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CHANGES IN INOSITOL-1,4,5-TRISPHOSPHATE BINDING TO HEPATIC PLASMA MEMBRANES CAUSED BY TEMPERATURE, *N*-ETHYLMALEIMIDE AND MENADIONE

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Abstract—We investigated the effects of the sulfhydryl-alkylating agent *N*-ethylmaleimide (NEM) and menadione—a sulfhydryl-arylating agent, which can undergo redox cycling—on the [^{3}H]inositol-1,4,5-trisphosphate ([^{3}H]IP_3) binding properties of rat plasma membranes. Rat liver plasma membranes were incubated for 15 min at 37° with 0.1 mM, 0.2 mM, 0.5 mM NEM or 0.3 mM menadione and subsequently diluted for use in [^{3}H]IP_3 binding studies. An incubation as such (15 min at 37°) already caused the dissociation constant (K_d) of [^{3}H]IP_3 binding to increase from 1.9 ± 0.2 nM to 3.4 ± 0.2 nM, with only a small change in the maximal number of IP_3 binding sites (B_{max}-values of 401 ± 32 and 349 ± 13 fmol/mg protein, respectively). Incubation with NEM (0.1, 0.2 and 0.5 mM) resulted in a dose dependent decrease in the B_{max} with 41, 87 and 99%, respectively, without a significant change in the K_d compared to the time matched controls. Menadione (0.3 mM) decreased the B_{max} with 54% without affecting the K_d. In contrast to our findings at 37°, incubation of the plasma membranes with NEM (0.5 mM) at 0° for 30 min did not affect [^{3}H]IP_3 binding. In order to account for this discrepancy, the reaction rate of NEM with glutathione was examined at both 0° and 37° by recording the changes in the UV-spectrum of NEM (λ_{max} = 302 nm) after addition of 1 mM NEM to 1 mM glutathione. A similar reaction rate was observed at both temperatures. These data suggest that alkylation of a sulfhydryl-moiety in the IP_3-receptor molecule causes inactivation of the receptor function. Since at 0° NEM is still able to react with sulfhydryl groups, but not able to inactivate the IP_3-receptor, it can be suggested that the sulfhydryl-moiety of the IP_3-receptor is masked and cannot be reached by a sulfhydryl-alkylating agent at 0°.

It is generally accepted that Ca^{2+}-mobilizing receptors induce rapid breakdown of phosphatidyl inositol in the plasma membrane by stimulation of phospholipase C [1–3]. The major metabolites generated by this breakdown are inositol-1,4,5-trisphosphate (IP_3) [4] and diacylglycerol [5]. Evidence is accruing in literature which shows that IP_3 mobilizes calcium by binding to a specific receptor [6–10]. Recent data indicate that plasma membranes prepared from rat liver are enriched in IP_3-receptors [8]. It has been suggested that the IP_3-receptor is located on a specific IP_3-sensitive organelle, called calciosome [11], which may co-sediment with the plasma membranes during preparation.

Guillemette and Segui [12] and Supattapone *et al.* [13] have shown that the IP_3-receptor has a protein nature containing disulfide bridges and free sulfhydryl group(s) which are both essential for the binding and response i.e. Ca^{2+}-mobilization, of IP_3. These studies were performed on bovine adrenal cortex microsomes and rat cerebellum.

Recently Mauger *et al.* [14] and Spät *et al.* [15] suggested that both in a crude hepatic membrane fraction and in permeabilized hepatocytes the IP_3-receptor can exist in two states with different affinities which are in equilibrium. This equilibrium can be changed by either a temperature-shift or pretreatment with hormones.

In this paper we describe the effects of the sulfhydryl alkylator *N*-ethylmaleimide (NEM) and menadione, which arylates sulfhydryl groups and can undergo redox cycling, on the binding of IP_3 to liver plasma membranes. Our results indicate that an essential free sulfhydryl group is hidden in the protein structure of the IP_3-receptor at low temperature and that it becomes available (e.g. for sulfhydryl-alkylators) at higher temperature.

