Oxidized glutathione causes sensitization of calcium release to inositol 1,4,5-trisphosphate in permeabilized hepatocytes

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The effects of GSSG on Ca^{2+} mobilization by $\text{Ins}(1,4,5)\text{P}_3$ were studied in permeabilized rat hepatocytes. Incubation with GSSG (2 mM) increased the sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ for Ca^{2+} release, with no effect on the size of the Ca^{2+} pool that could be released with maximal concentrations of $\text{Ins}(1,4,5)\text{P}_3$. GSSG decreased the EC_{50} for $\text{Ins}(1,4,5)\text{P}_3$ from a control value of 578 ± 23 nM to 137 ± 21 nM. GSSG had no effect on the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized cells, and sensitization of Ca^{2+} release was still observed when the poorly metabolizable analogue inositol 1,4,5-trisphosphorothioate was used. GSSG did not affect the ATP-dependent Ca^{2+} pump or the extent of loading of intracellular Ca^{2+} pools. In addition, the enhancement of $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity by GSSG occurred under conditions where the Ca^{2+} pumps were blocked with thapsigargin or by chelation of medium Ca^{2+} just before $\text{Ins}(1,4,5)\text{P}_3$ addition. The effect of GSSG was time- and dose-dependent, maximal effects being observed after 5 min incubation with 2 mM-GSSG. Cystine mimicked the GSSG-induced increase in $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity, and the effects could be reversed by dithiothreitol (DTT). DTT, GSH glutathione and cysteine had no effect when added alone. Other agents known to react with protein thiols, including *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and Ag^+ , did not affect the sensitivity to $\text{Ins}(1,4,5)\text{P}_3$, but were inhibitors of ATP-dependent Ca^{2+} uptake. The data suggest that the sensitivity of the intracellular Ca^{2+} pools to release by $\text{Ins}(1,4,5)\text{P}_3$ can be modulated by the formation of mixed disulphides with GSSG or other oxidized thiols.

INTRODUCTION

Stimulation of hepatocytes with a variety of hormones that activate inositol-lipid-specific phospholipase C leads to the generation of oscillatory increases in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) [1–3]. These $[\text{Ca}^{2+}]_i$ oscillations are dependent on a release of Ca^{2+} from intracellular stores that is triggered by $\text{Ins}(1,4,5)\text{P}_3$. We demonstrated recently that similar $[\text{Ca}^{2+}]_i$ oscillations can be induced by *t*-butyl hydroperoxide (TBHP) without a requirement for stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation [4]. Furthermore, in common with the actions of Ca^{2+} -mobilizing hormones in these cells, the $[\text{Ca}^{2+}]_i$ oscillations induced by TBHP occurred as $[\text{Ca}^{2+}]_i$ waves propagating from a specific subcellular locus in hepatocytes maintained in primary culture (conditions where they become functionally polarized). Previous studies of the effects of TBHP on Ca^{2+} homeostasis in hepatocytes have shown that this agent can inhibit microsomal and plasmalemmal Ca^{2+} pumps, and this effect was thought to underlie the elevation of $[\text{Ca}^{2+}]_i$ in these cells [5–8]. However, under conditions where it caused $[\text{Ca}^{2+}]_i$ oscillations, TBHP was relatively ineffective in inhibiting intracellular Ca^{2+} pumps [4]. In addition, other Ca^{2+} -pump inhibitors, such as thapsigargin, were unable to induce $[\text{Ca}^{2+}]_i$ oscillations in hepatocytes. In experiments where hepatocytes were preincubated with TBHP and then permeabilized, it was shown that the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from intracellular stores was enhanced by the TBHP pretreatment. This increased sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ was postulated to underlie the generation of $[\text{Ca}^{2+}]_i$ oscillations in response to TBHP by facilitating the action of basal levels of $\text{Ins}(1,4,5)\text{P}_3$ [4].

The effects of TBHP on Ca^{2+} pumps have been ascribed to the metabolic oxidation of cellular GSH, with the subsequent formation of mixed disulphides between protein thiol groups and GSSG (see [6,7] for reviews). It was therefore of interest to determine whether the effects of TBHP pretreatment of

hepatocytes on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, measured in subsequently permeabilized cells, could be reproduced by direct addition of GSSG to the permeabilized hepatocytes. In the present study we demonstrate that GSSG enhances the efficacy of $\text{Ins}(1,4,5)\text{P}_3$ in releasing Ca^{2+} by shifting the dose-response curve to the left, decreasing the EC_{50} by about 4-fold. Cystine has a similar effect on $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity, but the actions of GSSG are not mimicked by thiol-alkylating agents. The increased sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ in the presence of GSSG is not dependent on alterations of the ATP-dependent Ca^{2+} pump. These findings represent the identification of a novel mechanism by which $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release can be modulated.

