Effect of oxidized glutathione and temperature on inositol 1,4,5-trisphosphate binding in permeabilized hepatocytes

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The effect of oxidized glutathione (GSSG) on inositol 1,4,5-trisphosphate (IP₃) binding and the activity of IP₃-gated Ca²⁺ channels was examined in permeabilized hepatocytes. The permeability properties of the channel were measured by using Mn²⁺ quenching of compartmentalized fura-2 at 37 °C and at 4 °C for comparison with IP₃-binding measurements. GSSG (2 mM) increased the IP₃-sensitivity of Mn²⁺ quenching, consistent with previous studies based on Ca²⁺-release measurements [Renard, Seitz and Thomas (1992) *Biochem. J.* **284**, 507–512]. Measurements of [³H]IP₃ binding were made at 4 °C after preincubation of permeabilized hepatocytes at 37 °C in the absence or presence of GSSG. Under these conditions GSSG stimulated IP₃ binding by increasing the number of binding sites without changing the K_d. This effect was observed in the absence or presence of Ca²⁺, but was abolished when the preincubation with GSSG was carried out at 4 °C. Thimerosal also stimulated [³H]IP₃ binding, but this effect was mediated both by an increase in the maximum number of binding sites and by a decrease in the K_d. The effects of thimerosal and GSSG were not additive. Further analysis of the effect of GSSG revealed that preincubation of permeabilized

hepatocytes at 37 °C results in a progressive loss of [³H]IP₃-binding sites that can be prevented and reversed by inclusion of GSSG. A parallel loss of IP₃-sensitive Mn²⁺-quenchable stores was observed after incubation at 37 °C, and this could also be reversed by adding back GSSG. The loss of IP₃ binding was not the result of IP₃-receptor proteolysis, as judged by Western blotting of immunoreactive protein. The sensitivity of [³H]IP₃ binding in permeabilized hepatocytes to varied ratios of GSSG and GSH suggests that the IP₃ receptor responds to an oxidized redox environment such as that found in the lumen of the endoplasmic reticulum. GSSG had no direct effect on the ligand-binding activity of detergent-solubilized and partially purified IP₃ receptors. We conclude that GSSG exerts an indirect effect on the IP₃ receptors in permeabilized hepatocytes by preventing a temperature-dependent loss of IP₃-binding sites. We suggest that the hepatic IP₃ receptors may interact with a thiol–disulphide oxidoreductase that utilizes GSSG as a substrate and prevents inappropriate unfolding of the ligand-binding domain occurring after incubation of the receptor at 37 °C *in vitro*.

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP₃) is now recognized widely as a Ca²⁺-mobilizing intracellular messenger [1]. It is generated from phosphatidylinositol 4,5-bisphosphate as a consequence of receptor-mediated activation of phospholipase C and interacts with specific intracellular receptors to cause mobilization of sequestered intracellular Ca²⁺. By virtue of their activity, IP₃ receptors (IP₃R) occupy a central position in the regulation of intracellular Ca²⁺ and play a key role in the induction and maintenance of cellular Ca²⁺ oscillations [1,2]. These oscillations are often evoked by agonists that stimulate IP₃ formation, although they can also be triggered, in the absence of an increase of IP₃, by other agents that cause mobilization of intracellular Ca²⁺. These agents include compounds which oxidize cellular glutathione (such as t-butyl hydroperoxide [3]) or thiol reagents such as thimerosal [4–11]. Most of these studies on intact or permeabilized cells have reached the conclusion that the effects of thiol reagents are related to a sensitization of the IP₃R/ion channel to IP₃.

Glutathione plays an essential role in a number of important biochemical processes, including the maintenance of cell integrity and cytoskeletal organization, modulation of protein conformation and enzyme activity [12,13]. We have shown in a previous paper that GSSG enhances the efficacy of IP₃ in releasing Ca²⁺ by

shifting the dose–response curve to the left, decreasing the EC₅₀ by about 4-fold [6]. The quenching of compartmentalized fura-2 by Mn²⁺ has been used as a method for monitoring the activity of IP₃-gated Ca²⁺ channels [14,15]. The technique offers the advantage that IP₃-dependent Mn²⁺ fluxes can be monitored independently of the Ca²⁺-sequestration capacity of the internal stores at different temperatures and where ligand-binding and ion-fluxes can be measured under comparable experimental conditions. We have utilized this method to investigate further the mode of action of GSSG on the hepatic IP₃R. Our data indicate that there is a reversible change in IP₃R availability after incubation of permeabilized hepatocytes at 37 °C, which leads to masking of existing IP₃-binding sites by a process that can be reversed by GSSG. The lack of a direct effect of GSSG on solubilized receptor binding and the temperature requirement suggest the involvement of an enzyme-catalysed thiol modification of the IP₃ receptor itself or of ancillary regulatory proteins associated with the receptor.