METHODS AND MATERIALS

Preparation of membrane fractions. Plasma membranes and microsomal fractions were isolated basically following the procedures described by Guillemette *et al.* [8]. Livers from 180–250 g male Wistar rats (Harlan CPB, Zeist, The Netherlands) were minced and homogenized in an ice-cold buffer containing: 20 mM Hepes/KOH, pH 7.2, 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 2 mM EGTA and 1 mM dithiothreitol. After centrifugating the homogenate for 20 min at 1500 *g*, the pellet was resuspended in homogenization buffer and adjusted to 40% (w/w) sucrose. This was divided in equal portions over a number of centrifugation tubes and overlayed with 10 mL of homogenization buffer containing 250 mM sucrose (7% w/w). These tubes were centrifugated for 1.5 hr at 90,000 *g*. The interphase was then carefully removed, resuspended in the homogenization buffer without EGTA, centrifugated for 20 min at 140,000 *g* and this pellet was taken up in the same buffer.

The microsomal fraction was prepared by centrifugation of the 1500 *g* supernatant for 10 min

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at 8000 *g* and subsequent centrifugation of the obtained supernatant for 20 min at 35,000 *g*. The pellet was resuspended in the homogenization buffer without EGTA, recentrifuged for 20 min at 35,000 *g* and taken up in the same buffer.

The membrane fractions were frozen in small aliquots in liquid nitrogen and stored at −70° for receptor binding or cytochrome P450 analysis.

Characterization of membrane fractions. Cytochrome P450 (a marker for endoplasmic reticulum) was measured by the method of Omura and Sato [16] using a molar extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ (ΔAbs. 450–490 nm).

α_1-Adrenergic receptor concentration (a marker for plasma membrane) was determined by measuring [^{3}H]prazosin binding. This was performed by incubation of the membrane fractions (200 μg of protein) for 30 min at 37° in a buffer containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA in a final volume of 500 μL with increasing amounts of [^{3}H]prazosin (final concentrations of 15 pM up to 300 pM). Non-specific binding was determined in the presence of 10 μM phentolamine. Incubations were terminated by diluting the samples with 4 mL of ice-cold incubation medium, filtration through presoaked glass-fiber filters (Whatman GF/C) and washing the filters with 4 mL of ice-cold incubation medium.

IP_3-binding studies. Membrane fractions (300 μg of protein) were incubated in a buffer containing 25 mM Na_2HPO_4, 100 mM KCl, 20 mM NaCl, 1 mM EDTA, pH 7.4. Incubations were performed for 40 min at 0° in a final volume of 300 μL with [^{3}H]IP_3 (20,000 dpm; 0.8 nM) and increasing amounts of unlabeled IP_3 (final concentrations of 50 pM up to 1 μM). Non-specific binding was determined in the presence of 1 μM IP_3. Non-specific binding was about 10% of the total binding. Incubations were terminated by diluting the samples with 3 mL of ice-cold incubation medium, followed by immediate filtration through presoaked glass-fiber filters (Whatman GF/B) and washing with 3 mL of ice-cold incubation medium.

All the receptor bound radioactivities were measured by liquid scintillation spectrometry and analysed with the non-linear curve fitting program LIGAND. All incubations have been compared to their respective controls by fitting them together in one simultaneous fit. Statistical analysis of this simultaneous fit was used as criterium to conclude whether or not the binding sites (dissociation constants) had changed by the incubations compared to the controls. It should be noted that the dissociation constant and the maximal number of binding sites are two independent parameters obtained by analysis of the binding curves with LIGAND.

Preincubations. The effects of NEM and menadione on [^{3}H]IP_3-binding to the plasma membrane fraction were assessed by preincubations performed in the same buffer as used for the [^{3}H]IP_3-binding assay, pH 7.4, at 37° for 15 min with 3 mg of protein/mL and the concentrations of NEM or menadione as specified, followed by a three-fold dilution and subsequent [^{3}H]IP_3-binding at 0°.

