

Inositol 1,4,5-Trisphosphate Slowly Converts Its Receptor to a State of Higher Affinity in Sheep Cerebellum Membranes*

(Received for publication, April 20, 1995, and in revised form, December 4, 1995)

Jean-François Coquil[‡], Jean-Pierre Mauger, and Michel Claret

From the Unité de Recherche U.274, INSERM, Université Paris-Sud, 91405 Orsay Cedex, France

Incubation of cerebellar microsomes with D-myo-inositol 1,4,5-trisphosphate (InsP₃) (0.01–1 μ M), at 4 or 20 °C in a cytosolic-like medium devoid of Ca²⁺ and Mg²⁺, followed by InsP₃ removal, induced an increase in InsP₃ binding determined with 1 nM [³H]InsP₃. At 20 °C, and pH 7.1, maximal stimulation (1.5–2.5-fold) was obtained with 1 μ M InsP₃, and the EC₅₀ was 60 \pm 5 nM. Several lines of evidence suggested that the activating site is identical with the InsP₃ binding site: (i) activation and binding exhibited the same inositol phosphate specificity; (ii) addition of decavanadate, a competitive inhibitor of [³H]InsP₃ binding, to the preincubation mixture, prevented the activating effect of InsP₃; (iii) the concentration of InsP₃ giving half-maximal activation was close to that giving half-maximal InsP₃ binding. The time course of activation was found to be much slower than that of binding. While a *t*_{1/2} less than 0.4 s has been measured recently at neutral pH and 20 °C for binding of 0.5 nM [³H]InsP₃ (Hannaert-Merah, Z., Coquil, J.-F., Combettes, L., Claret, M., Mauger, J.-P., and Champeil, P. (1994) *J. Biol. Chem.* 269, 29642–29649), a 20-s preincubation with 1 μ M InsP₃ was required to half-maximally stimulate binding. Under the present conditions, the InsP₃-induced binding increase was only partially reversible. However, this effect was not blocked by anti-proteases suggesting that it did not involve proteolysis. Taking advantage of the marked difference in the kinetics of InsP₃ binding and InsP₃-dependent activation, we performed binding experiments on a short period (3 s) to determine the effect of InsP₃ pretreatment on the binding parameters. The data showed that this treatment increased the affinity of the receptor without changing the number of binding sites (control: *K*_D = 107 nM, *B*_{max} = 28 pmol/mg of protein; after preincubation with 1 μ M InsP₃: *K*_D = 53 nM, *B*_{max} = 32 pmol/mg of protein). The two states of the receptor bound InsP₃ with a Hill coefficient close to 1 on a 3-s scale. In agreement with the effect of InsP₃ pretreatment, equilibrium binding experiments performed on 10-min incubations revealed an apparent positive cooperative behavior (apparent Hill coefficient = 1.6; apparent *K*_D = 66 nM). These results report a new regulatory process of the InsP₃ receptor in cerebellum occurring independently of Ca²⁺ and on a relatively long time scale.

channel located in the endoplasmic reticulum (Taylor and Richardson, 1991; Ferris and Snyder, 1992; Berridge, 1993). This process may be crucial in the genesis of repetitive calcium spikes which characterize the cellular calcium response to many stimuli (Berridge, 1993). Several genes encoding a family of InsP₃ receptors have been identified, and the molecular diversity of the receptor is amplified further by alternative splicing. In many tissues, diverse isoforms of the receptor are produced in different amounts (Südhof *et al.*, 1991; Nakagawa *et al.*, 1991; Ross *et al.*, 1992; Blondel *et al.*, 1993; De Smedt *et al.*, 1994). In the cerebellum, most InsP₃ receptor mRNAs encode type I receptor (Furuichi *et al.*, 1989; De Smedt *et al.*, 1994). The high concentration of type I receptor in Purkinje cells of cerebellum, has facilitated its purification, and the receptor has been proposed to be a homotetramer (Supattapone *et al.*, 1988; Maeda *et al.*, 1990, 1991). Analysis of its cDNA sequence has suggested overall structural organization in three basic domains: an amino-terminal InsP₃ binding domain, a carboxyl-terminal Ca²⁺ channel domain, and a linking domain containing sites for regulatory processes. This structure has been subsequently confirmed for other isoforms (Furuichi *et al.*, 1989; Mignery and Südhof, 1990; Miyawaki *et al.*, 1991; Südhof *et al.*, 1991; Maranto, 1994).

Studies of InsP₃ binding and InsP₃-induced Ca²⁺ release (IICR) with permeabilized cells or diverse receptor preparations including microsomes have allowed the identification of a variety of regulatory mechanisms for the InsP₃ receptor. Ca²⁺ is centrally involved in the control of IICR: submicromolar concentrations of Ca²⁺ activate, whereas higher concentrations desensitize the channel (Iino, 1990; Finch *et al.*, 1991; Bezprozvanny *et al.*, 1991; Combettes *et al.*, 1994). In central nervous tissue, Ca²⁺ inhibits InsP₃ binding through a Ca²⁺-binding protein, calmodin (Danoff *et al.*, 1988), whereas, in peripheral tissues, high Ca²⁺ concentrations transform the receptor into an inactive form which displays a high affinity for InsP₃ (Pietri *et al.*, 1990; Rouxel *et al.*, 1992; Watras *et al.*, 1994). ATP has been shown to stimulate channel activity through specific sites on the receptor (Ferris *et al.*, 1990; Maeda *et al.*, 1991; Bezprozvanny *et al.*, 1991; Combettes *et al.*, 1994). Several protein kinases, including A, C, G, and Ca²⁺/calmodulin-dependent enzymes, phosphorylate the receptor (Ferris and Snyder, 1992; Joseph and Ryan, 1993; Komalavilas and Lincoln, 1994; Koga *et al.*, 1994). Oxidizing sulfhydryl reagents such as thimerosal have been shown to increase the sensitivity of the Ca²⁺ store by increasing the affinity of the InsP₃ receptor (Missiaen *et al.*, 1991; Renard *et al.*, 1992; Hilly *et al.*, 1993). In addition, recent observations suggest that InsP₃ exerts effects on its receptor different from the mere rapid opening of the Ca²⁺ channel. It has been shown that IICR activation in permeabilized hepatocytes is followed by a period of inactivity dependent on the duration of exposure to InsP₃ (Hajnóczky and Thomas, 1994). Furthermore, it was also observed that preincubation with InsP₃ potentiates the stimulation by thimerosal of the Ca²⁺

The second messenger D-myo-inositol 1,4,5-trisphosphate (InsP₃)¹ mobilizes intracellular Ca²⁺ by activating a receptor/

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] To whom correspondence should be addressed.