MATERIALS AND METHODS

Cell preparation

Isolated hepatocytes were prepared by collagenase perfusion of livers obtained from male Sprague–Dawley rats (250–300 g), as described previously [9]. After isolation, the hepatocytes were washed twice in a Hepes-buffered medium composed of 120 mM-NaCl, 20 mM-Hepes, 5 mM-KCl, 1 mM- KH_2PO_4 and 200 μM - MgCl_2 , pH 7.4. The cells were stored in this buffer on ice at a concentration of 50 mg of protein/ml.

Ca^{2+} measurements in permeabilized hepatocytes

For fluorimetric measurements of intracellular Ca^{2+} uptake and release, hepatocytes were permeabilized in medium composed of 120 mM-KCl, 20 mM-Hepes, 10 mM-NaCl, 1 mM- KH_2PO_4 , 200 μM - MgCl_2 , 75 μM -quin2 (free acid form), 20–40 μg of digitonin/ml, 5 μM -carbonyl cyanide *m*-chlorophenylhydrazone ('CCCP'), 5 μg of oligomycin/ml, 1 μg of rotenone/ml and 1 μg each of the proteinase inhibitors pepstatin, antipain and leupeptin/ml, with the pH adjusted to 7.2, as described by Thomas [9]. In some experiments, MgCl_2 was increased to 5 mM. Incubations were performed in the cuvette of a fluorimeter

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.; $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$, inositol 1,4,5-trisphosphorothioate; DTT, dithiothreitol; TBHP, *t*-butyl hydroperoxide; pCMB, *p*-chloromercuribenzoic acid; pCMBs, *p*-chloromercuriphenylsulphonic acid.

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(Photon Technology Deltascan) maintained at 37 °C with continuous stirring. The excitation wavelength was 340 nm, with emission at 495 nm. Cell permeabilization was initiated by adding hepatocytes (final concn. 4–6 mg of protein/ml) to prewarmed buffer containing digitonin. After cell permeabilization, CaCl_2 was added to give a free $[\text{Ca}^{2+}]$ of about 300 nM. This was followed by addition of 5 mM-phosphocreatine, 0.5 unit of creatine kinase/ml and 2 mM-ATPMg to initiate ATP-dependent Ca^{2+} uptake by intracellular non-mitochondrial stores. Calibration of quin2-calcium fluorescence in terms of free Ca^{2+} was achieved by determining the minimum and maximum fluorescence with EGTA/Tris and excess CaCl_2 , respectively, at the end of each run [9]. For measurement of changes in the total (buffered plus free) medium Ca^{2+} content, small calibration additions of standardized CaCl_2 were used. In the free Ca^{2+} concentration range used for the present experiments, changes in fluorescence with added Ca^{2+} were essentially linear (see [9]). Thus, in this experimental system, quin2 provides sufficient Ca^{2+} -buffering capacity to maintain a relatively constant level of free Ca^{2+} , while providing a direct read-out of both Ca^{2+} concentration and the magnitude of total Ca^{2+} changes in the medium (most of which is buffered by the relatively high concentration of the indicator) [9].

For some experiments, a similar protocol to that described above was used, except that $^{45}\text{Ca}^{2+}$ uptake and release were measured by a stopped-filtration assay and Ca^{2+} in the medium was buffered with EGTA (75 μM) instead of quin2. In addition, the incubation buffer also contained 4% poly(ethylene glycol), which helped to stabilize the cells after permeabilization and during filtration, giving more consistent values for the size of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool. For these experiments, $^{45}\text{CaCl}_2$ was added to give 0.5 $\mu\text{Ci}/\text{ml}$. Maximal uptake of $^{45}\text{Ca}^{2+}$ was achieved after 7 min incubation with ATPMg and was then stable for least 15 min. Incubations were terminated by diluting 0.5 ml portions of cell suspension with 3.0 ml of ice-cold stop

solution containing 310 mM-sucrose and 4 mM-EGTA, pH 7.4, followed by rapid filtration on glass-fibre filters (Whatman GF/C, 2.4 cm diam.). The filters were washed three times with the cold stopping buffer and then transferred to vials containing liquid-scintillation fluid for determination of the retained radioactivity. Blanks were obtained by incubating cells with ionomycin in the absence of ATP.