Analytical methods. The reaction between NEM and glutathione was followed by recording the changes in the UV-spectrum of NEM (λ_{max} = 302 nm) [17]. Incubations were performed in 115 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM Hepes, pH 7.2 either at 0° or at 37°. Glutathione was freshly prepared and neutralized before each experiment. The reaction was started by the addition of NEM (1 mM) to 1 mM glutathione and terminated by adding excess HCl, which caused a drop in the pH to *ca.* 2.0. At this pH the reaction between NEM and glutathione is very slow because the sulfhydryl group of glutathione is not ionized anymore (pK_a about 9.2). The amount of NEM unreacted was measured at 302 nm.

Protein concentrations were determined according to Bradford [18] using bovine serum albumin as standard.

Materials. [*7-methoxy*-^{3}H]Prazosin with a specific activity of 85 Ci/mmol and D-*myo*-[2-^{3}H]inositol-1,4,5-trisphosphate with a specific activity of 45 Ci/mmol were obtained from Amersham Corp. (Arlington Heights, IL). *N*-Ethylmaleimide was from Aldrich (Milwaukee, WI), menadione and D-*myo*-inositol-1,4,5-trisphosphate were obtained from the Sigma Chemical Co. (St Louis, MO), phentolamine was obtained from Ciba-Geigy (Summit, NJ) and all other chemicals used were of the highest purity available.

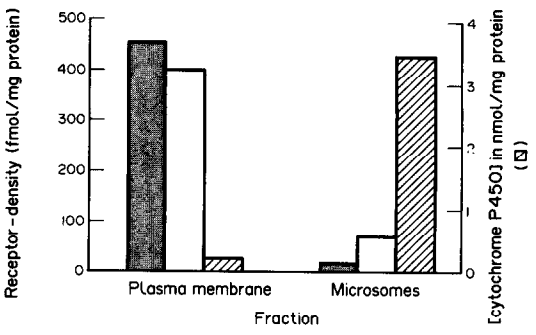

Fig. 1. Results of a typical membrane isolation showing the α_1-adrenergic receptor density (▨), the amount of IP_3-receptors (□) and the cytochrome P450 concentration (▨) in the plasma membrane fraction and the microsomal fraction.

RESULTS

In Fig. 1 the α_1-adrenergic receptor density, the amount of IP_3-receptors and the cytochrome P450 concentration in two different fractions (denoted as plasma membrane and microsomes) isolated from rat liver homogenate are shown. As can be seen from Fig. 1 there is a relatively high concentration of α_1-adrenergic receptors and a low concentration of cytochrome P450 in the plasma membrane fraction. On the contrary, the microsomal fraction has a low α_1-adrenergic receptor-density and is enriched in endoplasmic reticulum according to its high cytochrome P450 concentration. Moreover, it can be seen from the data depicted in Fig. 1, that the amount of IP_3-receptors in the plasma membrane

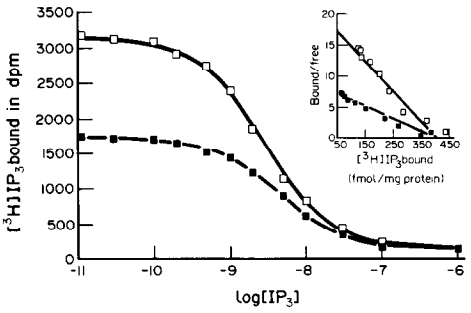

Fig. 2. A typical experiment, showing the effect of 15 min preincubation at 37° (■) compared to 0° (□) on the [^{3}H]IP_3-binding properties of the plasma membrane fraction. *Inset:* Scatchard plot of binding data.