¹ The abbreviations used are: InsP₃, D-myo-inositol-1,4,5-trisphosphate; IICR, InsP₃-induced Ca²⁺ release.

channel activity of the purified cerebellum receptor reconstituted in phospholipid vesicles (Kaplan *et al.*, 1994). The present studies report that occupancy of InsP₃ binding sites in sheep cerebellum microsomes induces a time-dependent increase in the affinity of InsP₃ receptor for its ligand. In contrast with results by Hajnóczky and Thomas (1994) on permeabilized hepatocytes, this InsP₃ effect was independent of Ca²⁺, suggesting that it represents a previously undescribed process.

EXPERIMENTAL PROCEDURES

Sheep cerebellar microsomes were prepared as described previously (Hannaert-Merah *et al.*, 1994). The microsomal preparation was resuspended in homogenization buffer (5 mM Hepes, 250 mM sucrose, 10 mM KCl, 1 mM β -mercaptoethanol, 10 μ g/ml leupeptin, 10 μ M pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4 at 4 °C) and then frozen in liquid N₂ and stored at -80 °C. Membranes were thawed and diluted in an ice-cold cytosolic-like medium (MI) containing 110 mM KCl, 20 mM NaCl, 1 mM NaH₂PO₄, 1 mM EDTA, 25 mM Hepes/KOH (pH 7.1), and 10 μ g/ml leupeptin. Where indicated, membranes were washed and resuspended in the same medium. Preincubation of membranes (0.2–1.0 mg/ml protein) with InsP₃ (1 nM to 1 μ M) was also conducted in MI, supplemented with other agents as indicated. In the first series of experiments, InsP₃ was removed by centrifuging the preincubation mixture at 36,000 $\times g$ for 1 h and washing membranes twice with ice-cold MI. A more rapid washing procedure was used in subsequent experiments. The membrane suspension preincubated with or without InsP₃ was layered onto a Whatman GF/C glass fiber filter and then washed with 10 ml of MI. The vacuum pump was adjusted to give an outflow rate of 1 ml/s, except where indicated. The binding activity of membranes was then measured directly on the filter, as described below.

[³H]InsP₃ binding was measured by mixing microsomes with an appropriate volume of a binding medium consisting of MI supplemented with 1 nM [³H]InsP₃, 0.1 mg/ml bovine serum albumin, and the indicated concentrations of unlabeled InsP₃. Nonspecific binding was determined in the presence of 10 μ M InsP₃. Binding was performed either in a test tube or on a GF/C glass fiber filter. In the former case, the binding mixture (final volume: 0.5 ml) was incubated for 10 min at 4 or 20 °C. Four hundred microliters of the mixture were transferred onto a GF/C glass fiber filter which was then rinsed with 1 ml of an ice-cold washing medium (25 mM Hepes, 250 mM sucrose, 1 mM NaH₂PO₄, 1 mM EDTA, pH 7.1). To measure [³H]InsP₃ binding to membranes adsorbed onto the GF/C filter, 0.5 ml of the binding medium was added to the filter, the vacuum pump being either off or running according to the desired contact time with [³H]InsP₃. Except where indicated, the filter was shortly rinsed with 1 ml of the ice-cold washing medium. As previously reported (Rouxel *et al.*, 1992; Hannaert-Merah *et al.*, 1994), InsP₃ dissociates very quickly from its receptor in a cytosolic-like medium at neutral pH and 20 °C ($t_{1/2} < 0.4$ s). Therefore, the rinsing step was not performed with MI but with a Hepes buffer containing 250 mM sucrose and cooled to 4 °C. In addition, the perfusion rate of outflow was adjusted so that the washing lasted less than 0.5 s. This procedure removed almost all [³H]InsP₃ trapped in the GF/C glass fiber filter. An adsorption of [³H]InsP₃ to this type of filter is possible, but was prevented by the addition of 1 mM EDTA to the binding medium. Excess fluid was removed from the filter under vacuum before transfer into a counting vial. Radioactivity was measured in a scintillation counter. Total binding and nonspecific binding were determined at least in triplicate, and the results were expressed as means \pm S.E. The competitive binding curves and the dose-response curve for InsP₃-dependent binding increase were fitted according to a one-site model, using Inplot Software (GraphPAD). Kinetics of InsP₃-dependent binding increase were fitted to a simple exponential behavior.

[³H]InsP₃ (17–21 Ci/mmol) was purchased from DuPont NEN. Non-radioactive InsP₃ was obtained from Calbiochem. Decavanadate was prepared as described previously (Föhr *et al.*, 1989). All other products were from Sigma or Boehringer Mannheim.

RESULTS

Preincubation of Cerebellar Microsomes with InsP₃ Increases [³H]InsP₃ Binding—When a microsomal fraction of sheep cerebellum was preincubated with 1 μ M InsP₃ in a cytosolic-like medium (MI) at 4 °C and then extensively washed by centrifugation in the same medium, its ability to subsequently bind [³H]InsP₃ at 1 nM was doubled (Table I). As this centrifugation

TABLE I
Effect of preincubation of cerebellar microsomes with InsP₃ on [³H]InsP₃ binding

The microsome preparation was thawed, diluted 12-fold in ice-cold MI, and centrifuged at 100,000 $\times g$ for 1 h. The pellet was resuspended and diluted in the same medium supplemented with bovine serum albumin at 0.1 mg/ml to 0.2–0.4 mg/ml membrane protein. One volume of this medium with or without 10 μ M InsP₃ was added to 9 volumes of the microsome suspension, and the mixture was incubated for 10 min at 4 °C (preincubation). Membranes were then washed either by centrifugation or by filtration on Whatman GF/C glass fiber filters. For the centrifugation method, the mixture was centrifuged at 100,000 $\times g$ and membranes were resuspended with ice-cold MI and recentrifuged at the same speed. The new pellet was resuspended in ice-cold MI and diluted to 0.5 ml with a binding mixture consisting of MI supplemented with 1 nM [³H]InsP₃ and 0.1 mg/ml bovine serum albumin (0.15–0.25 mg/ml membrane protein). After a 10-min incubation period at 4 °C, 0.4 ml of the binding mixture was then transferred to a Whatman GF/C glass fiber filter. The filter was rinsed with 1 ml of a washing medium containing 25 mM Hepes, 250 mM sucrose, 1 mM NaH₂PO₄, 1 mM EDTA, at pH 7.1 and 4 °C. In the filtration method, 0.5 ml of the preincubation mixture (0.1–0.2 mg of protein) was layered onto a GF/C glass fiber filter. Filters with adsorbed membranes were washed with 10 ml of ice-cold MI under vacuum. The vacuum was then broken and 0.5 ml of ice-cold binding mixture was layered on the filter. Following a 15-s incubation period, the vacuum was restored. Free ligand was removed by shortly perfusing 1 ml of the washing medium described above. *n* = number of experiments.

