Incubation of cerebellar microsomes with \( \text{d}-\text{myo-inositol 1,4,5-trisphosphate (InsP}_3 \) at 0.01-1 \( \mu \text{M} \), at 4 or 20 °C in a cytosolic-like medium devoid of \( \text{Ca}^{2+} \) and Mg\(^{2+} \), followed by InsP\(_3\) removal, induced an increase in InsP\(_3\) binding determined with 1 nm \([3H]\text{InsP}\(_3\) at 20 °C, and pH 7.1, maximal stimulation (1.5-2.5-fold) was obtained with 1 \( \mu \text{M} \text{InsP}_3 \) and the \( EC_{50} \) was 60 ± 5 nm. Several lines of evidence suggested that the activating site is identical with the InsP\(_3\) binding site: (i) activation and binding exhibited the same inositol phosphate specificity; (ii) addition of decavanadate, a competitive inhibitor of \([3H]\text{InsP}_3\) binding, to the preincubation mixture, prevented the activating effect of InsP\(_3\); (iii) the concentration of InsP\(_3\) giving half-maximal activation was close to that giving half-maximal InsP\(_3\) 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 \([3H]\text{InsP}_3\) (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 of 1 \( \mu \text{M} \text{InsP}_3\) was required to half-maximally stimulate binding. Under the present conditions, the InsP\(_3\)-induced binding increase was only partially reversible. However, this effect was not blocked by antiproteases suggesting that it did not involve proteolysis. Taking advantage of the marked difference in the kinetics of InsP\(_3\) binding and InsP\(_3\)-dependent activation, we performed binding experiments on a short period (3 s) to determine the effect of InsP\(_3\) 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: \( K_D = 107 \text{ nM} \), \( B_{\text{max}} \) = 28 pmol/mg of protein; after preincubation with 1 \( \mu \text{M} \text{InsP}_3\): \( K_D = 53 \text{ nM} \), \( B_{\text{max}} \) = 32 pmol/mg of protein). The two states of the receptor bound InsP\(_3\) with a Hill coefficient close to 1 on a 3-s scale. In agreement with the effect of InsP\(_3\) 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 InsP\(_3\) receptor in cerebellum occurring independently of \( \text{Ca}^{2+} \) and on a relatively long time scale.

The second messenger \( \text{d}-\text{myo-inositol 1,4,5-trisphosphate (InsP}_3\) mobilizes intracellular \( \text{Ca}^{2+} \) by activating a receptor/
channel activity of the purified cerebellum receptor reconstituted in phospholipid vesicles (Kaplin et al., 1994). The present studies report that occupancy of InsP<sub>3</sub> binding sites in sheep cerebellum microsomes induces a time-dependent increase in the affinity of InsP<sub>3</sub> receptor for its ligand. In contrast with results by Hajnóczky and Thomas (1994) on permeabilized hepatocytes, this InsP<sub>3</sub> effect was independent of Ca<sup>2+</sup>++, 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 suspended in homogenization buffer (5 mM Hepes, 250 mM sucrose, 10 mM KCl, 1 mM β-mercaptoethanol, 10 μg/ml leupeptin, 10 μM pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4 at 4°C) and then frozen in liquid N<sub>2</sub> and stored at -80°C. Membranes were thawed and dialyzed in an ice-cold cytosolic-like medium (MI) containing 110 mM KCl, 20 mM NaCl, 1 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, 25 mM Hepes/KOH (pH 7.1), and 10 μg/ml leupeptin. Where indicated, membranes were washed and resuspended in the same medium. Preincubation of membranes (0.2–1.0 mg/ml) with InsP<sub>3</sub> (1 mM to 3 μM) was also conducted in MI, supplemented with the other agents as indicated. In the first series of experiments, InsP<sub>3</sub> was removed by centrifuging the preincubation mixture at 36,000 × g for 1 h and washing membranes twice with ice-cold MI. A more rapid washing procedure was developed to remove the initially added unlabeled InsP<sub>3</sub>. Nonspecific binding was determined by centrifugation 4°C (preincubation). Membranes were then washed either by centrifugation method, the mixture was centrifuged at 100,000 × 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]InsP<sub>3</sub> 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 Na<sub>2</sub>PO<sub>4</sub>, 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 briefly perfusing 1 ml of the washing medium described above. n = number of experiments.