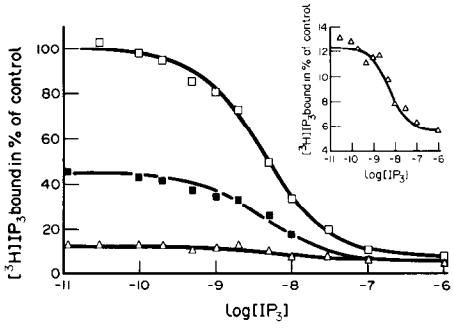

Fig. 3. The effect on the [^{3}H]IP_3-binding properties of the plasma membrane fraction of a preincubation with 100 μM (■) or 200 μM (△) *N*-ethylmaleimide at 37° for 15 min compared to the effect of a preincubation without *N*-ethylmaleimide (□). These data are representative of three independent experiments. *Inset:* the results obtained by treatment with 200 μM *N*-ethylmaleimide plotted on a different y-scale.

fraction is high in contrast to the microsomal fraction, which contains only low amounts of IP_3-receptors.

Fitting of the [^{3}H]prazosin saturation curves of the plasma membrane fraction revealed binding to a single binding site with a B_{max} of 456 ± 65 fmol/mg of protein and a K_d of 175 ± 35 pM. The [^{3}H]IP_3-displacement curves of the plasma membrane fraction were fitted assuming binding to a single binding site with a B_{max} of 401 ± 32 fmol/mg of protein and a K_d of 1.9 ± 0.2 nM. Neither [^{3}H]prazosin-binding nor [^{3}H]IP_3-binding could be fitted according to a two-receptor model. The plasma membrane fraction was used for further IP_3-binding studies.

After a preincubation for 15 min at 37°, a decrease in binding of [^{3}H]IP_3 was observed (Fig. 2). This is mainly caused by an increase of the K_d from 1.9 ± 0.2 nM to 3.4 ± 0.2 nM (Table 1) according to non-linear regression analysis of the [^{3}H]IP_3-displacement curves. After preincubation of the plasma membrane for 15 min at 37° in the presence of 0.5 mM or 1 mM NEM, almost no [^{3}H]IP_3 was bound specifically to the membranes anymore. Preincubation with 100 μM NEM or 200 μM NEM (Fig. 3) resulted in a decrease of the B_{max} of 41 and 87%, respectively (Table 1) without a significant change in the K_d compared to the respective control.

Neither exposure to 500 μM NEM at 0° nor exposure to 200 μM NEM at 0° after 15 min preincubation at 37° without NEM had any effect on the [^{3}H]IP_3-binding properties of the plasma membrane fraction (Table 1). To determine whether the lack of an effect of NEM at 0° was caused by a decreased reaction rate between NEM and sulfhydryl groups at 0°, the reaction between NEM and glutathione was followed by recording the changes in the UV-spectrum of NEM. As can be seen from Fig. 4 the reaction of NEM with glutathione was rapid at physiological pH and no differences between reaction rates and extents at 0° and 37° were determined.

Another sulfhydryl reagent is menadione, which is often used as a model compound in studies on oxidative stress. The left panel of Fig. 5 shows the effects of preincubation of the plasma membranes with increasing concentrations of menadione on the total binding and non-specific binding of [^{3}H]IP_3 to the membrane fraction. As can be seen from the figure, menadione causes a concentration-dependent decrease in the amount of [^{3}H]IP_3 bound to the plasma membranes at concentrations above 30 μM until [^{3}H]IP_3-binding is almost abolished at 1 mM menadione without any change in the non-specific binding of [^{3}H]IP_3. In the right panel of Fig. 5 the total binding of [^{3}H]IP_3 to the plasma membrane fraction is shown to be dependent on the duration of the incubation of the membranes in the presence of 500 μM menadione at 37°. The reaction is almost complete at 15 min. Subsequent experiments were performed with 300 μM menadione for 15 min at 37°. Under these experimental conditions a decrease of the B_{max} of 54% was observed (Fig. 6 and Table 1).