Data from $\text{Ins}(1,4,5)\text{P}_3$ dose-response experiments were fitted to the Hill equation by using the non-linear regression program ENZFITTER (Biosoft), with the following equation:

$$v = \frac{V_m}{1 + (K/[S])^h}$$

where V_m is the Ca^{2+} -release response at maximal $[\text{Ins}(1,4,5)\text{P}_3]$, v is the Ca^{2+} release given by $\text{Ins}(1,4,5)\text{P}_3$ concentration $[S]$, K is the EC_{50} value for $\text{Ins}(1,4,5)\text{P}_3$ and h is the Hill coefficient. For the data presented here, the only significant changes in the calculated parameters were in the EC_{50} values, which are given in the text.

Materials

$\text{Ins}(1,4,5)\text{P}_3$, ionomycin (free acid) and quin2 were obtained from Calbiochem. Inositol 1,4,5-trisphosphorothioate [$\text{Ins}(1,4,5)\text{P}_3\text{S}_3$] and D-[1- $^3\text{H}(\text{n})$]inositol 1,4,5-trisphosphate (^3H] $\text{Ins}(1,4,5)\text{P}_3$) were from New England Nuclear. $^{45}\text{CaCl}_2$ (20 mCi/mg) was from Amersham. All other biochemicals were obtained from Sigma or Boehringer Mannheim.

RESULTS

Hepatocytes were permeabilized in a KCl-based medium with free Ca^{2+} buffered with either quin2 or EGTA. In the presence of ATP, the permeabilized hepatocytes accumulated Ca^{2+} from the medium until a steady state was attained (Fig. 1). At this point

Fig. 1. Effects of GSH and GSSG on the sensitivity of Ca^{2+} release to $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized hepatocytes

Hepatocytes (5 mg of protein/ml) were permeabilized with digitonin, and Ca^{2+} uptake and release were monitored by using quin2 as described in the Materials and methods section. Traces show the fluorescence changes caused by alterations in quin2- Ca^{2+} complex-formation. Calibrated additions of CaCl_2 were used to calculate total Ca^{2+} changes. Free Ca^{2+} was buffered by the quin2 in the concentration range 250–300 nM, over which range the changes in fluorescence with added Ca^{2+} were essentially linear. The calibration bar in the Figure represents 10 nmol of $\text{Ca}^{2+}/2$ ml. Additions were as follows: ATP, 2 mM-ATPMg; IP_3 , $\text{Ins}(1,4,5)\text{P}_3$ at the indicated concentration; Iono, 4 μM -ionomycin. Immediately after permeabilization (3 min before ATP addition) the cells were treated with (b) 2 mM-GSSG, (c) 2 mM-GSH or (d) 2 mM-GSSG + 2 mM-DTT. The control condition with no pretreatments is shown in (a).

Fig. 2. Effect of GSSG on the concentration-dependence for Ins(1,4,5) P_3 -induced Ca^{2+} release in permeabilized hepatocytes

Hepatocytes were permeabilized with digitonin, and Ca^{2+} uptake and release were monitored by using quin2 as described in the Materials and methods section. The permeabilized cells were preincubated in the absence (●) or presence (▲) of 2 mM-GSSG, and the Ca^{2+} release in response to the indicated concentrations of Ins(1,4,5) P_3 was determined. In (a) the medium contained 5 mM-MgCl₂, and in (b) the concentration of MgCl₂ added to the medium was 200 μ M. Each point is the mean $\pm$ S.E.M. of values obtained from 3–6 separate experiments: * significantly different from equivalent Ins(1,4,5) P_3 concentration in control cells with $P < 0.05$, calculated by Student's t test.

further Ca^{2+} additions did not result in any further Ca^{2+} uptake, indicating that the intracellular non-mitochondrial Ca^{2+} stores were essentially full [9,10]. Under these conditions, after Ca^{2+} uptake the free $[Ca^{2+}]$ was about 250 nM. Fig. 1(a) shows the effects of sequential additions of sub-maximal (100 nM) and maximal (2 μ M) doses of Ins(1,4,5) P_3 . Further Ins(1,4,5) P_3 additions had no effect (results not shown), although there was a residual Ins(1,4,5) P_3 -insensitive Ca^{2+} pool that could be released by ionomycin. The Ins(1,4,5) P_3 -sensitive Ca^{2+} pool represented 35–40% of the total releasable Ca^{2+} stores. Under control conditions 100 nM-Ins(1,4,5) P_3 released 20% of the Ins(1,4,5) P_3 -sensitive Ca^{2+} store. When the cells were preincubated with GSSG (2 mM), the response to 100 nM-Ins(1,4,5) P_3 was greatly enhanced, such that about 75% of the Ins(1,4,5) P_3 -sensitive Ca^{2+} store was mobilized. However, the total size of this Ca^{2+} pool was unchanged, as judged by the subsequent responses to 2 μ M-Ins(1,4,5) P_3 and ionomycin (Fig. 1b). In earlier studies [4] we found that treatment of cells with TBHP or GSSG before permeabilization resulted in a decrease of about 25% in the maximal size of the Ins(1,4,5) P_3 -releasable Ca^{2+} pool. This effect was not observed consistently under the conditions of the present study. The earlier finding may have resulted from the presence of endogenous Ins(1,4,5) P_3 at sufficient levels to allow a partial