### TABLE I

<table>
<thead>
<tr>
<th>Washing procedure</th>
<th>n</th>
<th>Preincubation conditions</th>
<th>[3H]InsP&lt;sub&gt;3&lt;/sub&gt; binding (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>4</td>
<td>Control</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1 μM InsP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.17 ± 0.14</td>
</tr>
<tr>
<td>Filtration</td>
<td>4</td>
<td>Control</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1 μM InsP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.93 ± 0.10</td>
</tr>
</tbody>
</table>

**RESULTS**

Preincubation of Cerebellar Microsomes with InsP<sub>3</sub> Increases [3H]InsP<sub>3</sub> Binding—When a microsomal fraction of sheep cerebellum was preincubated with 1 μM InsP<sub>3</sub> 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]InsP<sub>3</sub> at 1 nM was doubled (Table I). As this centrifugation procedure was very time-consuming, another method was developed to remove the initially added unlabeled InsP<sub>3</sub>, this being washing the membranes with MI on a GF/C glass fiber filter. Following the washing step, [3H]InsP<sub>3</sub> binding was directly measured on the filter by adding 0.5 ml of MI containing 1 nM [3H]InsP<sub>3</sub>. The InsP<sub>3</sub>-dependent increase in [3H]InsP<sub>3</sub> 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 InsP<sub>3</sub> pretreatment were examined at 4°C. It was found that maximal activation was achieved with 0.1–0.3 μM InsP<sub>3</sub> and that the activating effect of InsP<sub>3</sub> was much slower to develop than InsP<sub>3</sub> binding. While the binding of 1 nM [3H]InsP<sub>3</sub> to cerebellar microsomes layered on a GF/C glass fiber filter reached equilibrium within 15 s, the activation due to preincubation with 1 μM InsP<sub>3</sub> 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 InsP<sub>3</sub> was generally performed for 10 min. The fact that the same level of activation was measured when membranes were washed free of InsP<sub>3</sub> 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 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 2 mM benzamidine, 1 μg/ml O-phenanthroline, 50 μg/ml trypsin inhibitor) to the preincubation medium, suggesting that the InsP<sub>3</sub>-induced binding increase did not involve proteolysis.

A crucial point in this type of experiment was to remove carefully the nonradioactive InsP<sub>3</sub> present in the preincubation
InsP₃ Increases Affinity of Its Cerebellar Receptor

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 unlabelled InsP₃ was expected. Washing the filter with M1 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 measured with 1 nM [³H]InsP₃. An additional experiment to determine the amount of residual InsP₃ after a washing with 10 ml of M1 (the standard protocol) was performed. In this experiment, the preincubation mixture containing both cerebellar membranes and 1 µM unlabelled 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 M1 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(1,3,4,5)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, 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 (Guillemet 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 (Führ et al., 1989; Taylor and Richardson, 1991). As illustrated in Fig. 1, A and B, 3 µM decavanadate inhibited InsP₃ binding measured with 0.1 µM 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 M1 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 M1 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-

### Table II

<table>
<thead>
<tr>
<th>Inositol phosphates</th>
<th>Decrease in [³H]InsP₃ binding due to competition</th>
<th>Increase in [³H]InsP₃ binding due to preincubation</th>
<th>%</th>
<th>µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ins(1,4,5)P₃</td>
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<td></td>
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<td>1</td>
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<td>Ins(2,4,5)P₃</td>
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<td></td>
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<td>10</td>
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<td>GroPIns(4,5)P₂</td>
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<td>1</td>
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<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₃</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