MATERIALS AND METHODS

Binding measurements in permeabilized cells

Isolated hepatocytes were prepared by collagenase perfusion of livers obtained from male Sprague–Dawley rats (250–300 g), as described previously [16]. After isolation, the hepatocytes were

Abbreviations used: IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; DTT, dithiothreitol; ER, endoplasmic reticulum.

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washed twice in Na⁺/Hepes-buffered medium, composed of 120 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM KH₂PO₄ and 0.2 mM MgCl₂, pH 7.4. The cells were stored in this buffer on ice at a concentration of 50 mg of protein/ml. For measurements of [³H]IP₃ binding, hepatocytes were permeabilized at 1–2.5 mg of protein/ml in medium (pH 7.2) composed of 120 mM KCl, 20 mM Hepes, 10 mM NaCl, 1 mM KH₂PO₄, 200 μ M MgCl₂, 75 μ M EGTA, 20–40 μ g of digitonin/ml, 5 μ M carbonyl cyanide *m*-chlorophenylhydrazide, 5 μ g of oligomycin/ml, 1 μ g of rotenone/ml, and 1 μ M each of the proteinase inhibitors pepstatin, antipain and leupeptin. Ca²⁺ (50 μ M) was added to give a free [Ca²⁺] of 300 nM, except for nominally Ca²⁺-free experiments, where Ca²⁺ was omitted (measured free [Ca²⁺] < 3 nM). The permeabilized hepatocytes were incubated with the different thiol compounds for 10 min at 4 °C or 37 °C. In some cases dithiothreitol (DTT) was added 3 min before addition of GSSG. When the experiments were carried out at 37 °C, the cells were cooled to 4 °C for 10 min before performing the binding assay. Binding was measured at 4 °C by incubating 1 ml of the permeabilized cells with (1–2) $\times$ 10⁴ c.p.m. of [³H]IP₃ (20 Ci/mmol) and the indicated concentrations of unlabelled ligand. After 10 min, the incubation was terminated by diluting the sample with 1 ml of ice-cold stop solution containing 50 mM Tris, 2 mM EDTA and 1 mg of BSA/ml, pH 7.4, followed by rapid filtration on glass-fibre filters (Whatman GF/B, 2.4 cm diam.). The filters were washed with 2 ml of the cold stop solution and then transferred to vials containing liquid-scintillation fluid for determination of the retained radioactivity. Non-specific binding was determined in the presence of 5 μ M IP₃. Added [³H]IP₃ was not metabolized over a 30 min interval at 4 °C (results not shown). The program LIGAND was used for the analysis of ligand-binding data, model fitting and parameter estimation (P. J. Munson, Analytical Biostatistical Section, Division of Computer Research and Technology, NIH, Bethesda, MD 20892, U.S.A.).

Measurement of IP₃-mediated Mn²⁺ quenching of compartmentalized fura-2

Intact hepatocytes (4 mg of protein/ml) were loaded with 5 μ M fura-2/AM for 35 min at 37 °C in a buffer (pH 7.4) containing 10 mM Na⁺/Hepes, 120 mM NaCl, 4.7 mM KCl, 5 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose and 2% BSA. After loading, the cells were washed and stored at 4 °C in Na⁺/Hepes buffer. Before use, the cells were washed once in Na⁺/Hepes buffer containing 100 μ M EGTA. The cells were then permeabilized at 4 °C or 37 °C in a buffer (pH 7.2) containing 120 mM KCl, 10 mM NaCl, 20 mM Tris/Hepes, 1 mM KH₂PO₄, 0.2 mM MgCl₂, 40 μ g of digitonin/ml, 5 μ M carbonyl cyanide *m*-chlorophenylhydrazide, 5 μ M oligomycin, 1 μ M rotenone, 2 mM MgATP, 5 mM phosphocreatine, 0.5 unit of creatine kinase/ml, and 1 μ g each of pepstatin, antipain and leupeptin/ml and 2 μ M thapsigargin. Incubations were performed in the cuvette of a fluorimeter (Photon Technology Inc. Deltascan) maintained at 4 °C or 37 °C with continuous stirring. The excitation wavelength was 360 nm with emission at 510 nm. The quenching of the fura-2 trapped within the organelles was monitored after addition of 40 μ M MnCl₂. The free [Ca²⁺] before Mn²⁺ addition was about 300 nM (measured by using the fura-2 released by the cells). Although it was not possible to measure the free [Ca²⁺] in this way after Mn²⁺ addition, the calculated increase in free [Ca²⁺] due to Mn²⁺ displacement was 80 nM (taking into account the released fura-2 and all other components of the buffer capable of binding Ca²⁺).