Fig. 3. GSSG increases the sensitivity to Ins(1,4,5) P_3

Quin2 was used to monitor Ca^{2+} uptake and release exactly as described for Fig. 1. The traces show only the Ca^{2+} -release phase after the completion of ATP-dependent Ca^{2+} sequestration. Permeabilized cells were incubated in the absence (a) or presence (b) of 2 mM-GSSG. Additions were as follows: IP₃-S, 0.25 μ M-Ins(1,4,5) P_3 ; IP₃, 2 μ M-Ins(1,4,5) P_3 ; Iono, 4 μ M-ionomycin.

release of Ca^{2+} in GSSG-pretreated cells, before addition of exogenous Ins(1,4,5) P_3 .

GSSG treatment had no effect on the extent or initial rate of ATP-dependent Ca^{2+} uptake. In a series of experiments similar to those shown in Fig. 1, control and GSSG-treated cells accumulated 2.92 ± 0.11 and 2.77 ± 0.19 nmol of Ca^{2+} /mg of cell protein respectively, and the initial rates of Ca^{2+} uptake were 2.19 ± 0.23 and 2.01 ± 0.20 nmol/min per mg of protein. The effect of GSSG on Ins(1,4,5) P_3 -sensitivity was not mimicked by GSH (Fig. 1c) and was prevented when cells were incubated simultaneously with GSSG and DTT (Fig. 1d). The sensitization by GSSG could also be reversed if DTT was added 3 min before Ins(1,4,5) P_3 , after the completion of the GSSG-induced sensitization process (see below for time-course studies).

Fig. 2(a) shows the effect of GSSG pretreatment on the dose-response to Ins(1,4,5) P_3 for Ca^{2+} release under conditions of high (5 mM, panel a) and low (200 μ M, panel b) levels of MgCl₂. These experiments were carried out essentially as shown in Fig. 1, except that for each experiment only a single Ins(1,4,5) P_3 dose was used. At higher Mg²⁺ levels the apparent sensitivity to Ins(1,4,5) P_3 was about 3-fold lower than under the low-Mg²⁺ condition used for most of the studies described here. However, under both conditions GSSG caused a substantial increase in sensitivity to Ins(1,4,5) P_3 for Ca^{2+} release, without affecting the maximal extent of Ca^{2+} release at saturating Ins(1,4,5) P_3 levels. In the presence of 5 mM-MgCl₂, the EC₅₀ for Ins(1,4,5) P_3 was decreased from 578 ± 23 nM in the control cells to 137 ± 21 nM after GSSG treatment. For the experiments carried out in the low-Mg²⁺ buffer, the EC₅₀ for Ins(1,4,5) P_3 in the absence of GSSG was 176 ± 4 nM, but GSSG treatment still

Fig. 4. Dose-response to GSSG for enhancement of $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity

Ca^{2+} uptake and release were measured in permeabilized hepatocytes by using $^{45}\text{Ca}^{2+}$ buffered with EGTA, in medium containing $200\ \mu\text{M-MgCl}_2$ and other addition as described in the Materials and methods section. Ca^{2+} mobilization by $\text{Ins}(1,4,5)\text{P}_3$ doses of $100\ \text{nM}$ and $2\ \mu\text{M}$ were measured in cells treated for 10 min with the indicated concentrations of GSSG. The data show only the Ca^{2+} responses to $100\ \text{nM-Ins}(1,4,5)\text{P}_3$. None of the GSSG concentrations affected the extent of Ca^{2+} release in response to $2\ \mu\text{M-Ins}(1,4,5)\text{P}_3$. Each point is the mean $\pm$ S.E.M. of values from four separate experiments.

resulted in a substantial enhancement of $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity, yielding an EC_{50} value of $49 \pm 4\ \text{nM}$. Similar effects of GSSG were observed by using $^{45}\text{Ca}^{2+}$ -loaded permeabilized hepatocytes to measure the $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity for Ca^{2+} release (results not shown).