Washing procedure	<i>n</i>	Preincubation conditions	
		Control	+1 μM InsP ₃
[³ H]InsP ₃ binding (pmol / mg protein)			
Centrifugation	4	0.66 ± 0.06	1.17 ± 0.14
Filtration	9	0.51 ± 0.05	0.93 ± 0.10

procedure was very time-consuming, another method was developed to remove the initially added unlabeled InsP₃, this being washing the membranes with MI on a GF/C glass fiber filter. Following the washing step, [³H]InsP₃ binding was directly measured on the filter by adding 0.5 ml of MI containing 1 nM [³H]InsP₃. The InsP₃-dependent increase in [³H]InsP₃ binding measured with this filtration method was the same as that with the centrifugation method (Table I). In initial studies, several characteristics of the effect of InsP₃ pretreatment were examined at 4 °C. It was found that maximal activation was achieved with 0.1–0.3 μ M InsP₃ and that the activating effect of InsP₃ was much slower to develop than InsP₃ binding. While the binding of 1 nM [³H]InsP₃ to cerebellar microsomes layered on the filter reached equilibrium within 15 s, the activation due to preincubation with 1 μ M InsP₃ was only maximal after 1–2 min of preincubation at 4 °C in MI. The activation remained unchanged for at least 2 h. In subsequent experiments, the pretreatment of cerebellar membranes with InsP₃ was generally performed for 10 min. The fact that the same level of activation was measured when membranes were washed free of InsP₃ by either filtration or by centrifugation, a much longer procedure, suggests slow reversibility of the activation under these conditions. Indeed, at 4 °C, the activation persisted for days. When washed membranes were placed in MI at 37 °C instead of being stored in this medium at 4 °C, the same qualitative results were obtained; however, within 5 min, a reduction to 48% of the initial activation occurred and remained at this level for at least 40 min. The degree of reversibility was not improved by the addition of an antiprotease mixture (10 μ g/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μ M pepstatin A, 2 mM benzamidine, 1 μ g/ml *O*-phenanthroline, 50 μ g/ml trypsin inhibitor) to the preincubation medium, suggesting that the InsP₃-induced binding increase did not involve proteolysis.

A crucial point in this type of experiment was to remove carefully the nonradioactive InsP₃ present in the preincubation

mixture, as [³H]InsP₃ binding might be reduced by residual InsP₃ leading to an underestimation of the InsP₃-dependent activation. An experiment was therefore performed in which the volume of the washing medium was varied. When membranes were preincubated with 1 μM InsP₃, the lowest subsequent binding of [³H]InsP₃ was observed in the absence of washing; that is, the condition for which the highest contamination of the filter with unlabeled InsP₃ was expected. Washing the filter with MI increased the binding of [³H]InsP₃ to the membranes, the maximal binding being attained at about 3–5 ml of washing medium. Increasing the volume of the washing medium to 30 ml did not change [³H]InsP₃ binding. When membranes were preincubated without InsP₃, washing the filter with up to 30 ml of MI had no effect on [³H]InsP₃ binding. These results indicate that unlabeled InsP₃ carried over from the preincubation mixture could be removed from the filter easily and the InsP₃ remaining after 5 ml of washing does not reduce the subsequent [³H]InsP₃ binding when measured with 1 nM [³H]InsP₃. An additional experiment to determine the amount of residual InsP₃ after a washing with 10 ml of MI (the standard protocol) was performed. In this experiment, the preincubation mixture containing both cerebellar membranes and 1 μM unlabeled InsP₃ was supplemented with 10 nM [³H]InsP₃. The results indicate that a two-thousandth of the initial [³H]InsP₃ remained on the filter, corresponding to 1 pmol of InsP₃ (final concentration of 2 nM after addition of binding mixture on the filter). From the [³H]InsP₃ displacement curve by nonradioactive InsP₃, we conclude that this residual InsP₃ was about 10-fold lower than the InsP₃ required to significantly reduce [³H]InsP₃ binding measured with 1 nM [³H]InsP₃. Thus, 10 ml of MI were adequate to wash membranes.

Specificity of the Activating Effect of InsP₃—The data reported above show that InsP₃ is both able to bind to cerebellar microsomes and to increase, by preincubation, its own binding. Therefore, we investigated whether these two processes involved the same binding site or two distinct types of sites on the cerebellar membranes. Firstly, we used several different inositol phosphates to compare the specificity of [³H]InsP₃ binding site with that of the site responsible for the activating effect of InsP₃. To do this, we examined the ability of inositol phosphates to inhibit the binding of [³H]InsP₃ to cerebellar membranes by simultaneous incubation, and their ability to increase binding of [³H]InsP₃ by preincubation with membranes. The results in Table II show that the increase in [³H]InsP₃ binding after preincubation with inositol phosphates is obtained with agonists of InsP₃ receptor, but by different degrees. The order of potency of these agents was the same for the two effects: Ins(1,4,5)P₃ > Ins(2,4,5)P₃ ≅ GroPIns(4,5)P₂ > Ins(1,3,4,5)P₄ > Ins(1,3,4)P₃. This specificity corresponds to that previously described for particulate or purified InsP₃ receptor preparations from cerebellum and peripheral tissues (Nahorski and Potter, 1989; Mourey *et al.*, 1990; Südhof *et al.*, 1991; Maeda *et al.*, 1991; Rouxel *et al.*, 1992).