Binding of IP₃ to solubilized hepatocyte extracts

Hepatocytes (10 mg) were resuspended in 2 ml of hepatocyte resuspension buffer, which contained 120 mM KCl, 20 mM Tris/Hepes (pH 7.2), 1 mM EDTA, 0.1 mM vanadate, 5 mM NaF, 4 nM okadaic acid, 1 mM phenylmethanesulphonyl fluoride and 10 μ g each of aprotinin, leupeptin and soybean trypsin inhibitor/ml. The cells were permeabilized with saponin on ice for 5 min (40 μ g/mg of cell protein). GSSG was added and the cells were preincubated at 37 °C for 10 min. The cells were centrifuged and resuspended in hepatocyte resuspension buffer containing 1% Triton X-100. As a control, a portion of saponin-permeabilized hepatocytes which had not been preincubated and kept at 4 °C was also solubilized with Triton X-100. After 20 min on ice, the samples were centrifuged at 25000 *g* for 20 min to remove insoluble debris. Binding was assayed on the Triton X-100 extracts by using a poly(ethylene glycol) precipitation assay [17]. For determination of IP₃R levels, the IP₃R was immunoprecipitated from Triton-X-100-solubilized hepatocyte extracts after incubation for 4 h with antibody to C-terminal Type-I IP₃ receptor and Protein A-Sepharose beads. Immunoprecipitated proteins were separated by SDS/PAGE on 5% gels, and immunoreactive proteins were revealed with an enhanced chemiluminescence kit (Amersham Corp.). Procedures for the partial purification of hepatocyte extracts on heparin-agarose columns and measurement of [³H]IP₃ binding to immunoprecipitated IP₃R have been described previously [17,18].

RESULTS

Mn²⁺ has been shown to permeate through IP₃-sensitive Ca²⁺ channels and quench the fluorescence of fura-2 compartmentalized in intracellular stores [14,15,19]. In the experiments described below, the Mn²⁺-quench measurements were made in the presence of thapsigargin to ensure that the intracellular stores were depleted of Ca²⁺. Thus the results are independent of the Ca²⁺ loading of the stores and can therefore be readily carried out at 4 °C in order to make comparisons with binding measurements, which have to be carried out at low temperatures to limit [³H]IP₃ metabolism. In addition, the ligand-induced inactivation of the IP₃R identified recently by using the Mn²⁺-quench approach [20] does not occur at this temperature. Figure 1 shows that the addition of 40 μ M Mn²⁺ induced a rapid phase of quenching that corresponds to interaction with the cytosolic dye that was released into the medium during cell permeabilization. After this rapid phase, the addition of a sub-maximal dose of IP₃ (50 nM) induced an additional rapid quench of fluorescence, which then slowed to its unstimulated rate (Figure 1a). When the permeabilized hepatocytes had first been preincubated for 10 min at 37 °C with 2 mM GSSG, the IP₃-stimulated Mn²⁺ quench was enhanced (Figure 1b). The releasable compartment size was measured as the ionomycin-quenchable fluorescence expressed as a percentage of the total fura-2 fluorescence. The total releasable compartment size was not affected by GSSG treatment (46.9 $\pm$ 0.9% for control cells versus 44.8 $\pm$ 0.9% for GSSG-treated cells). Furthermore, the addition of GSSG had no effect when Mn²⁺ quenching was initiated by addition of a saturating dose of 1 μ M IP₃ (Figures 1c and 1d). The IP₃ dose-response curve determined in the absence or presence of GSSG is shown in Figure 2(a). The EC₅₀ for IP₃ in control cells was 22.6 $\pm$ 5.8 nM (*h* = 1.1) and in GSSG-treated cells the EC₅₀ was 8.3 $\pm$ 1.2 nM (*h* = 1.3). The magnitude of the leftward shift in the IP₃ dose-response curve was comparable with that observed previously when Ca²⁺ release was measured with Quin-2 at 37 °C [6].

[³H]IP₃-binding assays were carried out on permeabilized hepatocytes at 4 °C after an initial preincubation at 37 °C for

Figure 1 Effect of GSSG on IP_3 -induced Mn^{2+} quench of compartmentalized fura-2 in permeabilized hepatocytes

Incubation conditions were as described in the Materials and methods section. Preincubation in the absence (a, c) or presence (b, d) of GSSG (2 mM) was carried out at 37 °C, and the Mn^{2+} quench was then measured at 4 °C. Mn^{2+} quench of fura-2 fluorescence was monitored with excitation at 360 nm and emission at 510 nm. Additions were as follows: Mn, 40 μM or 2 mM MnCl_2 ; IP_3 , 50 nM or 1 μM ; Iono, 2 μM ionomycin.