One possible explanation for the altered apparent sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ after GSSG treatment is that this agent could interfere with the metabolism of $\text{Ins}(1,4,5)\text{P}_3$. However, it was shown previously that TBHP treatment of cells does not interfere with $\text{Ins}(1,4,5)\text{P}_3$ metabolism [4]. Furthermore, previous studies [9] have indicated that $\text{Ins}(1,4,5)\text{P}_3$ metabolism by permeabilized hepatocytes is largely inhibited under the low- Mg^{2+} condition used for most of the work described here. In the buffer containing $5\ \text{mM-MgCl}_2$, $\text{Ins}(1,4,5)\text{P}_3$ breakdown (measured with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$) did occur, but there was no effect of GSSG on the rate of disappearance of $\text{Ins}(1,4,5)\text{P}_3$ or in the rate at which products were formed from it [primarily lower inositol phosphates, with no measurable $\text{Ins}(1,3,4,5)\text{P}_4$ production]. Under the conditions used in the experiments of Fig. 2(a), $\text{Ins}(1,4,5)\text{P}_3$ ($2.5\ \mu\text{M}$) was degraded at rates of 193 ± 21 and $200 \pm 17\ \text{pmol/min per mg}$ of protein in control and GSSG-treated cells respectively. As a further approach to examine the role of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, the ability of GSSG to enhance the Ca^{2+} release response to the poorly metabolizable $\text{Ins}(1,4,5)\text{P}_3$ analogue $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$ was examined. As shown in Fig. 3(a), $0.25\ \mu\text{M-Ins}(1,4,5)\text{P}_3\text{S}_3$ released only about 10% of the total $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} pool. After GSSG treatment, this dose of $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$ released approx. 5-fold more Ca^{2+} (Fig. 3b). Mean values for the Ca^{2+} release in response to $0.25\ \mu\text{M-Ins}(1,4,5)\text{P}_3\text{S}_3$ in the presence and absence of GSSG were 0.09 ± 0.02 and $0.54 \pm 0.12\ \text{nmol/mg}$ of cell protein respectively ($n = 3$).

A concentration of $2\ \text{mM-GSSG}$ was used in most of the studies described here, since this equates to the amount of GSSG formed in intact hepatocytes treated with TBHP under conditions where the latter compound causes oscillations of cytosolic free Ca^{2+} [4,6,11,12]. Fig. 4 shows the dose-dependence for the action of GSSG to sensitize the Ca^{2+} -release system in permeabilized hepatocytes to $\text{Ins}(1,4,5)\text{P}_3$. For these experiments, the permeabilized cells were preincubated with a range of GSSG

Fig. 5. Time course of GSSG-induced increase in $\text{Ins}(1,4,5)\text{P}_3$ sensitivity

$\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} -release was measured fluorimetrically by using quin2 in medium containing $200\ \mu\text{M-MgCl}_2$, as described in the Materials and methods section. GSSG was added at various times before $\text{Ins}(1,4,5)\text{P}_3$ addition, including up to 3 min before the addition of ATP [which was added 3 min after permeabilization and 12 min before $\text{Ins}(1,4,5)\text{P}_3$ treatment]. For each condition, cells were treated with $100\ \text{nM-Ins}(1,4,5)\text{P}_3$, followed by $2\ \mu\text{M-Ins}(1,4,5)\text{P}_3$. This allowed measurements of the magnitude of the Ca^{2+} release at $100\ \text{nM-Ins}(1,4,5)\text{P}_3$ (●) and the size of the residual $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store (▼) within a single experimental run. The graph also shows that the total $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} pool, measured as the sum of the two steps of Ca^{2+} release, was unaffected throughout the time course of GSSG treatment (■). Each point is the mean $\pm$ S.E.M. of 4–6 determinations; * significantly different from control with $P < 0.05$ calculated by Student's t test.

concentrations (0.1 – $10\ \text{mM}$), and then the Ca^{2+} release in response to sub-maximal ($100\ \text{nM}$) and maximal ($2\ \mu\text{M}$) $\text{Ins}(1,4,5)\text{P}_3$ doses was determined, essentially as shown in Fig. 1. The GSSG-induced increase in $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity was manifest in an enhancement of the Ca^{2+} release at $100\ \text{nM-Ins}(1,4,5)\text{P}_3$. The EC_{50} value for GSSG was about $1.5\ \text{mM}$, and maximal effects were observed with 2 – $10\ \text{mM-GSSG}$. There was no effect of any of the GSSG concentrations tested on the Ca^{2+} release observed with maximal $\text{Ins}(1,4,5)\text{P}_3$ (results not shown).

