Subcellular distribution of the calcium-storing inositol 1,4,5-trisphosphate-sensitive organelle in rat liver

Possible linkage to the plasma membrane through the actin microfilaments

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The role of $\text{Ins}(1,4,5)\text{P}_3$ in the mobilization of Ca^{2+} from intracellular stores of non-muscle cells has been extensively demonstrated; however, the nature of the organelle releasing the Ca^{2+} is still poorly understood. The distributions of the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites and of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool were investigated in subcellular fractions obtained from rat liver and compared with those of other markers. The $\text{Ins}(1,4,5)\text{P}_3$ -binding vesicles appeared to be completely distinct from the endoplasmic-reticulum-derived microsomes and were enriched in the same fractions which were enriched in alkaline phosphodiesterase I activity. This co-purification of the plasma-membrane marker with the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites was dramatically altered after freezing or after treatment of the homogenate with the microfilament-disruptive drug cytochalasin B, suggesting that the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive organelle may be linked to the plasma membrane through the actin microfilaments. No correlation was observed between the $\text{Ins}(1,4,5)\text{P}_3$ -binding capacity and the portion of the Ca^{2+} pool that was released by $\text{Ins}(1,4,5)\text{P}_3$. This may result from the disruption of the native organelle during homogenization, leading to the formation of vesicles containing the $\text{Ins}(1,4,5)\text{P}_3$ receptor, but lacking the Ca^{2+} pump. These results are consistent with the idea of a specialized $\text{Ins}(1,4,5)\text{P}_3$ -regulated organelle distinct from the endoplasmic reticulum, and we propose a model of the structural organization of this organelle, in which the anchorage to the cytoskeleton as well as the spatial separation of the Ca^{2+} pump from the $\text{Ins}(1,4,5)\text{P}_3$ receptor have important functional significance.

INTRODUCTION

The role of $\text{Ins}(1,4,5)\text{P}_3$ as mediator of intracellular Ca^{2+} mobilization in response to various extracellular stimuli is now well established, and has been extensively documented in permeabilized cells and microsomal preparations (for a review, see Berridge, 1987). According to the current models, $\text{Ins}(1,4,5)\text{P}_3$ acts through specific intracellular receptors; indeed, high-affinity binding sites for $\text{Ins}(1,4,5)\text{P}_3$ have been described in various tissues (Baukal *et al.*, 1985; Hirata *et al.*, 1985; Spat *et al.*, 1986a,b, 1987; Worley *et al.*, 1987a; Willcocks *et al.*, 1987). The purification and characterization of a 260 kDa $\text{Ins}(1,4,5)\text{P}_3$ -binding protein from rat cerebellum (Supattapone *et al.*, 1988) and its functional reconstitution in lipid vesicles (Ferris *et al.*, 1989) have previously been reported. A protein of similar size appears to be responsible for the high-affinity binding of $\text{Ins}(1,4,5)\text{P}_3$ observed in liver (Nunn *et al.*, 1990), and more recently Nunn & Taylor (1990) have demonstrated that this liver $\text{Ins}(1,4,5)\text{P}_3$ -binding site is the Ca^{2+} -mobilizing receptor.

However, the nature and the location of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool are still controversial. The use of specific inhibitors of mitochondrial function have unequivocally excluded the mitochondria as the site of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release (Berridge, 1987; Carafoli, 1987), but at least two non-mitochondrial organelles have been proposed to fulfil this function in non-muscle cells: the endoplasmic reticulum and a newly described organelle, the calciosome (Volpe *et al.*, 1988). A role for endoplasmic reticulum was primarily supported by early subcellular fractionation of exocrine pancreas (Bayerdörffer *et al.*, 1984), a tissue where this organelle is particularly abundant, and more recently by immunocytochemical labelling of the $\text{Ins}(1,4,5)\text{P}_3$ receptor in cerebellar Purkinje neurons (Ross *et al.*,

1989). However, the identity of the organelle sensitive to $\text{Ins}(1,4,5)\text{P}_3$ as endoplasmic reticulum has been strongly challenged, on the basis of results obtained from subcellular-fractionation studies of human neutrophils (Krause & Lew, 1987), rat liver (Guillemette *et al.*, 1988), bovine adrenal cortex (Rossier *et al.*, 1989) and dog brain (Alderson & Volpe, 1989). The concept of an organelle distinct from the endoplasmic reticulum, and specifically involved in the control of $[\text{Ca}^{2+}]_i$ by $\text{Ins}(1,4,5)\text{P}_3$ in non-muscle cells, was suggested by morphological studies of HL60 and PC12 cells (Volpe *et al.*, 1988). This organelle, named the calciosome, was mainly characterized by its content of a calsequestrin-like protein (Pozzan *et al.*, 1988). However, the presence of the $\text{Ins}(1,4,5)\text{P}_3$ receptor on this organelle has not been demonstrated, and the presence of a calsequestrin-like protein in rat liver is still controversial (Damiani *et al.*, 1988; Van *et al.*, 1989).

In this paper, we present evidence that the $\text{Ins}(1,4,5)\text{P}_3$ receptors in rat liver are not located on the endoplasmic reticulum, and we show that the vesicles containing the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites are enriched in parallel with the plasma-membrane marker during subcellular fractionation. This correlation is possibly due to actin microfilaments linking the $\text{Ins}(1,4,5)\text{P}_3$ -binding organelle to the peripheral membrane. We propose a model of the structural and functional organization of this organelle.

MATERIALS AND METHODS

Materials

Cytochalasin B, colchicine, heparin, BSA, EGTA, PMSF, dTMP *p*-nitrophenyl ester, cytochrome *c*, NADPH, $\text{Na}_2\text{S}_2\text{O}_4$, Na_2ATP , phosphocreatine, creatine kinase and DNAase were obtained from Sigma. Oligomycin, antimycin and $\text{Ins}(1,4,5)\text{P}_3$

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; PMSF, phenylmethanesulphonyl fluoride.