10 min in the absence or presence of 2 mM GSSG (Figure 2b). The data show that GSSG pretreatment doubled the amount of $[\text{H}]\text{IP}_3$ bound when measured with 1 nM $[\text{H}]\text{IP}_3$. Displacement curves obtained with increasing concentrations of unlabelled IP_3 were used to analyse changes in the binding parameters [21]. Scatchard plots of the data are shown in Figure 2(c) and the binding parameters measured under various conditions are summarized in Table 1. The analysis shows that the enhancement of $[\text{H}]\text{IP}_3$ binding observed after GSSG pretreatment was due to an increase in the B_{max} of the binding sites, and that there was no change in the K_d . The data in the absence or presence of GSSG was best fitted by a single-site model of ligand binding. Cystine (2 mM) was able to mimic the effects of GSSG to increase the B_{max} of $[\text{H}]\text{IP}_3$ -binding sites (Table 1) and has previously been shown to reproduce the effects of GSSG on IP_3 -mediated Ca^{2+} release [6]. Cysteine or GSH had no effect on the binding parameters (Table 1). DTT was also without effect on its own (results not shown), but reversed the action of GSSG (Table 1).

It should be noted that the Mn^{2+} -quench measurements and the $[\text{H}]\text{IP}_3$ -binding assays shown above were carried out at a free Ca^{2+} concentration of 300–400 nM. In permeabilized hepatocytes, it has been reported that Ca^{2+} shifts the IP_3R from a low-affinity to a high-affinity form [22]. To examine the dependence of the GSSG effect on Ca^{2+} concentration, we repeated the measurements of the binding parameters with only EGTA present in the incubation medium. In the absence of added Ca^{2+} , the measured K_d and B_{max} of the binding site was higher than in the presence of Ca^{2+} (Figure 3 and Table 1). Under these conditions, GSSG pretreatment again increased the B_{max} without affecting

the K_d (Figure 3 and Table 1). In this series of experiments, we also compared the effects of GSSG with those of thimerosal. Like GSSG, thimerosal also stimulated $[\text{H}]\text{IP}_3$ binding. However, the effect of thimerosal on $[\text{H}]\text{IP}_3$ binding was due to both a decrease in K_d and an increase in B_{max} (Figure 3 and Table 1). Table 2 shows that, when both agents were added together at maximal concentrations, their effects on $[\text{H}]\text{IP}_3$ binding were not additive.

The role of temperature on the effect of GSSG is shown in Figure 4. We found that preincubation of permeabilized hepatocytes at 4 °C with 2 mM GSSG did not enhance IP_3 -mediated Mn^{2+} quench at any concentration of added IP_3 (Figure 4a). Measurements of the binding parameters under these conditions also showed that GSSG did not significantly affect the K_d or B_{max} of the binding sites (Figures 4b and 4c, Table 1). However, it was noted that preincubation of the cells at 37 °C itself caused a 67% decrease in the maximal number of $[\text{H}]\text{IP}_3$ -binding sites compared with cells preincubated at 4 °C (Table 1). The requirement for preincubation at 37 °C to observe effects of GSSG was investigated in more detail in Figure 5. In these experiments permeabilized hepatocytes were incubated in the absence of added Ca^{2+} at 37 °C. Samples were removed at different times and assayed for binding by dilution into a medium at 4 °C containing $[\text{H}]\text{IP}_3$. The results show a progressive decline in binding activity during the incubation at 37 °C, reaching a final value that is approx. 30% of that present at the start of the incubation. Since the binding measurements were made at 4 °C, it can be concluded that the effect of incubation at 37 °C are not readily reversible upon returning the permeabilized cells to 4 °C. Inclusion of

Figure 2 Effect of preincubation with GSSG at 37 °C on Mn²⁺ quench and IP₃ binding

(a) IP₃-activated Mn²⁺ quench of compartmentalized fura-2 was measured at 4 °C in suspensions of hepatocytes preincubated with (○) or without (●) 2 mM GSSG at 37 °C as shown in Figure 1. Each point is the mean $\pm$ S.E.M. of values obtained from eight separate experiments. (b) Displacement analysis of [³H]IP₃ binding to permeabilized hepatocytes preincubated with (○) or without (●) 2 mM GSSG at 37 °C. The binding assay was carried out at 4 °C as indicated in the Materials and methods section; the cell protein concentration used was 1 mg/ml. (c) Scatchard plot of the binding data. Points are means of triplicate determinations in one experiment, and are representative of data obtained with seven separate cell preparations.

Figure 3 Effect of GSSG and thimerosal on IP₃ binding to permeabilized hepatocytes in the absence of Ca²⁺

Preincubation was carried out at 37 °C and the binding assay at 4 °C, as indicated in the Materials and methods section. The cell protein concentration used was 2.5 mg/ml. The cells were preincubated for 10 min at 37 °C in the absence (●) or in the presence of 2 mM GSSG (○) or 100 μ M thimerosal (■). The inset shows the Scatchard plot of the data. Points are means of triplicate determinations in one experiment and are representative of data obtained with four separate cell preparations.