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**Note:** The table above lists the decrease in [³H]InsP₃ binding due to competition and the increase in [³H]InsP₃ binding due to preincubation for various inositol phosphates at different concentrations.
sessions. At the longer time of 5 min, preincubations were conducted within test tubes. In all cases, InsP$_3$ pretreatment was stopped by washing the membranes with 10 ml of MI. Fig. 3 illustrates the results of these experiments. The maximal increase of [H]InsP$_3$ 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 time. This condition is satisfied in MI at 20°C, a medium in which equilibrium for [H]InsP$_3$ 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 with the various ligands at concentrations from 0.1 to 100 nM. The Hill coefficient was calculated for each concentration of InsP$_3$ and was found to be close to 1 for membranes preincubated with or without 1 μM InsP$_3$. This result indicates that the observed activation by InsP$_3$ is a much slower process than InsP$_3$ 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 InsP$_3$ Binding Characteristic Modified by InsP$_3$ Pretreatment—We then examined whether the preexposure of microsomes to InsP$_3$ affected the affinity or the number of binding sites for [H]InsP$_3$. As the determination of $K_D$ and $B_{max}$ requires an equilibrium binding experiment with increasing InsP$_3$ concentrations, it was a prerequisite to measure the level of [H]InsP$_3$ binding within a sufficiently short time to avoid InsP$_3$-dependent activation. This condition is satisfied in MI at 20°C, a medium in which equilibrium for [H]InsP$_3$ 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 with the various ligands at concentrations from 0.1 to 100 nM. The Hill coefficient was calculated for each concentration of InsP$_3$ and was found to be close to 1 for membranes preincubated with or without 1 μM InsP$_3$. This result indicates that the observed activation by InsP$_3$ is a much slower process than InsP$_3$ 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).
InsP₃ with the same receptor. Firstly, the selectivity of the 
activating site was the same as that of the [³H]InsP₃ binding 
parameter. As the total number of InsP₃ binding sites was not changed by 
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 (B₀). 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 value close to 1 determined with untreated 
membranes (Fig. 4, B). These results support the possibility 
that the activation process results from prolonged occupancy of the 
InsP₃ binding site.

**DISCUSSION**

The present studies show that in sheep cerebellar micro- 
somes 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; Maurey et al., 1990; Südhof et al., 1991; 
Maeda et al., 1991; Rouxel et al., 1992). Secondly, decavanadate, a competitive inhibitor of InsP₃ receptor (Förh et al., 
1989; Taylor and Richardson, 1991), prevented the activation 
by InsP₃. Thirdly, the EC₅₀ value for activation at 20 °C (60 nM) 
was close to the apparent Kᵦ 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.
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 Sudhof, 1990). Recently, this conformational change has been suggested to alter accessibility of thimerosal to certain sulphydryl 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 μM, 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; Hannonet-Merah et al., 1994), an effect which has been proposed to be mediated by the Ca2+-binding protein, calmodulin (Danoff et al., 1988). However, the InsP3 activating effect cannot be due to removal of inhibitory influence by calmodulin, 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 μM and then washed with 10 ml of M1 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 M1, 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 calmodulin 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 immunocytochemical 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 (t1/2 = 20 s; Fig. 3) as compared with that of the InsP3 binding (see above) and IICR (t1/2 = 0.2 s with 0.15 μM 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 (Hajnoczky and Thomas, 1994). The time course for [3H]InsP3 binding activation in the present studies (t1/2 = 20 s, Fig. 3) is very similar to that of the InsP3-induced inactivation described by Hajnoczky and Thomas (t1/2 = 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 μM. 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.

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Inositol 1,4,5-Trisphosphate Slowly Converts Its Receptor to a State of Higher Affinity in Sheep Cerebellum Membranes
Jean-François Coquil, Jean-Pierre Mauger and Michel Claret

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