Secondly, as heparin is a potent inhibitor of InsP₃ binding to its receptor (Taylor and Richardson, 1991), we examined whether this agent was able to block the activating effect of InsP₃. Unfortunately, pre-exposure of the cerebellar membranes to heparin led to an irreversible inhibition of InsP₃ binding activity. A similar effect was reported previously for microsomes from bovine adrenal cortex at 4 °C in InsP₃ binding assays (Guillemette *et al.*, 1989). However, in contrast with these studies, we were unable to recover more than 30% of [³H]InsP₃ binding by diluting and washing cerebellar microsomes at 37 °C. Therefore, we repeated the same type of experiments with decavanadate, another agent described as a competitive antagonist of InsP₃ receptor (Föhr *et al.*, 1989; Taylor

TABLE II
Identity of the specificity of InsP₃ binding sites and InsP₃-dependent activating sites

Microsomes were thawed and washed with ice-cold MI as described in Table I. Membrane preincubation and measurement of [³H]InsP₃ binding were performed at 4 °C. The specificity of InsP₃ binding sites was examined by measuring the ability of various inositol phosphates to inhibit the binding of 1 nM [³H]InsP₃ during incubation in the binding medium. Incubation was performed in tubes for 10 min, and the amount of bound [³H]InsP₃ was measured by filtering 0.4 ml of the binding mixture on a GF/C glass fiber filter as indicated under "Experimental Procedures." The specificity of the regulatory sites was examined by comparing the increases of [³H]InsP₃ binding activity resulting from a 10-min preincubation of membranes with the various inositol phosphates. Preincubation was performed in tubes and the membrane washing and 1 nM [³H]InsP₃ binding on GF/C glass fiber filters as described under "Experimental Procedures" and in Table I. Data are means of one representative experiment.

Inositol phosphates	Increase in [³ H]InsP ₃ binding due to preincubation		Decrease in [³ H]InsP ₃ binding due to competition	
	μM	%	μM	%
Ins(1,4,5)P ₃	1	71 ± 5		96 ± 1
	10	67 ± 5		100
Ins(1,3,4)P ₃	1	0 ± 6		0 ± 2
	10	0 ± 4		0 ± 2
Ins(2,4,5)P ₃	1	43 ± 4		55 ± 4
	10	63 ± 7		96 ± 1
GroPIns(4,5)P ₂	1	60 ± 2		46 ± 1
	10	59 ± 3		92 ± 1
Ins(1,3,4,5)P ₄	1	29 ± 10		0 ± 4
	10	40 ± 5		64 ± 1

and Richardson, 1991). As illustrated in Fig. 1, A and B, 3 μM decavanadate inhibited InsP₃ binding measured with 0.1 μM [³H]InsP₃ by 91% (A) and greatly reduced the activation by 0.1 μM InsP₃ (B). Cerebellar membranes preincubated with 3 μM decavanadate alone did not exhibit a modified binding activity. Thus, decavanadate was entirely removed by washing membranes with MI and was unable to mimic the activating effect of InsP₃.

The Activating Effect of Preincubation with InsP₃ Occurs at 20 °C—All studies described above were performed at 4 °C, the temperature most often used in InsP₃ binding assays. However, it was important to know whether activation by InsP₃ could also occur at higher temperatures. Therefore, we then performed a series of experiments in which membrane preincubation, membrane washing, and InsP₃ binding assay were done at 20 °C. This temperature has been found to be suitable for the study of IICR (Combettes *et al.*, 1994) and InsP₃ binding (Hannaert-Merah *et al.*, 1994) in cerebellar microsomes. In this latter study, equilibrium for InsP₃ binding was shown to be attained more quickly at 20 °C than at 4 °C. Fig. 2 exhibits the dependence of [³H]InsP₃ binding on the concentration of InsP₃ in the preincubation mixture at 20 °C. The maximal amplitude of binding activation was obtained at about 1 μM InsP₃ and was identical with that measured at 4 °C. Stimulation of 82 ± 5% was determined in 21 experiments at 20 °C and of 83 ± 7% at 4 °C (results from Table I). The half-maximal response was observed at 60 ± 5 nM InsP₃.

Time Course of the InsP₃-dependent Activation—In order to compare further the characteristics of [³H]InsP₃ binding and the activating effect of InsP₃, we determined the kinetics of activation by preincubation with 1 μM InsP₃ at 20 °C. For technical reasons, the method of InsP₃ pretreatment differed according to its duration. For the shortest times (2 and 5 s), membranes were adsorbed onto the filter and perfused with an appropriate volume of MI with or without 1 μM InsP₃. The rate of outflow through the filter was adjusted to 0.5 ml/s. For 10-s to 2-min periods, membranes were also treated with InsP₃ on the filter, except incubations were performed instead of perfu-

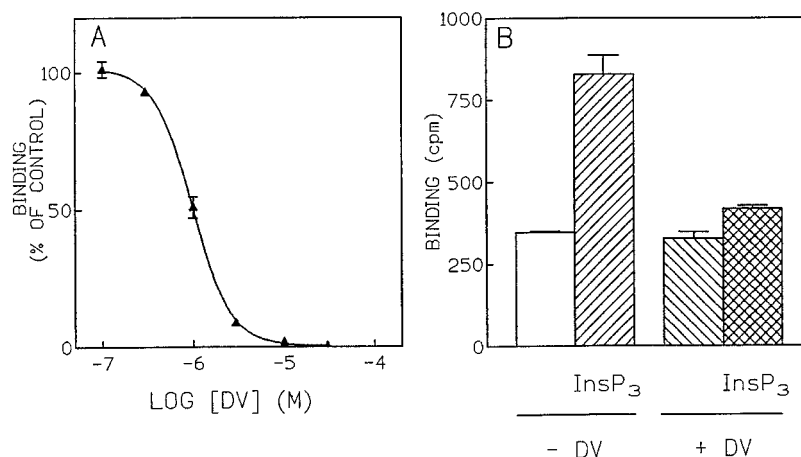


FIG. 1. **Blockade by decavanadate of the increase in binding induced by InsP₃.** Experiments were performed at 4 °C. Microsomes were prepared as described under "Experimental Procedures" and washed with ice-cold MI as indicated in Table I. A, [³H]InsP₃ binding was performed in a test tube by incubating microsomes for 10 min in binding medium containing 20 nCi/ml [³H]InsP₃, 0.1 μM InsP₃, and decavanadate (DV) as specified. Bound [³H]InsP₃ was measured by filtering 0.4 ml of the incubation mixture. Results are expressed as percent of [³H]InsP₃ binding measured in the absence of decavanadate. B, microsomes were preincubated for 10 min in MI with or without 0.1 μM InsP₃ and 3 μM decavanadate as indicated. Five hundred-microliter aliquots of the preincubation mixtures were transferred onto GF/C glass fiber filters, membranes were washed, and InsP₃ binding was measured on a filter with 1 nM [³H]InsP₃. Data are means of one experiment representative of two.