GSSG (5 mM) in the incubation mixture completely prevented the temperature-dependent decrease in [³H]IP₃-binding sites. Furthermore, addition of GSSG at a later time during the incubation at 37 °C returned binding activity to that observed initially. Samples removed at the end of the incubation were immunoprecipitated with antibody to C-terminal type-I IP₃R. The data show that the total amount of immunoreactive receptor was the same under all conditions (Figure 5b). The reversibility of the temperature effect, together with the immunoprecipitation experiments, suggest that the observed loss of binding sites is not due to proteolysis of the receptor. The temperature-dependent decrease in binding activity was seen even when the receptors were transformed into a high-affinity state by adding Ca²⁺ (Table 3). Qualitatively, the effect of GSSG was the same as observed

Table 1 Effect of thiol- and disulphide-containing compounds on IP₃-binding parameters at different Ca²⁺ concentrations and temperatures

IP₃-binding parameters were measured in permeabilized hepatocytes by using displacement of [³H]IP₃ as described in the Materials and methods section. The temperature represents that of the preincubation. In all cases the binding measurements were performed at 4 °C. Values are means $\pm$ S.E.M. of K_d and B_{max} values calculated from 2–8 separate experiments. Significances of differences from the control values obtained under the same condition of incubation were calculated by Student's *t* test: ***P* < 0.01, **P* < 0.05.

Free [Ca ²⁺] (nM)	Temperature (°C)	Treatment	K_d (nM)	B_{max} (fmol/mg)
300	37	Control	3.3 $\pm$ 0.5	45.0 $\pm$ 0.6
300	37	GSSG (2 mM)	3.3 $\pm$ 0.4	71.0 $\pm$ 5.0**
300	37	GSH (2 mM)	3.3 $\pm$ 0.6	42.4 $\pm$ 4.9
300	37	DTT (2 mM) + GSSG (2 mM)	5.1	42.5
300	37	Cystine (2 mM)	3.1 $\pm$ 0.5	65.3 $\pm$ 7.0*
300	37	Cysteine (2 mM)	3.0 $\pm$ 0.2	41.0 $\pm$ 7.6
< 3	37	Control	15.6 $\pm$ 1.7	80.2 $\pm$ 9.5
< 3	37	GSSG (2 mM)	12.0 $\pm$ 2.9	128.9 $\pm$ 8.1**
< 3	37	Thimerosal (100 μ M)	4.5 $\pm$ 0.9**	106.6 $\pm$ 7.2*
300	4	Control	4.6 $\pm$ 0.1	136.2 $\pm$ 24.6
300	4	GSSG (2 mM)	3.4 $\pm$ 0.8	116.0 $\pm$ 18.4

Table 2 Non-additive effect of thimerosal and GSSG on $[\text{^3H}]\text{IP}_3$ binding

Permeabilized hepatocytes were preincubated as indicated for 10 min at 37 °C in the absence of added Ca^{2+} , and the binding assays were carried out at 4 °C in the presence of 1 nM $[\text{^3H}]\text{IP}_3$ as indicated in the Materials and methods section. The cell protein concentration used was 2.5 mg/ml. Each point is the mean $\pm$ S.E.M. of values obtained from three independent experiments.

Additions	$[\text{^3H}]\text{IP}_3$ bound (fmol/mg of protein)
None	5.1 ± 0.9
Thimerosal (100 μM)	16.6 ± 2.1
Thimerosal (200 μM)	13.3 ± 2.1
GSSG (2 mM)	9.4 ± 1.9
GSSG (5 mM)	11.0 ± 1.9
Thimerosal (100 μM) + GSSG (2 mM)	17.7 ± 2.1
Thimerosal (200 μM) + GSSG (5 mM)	14.8 ± 0.7

Figure 4 Effect of preincubation with GSSG at 4 °C on Mn^{2+} quench and IP_3 binding

(a) IP_3 -activated Mn^{2+} quench of compartmentalized fura-2 was measured at 4 °C in suspensions of hepatocytes preincubated with (○) or without (●) 2 mM GSSG at 4 °C. Each point is the mean $\pm$ S.E.M. of values obtained from seven separate experiments. (b) Displacement analysis of $[\text{^3H}]\text{IP}_3$ binding to permeabilized hepatocytes preincubated with (○) or without (●) GSSG at 4 °C. The cell protein concentration was 1 mg/ml. (c) Scatchard plot of the binding data. Points are means of triplicate determinations in one experiment and are representative of data obtained with seven separate cell preparations.

Figure 5 Time course of GSSG effect on IP_3 binding in permeabilized hepatocytes

(a) Permeabilized hepatocytes were incubated at 37 °C for the indicated times in the presence (○) or absence (●) of 5 mM GSSG. Some samples were also incubated for 5 min at 37 °C before GSSG addition (■). At the end of the incubation, the permeabilized cells were cooled on ice for 5 min and then assayed for $[\text{^3H}]\text{IP}_3$ binding in the absence of added Ca^{2+} . (b) Western blot of IP_3R . Permeabilized hepatocytes (10 mg of cell protein/ml) were kept on ice (lane 1) or incubated at 37 °C for 10 min in the absence (lane 2) or presence (lane 3) of GSSG (5 mM). The IP_3R was located as indicated in the Materials and methods section. Arrowhead shows position of myosin standard.

