]). The hydropathy profile of the amino acid sequence of the Ins(1,4,5)P_3 receptor indicates that it is a unique protein composed of the large *N*-terminal domain, the membrane-spanning domains clustered near the *C*-terminus and the short *C*-terminal domain [20]. A transfection experiment with a mutant in which the 3′-portion of the cDNA is deleted demonstrated that the mutant protein is recovered in the soluble fraction, indicating that the *C*-terminal region has a membrane-anchoring action [30]. Recently it has been demonstrated that the cerebellar Ins(1,4,5)P_3 receptor itself has Ca^{2+}-release-channel activity, revealed by the liposome vesicle system [31] and the planar lipid bilayer system [22]. A cross-linking experiment showed that the Ins(1,4,5)P_3 receptor forms a tetrameric structure [22], and this finding corresponds well with the negative staining image obtained by electron microscopy [19]. Since the *C*-terminus is near the membrane-spanning domains of four subunits which seem to form a single membrane pore, we examined how the *C*-terminal portion of the receptor is involved in Ca^{2+} release by using mAbs.

mAb18A10 had two effects. It suppressed Ins(1,4,5)P_3-induced Ca^{2+} release, and increased the ability of the Ins(1,4,5)P_3 receptor to bind to Ins(1,4,5)P_3: the EC_{50} increased from 112 ± 8 nM to 460 ± 50 nM for Ins(1,4,5)P_3 and the K_d value for Ins(1,4,5)P_3 binding decreased from 43 ± 12 nM to 25 ± 4 nM in the presence of 50 μg of mAb18A10/ml. The dose–response effect of mAb18A10

on the stimulation of Ins(1,4,5)P_3 binding and on Ca^{2+}-release inhibition occurred with a similar range of antibody concentrations. Thus it seems that the two effects of mAb18A10 are closely related phenomena. The question then arises as to what part of the Ins(1,4,5)P_3 receptor the mAb18A10 recognizes. Does it recognize the complex tertiary structure or only a primary peptide sequence? Since mAb18A10 reacted with a Ins(1,4,5)P_3 receptor that had been denatured by SDS and the reducing agent in Western blotting, we presumed that mAb18A10 does not recognize the specific higher-order conformation, but recognizes the specific sequence in the Ins(1,4,5)P_3 receptor. We have identified the sequence recognized by mAb18A10 to include amino acid residues 2736–2747, which is the most *C*-terminal region of the receptor.

How does the antibody suppress the channel activity? Binding of mAb18A10 to the *C*-terminus presumably located close to the central membrane pore may allosterically change the channel conformation to suppress channel activity, or it may simply occupy the membrane pore site in the channel to cause steric hindrance to Ca^{2+} flow. If this is the case, how does the antibody increase Ins(1,4,5)P_3 binding? One possible explanation is that the antibody works as if it is a ligand or modulator and allosterically changes the conformation to increase Ins(1,4,5)P_3 binding. Recently Pietri *et al.* [32] indicated that Ca^{2+} induces the interconversion of the liver Ins(1,4,5)P_3 receptor from a low-affinity state to a high-affinity state for Ins(1,4,5)P_3 binding, which is associated with the inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release. The effects of mAb18A10 on the binding affinity of the Ins(1,4,5)P_3 receptor to Ins(1,4,5)P_3 and Ins(1,4,5)P_3-induced Ca^{2+} release mimic the effects of Ca^{2+} on the interconversion of the liver Ins(1,4,5)P_3 receptor. In the case of the liver Ins(1,4,5)P_3 receptor, Ca^{2+} itself or the mediator of Ca^{2+} affects the receptor and causes its interconversion. mAb18A10 may play a similar role to that of Ca^{2+} or its mediator for the cerebellar Ins(1,4,5)P_3 receptor.