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were purchased from Calbiochem, dithiothreitol and Hepes from Boehringer Mannheim Biochemicals, and Percoll was from Pharmacia. Glycine and Coomassie Blue were obtained from Bio-Rad, $^{45}\text{CaCl}_2$ (10–75 Ci/g) was Du Pont–New England Nuclear, and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (21–52 Ci/mmol) was from Amersham.

Preparation of subcellular fractions

Livers from sodium pentobarbital-anaesthetized male Sprague–Dawley rats (200–350 g) were perfused *in situ* with 200 ml of Krebs–Ringer solution and fractionated by a modification of methods previously described for liver microsomes (Dawson, 1982) and plasma-membrane (Prpic *et al.*, 1984) preparation. After removal, the liver was minced in a buffer containing 250 mM-sucrose, 5 mM-Hepes/KOH, 1 mM-EGTA and 1 mM-dithiothreitol, pH 7.4 (Buffer A), homogenized at 4 °C with a glass Potter homogenizer (four strokes, 0.15 mm clearance), and then centrifuged for 10 min at 500 *g*. The pellet from this first centrifugation step (named 'HS' for high sedimentation coefficient) was resuspended in Buffer A at a concentration of 10–15 mg of protein/ml, supplemented with Percoll (14 %, v/v), and centrifuged for 30 min at 36000 *g*. Three distinct layers were obtained after formation of the density gradient: the top layer ('HSLD', of low density) contained flake-like structures floating in the medium, whereas both the intermediate ('HSMD', of medium density) and the bottom ('HSHD', of high density) layers appeared to be more compact. The latter fraction, HSHD, was contaminated with erythrocytes. The supernatant from the 500 *g* centrifugation step was centrifuged for 10 min at 2000 *g* to obtain a pellet with a medium sedimentation coefficient ('MS'), and the resulting supernatant was further centrifuged for 40 min at 36000 *g* to sediment the microsomes ('LS', of low sedimentation coefficient). Finally, the LS pellet was resuspended in Buffer A containing 35 % (v/v) Percoll and further fractionated by centrifugation at 36000 *g* for 30 min to produce low-density ('LSLD') and high-density ('LSHD') fractions. All the fractions obtained by centrifugation on Percoll gradients were washed in 10 vol. of a medium containing 120 mM-KCl and 20 mM-Hepes (pH 7.4). After a 40 min centrifugation at 36000 *g*, the membranes remaining above the hard Percoll pellet formed were removed and resuspended in 250 mM-sucrose/20 mM-Hepes buffer (pH 7.4) supplemented with 1 mM-dithiothreitol and 0.2 mM-PMSF. Samples of each fraction were stored at –20 °C until assayed for various subcellular markers.

Assays for marker enzymes

All enzyme assays were carried out at 37 °C.

Alkaline phosphodiesterase I activity was used as a marker for plasma membrane, and determined by spectrometric assay of the dephosphorylation of dTMP *p*-nitrophenyl ester in a glycine buffer at pH 9.0 (Aronson & Touster, 1974).

NADPH-dependent cytochrome *c* reductase activity was determined by colorimetric measurement of cytochrome *c* reduction in the presence of KCN and NADPH (Fleischer & Fleischer, 1967). Sulphatase C activity was determined as previously described (Rossier *et al.*, 1989). Both activities were used as independent markers of the endoplasmic reticulum.

The mitochondrial cytochrome *c* oxidase activity (Wikstrom *et al.*, 1981) was measured by colorimetric recording of cytochrome *c* oxidation after reduction of the substrate with $\text{Na}_2\text{S}_2\text{O}_4$.

Protein concentration was measured by the Coomassie Blue method of Bradford (1976), with BSA as standard.

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding

The specific $\text{Ins}(1,4,5)\text{P}_3$ -binding capacity of the various subcellular fractions was determined by a modification of the method

of Guillemette *et al.* (1987). The membranes (0.5–1 mg of protein in 500 μl) were incubated for 10 min at 4 °C, in the presence of 50000 d.p.m. of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (approx. 1.5 nM), as previously described (Rossier *et al.*, 1989), except that the pH was adjusted to 8.4 instead of 7.4 unless otherwise specified. Non-specific binding was estimated in the presence of an excess of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ (5 μM), and appeared to be similar in each fraction. The specific binding of $\text{Ins}(1,4,5)\text{P}_3$ was linear in the range of 0.1–2 mg of protein/ml of incubation medium. An alternative method to separate free from bound $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, which did not use GF/B glass-fibre filters, but involved high-speed centrifugation of the membranes for 10 min in a Beckman Airfuge (Alderson & Volpe, 1989), gave qualitatively similar results. The fractions were frozen and thawed at least twice before determination of their $\text{Ins}(1,4,5)\text{P}_3$ -binding capacity.

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

The capacity of the various fractions to accumulate Ca^{2+} in an ATP-dependent manner was measured by incubating portions (0.25 mg of protein in 25 μl) with 75 μl of a medium containing 100 mM- MgCl_2 , 2 mM- KH_2PO_4 and 25 mM-Hepes, at pH 7.2, and supplemented with 1 μg of oligomycin/ml, 10 μM -antimycin, 1 mM-EGTA, 0.453 mM- CaCl_2 (0.15 μM free Ca^{2+}), and 1 μCi of $^{45}\text{CaCl}_2$ /ml. After a 5 min preincubation at 37 °C, ATP-dependent $^{45}\text{Ca}^{2+}$ uptake was initiated by addition of 3 mM- Na_2ATP and an ATP-regenerating system (10 mM-phosphocreatine and 8 units of creatine kinase/ml). The reaction was terminated after reaching the steady state (20 min) by filtration through GF/C glass-fibre filters pre-soaked in 230 mM-sucrose and 40 mM-NaCl (washing medium). The filters were rinsed with 2 $\times$ 4 ml of washing medium, dried, and the bound $^{45}\text{Ca}^{2+}$ was measured by liquid-scintillation counting. The non-specific binding was estimated by omitting the ATP during the incubation, and the inhibitory effect of $\text{Ins}(1,4,5)\text{P}_3$ on Ca^{2+} uptake was measured by adding 20 μM - $\text{Ins}(1,4,5)\text{P}_3$ either immediately before ATP or 30 s before the end of the incubation.