Table 3 Ca^{2+} -induced alteration in IP_3 binding at 4 °C and 37 °C

Permeabilized hepatocytes were kept on ice or incubated at 37 °C for 10 min in the absence or presence of 5 mM GSSG in the absence of Ca^{2+} . $[\text{^3H}]\text{IP}_3$ binding was then measured at 4 °C in the absence of Ca^{2+} or with the free Ca^{2+} concentration buffered at 10 μM . The data shown are means $\pm$ S.E.M. of four independent experiments.

Preincubation conditions	Ca^{2+} in binding assay	$[\text{^3H}]\text{IP}_3$ bound (fmol/mg of protein)
Control, 4 °C	0	23.2 ± 2.5
	10 μM	53.3 ± 6.5
Control, 37 °C	0	9.1 ± 1.5
	10 μM	30.1 ± 3.0
GSSG, 37 °C	0	26.9 ± 5.9
	10 μM	44.8 ± 7.9

without Ca^{2+} , i.e. GSSG decreased the decline in binding activity seen as a result of incubation of permeabilized hepatocytes at 37 °C.

We have examined the effects of GSSG and incubation at 37 °C on the $[\text{^3H}]\text{IP}_3$ -binding activity of several partially purified preparations of the hepatic IP_3R . $[\text{^3H}]\text{IP}_3$ -binding sites can be solubilized from permeabilized hepatocytes with Triton X-100 [17,23]. It has also been shown previously that $[\text{^3H}]\text{IP}_3$ binding to the IP_3R can be measured directly in anti- IP_3R antibody immunoprecipitates [18]. The binding activity in both these preparations

Table 4 Effect of temperature and GSSG on IP₃ binding to solubilized receptors

Permeabilized hepatocytes (5 mg/ml) were incubated at 37 °C for 10 min in the absence or presence of GSSG (5 mM). Binding of [³H]IP₃ was then measured at 4 °C in the absence of Ca²⁺, as described in the Materials and methods section. The same protocol was used for Triton X-100 extracts of hepatocytes, except that [³H]IP₃ binding was assayed by a poly(ethylene glycol) precipitation assay. Binding of [³H]IP₃ to the immunoprecipitated IP₃R was assayed as described previously [18]. In all cases the binding data were expressed as a percentage of the binding activity measured when the sample was maintained at 4 °C. The values for permeabilized cells are means ± S.E.M. of four separate experiments, with the 100% value for binding being 23.2 ± 2.5 fmol/mg of protein. The values for extracts and immunoprecipitated IP₃R are means of two separate experiments. The 100% value for the Triton X-100 extracts and immunoprecipitated IP₃R was 70 fmol/mg of protein and 237 c.p.m./70 µl of beads, respectively.

Experimental system	Temp.	GSSG	[³ H]IP ₃ bound relative to 4 °C (%)
Permeabilized hepatocytes	4 °C	—	100
	37 °C	—	39 ± 6
	37 °C	+	115 ± 15
Triton X-100 extracts	4 °C	—	100
	37 °C	—	8
	37 °C	+	9
Immunoprecipitates	4 °C	—	100
	37 °C	—	29
	37 °C	+	24

was also substantially decreased when incubated at 37 °C (Table 4). The latter findings suggest that the loss of binding activity occurring as the temperature is raised may result from an altered conformation of the receptor itself or of a regulatory protein. In contrast with the observations made with the receptor in its membrane environment, the addition of GSSG has no protective effect on the IP₃R in Triton X-100 extracts or in immunoprecipitates. When permeabilized hepatocytes were treated with GSSG and then solubilized, the binding activity in the extracts was not different from that obtained from untreated hepatocytes (results not shown).

GSSG at concentrations greater than 2 mM is maximally effective in sensitizing IP₃-mediated Ca²⁺ release. With estimated ratios of cytoplasmic GSH/GSSG of 60 and total glutathione concentrations of 10 mM [13,24], it seems unlikely that the concentration of cytoplasmic GSSG exerts any effects on the IP₃R under physiological conditions. However, the lumen of the endoplasmic reticulum (ER) is also estimated to contain 10 mM total glutathione, but at a ratio of GSH/GSSG of 2. The oxidized ratio of GSH/GSSG is maintained by the selective, temperature-dependent, transport of GSSG across the ER membrane [24]. The dependence of [³H]IP₃ binding in permeabilized hepatocytes was measured at different ratios of GSSG/GSH and as a function of total glutathione concentration (Figure 6). The data show that [³H]IP₃ binding is sensitive to changes in the ratio of GSSG/GSH below 2 (or GSH/GSSG ratios > 0.5). These values are more appropriate to those found in the ER lumen than in the cytosolic compartment. Figure 6 also shows that the relationship between ligand binding and imposed GSSG/GSH ratio is also a function of the total glutathione concentration. Maximal levels of binding and the half-maximal ratios of GSSG/GSH were increased as total glutathione concentration was increased. At a GSSG/GSH ratio of 9, IP₃ binding was increased substantially in the range of total glutathione concentrations of 2–10 mM. Since maximal effects of GSSG were obtained at 2 mM GSSG in the absence of GSH [6]; results not