We have recently determined the exact subcellular location and topology of the Ins(1,4,5)P_3 receptor in cerebellar Purkinje cells by pre-embedded Immunogold labelling of frozen ultrathin sections [33]. We found that the Ins(1,4,5)P_3 receptor is located in various areas of the smooth endoplasmic reticulum (SER): stacked ER membranes, subsurface cisternae and the dendritic spine apparatus [33]. Similar results were reported recently by Satoh *et al.* [34]. As for the topology of the receptor on the SER membrane, we concluded that both *N*- and *C*-termini are on the cytoplasmic side of the SER [33], since the epitopes recognized by the three mAbs were all located on the side. This is consistent with our finding that mAb18A10 interfered with the Ca^{2+}-release function of the Ins(1,4,5)P_3 receptor when the antibody was added to the outside of the microsomal vesicles.

mAb18A10 inhibited Ca^{2+} release from the microsomes at low concentrations of Ins(1,4,5)P_3, but it did not suppress Ca^{2+} release at higher Ins(1,4,5)P_3 concentrations, at which maximal Ca^{2+} release occurred. The high concentration of Ins(1,4,5)P_3 might induce a conformational change in the Ins(1,4,5)P_3 receptor subunit, thereby preventing mAb18A10 from recognizing the epitope. Alternatively, Ins(1,4,5)P_3 might allosterically alter the homotetrameric structure, decreasing the affinity between the receptor and the antibody. However, e.l.i.s.a. indicated that the affinity of mAb18A10 for the cerebellar microsomal fraction was not changed by the addition of 10 μM-Ins(1,4,5)P_3 (results not shown), eliminating these possibilities. An alternative explanation is that the binding of mAb18A10 causes a delay in channel opening after Ins(1,4,5)P_3 binds to the receptor. Meyer *et al.* [35] found by the rapid mixing technique that Ca^{2+} flux from permeabilized basophilic leukaemia cells was delayed for 1 s after the addition of 15 nM-Ins(1,4,5)P_3. In our Ca^{2+}-release assay system, at a low concentration of Ins(1,4,5)P_3 the added Ins(1,4,5)P_3 is rapidly degraded by the Ins(1,4,5)P_3 phosphatase abundant in the cerebellum, and the opened channel is also rapidly closed. If the binding of mAb18A10 delays channel opening, Ins(1,4,5)P_3-induced Ca^{2+} release may be inhibited at low Ins(1,4,5)P_3 concentrations, because Ins(1,4,5)P_3 is degraded immediately and its concentration may become lower than the threshold level before channel opening can occur. However, at higher concentrations, Ins(1,4,5)P_3 may remain at a high enough concentration to open the channel, although the degradation of Ins(1,4,5)P_3 continues. Further detailed kinetic studies on the Ins(1,4,5)P_3 receptor channel are necessary to examine this hypothesis.

Inhibitors, either natural or synthetic, have provided a great deal of information on the structure–function relationships of receptors and channels [36–38]. TMB-8, PCMB and cinnarizine are known to inhibit Ins(1,4,5)P_3-induced Ca^{2+} release. They do this via blockade of Ca^{2+}-channel opening or by inhibiting Ins(1,4,5)P_3 binding to the receptor [39], but they also act as general Ca^{2+} antagonists on the Ca^{2+} channel located in the plasma membrane or intracellular vesicle. Heparin is also a well-known inhibitor of Ins(1,4,5)P_3 binding to the receptor and it suppresses Ins(1,4,5)P_3-induced Ca^{2+} release [40]. However, it is not a specific inhibitor of the Ins(1,4,5)P_3 receptor. Heparin exerts a variety of effects, because there are numerous heparin-binding proteins in the cell. In this sense, mAb18A10 is considered to be a specific molecule that recognizes the Ins(1,4,5)P_3 receptor and changes the Ins(1,4,5)P_3-induced Ca^{2+}-release and Ins(1,4,5)P_3-binding activities.

Antibodies against the various regions of the receptor will give us more information on its structure and function. Addition of mAbs to cultured cells will give us information about the physiological role of the Ins(1,4,5)P_3 receptor.

We thank Dr. Michio Niinobe, Dr. Astushi Miyawaki, Dr. Michisuke Yuzaki and Dr. Testuro Kondo for useful discussions, Dr. Eiichi Ozeki for his comments, and Dr. Shigehisa Okuyama for synthesizing the peptides. We are grateful to Dr. Takehisa Matsuda for kind permission to use a spectrofluorimeter. This work was supported by a grant for specially promoted research from the Japanese Ministry of Education, Science and Culture.