RESULTS

Distribution of markers among the various subcellular fractions

Freshly removed rat livers were homogenized and fractionated by differential centrifugation and by centrifugation on Percoll density gradients as described in the Materials and methods section. The specific activities of selected markers were measured in these fractions and expressed as a percentage of the activity present in the homogenate (H). Fig. 1(b) shows the distribution of the alkaline phosphodiesterase I, a marker for the plasma membrane, the NADPH-dependent cytochrome *c* reductase, specific for the endoplasmic reticulum, and the cytochrome *c* oxidase, which is associated with the mitochondria. As expected, the marker for the endoplasmic reticulum, which is mostly disrupted into microsomes during the homogenization, is increased in fractions of lower sedimentation coefficient (LS and LSLD), whereas the marker for mitochondria is increased in fractions of higher sedimentation coefficient, characteristic of heavier and denser organelles (MS and HSHD). The fact that the alkaline phosphodiesterase I is enriched in both the heaviest (HSLD; 30 % recovery) and lightest (LSLD; 7 % recovery) fractions suggests that the plasma membrane is present in the form of large membrane sheets as well as smaller vesicles.

The specific $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding capacity of the various fractions was also determined (Fig. 1a). This revealed a 5.6-fold enrichment of the binding sites in the HSLD fraction (45 % recovery), whereas the microsomal fraction (LSLD) showed only a 2.2-fold increase in its capacity to bind $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (5 % recovery). Because the $\text{Ins}(1,4,5)\text{P}_3$ binding is generally charac-

Fig. 1. Distribution of the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites and markers in rat liver subcellular fractions

Rat livers were homogenized and fractionated as indicated in the Materials and methods section. The $\text{Ins}(1,4,5)\text{P}_3$ -binding capacity (panel a) and the specific activities of the various markers (panel b) were determined (per mg of protein) in each fraction, and expressed as percentage of the specific activity present in the homogenate (H). The non-specific binding was estimated for each fraction in the presence of $5\text{ }\mu\text{M}$ unlabelled $\text{Ins}(1,4,5)\text{P}_3$, and subtracted from the total binding. Each value was determined in duplicate, and the result is representative of at least four different preparations in which similar patterns of distribution were obtained. In (b): $\square$, alkaline phosphodiesterase I (plasma membrane); $\square$ (hatched), NADPH-dependent cytochrome *c* reductase (endoplasmic reticulum); $\blacksquare$, cytochrome *c* oxidase (mitochondria). The various fractions are defined in the Materials and methods section.

Table 1. Characteristics of the $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding

The total $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ bound to HSLD membranes (0.7 mg of protein/500 μl) was determined under conditions of different pH, and at pH 7.4 in the presence of an excess ($5\text{ }\mu\text{M}$) of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ or 0.1 mg of heparin/ml. Each value represents the mean of triplicates.

pH	Bound $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (d.p.m./mg of protein)		
	Control	+ $\text{Ins}(1,4,5)\text{P}_3$	+ heparin
5.2	1720	—	—
7.4	3680	582	955
9.1	4128	—	—

terized in microsome preparations, properties of the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites in the HSLD fraction were further investigated by incubating the membranes in the presence of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ for

Fig. 2. Correlation between the distribution of the plasma-membrane markers and the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites

The alkaline phosphodiesterase I (APDE I; a) and the NADPH-dependent cytochrome *c* reductase (Cyt. *c* red; b) activities present in the various fractions were determined and compared with the specific $\text{Ins}(1,4,5)\text{P}_3$ -binding capacity of the same fraction. The correlations were established by least-squares linear-regression analysis. Highly significant correlations (*r* values between 0.73 and 0.92) between the plasma-membrane marker and the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites were observed in each of eight independent preparations.

10 min at 4°C under various conditions (Table 1). The total amount of bound $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was sensitive to the pH, increasing 2.4-fold when the pH rose from 5.2 to 9.1. At pH 7.4, the presence of an excess of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ ($5\text{ }\mu\text{M}$) in the medium displaced 85% of the tracer, whereas 0.1 mg of heparin/ml, a known inhibitor of the $\text{Ins}(1,4,5)\text{P}_3$ binding on its receptor (Worley *et al.*, 1987b), decreased the amount of bound tracer by 75%.

Because $\text{Ins}(1,4,5)\text{P}_3$ -binding sites did not co-purify with the NADPH-dependent cytochrome *c* reductase activity (Fig. 2b), another marker of the endoplasmic reticulum, the sulphatase C activity, was also determined in the same fractions. We found a strong correlation ($r = 0.949$ for $n = 10$, $P < 0.0001$) between the distribution of both endoplasmic-reticulum markers, with a slope of almost 1 when the activities were expressed as percentage of homogenate activity (results not shown). This confirms the specificity of these markers for the endoplasmic reticulum and demonstrates that the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites are distinct from this organelle.