Figure 6 Effect of different ratios of GSSG/GSH on IP₃ binding

The preincubation with GSSG and GSH was carried out at 37 °C and the binding assay at 4 °C in the absence of Ca²⁺, as indicated in the Materials and methods section. The cell protein concentration used was 2.5 mg/ml. The permeabilized hepatocytes were preincubated for 10 min at 37 °C in the presence of 2 mM (○), 5 mM (■) or 10 mM (□) total glutathione adjusted to the indicated ratios of GSSG/GSH. The 100% control value for binding was 3.55 ± 0.45 fmol/mg of protein. The data shown are means ± S.E.M. of values from two to four independent experiments.

shown), these data suggest that the level of GSH itself may also play a role in setting the sensitivity to GSSG [25].

DISCUSSION

In liver, the IP₃R/ion channel is proposed to exist in at least three conformational states: an active ion-conducting, low-affinity ligand-binding, state (I); an active ion-conducting, high-affinity ligand-binding, state (II); and an inactive ion-conducting, high-affinity ligand-binding, state (III) [7,26,27]. Interconversion between these states is facilitated by several factors. Low concentrations of Ca²⁺ stabilize the receptor in state II, and higher concentrations favour conversion of the receptor into state III. As observed in other studies [17,27] using comparable experimental conditions, only a single class of binding sites was detected in the presence or absence of Ca²⁺.

The main observation made in this study was that the effects of GSSG on IP₃R binding or ion fluxes require preincubation with the thiol agent at 37 °C and that preincubation of the permeabilized hepatocytes at this temperature itself led to a loss of binding sites that was prevented by inclusion of GSSG. Previous studies have shown that incubation of permeabilized hepatocytes at 37 °C favours the low-affinity form of the receptor (state I) [22,28]. The dissociation rate of IP₃ from the receptor is increased at 37 °C compared with 4 °C [29]. It is therefore possible that the apparent decrease in binding sites observed after preincubation at 37 °C is the result of loss of a rapidly dissociating component of the labelled ligand during the membrane-filtration assay employed in these experiments. This seems unlikely, for several reasons. Firstly, the actual binding measurements were conducted at 4 °C and after an initial equilibration period at 4 °C, a temperature where the dissociation rate of label over the estimated 2 s wash period would produce a minimal loss of label. Secondly, a loss of binding sites was also observed in the presence of Ca²⁺. Again, the dissociation rate of IP₃ from the high-affinity form at 4 °C has been shown to be extremely slow

[29]. Finally, it should be noted that it is unlikely that the loss of binding sites at 37 °C reflects the transition of the receptor to a low-affinity form that we cannot detect, because the K_d values obtained for the low- and high-affinity receptor forms in the present study are in reasonable agreement with the values in the literature (cf. [22,27]).

Preincubation of permeabilized hepatocytes or liver membranes has been shown previously to lead to the complete loss of high-affinity IP_3 -binding sites [22,28]. In our hands, this loss reflected a decrease in B_{max} of the binding sites and occurred irrespective of whether the receptor was in the high- or low-affinity form. Loss of binding sites was not the result of receptor proteolysis, as judged by its reversibility by GSSG and by direct immunoblotting measurements. Preincubation of hepatocytes at 37 °C in the presence of GSSG produced a shift in the EC_{50} for IP_3 -mediated Mn^{2+} quench, whereas binding measurements made under the same experimental conditions show that preincubation with GSSG increased the B_{max} of the binding sites without altering the K_d . If bivalent-cation flux is proportional to the number of occupied IP_3R , it would be expected that the greater the density of functional receptors, the lower the IP_3 concentration required to attain any given concentration of occupied receptor [30,31]. Thus the higher number of functional binding sites present after GSSG treatment is compatible with a lower EC_{50} for IP_3 -gated Ca^{2+} fluxes. On this basis it would also be predicted that the EC_{50} for IP_3R -gated Mn^{2+} quench should be higher in cells preincubated at 37 °C (Figure 2a) than at 4 °C (Figure 4a). The calculated EC_{50} values of 22.6 ± 5.8 nM at 37 °C and 14.1 ± 2.0 nM at 4 °C do indeed reflect this trend, although these parameter values were not significantly different.