REFERENCES

1. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
2. Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193
3. Hess, P. (1990) Annu. Rev. Neurosci. **13**, 337–356
4. Macdermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J. & Barker, J. L. (1986) Nature (London) **321**, 519–522
5. Sugiyama, H., Ito, I. & Hirono, C. (1987) Nature (London) **325**, 531–533
6. Nishizuka, Y. (1984) Science **225**, 1365–1370
7. Fisher, S. K., Van Rooijen, L. A. A. & Agranoff, B. W. (1984) Trends Biochem. Sci. **9**, 53–56
8. Hokin, L. E. (1985) Annu. Rev. Biochem. **54**, 205–235
9. Smith, J. B., Smith, L. & Higgins, B. L. (1985) J. Biol. Chem. **260**, 14413–14416
10. Suematsu, E., Hirata, M., Sasaguri, T., Hashimoto, T. & Kuriyama, H. (1985) Comp. Biochem. Physiol. **82A**, 645–649
11. Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. (1989) Nature (London) **340**, 236–239
12. Chow, S. K. & Jondal, M. (1990) J. Biol. Chem. **265**, 902–907
13. Joseph, S. K., Rice, H. L. & Williamson, J. R. (1989) Biochem. J. **258**, 261–265
14. Tsien, R. Y. & Poenie, M. (1986) Trends Biochem. Sci. **11**, 450–455
15. Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. & Hiramoto, Y. (1986) Dev. Biol. **118**, 259–267
16. Berridge, M. J. & Irvine, R. F. (1989) Nature (London) **341**, 197–205
17. Tsien, R. Y. (1988) Trends Neurosci. **11**, 419–424

18. Berridge, M. J. (1990) J. Biol. Chem. **265**, 9583–9586
19. Maeda, N., Niinobe, M. & Mikoshiba, K. (1990) EMBO J. **9**, 61–67
20. Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) Nature (London) **342**, 32–38
21. Miyawaki, A., Furuichi, T., Maeda, N. & Mikoshiba, K. (1990) Neuron **5**, 11–18
22. Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M. & Mikoshiba, K. (1991) J. Biol. Chem. **266**, 1109–1116
23. Maeda, N., Niinobe, M., Nakahira, K. & Mikoshiba, K. (1988) J. Neurochem. **51**, 1724–1730
24. Maeda, N., Niinobe, M., Inoue, Y. & Mikoshiba, K. (1989) Dev. Biol. **133**, 67–76
25. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
26. Richardson, C. D., Berkovich, A., Rozenblatt, S. & Bellini, W. J. (1985) J. Virol. **54**, 186–193
27. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
28. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
29. Stauderman, K. A., Harris, G. D. & Lovenberg, W. (1988) Biochem. J. **255**, 677–683
30. Mignery, G. A. & Sudhof, T. C. (1990) EMBO J. **9**, 3893–3898
31. Ferris, C. D., Huganir, R. L., Supattapone, S. & Snyder, S. H. (1989) Nature (London) **342**, 87–89
32. Pietri, F., Hilly, M. & Mauger, J. P. (1990) J. Biol. Chem. **265**, 17478–17485
33. Otsu, H., Yamamoto, A., Maeda, N., Mikoshiba, K. & Tashiro, Y. (1990) Cell Struct. Funct. **15**, 163–173
34. Satoh, T., Ross, C. A., Villa, A., Supattapone, S., Pozzan, T., Snyder, S. H. & Meldolesi, J. (1990) J. Cell Biol. **111**, 615–624
35. Meyer, T., Wensel, T. & Stryer, L. (1990) Biochemistry **29**, 32–37
36. Changeux, J. P., Devillers-Thiery, A. & Chemouilli, P. (1984) Science **225**, 1335–1345
37. Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H. & Barnard, E. A. (1987) Nature (London) **328**, 221–227
38. Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E. D. & Betz, H. (1987) Nature (London) **328**, 215–220
39. Palade, P., Dettbarn, C., Alderson, B. & Volpe, P. (1989) Mol. Pharmacol. **36**, 673–680
40. Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. & Gill, D. L. (1988) J. Biol. Chem. **263**, 11075–11079

Received 27 December 1990/19 February 1991; accepted 25 February 1991