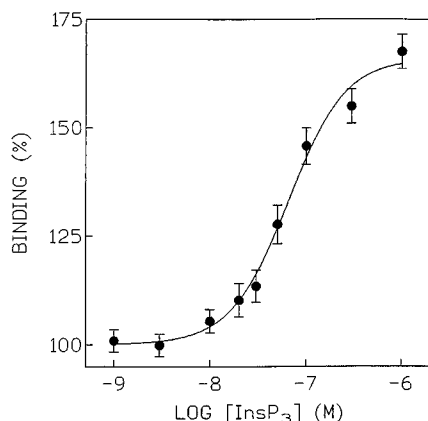


FIG. 2. **Effect of increasing InsP₃ concentrations in the preincubation mixture on subsequent [³H]InsP₃ binding.** Experiments were performed at 20 °C. Microsomes were preincubated for 10 min in MI in the absence or presence of InsP₃ at the specified concentration. Five hundred-microliter aliquots of the preincubation mixture were used to measure the InsP₃ binding on GF/C glass fiber filters with 1 nM [³H]InsP₃, as described under "Experimental Procedures" and in Table I. Results are expressed as percent of [³H]InsP₃ binding of microsomes preincubated without InsP₃. Data points are means of three different experiments.

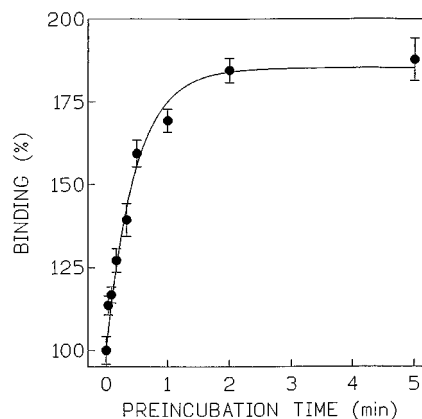


FIG. 3. **Time course of activation of [³H]InsP₃ binding during preincubation of microsomes with InsP₃.** The experiment was carried out at 20 °C. For 2-s and 5-s pretreatments, 1-ml aliquots of the diluted microsome preparation were layered on GF/C glass fiber filters, and adsorbed microsomes were perfused manually at 0.5 ml/s with 1 ml or 2.5 ml of MI containing 1 μM InsP₃. Pretreatments of 10 s to 2 min were performed by applying 1 ml of the InsP₃ solution onto the filters with the vacuum pump being off. Five-minute pretreatments were conducted in test tubes. After the 5-min incubation, 1 ml of preincubation mixture was transferred onto GF/C filters. In all cases, microsome pretreatment was stopped by passing 10 ml of MI through the filter. [³H]InsP₃ binding to microsomes was measured on the filters as described under "Experimental Procedures" and in Table I. Controls were performed according to the same protocols except that InsP₃ was omitted from the preincubation medium. Results are expressed as percent increase of [³H]InsP₃ binding as compared to the corresponding control. Data points are means of one experiment representative of two.

sions. At the longer time of 5 min, preincubations were conducted within test tubes. In all cases, InsP₃ pretreatment was stopped by washing the membranes with 10 ml of MI. Fig. 3 illustrates the results of these experiments. The maximal increase of [³H]InsP₃ binding activity was reached within 2 min and remained unchanged up to 5 min. The experimental data points were fitted to a simple exponential with a half-time ($t_{1/2}$) of 20 s. Additional experiments at 20 °C showed that binding activation remained unchanged for at least a 20-min preincubation (data not shown). Furthermore, we observed that the activation was also completed within 1–3 min with lower InsP₃ concentrations even if the maximal extent of activation was not greater than 15%. Thus, the observed activation by InsP₃ is a much slower process than InsP₃ binding itself, the latter having a half-time shorter than 0.4 s under the same experimental conditions as employed here (Hannaert-Merah *et al.*, 1994).

Determination of the InsP₃ Binding Characteristic Modified by InsP₃ Pretreatment—We then examined whether the pre-exposure of microsomes to InsP₃ affected the affinity or the

number of binding sites for [³H]InsP₃. As the determination of K_D and B_{max} requires an equilibrium binding experiment with increasing InsP₃ concentrations, it was a prerequisite to measure the level of [³H]InsP₃ binding within a sufficiently short time to avoid InsP₃-dependent activation. This condition is satisfied in MI at 20 °C, a medium in which equilibrium for [³H]InsP₃ binding is attained in less than 2 s (Hannaert-Merah *et al.*, 1994). Competitive binding experiments were performed by perfusing membranes adsorbed onto the filter for periods of 2–3 s with the binding medium containing 1 nM [³H]InsP₃ and increasing concentrations of unlabeled ligand (Fig. 4A). Non-linear regression analysis for a one-site model gave a Hill coefficient close to 1 for membranes preincubated with or without 1 μM InsP₃ ($n_H = 1.07$ and 0.95, respectively). Assuming n_H

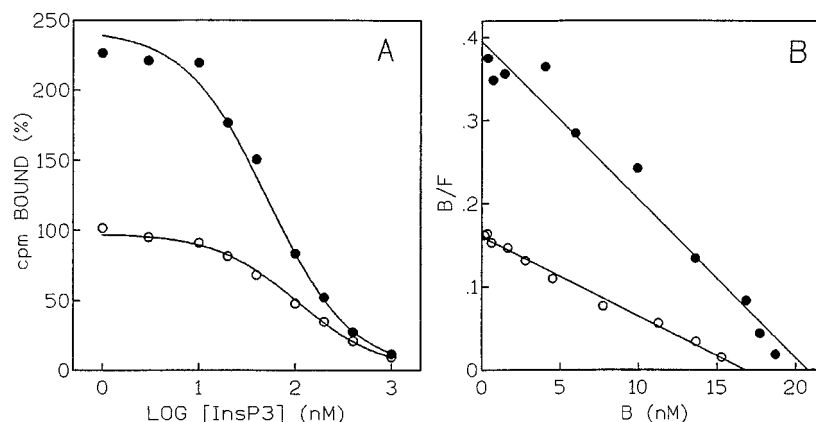


FIG. 4. Effect of pre-exposure of microsomes to InsP₃ on [³H]InsP₃ binding parameters. A, the experiment was carried out at 20 °C. Microsomes were preincubated for 10 min in MI with (●) or without (○) 1 μM InsP₃. One-ml aliquots of the preincubation mixtures were layered onto GF/C glass fiber filters. Adsorbed microsomes were washed with 10 ml of MI and then perfused for 3 s with 0.5 ml of binding mixture containing 1 nM [³H]InsP₃ (20 nCi/ml) and the indicated unlabeled InsP₃ concentrations. The filters were not rinsed with ice-cold Hepes/sucrose buffer to remove free ligand; however, the data were corrected for nonspecific binding determined with 10 μM InsP₃. Results are expressed as percent of [³H]InsP₃ binding determined for control membranes without unlabeled InsP₃. Simulated curves were constructed for a one-site model and $n_H = 1$. B, Scatchard plots obtained from the same data as in A. Data points are means of one experiment representative of two.