Co-purification of the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites with the plasma membrane

In contrast with what has been observed for the endoplasmic-reticulum markers, the alkaline phosphodiesterase activity appeared to be enriched in the same fractions which showed an increase in the $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ -binding capacity. Moreover, a significant correlation between the distribution of the $\text{Ins}(1,4,5)\text{P}_3$ -binding sites and the plasma-membrane marker was reproducibly obtained when the fractions were prepared as described in the Materials and methods section (Fig. 2a). Such a correlation can be due to several reasons: (1) the $\text{Ins}(1,4,5)\text{P}_3$ receptors are mainly located on the plasma membrane, (2) the weight and the density of the plasma membrane and of the $\text{Ins}(1,4,5)\text{P}_3$ -binding vesicles are very similar, or (3) there is a

Table 2. Effect of various treatments on the correlation between the Ins(1,4,5) P_3 -binding sites and the plasma membrane

The homogenates from five independent preparations (I–V) were divided, mixed 1:1 (v/v) with a buffer containing 100 mM-KCl, 10 mM-MgCl₂, 25 mM-Hepes, 1 mM-dithiothreitol, 20 μ M-PMSF, 0.5 mM-EGTA and 0.8 mM-CaCl₂, at pH 7.6, and incubated for 60 min at 37 °C with 57 units of DNAase/ μ l, 1 μ M-cytochalasin B (CB), or 2 μ M-colchicine (CC). Control homogenates were incubated without added agent, and in one experiment a part of the homogenate was frozen (at –80 °C) and thawed (at +20 °C) three times before being incubated as the control. The membranes were then fractionated in parallel, and the markers determined in each fraction. The correlation between the Ins(1,4,5) P_3 -binding capacity and the plasma-membrane marker activity was analysed in each case as in Fig. 2, and the *r* values, with their significance (**P* < 0.05, ***P* < 0.01), were determined: n.d. means that the effect of a particular treatment was not determined in a particular experiment.

Preparation	Treatment before fractionation ...	Correlation (<i>r</i> values) between distribution of Ins(1,4,5) P_3 -binding sites and plasma-membrane marker					
		Control	DNAase	CB	CC	CB + CC	Freezing
I		0.893**	n.d.	n.d.	n.d.	0.542	n.d.
II		0.878**	0.886**	0.406	0.617	n.d.	n.d.
III		0.857**	0.760*	0.615	0.698*	n.d.	n.d.
IV		0.726*	0.694	0.621	0.654	n.d.	n.d.
V		0.911**	n.d.	0.903**	n.d.	n.d.	0.357

Fig. 3. Effect of cytochalasin B treatment on the distribution of the Ins(1,4,5) P_3 -binding sites and plasma-membrane marker

Rat liver homogenate was split and incubated in the presence or absence of cytochalasin B as described in the legend of Table 2. The distributions of the Ins(1,4,5) P_3 -binding sites (a) and the alkaline phosphodiesterase I activity (APDE I; b) in treated fractions were compared with the distributions of these markers in corresponding untreated fractions. The marker activities were determined in duplicate, and the results from two independent preparations were pooled for analysis.

physical link between the plasma membrane and these vesicles. In order to investigate this last possibility, the homogenates from five different preparations were split and treated with various agents which could potentially disrupt that linkage. The membranes were immediately fractionated in parallel after the treatment, and the marker activities determined in each fraction. Treatment of the membranes with 57 units of DNAase/ml decreased by 50% the amount of nucleic acid measured by ethidium bromide fluorescence, which was particularly abundant

in the HSLD fraction (0.6–1.2 mg/mg of protein). However, DNAase did not affect the distribution of either the plasma-membrane marker or the [³H]Ins(1,4,5) P_3 -binding sites, and the correlation between these markers was essentially the same after treatment (Table 2). In contrast, treatment of the homogenate with 1 μ M-cytochalasin B (or, to a lesser extent, with 2 μ M-colchicine) markedly decreased this correlation, which in all cases but one was no longer significant. Freezing of the membranes before fractionation also resulted in a clear-cut separation of the Ins(1,4,5) P_3 -binding sites from the plasma membrane marker (Table 2, preparation V). A net redistribution of the Ins(1,4,5) P_3 -binding sites from one specific fraction to another after freezing or cytochalasin B treatment was not reproducibly obtained, except for the 36000 *g* supernatant, which was always significantly enriched (results not shown). This may be due in part to the effect of these treatments on the Ins(1,4,5) P_3 -binding capacity of the membranes (see below). However, the correlation between the Ins(1,4,5) P_3 -binding sites and the plasma-membrane marker was in each preparation highly significant and greater in control than in frozen or cytochalasin B-treated fractions, in which case it was no longer significant. In order to determine if cytochalasin B treatment was affecting the distribution of either the plasma membrane or the Ins(1,4,5) P_3 -binding vesicles, both marker activities were compared in each treated and untreated fraction. Fig. 3(a) shows that cytochalasin B induced substantial changes in the distribution of the Ins(1,4,5) P_3 -binding sites. In contrast, cytochalasin B treatment did not modify the distribution of the alkaline phosphodiesterase I activity (Fig. 3b). This effect on Ins(1,4,5) P_3 -receptor distribution cannot be explained by the variability within the assay. Indeed, when the distribution of Ins(1,4,5) P_3 -binding sites was compared in independent control preparations, the data were highly significantly correlated (*r* = 0.887, *P* < 0.005).

Effect of freezing and cytochalasin B treatment on membrane Ins(1,4,5) P_3 binding capacity

Treatment of the membranes with cytochalasin B also resulted in an increase in their specific [³H]Ins(1,4,5) P_3 -binding capacity, particularly in membranes which previously had been frozen only once (Table 3). A second freeze/thaw cycle by itself increased dramatically the capacity of control membranes to bind Ins(1,4,5) P_3 , and the subsequent cytochalasin B effect on [³H]-Ins(1,4,5) P_3 binding was then much less. Further freezing did not increase the binding capacity, and in all cases the non-specific

Table 3. Effect of cytochalasin B treatment and membrane freezing on the Ins(1,4,5) P_3 -binding capacity

The HSLD fraction was prepared from rat liver as described in the Materials and methods section, split and incubated for 40 min in the absence (Control) or in the presence (CB) of 2 μ M-cytochalasin B. The membranes were then washed in parallel and resuspended in buffer A before being split again and frozen at -20°C overnight. Half of these samples were thawed at room temperature and processed again through a freeze/thaw cycle before determination of their Ins(1,4,5) P_3 -binding capacity. Each value is the mean ($\pm$ S.E.M.) of four determinations. Although not always analysed quantitatively, the effect of freezing and/or cytochalasin B treatment on the Ins(1,4,5) P_3 -binding capacity was qualitatively observed in each fraction.