DISCUSSION

The method to prepare plasma membranes described in this study is similar to the method described in an earlier study in which the membranes were used for measuring α_1-adrenergic receptors [19]. The membranes obtained in this way are enriched in activity of Na^+/K^+-ATPase and 5′-nucleotidase, which are markers for plasma membrane. The plasma membranes used in this study have high amounts of α_1-adrenergic receptors, used as a marker of plasma membrane, and have low amounts of cytochrome P450, used as a marker of endoplasmic reticulum. In contrast, the microsomal preparation has high levels of cytochrome P450 and almost non-detectable levels of α_1-adrenergic receptors. The density of IP_3-receptors parallels the α_1-adrenergic receptor density suggesting that either the IP_3-receptor is embedded in the plasma membrane itself or that the IP_3-receptor is located on a membrane fraction closely associated with the plasma membrane. This confirms data recently reported by Guillemette *et al.* [8], who showed that a plasma membrane fraction prepared from rat liver is enriched in angiotensin II binding and IP_3-receptors.

Table 1. Effect of different preincubations on the IP_3-binding properties of hepatic plasma membranes

15 min preincubation	K_d (nM)	B_{max} (fmol/mg of protein)
0°	1.9 ± 0.2	401 ± 32 (3)
37°	3.4 ± 0.2	349 ± 13 (6)
37° without NEM	4.2 ± 0.5	432 ± 70 (2)
37° with 100 μM NEM	4.2 ± 0.5*	286 ± 70 (2)
37° without NEM	4.1 ± 0.4	375 ± 37 (2)
37° with 200 μM NEM	4.1 ± 0.4*	40 ± 14 (2)
0° with 500 μM NEM	2.1 ± 0.2	393 ± 24 (2)
0° with 200 μM NEM *after* 15 min at 37°	2.8 ± 0.4	350 ± 29 (2)
37° without menadione	4.5 ± 0.6	360 ± 65 (2)
37° with 300 μM menadione	4.5 ± 0.6*	167 ± 46 (2)

* These K_d values have been obtained by fitting the controls simultaneously with the incubations (see Materials and Methods).

Plasma membranes were preincubated for 15 min at the specified temperature in the presence of the indicated concentrations of *N*-ethylmaleimide or menadione. In the case of menadione both the controls and the samples were preincubated in the presence of 0.3% (v/v) ethanol. After the preincubation the plasma membrane fractions were put on ice and three-fold diluted for the subsequent [^{3}H]IP_3-binding assay at 0°. The data are expressed as the mean ± SD as estimated by LIGAND. The numbers between parentheses denote the number of independent experiments.

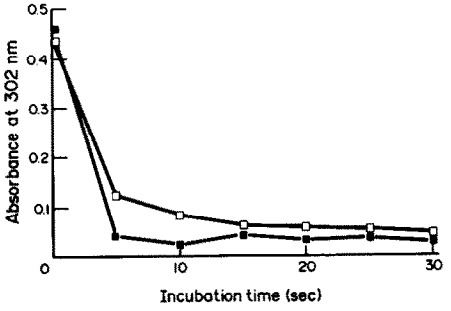

Fig. 4. The reaction between 1 mM *N*-ethylmaleimide and 1 mM glutathione in Hepes/KCl medium, pH 7.4 at 0° (□) and 37° (■). For more details see Materials and Methods. These data represent the results of one single experiment.

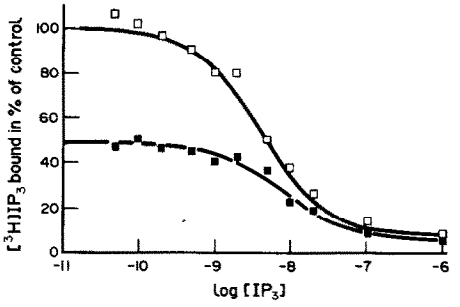

Fig. 6. The effects of a 15 min preincubation at 37° with (■) and without (□) 300 μM menadione on the [^{3}H]IP_3-binding properties of the plasma membrane fraction. These data are representative of three independent experiments.