= 1, a K_D of 107 nM was determined for control membranes and 53 nM for InsP₃-pretreated membranes. Corresponding B_{max} values of 28 and 32 pmol/mg of protein were calculated as suggested by Swillens (1992). Therefore, it appears that, during preincubation, InsP₃ increases the affinity of the receptor without changing the number of binding sites. Scatchard plots constructed from the same data are shown in Fig. 4B.

From the data reported above, we anticipated that measurement of [³H]InsP₃ binding in the presence of increasing InsP₃ concentrations on a time long enough to allow activation by InsP₃ should express positive cooperativity. As illustrated by the squares in Fig. 5, binding values (B) measured at InsP₃ concentrations, shown previously to trigger the activation process during preincubation, were higher than that determined with 1 nM [³H]InsP₃ alone (B_0). These results proved such positive cooperativity directly. When transformed into a direct coordinate system and fitted to the Hill equation, half-maximal binding was calculated to be 66 nM InsP₃ and the apparent Hill coefficient to be 1.6 (data not shown). In 5 different experiments performed at 4 or 20 °C, we measured a maximal B/B_0 value of 1.31 ± 0.06 .

DISCUSSION

The present studies show that in sheep cerebellar microsomes the affinity for InsP₃ of its receptor was markedly increased during exposure to InsP₃ over a period of 2 s to 2 min. As the total number of InsP₃ binding sites was not changed by this treatment, it appears that these sites were converted to a state of higher affinity. Several lines of evidence indicate that the activating effect of InsP₃ resulted from an interaction of InsP₃ with the same receptor. Firstly, the selectivity of the activating site was the same as that of the [³H]InsP₃ binding site and that previously reported for the InsP₃ receptor in cerebellum from other species and peripheral tissues (Nahorski and Potter, 1989; Mourey *et al.*, 1990; Südhof *et al.*, 1991; Maeda *et al.*, 1991; Rouxel *et al.*, 1992). Secondly, decavanadate, a competitive inhibitor of InsP₃ receptor (Föhr *et al.*, 1989; Taylor and Richardson, 1991), prevented the activation by InsP₃. Thirdly, the EC_{50} value for activation at 20 °C (60 nM) was close to the apparent K_D value determined for [³H]InsP₃ binding in a 10-min incubation (66 nM). Whereas the sites for activation and binding appear to be identical, the former effect developed much more slowly than the latter. We conclude from these observations that the activation process results from prolonged occupancy of the InsP₃ binding site.

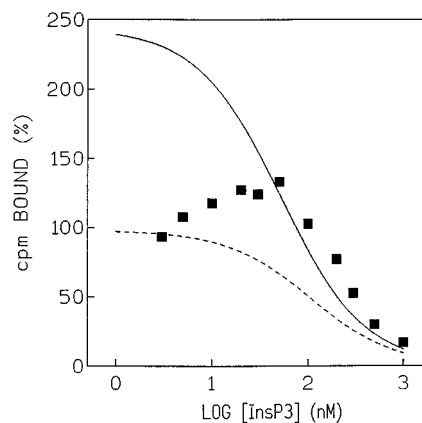


FIG. 5. [³H]InsP₃ displacement curve by unlabeled InsP₃ obtained from 10-min incubations. Comparison with displacement curves obtained from 3-s incubations of microsomes pretreated with or without 1 μM InsP₃. Experiments were performed at 20 °C. For 10-min incubations (■), cerebellar microsomes were added to a binding medium (final volume, 0.5 ml), consisting of MI supplemented with 1 nM [³H]InsP₃ and increasing concentrations of unlabeled InsP₃. Four hundred microliters of the incubation mixture was then transferred on GF/C glass fiber filters to measure the amount of bound [³H]InsP₃. Results are expressed as percent of [³H]InsP₃ binding determined without the addition of unlabeled ligand. Displacement curves obtained from 3-s perfusions of microsomes pretreated for 10 min with (solid line) or without (dashed line) InsP₃ were determined from data presented in Fig. 4. Data points are means of one representative experiment.

Most of the experiments in the present work were performed with a 10-min preincubation of cerebellar microsomal membranes with nonradioactive InsP₃, followed by washing and determination of [³H]InsP₃ binding after a short incubation period. The resulting increase of affinity observed in these two-step experiments was confirmed in binding measurements performed after longer (10 min) incubation periods, which revealed an apparent positive cooperative behavior (Fig. 5) and hence also indicate that InsP₃ stimulates its own binding. Consistent with the slow activation by preincubation with InsP₃ (Fig. 3), this behavior was not observed in experiments in which this incubation was only 2–3 s at 20 °C, as indicated by the Hill coefficient value close to 1 determined with untreated membranes (Fig. 4, A and B). Considered together, these characteristics of InsP₃ binding to its cerebellar receptor are reminiscent of properties of a hysteretic protein for which the apparent cooperative behavior results from slow conformational

transition upon binding of its ligand. Positive cooperative behavior has not been reported previously for InsP₃ binding to cerebellum receptor. However, it has been proposed that upon interaction with its binding site, InsP₃ elicits a large conformational change in its receptor (Mignery and Südhof, 1990). Recently, this conformational change has been suggested to alter accessibility of thimerosal to certain sulfhydryl groups (Kaplin *et al.*, 1994).

In peripheral tissues, elevation of cytosolic Ca²⁺ above its resting concentration (100–200 nM) increases the affinity of the InsP₃ receptor for its ligand (Hilly *et al.*, 1993; Marshall and Taylor, 1993) by reducing the dissociation rate constant (Hilly *et al.*, 1993). When the free Ca²⁺ concentration reaches 0.5–1 μ M, these receptors are converted into a high affinity inactive state characterized by low rates of association and dissociation of InsP₃ (Pietri *et al.*, 1990; Hilly *et al.*, 1993; Watras *et al.*, 1994). In contrast, the InsP₃-dependent conversion of sheep cerebellum InsP₃ receptor to a higher affinity state was observed in the presence of 1 mM EDTA, that is, at nanomolar free Ca²⁺ concentrations. In this tissue, Ca²⁺ inhibits InsP₃ binding (Worley *et al.*, 1987; Hannaert-Merah *et al.*, 1994), an effect which has been proposed to be mediated by the Ca²⁺-binding protein, calmodin (Danoff *et al.*, 1988). However, the InsP₃ activating effect cannot be due to removal of inhibitory influence by calmodin, since 1 mM EDTA has been reported to prevent and reverse the inhibition by this protein (Worley *et al.*, 1987; Joseph *et al.*, 1989). Furthermore, we found that sheep cerebellar microsomes preincubated with free Ca²⁺ concentrations up to 100 μ M and then washed with 10 ml of MI containing 1 mM EDTA did not exhibit a lower [³H]InsP₃ binding as compared with membranes exposed to nanomolar Ca²⁺ concentrations (data not shown). Therefore, we conclude that the activation by InsP₃ occurs independently of Ca²⁺. However, this does not preclude any regulatory influence of Ca²⁺ on this process.