The experiment of Fig. 5 demonstrates that the effects of GSSG on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release were time-dependent. Permeabilized hepatocytes were allowed to accumulate Ca^{2+} in the presence of ATP for a fixed period and then were treated sequentially with $100\ \text{nM-}$ and $2\ \mu\text{M-Ins}(1,4,5)\text{P}_3$. As described above, the relative efficacies of the sub-maximal and maximal $\text{Ins}(1,4,5)\text{P}_3$ doses can be used as an index of the GSSG-induced shift in $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity. The effect of incubating the cells with GSSG ($2\ \text{mM}$) for various times before the addition of $\text{Ins}(1,4,5)\text{P}_3$ is shown in Fig. 5. A significant enhancement of $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity was observed when GSSG was present for as little as 1 min before $\text{Ins}(1,4,5)\text{P}_3$ addition, and this effect was maximal in cells exposed to GSSG for 5 min.

It has been shown that other agents which modify protein thiol groups can inhibit $\text{Ins}(1,4,5)\text{P}_3$ binding, $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release and also the Ca^{2+} -uptake pump [13–17]. Since the most likely mechanism by which GSSG might modify the properties of the $\text{Ins}(1,4,5)\text{P}_3$ -release mechanism is through interactions with protein thiol groups, we examined the effects of some of these other agents on $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity. Unlike GSSG, most of the other agents tested interfered with the activity of the ATP-dependent Ca^{2+} pump. N -Ethylmaleimide (0.1 – $2\ \text{mM}$) and the organic mercurials p -chloromercuribenzoic acid (pCMB) and p -chloromercuriphenylsulphonic acid (pCMBS) (10 – $100\ \mu\text{M}$),

Table 1. Effects of thiol- and disulphide-containing compounds on Ins(1,4,5) P_3 -sensitivity

Ca^{2+} uptake and Ins(1,4,5) P_3 -induced Ca^{2+} release were measured by using quin2 in the presence of 200 μM -MgCl₂ as described in the Materials and methods section. After permeabilization, the cells were incubated with the indicated test agents before initiating ATP-dependent Ca^{2+} uptake. The Ca^{2+} release responses to 100 nM- and 2 μM -Ins(1,4,5) P_3 were measured sequentially, and the ratio of the amount of Ca^{2+} released by 100 nM to the total Ins(1,4,5) P_3 -releasable Ca^{2+} pool [the sum of the responses to the two doses of Ins(1,4,5) P_3] was calculated (Release ratio). Total ATP-dependent Ca^{2+} uptake under control conditions was 3.09 ± 0.15 nmol/mg of cell protein, and this was unaffected by the pretreatments used, except for 2 mM-cystine, which decreases the total sequestered Ca^{2+} to 1.68 ± 0.07 nmol/mg of cell protein (at 0.5 mM-cystine ATP-dependent Ca^{2+} uptake was 2.88 ± 0.22 nmol/mg, which was not significantly different from the control). Values are means $\pm$ S.E.M. of data from 3–6 separate experiments, with significance of differences from the control calculated by Student's *t* test: **P* < 0.01, ***P* < 0.001.

Condition	Ca ²⁺ release by Ins(1,4,5) P_3 (nmol/mg of protein)		Release ratio (0.1 μM /Total)
	0.1 μM -Ins P_3	Total	
Control	0.21 ± 0.02	0.98 ± 0.11	0.21 ± 0.04
2 mM-GSSG	$0.66 \pm 0.13^*$	0.88 ± 0.14	$0.75 \pm 0.05^{**}$
2 mM-GSSG + 2 mM-DTT	0.17 ± 0.03	0.81 ± 0.18	0.21 ± 0.02
2 mM-GSH	0.16 ± 0.03	0.93 ± 0.22	0.17 ± 0.02
1 mM-GSSG + 1 mM-GSH	$0.41 \pm 0.05^*$	0.86 ± 0.23	$0.48 \pm 0.03^{**}$
0.2 mM-GSSG + 1.8 mM-GSH	0.18 ± 0.08	0.83 ± 0.22	0.20 ± 0.01
2 mM-Cysteine	0.28 ± 0.01	1.22 ± 0.09	0.23 ± 0.02
2 mM-Cystine	0.33 ± 0.05	$0.50 \pm 0.07^*$	$0.66 \pm 0.03^{**}$
0.5 mM-Cystine	$0.44 \pm 0.05^*$	0.71 ± 0.10	$0.62 \pm 0.02^{**}$