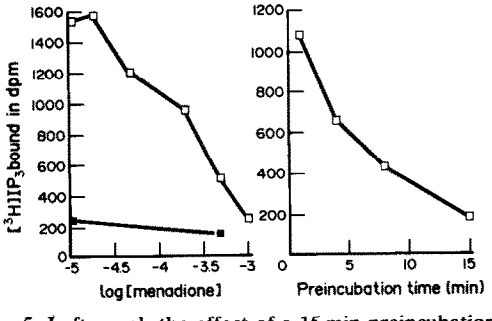

Fig. 5. Left panel: the effect of a 15 min preincubation at 37° with increasing concentrations of menadione on the total binding (□) and non-specific binding (■) of [^{3}H]IP_3 to the plasma membrane fraction. Right panel: the total amount of [^{3}H]IP_3 bound (□) to the plasma membrane fraction after a preincubation with 500 μM menadione at 37° for different times. One typical experiment is shown.

Binding of IP_3 to the plasma membranes corresponds with binding to a single site, which is saturable according to non-linear regression analysis with LIGAND. The occurrence of one IP_3 binding site is in good agreement with data reported by Guillemette *et al.* [8] but not according to Spät *et al.* [15] and Mauger *et al.* [14] who described interaction with two binding sites with high and low affinity. The difference between the observed binding characteristics of IP_3 could be due to the different preparations used in the binding assay; our isolation procedure for preparing plasma membranes was basically according to Guillemette *et al.* [8] whereas Mauger *et al.* [14] either used a crude membrane fraction or permeabilized hepatocytes and Spät *et al.* used a microsomal fraction [15]. A second explanation could be the presence of Mg^{2+} before and during the incubation with [^{3}H]IP_3; Mauger *et al.* used 1.5 mM $MgCl_2$, Spät *et al.* used 5 mM $MgSO_4$. This may also explain the difference

observed in the rate of association of [^{3}H]IP_3 to its receptor; maximal saturation was attained in about 2 min in the experiments of Mauger *et al.* and Spät *et al.* This differs largely from our data (not shown) and those obtained by Guillemette *et al.* [8] because in these cases the time needed for maximal saturation was 20–30 min.

When the plasma membranes were incubated for 15 min at 37°, an increase in the dissociation constant was observed without a change in the maximal number of receptors suggesting that some irreversible change(s) has occurred in the receptor protein and/or in the lipid membrane. This temperature effect has some similarities with the temperature effect observed by Mauger *et al.* [14]. They found binding to a high and low affinity binding site at 4° and after a preincubation at 37° only the low affinity site could be detected. However, as mentioned we could detect only one binding site with high affinity.

NEM caused a marked reduction in the amount of detectable receptors without a change in the affinity (Table 1 and Fig. 3). This reduction was concentration dependent and is explained by alkylation of an essential free sulfhydryl group by NEM. This is in agreement with the concentration-dependent effect of NEM on IP_3-receptors on bovine adrenal cortex microsomes [12]. At 0° NEM did not cause any inhibition of IP_3-binding even at a concentration of 500 μM. Because the reaction rate between NEM and glutathione is equal at 0° and 37°, a possible explanation for our findings is that NEM cannot reach the essential free sulfhydryl group of the IP_3-receptor at low temperature. This might be caused by a change in the conformation of the receptor protein induced by low(er) temperatures. The sulfhydryl group of the IP_3-receptor appears to be more reactive towards NEM than the sulfhydryl group of the α_1-adrenergic receptor.* Also menadione reduced the number of detectable IP_3-receptors, but was less potent than NEM. This difference in reactivity towards sulfhydryl groups between NEM and menadione is also observed in the effects of NEM and menadione on α_1-adrenergic receptor density in isolated plasma membranes or intact hepatocytes.*

In fact menadione has a relatively low reactivity towards sulfhydryl groups when compared to other quinones [20].