The ability of InsP₃ to increase its own binding in microsomes washed with MI, indicates that molecules involved in this process are tightly associated with membranes. Several membrane-associated proteins have been proposed to interact with the cerebellum InsP₃ receptor, including ankyrin (Joseph and Samanta, 1993; Bourguignon *et al.*, 1993) and calmodin which, however, is not involved as discussed above. Another possibility is that the transition induced by InsP₃ involves changes in interaction between the InsP₃ receptor subunits. Evidence for an association between subunits of InsP₃ receptors of adjacent cisternae of smooth endoplasmic reticulum has been obtained in immunocytological studies of Purkinje cells (Satoh *et al.*, 1990; Otsu *et al.*, 1990; Villa *et al.*, 1991; Takei *et al.*, 1992, 1994). Alternatively, it may be possible that the activating effect of InsP₃ might involve a covalent modification of the InsP₃ receptor, *e.g.* a change in the phosphorylation state. Cerebellum InsP₃ receptor has been shown to be phosphorylated by several protein kinases (Ferris and Snyder, 1992; Koga *et al.*, 1994).

A major characteristic of the affinity increase of the cerebellum InsP₃ receptor following InsP₃ pretreatment is the slowness of its kinetics ($t_{1/2}$ = 20 s; Fig. 3) as compared with that of the InsP₃ binding (see above) and IICR ($t_{1/2}$ = 0.2 s with 0.15 μ M InsP₃; Combettes *et al.* (1994)). This difference implies that the increase in InsP₃ receptor affinity, resulting from InsP₃ binding, occurs after Ca²⁺ efflux has been completed and thus affects subsequent events only. Recently, it has been reported that pre-exposure of permeabilized hepatocytes to InsP₃ is followed by a time-dependent inactivation of IICR (Hajnóczky and Thomas, 1994). The time course for [³H]InsP₃ binding activation in the present studies ($t_{1/2}$ = 20 s, Fig. 3) is very similar to

that of the InsP₃-induced inactivation described by Hajnóczky and Thomas ($t_{1/2}$ = 15 s). However, in contrast to our results on cerebellar microsomes, inactivation by InsP₃ in permeabilized hepatocytes was dependent on the presence of Ca²⁺ and was accelerated by increasing the Ca²⁺ concentration up to 1 μ M. With respect to the effect of InsP₃ pretreatment of cerebellar microsomes on the function of the InsP₃ receptor (IICR), further studies will be required. The slow kinetics of the increase in InsP₃ affinity suggests that it will be dependent on a prolonged increase in the level of InsP₃ in intact cells, and that, therefore, it reflects a long-term regulation process. The same effect might also be attained with repetitive increases in the cellular level of InsP₃ if the InsP₃ binding activation is slowly reversible in intact cells, as suggested by the present *in vitro* conditions. Interestingly, such a situation might be encountered in long-term potentiation and long-term depression, two important models of synaptic plasticity, induced by tetanic and/or repetitive or prolonged stimulation of synapses (Madison *et al.*, 1991; Bliss and Collingridge, 1993; Artola and Singer, 1993). In cerebellum, long term depression is well known to occur at the parallel fiber-Purkinje cell synapses (Ito, 1989; Daniel *et al.*, 1992; Conquet *et al.*, 1994). Evidence has been obtained for involvement of the InsP₃/Ca²⁺ signaling system in these processes (Kato, 1993; Kasai and Petersen, 1994). Many different cell types respond by repetitive Ca²⁺ spikes to sustained application of agonists acting through InsP₃. Characteristically, the agonist-stimulated accumulation of InsP₃ consists in a rapid peak followed by a much lower but sustained phase (Willars and Nahorski, 1995). We hypothesize that such a pattern of InsP₃ accumulation may lead to an increase in the affinity of the InsP₃ receptor for InsP₃ and therefore facilitate the generation of Ca²⁺ oscillations while InsP₃ level is increased only slightly. Repetitive Ca²⁺ spikes have been observed at basal InsP₃ concentration following intracellular injection of thimerosal, a thiol alkylating agent which sensitizes the Ca²⁺ stores to InsP₃ (Bootman *et al.*, 1992), by increasing the affinity of the InsP₃ receptor (Hilly *et al.*, 1993). Consistent with our hypothesis, studies in intact cells have shown that InsP₃-dependent Ca²⁺ mobilization may also be sensitized by a prior InsP₃ injection or agonist activation (Parker and Miledi, 1989).

In summary, the present studies show that in sheep cerebellar microsomes a prolonged exposure of InsP₃ to its receptor, converts this protein to a state exhibiting higher affinity. This phenomenon indicates that, upon binding, InsP₃ not only opens the Ca²⁺ channel (a rapid process) but also initiates a slower regulation of the protein.

Acknowledgments—We thank J. Simon for excellent technical help, D. Reuter for expert secretarial assistance, and Drs. P. Champeil and F. Crépel for helpful discussions and critical reading of the manuscript.