which form covalent adducts with protein thiols, both inhibited Ca^{2+} uptake without shifting the sensitivity to Ins(1,4,5) P_3 (measured at concentrations of inhibitor where Ca^{2+} uptake was not completely blocked). Ag⁺ ions have been shown to enhance Ca^{2+} release from skeletal-muscle sarcoplasmic reticulum by interacting with protein thiol groups [18]. In permeabilized hepatocytes, Ag⁺ (20–50 μM) inhibited the Ca^{2+} pump, but had no effect on Ins(1,4,5) P_3 sensitivity. By contrast, cystine (2 mM), which also caused a partial inhibition of ATP-dependent Ca^{2+} uptake, was able to shift the apparent sensitivity to Ins(1,4,5) P_3 , as judged from the ratio of Ca^{2+} release at 100 nM-Ins(1,4,5) P_3 to the maximal release with 2 μM -Ins(1,4,5) P_3 (Table 1). Cysteine and GSH at similar concentrations had no effect. The effect of cystine on Ins(1,4,5) P_3 -sensitivity was more potent than its effect on the Ca^{2+} pump, since at 0.5 mM-cystine the effect of 100 nM-Ins(1,4,5) P_3 was enhanced (Table 1) without a significant alteration in the extent of ATP-dependent Ca^{2+} sequestration (see the legend to Table 1). Thus it appears that the GSSG-induced enhancement of Ins(1,4,5) P_3 -sensitivity may be dependent on the presence of the cysteine–cysteine linkage and that this most probably forms a mixed disulphide with a protein component involved in the Ins(1,4,5) P_3 -triggered Ca^{2+} -release mechanism. The ability of DTT to reverse the effects of GSSG supports this suggestion.

Under physiological conditions, intracellular GSSG levels are maintained low by the activity of glutathione reductase, which keeps the GSH/GSSG couple in a reduced state. Since the data suggest that GSSG may act via protein thiol oxidation, we attempted to examine the effects of different GSH/GSSG redox states imposed in the permeabilized cells by mixing the two

Table 2. Effect of GSSG on Ins(1,4,5) P_3 -sensitivity under conditions of Ca^{2+} -pump inhibition

Ca^{2+} uptake and Ins(1,4,5) P_3 -induced Ca^{2+} release were measured in permeabilized hepatocytes incubated in the absence or presence of 2 mM-GSSG, by using quin2 buffer containing 200 μM -MgCl₂ as described in the Materials and methods section. Just before Ins(1,4,5) P_3 addition, the ATP-dependent Ca^{2+} pump was inhibited either by adding 1 μM -thapsigargin (Thapsi.) 1 min before Ins(1,4,5) P_3 or by adding an excess of quin2 sufficient to decrease the medium free [Ca^{2+}] to less than 10 nM (Low Ca^{2+}) 10 s before Ins(1,4,5) P_3 . In both cases there was a substantial rate of Ca^{2+} leak independent of Ins(1,4,5) P_3 addition, but this leak was not affected by GSSG. The Ca^{2+} -release responses to 100 nM- and 2 μM -Ins(1,4,5) P_3 were measured (in separate incubations) and the 'Release ratio' was calculated as described for Table 1. Values are means $\pm$ S.E.M. from experiments carried out using at least six separate hepatocyte preparations. Statistical significance of differences from the equivalent condition in the absence of GSSG were calculated by Student's *t* test: **P* < 0.01, ***P* < 0.001.

Condition	Ca ²⁺ release (nmol/mg of protein)		Release ratio (0.1 μM /2.0 μM)
	0.1 μM -Ins P_3	2.0 μM -Ins P_3	
Thapsi.	0.18 ± 0.06	0.56 ± 0.06	0.32 ± 0.04
Thapsi. + GSSG	$0.39 \pm 0.01^*$	0.45 ± 0.03	$0.87 \pm 0.08^{**}$
Low Ca^{2+}	0.05 ± 0.02	0.58 ± 0.04	0.09 ± 0.02
Low Ca^{2+} + GSSG	$0.39 \pm 0.05^{**}$	0.46 ± 0.08	$0.85 \pm 0.07^{**}$

components in varying proportions but maintaining total glutathione at 2 mM [19]. With a 1:1 mixture there was still a significant enhancement of Ins(1,4,5) P_3 sensitivity (Table 1), but at a 1:9 ratio (GSSG:GSH) this effect was lost. These data suggest that glutathione-mediated oxidation would not affect Ins(1,4,5) P_3 -sensitivity under physiological conditions. These experiments are somewhat limited, however, by the complexity of the permeabilized hepatocyte system, where both enzymic and non-enzymic mechanisms may significantly modify the applied GSH/GSSG ratio when one of the components is present at a low concentration. When GSSG was maintained at 2 mM and GSH:GSSG ratio adjusted with increasing amounts of GSH, Ca^{2+} uptake was inhibited.