In summary, the data presented here indicate the presence of relatively high concentrations of IP_3-receptors in a partially purified rat liver plasma membrane fraction. Moreover the data indicate the occurrence of an irreversible change in the IP_3-receptor interaction, which is induced by a shift from 0° to 37°. This is indicated by a decrease in affinity of the receptor for IP_3 after an incubation at 37° compared to 0°. The IP_3-receptor protein has at least one essential free sulfhydryl group which can be alkylated by NEM or menadione at 37°. At 0° this sulfhydryl group is not vulnerable to the sulfhydryl-alkylators tested, possibly because of a change in the conformation of the receptor protein at 0°.

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* F. B. Pruijn and A. Bast, unpublished results.

REFERENCES

1. Michell RH, Inositol phospholipids and cell surface receptor function. *Biochim Biophys Acta* **415**: 81–87, 1975.
2. Berridge MJ, Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* **220**: 345–360, 1984.
3. Williamson JR, Cooper RH, Joseph SK and Thomas AP, Inositol trisphosphate and diacylglycerol as intracellular second messengers in liver. *Am J Physiol* **248**: C203–C216, 1985.
4. Berridge MJ and Irvine RF, Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**: 315–321, 1984.
5. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**: 693–698, 1984.
6. Stauderman KA, Harris GD and Lovenberg W, Characterization of inositol 1,4,5-trisphosphate-stimulated calcium release from rat cerebellar microsomal fractions. *Biochem J* **255**: 677–683, 1988.
7. Worley PF, Baraban JM, Supattapone S, Wilson VS and Snyder SH, Characterization of inositol trisphosphate receptor binding in brain. *J Biol Chem* **262**: 12132–12136, 1987.
8. Guillemette G, Balla T, Baukal AJ and Catt KJ, Characterization of inositol 1,4,5-trisphosphate receptors and calcium mobilization in a hepatic plasma membrane fraction. *J Biol Chem* **263**: 4541–4548, 1988.
9. Guillemette G, Balla T, Baukal AJ and Cat KJ, Inositol 1,4,5-trisphosphate binds to a specific receptor and releases microsomal calcium in the anterior pituitary gland. *Proc Natl Acad Sci USA* **84**: 8195–8199, 1987.
10. Guillemette G, Balla T, Baukal AJ, Spät A and Catt KJ, Intracellular receptors for inositol 1,4,5-trisphosphate in angiotension II target tissues. *J Biol Chem* **262**: 1010–1015, 1987.
11. Volpe P, Krause K-H, Hashimoto S, Zorzato F, Pozzan T, Meldolesi J and Lew DP, "Calciosome", a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? *Proc Natl Acad Sci USA* **85**: 21091–21095, 1988.
12. Guillemette G and Segui JA, Effects of pH, reducing and alkylating reagents on the binding and Ca^{2+} release activities of inositol 1,4,5-triphosphate in the bovine adrenal cortex. *Mol Endocrinol* **2**: 1249–1255, 1988.
13. Supattapone S, Worley PF, Baraban JM and Snyder SH, Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J Biol Chem* **263**: 1530–1534, 1988.
14. Mauger J-P, Claret M, Pietri F and Hilly M, Hormonal regulation of inositol 1,4,5-trisphosphate receptor in rat liver. *J Biol Chem* **264**: 8821–8826, 1989.
15. Spät A, Fabiato A and Rubin RP, Binding of inositol trisphosphate by a liver microsomal fraction. *Biochem J* **233**: 929–932, 1986.
16. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
17. Gregory JD, The stability of *N*-ethylmaleimide and its reaction with sulfhydryl groups. *J Am Chem Soc* **77**: 3922–3923, 1955.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 246–254, 1976.
19. Pruijn FB, van Gelderen JG and Bast A, The effects

of radical stress and *N*-ethylmaleimide on rat hepatic α_1-adrenergic receptors. *Toxicol Lett* **45**: 73–82, 1989.
20. Wilson I, Wardman P, Lin T-S and Sartorelli AC, Reactivity of thiols towards derivatives of 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents. *Chem Biol Interact* **61**: 229–240, 1987.