No. of freeze/thaw cycles	Specific Ins(1,4,5) P_3 -binding capacity (fmol/mg of protein)	
	Control	CB (2 μ M)
1	10.9 ($\pm$ 3.7)	31.8 ($\pm$ 4.2)
2	51.7 ($\pm$ 0.7)	55.4 ($\pm$ 1.9)

Table 4. Ca^{2+} uptake by rat liver subcellular fractions

The non-mitochondrial $^{45}\text{Ca}^{2+}$ uptake was determined in the fractions presented in Fig. 1. Each fraction was incubated as described in the Materials and methods section, either without ATP or with ATP and an ATP-regenerating system. Some fractions were supplemented with 20 μ M-Ins(1,4,5) P_3 . The portion of the Ca^{2+} pool maintained depleted by Ins(1,4,5) P_3 was estimated in each fraction and expressed as a percentage of the ATP-dependent Ca^{2+} pool in that fraction. Each value represents the mean of triplicates from one of two independent experiments, each of which gave similar results.

Treatment	Ca^{2+} content (nmol/mg of protein)			
	H	HS	MS	LS
No ATP	0.058	0.211	0.074	0.094
ATP alone	1.242	1.473	1.241	2.413
ATP + Ins(1,4,5) P_3	1.128	1.415	1.001	2.089
Portion of the pool depleted by Ins(1,4,5) P_3	10 %	5 %	21 %	14 %

binding remained unchanged. This suggests that rupture of the microfilaments, either by a specific agent such as cytochalasin B or by repetitive freezing and thawing of the membranes, can reveal previously hidden Ins(1,4,5) P_3 -binding sites. In contrast, treatment of the same membranes with colchicine partially decreased (by about 40 %) their specific Ins(1,4,5) P_3 -binding capacity (results not shown).

Distribution of the Ins(1,4,5) P_3 -sensitive Ca^{2+} stores

The ATP-dependent $^{45}\text{Ca}^{2+}$ uptake by the fractions was also investigated, as well as the ability of Ins(1,4,5) P_3 to prevent this uptake by increasing Ca^{2+} efflux from the vesicles. Because the Percoll, even after washing, strongly inhibited the Ca^{2+} -pumping activity of the vesicles, only the fractions obtained by differential centrifugation were analysed. The microsomal (LS) fraction had clearly the highest Ca^{2+} -pumping activity (Table 4), but only 14 % of the accumulated Ca^{2+} in the presence of ATP was Ins(1,4,5) P_3 -sensitive. The HS fraction, the most enriched in Ins(1,4,5) P_3 -binding sites (Fig. 1a), had only 5 % of its Ca^{2+} -uptake activity prevented by the presence of Ins(1,4,5) P_3 . It is noteworthy that the difference observed between the fractions is

not due to different metabolic rates of Ins(1,4,5) P_3 , because the same Ins(1,4,5) P_3 concentration (20 μ M) had a similar effect if added to the medium only 30 s before the end of the incubation (results not shown). There was no correlation between the Ins(1,4,5) P_3 -binding capacity of the fractions and the amount of Ca^{2+} released in these fractions by Ins(1,4,5) P_3 . This result suggests either that some Ins(1,4,5) P_3 receptors, although able to bind their ligand, are not functional, or that the stoichiometry between the Ca^{2+} -pumping and Ca^{2+} -releasing sites on the same vesicle is different from one fraction to the other.

DISCUSSION

The most important finding of this study is that during subcellular fractionation of liver the Ins(1,4,5) P_3 -binding vesicles appear to be associated with the plasma membrane, and that this association is altered by freezing or treatment with the microfilament-disruptive drug cytochalasin B.

The fraction characterized by sedimentation at low-speed centrifugation and low density (HSLD) showed the highest Ins(1,4,5) P_3 -binding capacity (Fig. 1). The characteristics of [^3H]Ins(1,4,5) P_3 binding in this fraction (Table 1) were consistent with those observed in microsomes from liver and other tissues; the binding was pH-dependent (Worley *et al.*, 1987b; Rossier *et al.*, 1989) and inhibited by heparin (Worley *et al.*, 1987b; Hill *et al.*, 1987; Kobayashi *et al.*, 1988; Ghosh *et al.*, 1988; Guillemette *et al.*, 1989; Tones *et al.*, 1989).

By analysis of the distribution of the other markers, it appeared that the HSLD fraction was enriched in plasma membrane, whereas its content of endoplasmic reticulum was lower than that of the homogenate, and mitochondria were almost completely absent (Fig. 1). These observations are consistent with the finding by Guillemette *et al.* (1988) that the fraction enriched in plasma membrane showed higher Ins(1,4,5) P_3 -binding capacity than those enriched in endoplasmic reticulum or mitochondria. In addition, the lack of correlation between the Ins(1,4,5) P_3 -binding capacity and the content of endoplasmic reticulum, measured by either the NADPH-dependent cytochrome *c* reductase or the sulphatase C activity, strongly suggests that the Ins(1,4,5) P_3 receptors in liver are not located on the endoplasmic-reticulum membrane. This result agrees with those obtained in neutrophils (Krause & Lew, 1987), parotid cells (Henne *et al.*, 1987), adrenal cortex (Rossier *et al.*, 1989) and brain (Alderson & Volpe, 1989), but is in contradiction with early studies performed in pancreatic (Bayerdörffer *et al.*, 1984) and insulinoma cells (Prentki *et al.*, 1984). It is not clear if this discrepancy is due to tissue specificity or to different fractionation protocols, because generally only the microsomal fractions have been analysed. Moreover, immunocytochemical detection of the Ins(1,4,5) P_3 receptor on the rough and smooth endoplasmic reticulum of neuronal cells (Ross *et al.*, 1989), a tissue where this protein is particularly abundant, may indicate newly synthesized receptor. It is noteworthy that the immunoreactive staining was also present in other structures, such as the *cis* cisternae of the Golgi apparatus, the nuclear membrane, and sub-plasmalemmal cisternae.