REFERENCES

- Artola, A., and Singer, W. (1993) *Trends Neurosci.* **16**, 480–487
- Berridge, M. J. (1993) *Nature* **361**, 315–325
- Bezprozvanny, I., Watras, J., and Ehrlich, B. E. (1991) *Nature* **351**, 751–754
- Bliss, T. V. P., and Collingridge, G. L. (1993) *Nature* **361**, 31–39
- Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) *J. Biol. Chem.* **268**, 11356–11363
- Bootman, M. D., Taylor, C. W., and Berridge, M. J. (1992) *J. Biol. Chem.* **267**, 25113–25119
- Bourguignon, L. Y. W., Jin, H., Iida, N., Brandt, N. R., and Zhang, S. H. (1993) *J. Biol. Chem.* **268**, 7290–7297
- Combettes, L., Hannaert-Merah, Z., Coquil, J.-F., Rousseau, C., Claret, M., Swillens, S., and Champeil, P. (1994) *J. Biol. Chem.* **269**, 17561–17571
- Conquet, F., Bashir, Z. I., Davies, C. H., Daniel, H., Ferraguti, F., Bordini, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Condé, F., Collingridge, G. L., and Crépel, F. (1994) *Nature* **372**, 237–243
- Daniel, H., Hémar, N., Jaillard, D., and Crépel, F. (1992) *Exp. Brain Res.* **90**, 327–331
- Danoff, S. K., Supattapone, S., and Snyder, S. H. (1988) *Biochem. J.* **254**, 701–705
- De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van Den Bosch, L., and Casteels, R. (1994) *J. Biol. Chem.* **269**, 21691–21698
- Ferris, C. D., and Snyder, S. H. (1992) *Annu. Rev. Physiol.* **54**, 469–488

- Ferris, C. D., Haganir, R. L., and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2147–2151
- Finch, E. A., Turner, T. J., and Goldin, S. M. (1991) *Science* **252**, 443–446
- Föhr, K. J., Scott, J., Ahnert-Hilger, G., and Gratzl, M. (1989) *Biochem. J.* **262**, 83–89
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989) *Nature* **342**, 32–38
- Guillemette, G., Lamontagne, S., Boulay, G., and Mouillac, B. (1989) *Mol. Pharmacol.* **35**, 339–344
- Hajnoczky, G., and Thomas, A. P. (1994) *Nature* **370**, 474–477
- Hannaert-Merah, Z., Coquil, J.-F., Combettes, L., Claret, M., Mauger, J.-P., and Champeil, P. (1994) *J. Biol. Chem.* **269**, 29642–29649
- Hilly, M., Piétri-Rouxel, F., Coquil, J.-F., Guy, M., and Mauger, J.-P. (1993) *J. Biol. Chem.* **268**, 16488–16494
- Iino, M. (1990) *J. Gen. Physiol.* **95**, 1103–1122
- Ito, M. (1989) *Annu. Rev. Neurosci.* **12**, 85–102
- Joseph, S. K., and Ryan, S. V. (1993) *J. Biol. Chem.* **268**, 23059–23065
- Joseph, S. K., and Samanta, S. (1993) *J. Biol. Chem.* **268**, 6477–6486
- Joseph, S. K., Rice, H. L., and Williamson, J. R. (1989) *Biochem. J.* **258**, 261–265
- Kaplin, A. I., Ferris, C. D., Voglmaier, S. M., and Snyder, S. H. (1994) *J. Biol. Chem.* **269**, 28972–28978
- Kasai, H., and Petersen, O. H. (1994) *Trends Neurosci.* **17**, 95–101
- Kato, N. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3650–3654
- Koga, T., Yoshida, Y., Cai, J.-Q., Islam, M. O., and Imai, S. (1994) *J. Biol. Chem.* **269**, 11640–11647
- Komalavilas, P., and Lincoln, T. M. (1994) *J. Biol. Chem.* **269**, 8701–8707
- Madison, D. V., Malenka, R. C., and Nicoll, R. A. (1991) *Annu. Rev. Neurosci.* **14**, 379–397
- Maeda, N., Niinobe, M., and Mikoshiba, M. (1990) *EMBO J.* **9**, 61–67
- Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991) *J. Biol. Chem.* **266**, 1109–1116
- Maranto, A. R. (1994) *J. Biol. Chem.* **269**, 1222–1230
- Marshall, I. C. B., and Taylor, C. W. (1993) *J. Biol. Chem.* **268**, 13214–13220
- Mignery, G. A., and Südhof, T. C. (1990) *EMBO J.* **9**, 3893–3898
- Missiaen, L., Taylor, C. W., and Berridge, M. J. (1991) *Nature* **352**, 241–244
- Miyawaki, A., Furuichi, T., Ryou, Y., Yoshikawa, S., Nakagawa, T., Saitoh, T., and Mikoshiba, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4911–4915
- Mourey, R. J., Verma, A., Supattapone, S., and Snyder, S. H. (1990) *Biochem. J.* **272**, 383–389
- Nahorski, S. R., and Potter, B. V. L. (1989) *Trends Pharmacol. Sci.* **10**, 139–144
- Nakagawa, T., Okano, H., Furuichi, T., Aruga, J., and Mikoshiba, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6244–6248
- Otsu, H., Yamamoto, A., Maeda, N., Mikoshiba, K., and Tashiro, Y. (1990) *Cell Struct. Funct.* **15**, 163–173
- Parker, I., and Miledi, R. (1989) *J. Neurosci.* **9**, 4068–4077
- Pietri, F., Hilly, M., and Mauger, J.-P. (1990) *J. Biol. Chem.* **265**, 17478–17485
- Renard, D. C., Seitz, M. B., and Thomas, A. (1992) *Biochem. J.* **284**, 507–512
- Ross, C. A., Danoff, S. K., Schell, M. J., Snyder, S. H., and Ullrich, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4265–4269
- Rouxel, F. P., Hilly, M., and Mauger, J.-P. (1992) *J. Biol. Chem.* **267**, 20017–20023
- Satoh, T., Ross, C. A., Villa, A., Supattapone, S., Pozzan, T., Snyder, S. H., and Meldolesi, J. (1990) *J. Cell Biol.* **111**, 615–624
- Südhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., and Mignery, G. A. (1991) *EMBO J.* **10**, 3199–3206
- Supattapone, S., Worley, P. F., Baraban, J. M., and Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Swillens, S. (1992) *Trends Pharmacol. Sci.* **13**, 430–434
- Takei, K., Stukenbrok, H., Metcalf, A., Mignery, G. A., Südhof, T. C., Volpe, P., and De Camilli, P. (1992) *J. Neurosci.* **12**, 489–505
- Takei, K., Mignery, G. A., Mugnaini, E., Südhof, T. C., and De Camilli, P. (1994) *Neuron* **12**, 327–342
- Taylor, C. W., and Richardson, A. (1991) *Pharmacol. Ther.* **51**, 97–137
- Villa, A., Podini, P., Clegg, D. O., Pozzan, T., and Meldolesi, J. (1991) *J. Cell Biol.* **113**, 779–791
- Watras, J., Moraru, I., Costa, D. J., and Kindman, L. A. (1994) *Biochemistry* **33**, 14359–14367
- Willars, G. B., and Nahorski, S. R. (1995) *Br. J. Pharmacol.* **114**, 1133–1142
- Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S., and Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136