The thiol reagent thimerosal has also been shown to enhance the affinity of IP_3 -mediated Ca^{2+} release in permeabilized hepatocytes [7]. However, there are several differences in the mode of action of thimerosal and GSSG. Firstly, the effect of thimerosal does not require preincubation at 37 °C [7]. Secondly, preincubation of the permeabilized hepatocytes at 37 °C with thimerosal changes the K_d and B_{max} of the binding site, whereas GSSG affects only the latter parameter (Table 1). In a recent paper, Kaplin et al. [25] showed that thimerosal has a direct effect on the purified IP_3R , increasing the affinity of the receptor without changing the B_{max} . Taken together, these data suggest two distinct sites of action: one, common to GSSG and thimerosal, results in an increase in B_{max} , and the other, perhaps more direct, effect, which is specific for thimerosal, results in a decrease in K_d . Recently, we have shown that mersalyl and thimerosal, which are related organomercurial thiol agents, have distinct effects on the conformation of the IP_3R , as judged from its sensitivity to proteolysis [32]. These studies underscore the view that the IP_3R may possess several reactive thiol groups that are differentially reactive with added thiol agents.

All known IP_3R isoforms are believed to exist in membranes as homotetramers. The six putative transmembrane domains present at the C-terminus of each monomer are proposed to be involved in oligomerization of the protein and in formation of a Ca^{2+} channel [33,34]. A conserved sequence (TTCFICG) present in the C-terminal tail of types I, II and III IP_3R has been proposed to be the target of thiol reagents that enhance IP_3 -mediated Ca^{2+} release [1]. However, we found that binding sites were not decreased upon preincubation of cerebellum microsomes at 37 °C and there were no effects of GSSG (results not shown). Furthermore, although the temperature-dependent loss of binding sites could be observed in several disrupted systems, the protective effect of GSSG could only be observed in native membranes. These data imply that GSSG does not interact directly with the receptor, but may alter the activity of an

associated regulatory protein. The following hypothesis is advanced to account for our experimental data. Loss of the ligand-binding activity of permeabilized hepatocytes at 37 °C is the result of inappropriate unfolding of the ligand-binding domain and that this process is prevented or reversed by GSSG as a result of an enzymic process which utilizes or is regulated by GSSG. Unfolding of the ligand-binding domain may be the result of a disruption of inter-molecular non-covalent interactions, formation of inappropriate disulphide bonds or partial disassembly of oligomers. There is some evidence in the literature that the latter process is facilitated by reduction of disulphide bonds [35]. An enzymic process that could play a role in mediating the effect of GSSG would be any thiol-disulphide oxidoreductase that interacts with the IP_3R . Any candidate enzyme would have to be tightly associated with the membrane or be intraluminal, since thorough washing of permeabilized hepatocytes does not prevent the effect of GSSG (results not shown). Potential candidates are intraluminal enzymes such as protein disulphide-isomerase [36,37]. A system *in vitro* which retains the interaction between IP_3R and added purified oxidoreductases is necessary to test this hypothesis further.

The physiological implications of our observations regarding the effects of temperature and GSSG are unclear. When intact hepatocytes are preincubated at 37 °C and then permeabilized, $[\text{H}]\text{IP}_3$ -binding activity is not significantly different from that in hepatocytes permeabilized after preincubation at 4 °C (results not shown). This implies that the temperature-dependent loss of binding sites does not occur in an intact cell, possibly because the glutathione redox state is poised at a favourable ratio. Recently, Missiaen et al. [38] showed that decreasing the total cellular glutathione by 80 % in HeLa cells with an inhibitor of glutathione biosynthesis had no effect on Ca^{2+} spiking induced in these cells by histamine. These workers also showed that DTT had no effect on Ca^{2+} spiking. They concluded that glutathione plays no role in agonist-induced Ca^{2+} signalling in HeLa cells [38]. Our own data suggest that the hepatic IP_3R is sensitive to changes in GSH/GSSG ratios that are much lower than those thought to exist in the cytosol. The underlying assumption in these experiments is that changing the ratio of GSH/GSSG in the medium of permeabilized cells would produce a parallel change in the ER luminal ratio. If this assumption is valid it can be concluded that the hepatic IP_3R is sensitive to GSH/GSSG ratios that are similar to those reported to exist in the ER lumen [24]. It is therefore possible that the glutathione redox ratio in this compartment and the activity of luminal thiol-disulphide oxidoreductases are important parameters in the regulation of the IP_3R . It should be noted that two conserved cysteine residues are found in a putative intraluminal loop of all cloned IP_3R isoforms [39]. Although the physiological significance of the present findings remain to be established, these data do have relevance for studies of IP_3 -mediated responses in permeabilized cells. Such studies are customarily carried out after a period of preincubation at 37 °C in the absence of a glutathione redox buffer, and it is clear that both these factors have important effects regulating the function of the liver IP_3R .

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