In rat liver, we have shown that the Ins(1,4,5) P_3 -binding sites are distinct from the endoplasmic reticulum. However, demonstration of the presence of a calsequestrin-like protein associated with the Ins(1,4,5) P_3 -receptor-containing vesicles will be necessary before concluding that this organelle is identical with the calciosomes previously described in HL60 and PC12 cells (Volpe *et al.*, 1988).

During subcellular fractionation, a significant correlation was observed between the Ins(1,4,5) P_3 -binding capacity and the plasma-membrane marker activity measured in the various

Fig. 4. Structural and functional organization of the Ins(1,4,5)P₃-sensitive organelle

Our results suggest that the Ins(1,4,5)P₃-sensitive organelle in rat liver is divided into two functionally distinct regions, one being involved in Ca²⁺ uptake and the other in Ca²⁺ release. Another important feature is the anchorage of this organelle to the plasma membrane (PM) through actin microfilaments, which is suggested from the effect of cytochalasin B on the recovery of the Ins(1,4,5)P₃-binding sites with plasma-membrane markers after homogenization. See the Discussion section for more details.

fractions (Fig. 2). The possibility that a small portion of the alkaline phosphatase activity is present on the Ins(1,4,5)P₃-sensitive organelle cannot be ruled out, but we are confident that the Ins(1,4,5)P₃ receptor is not located on the plasma membrane. Indeed, freezing of the homogenate or treatment with cytoskeleton-disruptive drugs dramatically decreased this correlation (Table 2). In addition, fractionation of microsomes (but not of the HSLD fraction) on a high-resolution Percoll density gradient led to a partial separation between the Ins(1,4,5)P₃-binding sites and the plasma-membrane marker (results not shown), suggesting that these organelles are less tightly bound in the microsomal fraction. A similar separation has been previously obtained with HL60-cell (Volpe *et al.*, 1988) and adrenal cortex (Rossier *et al.*, 1989) microsomes. Freezing and cytochalasin B treatment, perhaps by a common mechanism, resulted in two different effects: (1) a redistribution of the Ins(1,4,5)P₃-binding sites, decreasing their correlation with the plasma membrane, and (2) a significant increase in the Ins(1,4,5)P₃-binding capacity of the various fractions. Both effects support the idea that the Ins(1,4,5)P₃-binding organelle is associated with actin microfilaments. Interactions between the microfilament network and various intracellular organelles have been described previously; for example, actin has been found in association with isolated secretory granules (Jockusch *et al.*, 1977), and has been shown to induce cross-linking between lysosomal membranes (Mehrabian *et al.*, 1984). Microfilaments also appear to link the mitochondria to the endoplasmic reticulum in some steroidogenic cells (Aguas, 1981). More recently, translocation of isolated organelles along actin filaments could be directly observed and the mechanism for this movement elucidated (Adams & Pollard, 1986).

Colchicine, although less effective than cytochalasin B, also caused a dissociation of the Ins(1,4,5)P₃-binding sites from the plasma membrane. It is less likely that microtubules are similarly bound to this organelle, because the effect of both drugs does not seem to be additive, but rupture of the microtubules, which can run closely in parallel to the microfilaments (Koonce & Schliwa, 1986), may be responsible for a destabilization of the F-actin.

If actin molecules are able to bind to the surface of the organelle in a specific way, we may hypothesize the presence of anchorage sites such as those present in the plasma membrane. The dramatic effect of cytochalasin B treatment on the Ins(1,4,5)P₃-binding capacity of the vesicles (Table 3) suggests that these sites may be close to, or associated with, the Ins(1,4,5)P₃ receptor. This hypothesis is supported by the communication by Guillemette *et al.* (1990) that the Ins(1,4,5)P₃ receptors in adrenal cortex and liver are partly resistant to solubilization with Triton X-100.

The microfilaments may provide a physical framework to maintain the Ins(1,4,5)P₃-sensitive organelles at specific intracellular loci. The location of Ins(1,4,5)P₃-sensitive Ca²⁺ pools near the plasma membrane, reminiscent of the morphology of the junctional sarcoplasmic reticulum in the smooth muscle (Somlyo, 1985), is suggested by some current models relating intracellular Ca²⁺ release to plasma-membrane permeability (Putney, 1986; Mullaney *et al.*, 1987; Takemura *et al.*, 1989; Irvine, 1990). In addition, several experimental observations support a close association between the plasma membrane and the Ins(1,4,5)P₃-sensitive organelle; (1) serial Ins(1,4,5)P₃ injections in *Limulus* photoreceptor cells (Payne & Fein, 1987) and immature *Xenopus* oocytes (Busa *et al.*, 1985; Berridge, 1988)

produce different responses, depending on the position of the micropipette within the cell; (2) following lysis and sonication of pancreatic islet cells attached to positively charged Sephadex beads, a significant part of the Ins(1,4,5) P_3 -sensitive Ca^{2+} pool remains associated with the beads (Dunlop & Larkins, 1988); (3) permeabilization of hepatocytes with saponin releases part of the Ins(1,4,5) P_3 -sensitive structures into the medium (Champeil *et al.*, 1989); (4) muscarinic stimulation of parotid acinar cells induces opening of Ca^{2+} -sensitive K^+ channels before fura-2-detectable $[Ca^{2+}]_i$ begins to rise (Foskett *et al.*, 1989); and (5) immunocytochemical localization of the Ins(1,4,5) P_3 receptor in Purkinje cells has revealed the presence of this protein, at least in part, in structures just beneath the plasma membrane (Ross *et al.*, 1989). Because this evidence is largely indirect, it is hoped that high-resolution spatial imaging of the intracellular Ca^{2+} pools will soon provide more reliable information concerning the intracellular location of this important organelle.

The ability of Ins(1,4,5) P_3 to prevent $^{45}Ca^{2+}$ accumulation into the vesicles, by maintaining a constant efflux of Ca^{2+} , was measured in some fractions (Table 4). Two important observations have been drawn from these experiments: (1) only a small fraction of the Ca^{2+} pool is releasable by Ins(1,4,5) P_3 , and (2) there is no correlation between the amount of Ca^{2+} released by Ins(1,4,5) P_3 and the capacity of the vesicles to bind this agent. On the basis of kinetic studies of $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes (Taylor & Putney, 1985), it has been estimated that Ins(1,4,5) P_3 could discharge up to 40% of the total ATP-dependent $^{45}Ca^{2+}$ pool. After homogenization of the tissue, the size of the Ins(1,4,5) P_3 -releasable pool was markedly decreased to less than 10% (see H in Table 4). In this study, after fractionation of the homogenate, the MS fraction appeared to be the most sensitive to Ins(1,4,5) P_3 with approx. 21% of its accumulated Ca^{2+} mobilizable by this agent. This result is consistent with the observation by Dawson & Irvine (1984) that, in the absence of GTP, the 'crude mitochondrial' fraction is more sensitive to Ins(1,4,5) P_3 than is the microsomal fraction.

These results, as well as the lack of correlation between the binding and the effect of Ins(1,4,5) P_3 , are consistent with the model of fragmentation of the Ins(1,4,5) P_3 -sensitive Ca^{2+} pool recently proposed by Dawson & Comerford (1989). This model proposes that, as in the sarcoplasmic reticulum of skeletal muscle, the Ca^{2+} -pumping sites (Ca/Mg-ATPases) and the Ca^{2+} -release sites [Ins(1,4,5) P_3 receptors] are spatially separated on the surface of the Ins(1,4,5) P_3 -sensitive organelle of non-muscle cells. Splitting of such an organelle, caused by shearing forces during homogenization, could produce different types of vesicles: some bearing only the Ca^{2+} pump, others only the Ins(1,4,5) P_3 receptor, and only a small portion being able to accumulate and release the cation.

Considering the results presented in this study and other reports discussed above, we can propose a model for the structural and functional organization of the Ins(1,4,5) P_3 -sensitive organelle in rat liver (Fig. 4). We believe that this organelle is divided into two functionally distinct regions, one involved in Ca^{2+} uptake and the other in Ins(1,4,5) P_3 -induced Ca^{2+} release. This organelle is maintained at an appropriate location in the cytosol by actin microfilaments, cross-linking the plasma membrane to the organelle, possibly to the Ins(1,4,5) P_3 receptor itself. We suspect that during homogenization this organelle is split and that the region enriched in Ins(1,4,5) P_3 receptors remains associated with the large plasma-membrane sheets, leading to the recovery of both markers in the same fractions, and also decreasing the ability of the Ins(1,4,5) P_3 -binding vesicles to accumulate Ca^{2+} .

Interaction between the cytoskeleton and the Ins(1,4,5) P_3 -sensitive organelle could have functional implications other than to maintain the Ca^{2+} pool in place. For example, it has been

proposed that the integrity of the microfilaments is necessary for the function of the Ins(1,4,5) P_3 -sensitive pool in parietal cells (Tsunoda, 1986). It has also been suggested that a decrease in Ca^{2+} content of the Ins(1,4,5) P_3 -sensitive pool leads to activation of Ca^{2+} influx through an unknown signalling mechanism (Take-mura *et al.*, 1989), and perhaps microfilaments could be involved in that mechanism. The cytoskeleton is also a dynamic structure continuously changing its organization, and is involved in cell motility. Transportation of the Ca^{2+} pools to various places in the cytosol would be a useful way to modulate intracellular response, especially if the distance between the Ins(1,4,5) P_3 receptor and the plasma membrane, where Ins(1,4,5) P_3 is produced and metabolized (Storey *et al.*, 1984; Shears *et al.*, 1988), is critical in controlling the concentration of Ins(1,4,5) P_3 reaching the receptor. In neutrophils, translocation of casequestrin-like protein-containing vesicles during cell activation has been observed (Krause *et al.*, 1989). Finally, the accessibility of the Ins(1,4,5) P_3 receptor to the ligand could be regulated by steric effects, owing to the proximity of the Ins(1,4,5) P_3 -binding site to the anchorage site for the actin filaments.

In conclusion, it is becoming increasingly apparent that Ca^{2+} signalling and cytoskeletal dynamics are closely interrelated. The effect of Ca^{2+} ions on the assembly and disruption of the cytoskeleton has been known for some time; in the future, perhaps the influence of the cytoskeleton state on Ca^{2+} signalling will come to be equally appreciated.

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REFERENCES

- Adams, R. J. & Pollard, T. D. (1986) *Nature* (London) **322**, 754–756
- Aguiar, A. P. (1981) *J. Ultrastruct. Res.* **74**, 175–182
- Alderson, B. H. & Volpe, P. (1989) *Arch. Biochem. Biophys.* **272**, 162–174
- Aronson, N. N., Jr. & Touster, O. (1974) *Methods Enzymol.* **31**, 90–102
- Baukal, A. J., Guillemette, G., Rubin, R., Spat, A. & Catt, K. J. (1985) *Biochem. Biophys. Res. Commun.* **133**, 532–538
- Bayerdörffer, E., Streb, H., Eckhardt, L., Haase, W. & Schulz, I. (1984) *J. Membr. Biol.* **81**, 69–82
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Berridge, M. J. (1988) *Proc. R. Soc. London B* **234**, 359–378
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Busa, W. B., Fergusson, J. E., Joseph, S. K., Williamson, J. R. & Nuccitelli, R. (1985) *J. Cell Biol.* **101**, 677–682
- Carafoli, E. (1987) *Annu. Rev. Biochem.* **56**, 395–433
- Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orłowski, S. & Claret, M. (1989) *J. Biol. Chem.* **264**, 17665–17673
- Damiani, E., Spamer, C., Heilman, C., Salvatori, S. & Margreth, A. (1988) *J. Biol. Chem.* **263**, 340–343
- Dawson, A. P. (1982) *Biochem. J.* **206**, 73–79
- Dawson, A. P. & Comerford, J. G. (1989) *Cell Calcium* **10**, 343–350
- Dawson, A. P. & Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120**, 858–864
- Dunlop, M. E. & Larkins, R. G. (1988) *Biochem. J.* **253**, 67–72
- Ferris, C. D., Haganir, R. L., Supattapone, S. & Snyder, S. H. (1989) *Nature* (London) **342**, 87–89
- Fleischer, S. & Fleischer, B. (1967) *Methods Enzymol.* **10**, 406–433
- Foskett, J. K., Gunter-Smith, P. J., Melvin, J. E. & Turner, R. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 167–171
- Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. & Gill, D. L. (1988) *J. Biol. Chem.* **263**, 11075–11079
- Guillemette, G., Balla, T., Baukal, A. J., Spat, A. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1010–1015

- Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) *J. Biol. Chem.* **263**, 4541–4548
- Guillemette, G., Lamontagne, S., Boulay, G. & Mouillac, B. (1989) *Mol. Pharmacol.* **35**, 339–344
- Guillemette, G., Favreau, I., Boulay, G. & Potier, M. (1990) *Mol. Pharmacol.* **38**, 841–847
- Henne, V., Piiper, A. & Söling, H.-D. (1987) *FEBS Lett.* **218**, 153–158
- Hill, T. D., Berggren, P.-O. & Boynton, A. L. (1987) *Biochem. Biophys. Res. Commun.* **149**, 897–901
- Hirata, M., Sasaguri, T., Hamachi, T., Hashimoto, T., Kukita, M. & Koga, T. (1985) *Nature (London)* **317**, 723–725
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Jockusch, B. M., Burger, M. M., DaPrada, M., Richards, J. G., Chaponnier, C. & Gabbiani, G. (1977) *Nature (London)* **270**, 628–629
- Kobayashi, S., Somlyo, A. V. & Somlyo, A. P. (1988) *Biochem. Biophys. Res. Commun.* **153**, 625–631
- Koonce, M. P. & Schliwa, M. (1986) *J. Cell Biol.* **103**, 605–612
- Krause, K.-H. & Lew, D. P. (1987) *J. Clin. Invest.* **80**, 107–116
- Krause, K.-H., Pittet, D., Volpe, P., Pozzan, T., Meldolesi, J. & Lew, D. P. (1989) *Cell Calcium* **10**, 351–361
- Mehrabian, M., Bame, K. J. & Rome, L. H. (1984) *J. Cell. Biol.* **99**, 680–685
- Mullaney, J. M., Chueh, S.-H., Ghosh, T. K. & Gill, D. L. (1987) *J. Biol. Chem.* **262**, 13865–13872
- Nunn, D. L. & Taylor, C. W. (1990) *Biochem. J.* **270**, 227–232
- Nunn, D. L., Potter, B. V. L. & Taylor, C. W. (1990) *Biochem. J.* **265**, 393–398
- Payne, R. & Fein, A. (1987) *J. Cell Biol.* **104**, 933–937
- Pozzan, T., Volpe, P., Zorzato, F., Bravin, M., Lew, D. P., Krause, K. H., Hashimoto, S., Bruno, B. & Meldolesi, J. (1988) *J. Cardiovasc. Pharmacol.* **12**, S80–S84
- Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Nature (London)* **309**, 562–564
- Prpic, V., Green, K. C., Blackmore, P. F. & Exton, J. H. (1984) *J. Biol. Chem.* **259**, 1382–1385
- Putney, J. W., Jr. (1986) *Cell Calcium* **7**, 1–12
- Ross, C. A., Meldolesi, J., Milner, T. A., Satoh, T., Supattapone, S. & Snyder, S. H. (1989) *Nature (London)* **339**, 468–470
- Rossier, M. F., Capponi, A. M. & Vallotton, M. B. (1989) *J. Biol. Chem.* **264**, 14078–14084
- Shears, S. B., Evans, W. H., Kirk, C. J. & Michell, R. H. (1988) *Biochem. J.* **256**, 363–369
- Somlyo, A. P. (1985) *Circ. Res.* **57**, 497–507
- Spat, A., Fabiato, A. & Rubin, R. P. (1986a) *Biochem. J.* **233**, 929–932
- Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W., Jr. (1986b) *Nature (London)* **319**, 514–516
- Spat, A., Lukacs, G. L., Eberhardt, I., Kiesel, L. & Runnebaum, B. (1987) *Biochem. J.* **244**, 493–496
- Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) *Nature (London)* **312**, 374–376
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Takemura, H., Hughes, A. R., Thastrup, O. & Putney, J. W., Jr. (1989) *J. Biol. Chem.* **264**, 12266–12271
- Taylor, C. W. & Putney, J. W., Jr. (1985) *Biochem. J.* **232**, 435–438
- Tones, M. A., Bootman, M. D., Higgins, B. F., Lane, D. A., Pay, G. F. & Lindahl, U. (1989) *FEBS Lett.* **252**, 105–108
- Tsunoda, Y. (1986) *FEBS Lett.* **207**, 47–52
- Van, P. N., Peter, F. & Söling, H.-D. (1989) *J. Biol. Chem.* **264**, 17494–17501
- Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Lew, D. P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1091–1095
- Wikstrom, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase: a Synthesis*, Academic Press, London
- Willcocks, A. L., Cooke, A. M., Potter, B. V. L. & Nahorski, S. R. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1071–1078
- Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987a) *Nature (London)* **325**, 159–161
- Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987b) *J. Biol. Chem.* **262**, 12132–12136

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