To exclude the possibility that the effects of GSSG on Ins(1,4,5) P_3 -induced Ca^{2+} release might be mediated at the level of the Ca^{2+} -uptake pathway, experiments were carried out in which Ca^{2+} pumping was blocked just before addition of the Ins(1,4,5) P_3 (Table 2). This was achieved in two ways; thapsigargin [20] was added to inhibit the pump, or Ca^{2+} in the medium was decreased below the threshold for uptake using an excess of quin2 to decrease Ca^{2+} to < 10 nM. Both of these approaches caused a substantial rate of basal Ca^{2+} release, owing to the presence of a leak pathway. However, although somewhat decreased, the amount of Ca^{2+} available for release by Ins(1,4,5) P_3 was reproducible when Ins(1,4,5) P_3 was added at a fixed time after initiation of Ca^{2+} -pump inhibition. Under these conditions GSSG was still effective in enhancing the sensitivity to Ins(1,4,5) P_3 , especially when the Ca^{2+} released by 100 nM-Ins(1,4,5) P_3 was expressed as a ratio of the total Ca^{2+} which could be released by a maximal Ins(1,4,5) P_3 dose ('Release ratio' of Table 2).

DISCUSSION

The ability of GSSG to increase Ins(1,4,5) P_3 -sensitivity represents a new mechanism by which the Ins(1,4,5) P_3 -operated Ca^{2+} release system can be modulated. The time course of the effect of GSSG is consistent with the observed latent period

(2–5 min) before the initiation of Ca^{2+} oscillations after TBHP addition to hepatocytes [4]. Furthermore, the concentration range over which GSSG is effective in permeabilized cells is similar to the amounts of GSSG generated in TBHP-treated hepatocytes, as well as under other conditions which result in high glutathione peroxidase activity [6,7,11,12]. Thus it is possible that $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity in intact cells could be modulated in response to changes in cellular redox state. The extent to which this might occur under physiological conditions will depend on the redox potential of the relevant thiol group(s). Although it is difficult to obtain an accurate measure of this in permeabilized hepatocytes, our data suggest that $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity is most likely to be affected under conditions of oxidative stress. Smaller shifts in the GSH/GSSG ratio, such as occur during starvation [19], probably would not modify $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity, but a definitive answer to this question will depend on the isolation of the relevant GSSG-sensitive component for studies of the redox potential of the relevant thiol group. In addition to the possible physiological significance of the present findings, these data also provide a new tool for mechanistic studies of the $\text{Ins}(1,4,5)\text{P}_3$ -activated Ca^{2+} -release mechanism. Indeed, Missiaen *et al.* [21] recently utilized GSSG in a study of $\text{Ins}(1,4,5)\text{P}_3$ -dependent spontaneous Ca^{2+} release to facilitate the actions of endogenous $\text{Ins}(1,4,5)\text{P}_3$.

In principle, GSSG could enhance the apparent sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ in three ways. It could modify the $\text{Ins}(1,4,5)\text{P}_3$ receptor directly, it could alter the effective concentration of $\text{Ins}(1,4,5)\text{P}_3$ by inhibiting the metabolism of this second messenger, or it could inhibit the ATP-dependent Ca^{2+} pump which opposes the Ca^{2+} -release process. Since GSSG had no effect on $\text{Ins}(1,4,5)\text{P}_3$ metabolism and was still effective under conditions where the Ca^{2+} pumps were inactive, it appears most likely that GSSG acts at the level of the $\text{Ins}(1,4,5)\text{P}_3$ receptor/channel. The effect of GSSG on $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity can be mimicked by cystine, but not by GSH or cysteine. Together with the finding that DTT reverses the GSSG effect, these data suggest that the mechanism of GSSG action is dependent on the formation of mixed disulphides with a protein component of the Ca^{2+} -release system. Several studies have provided evidence for critical thiol groups involved in $\text{Ins}(1,4,5)\text{P}_3$ action. The alkylating agents *N*-ethylmaleimide and iodoacetamide, as well as organic mercurials such as pCMB and pCMBs, decrease $\text{Ins}(1,4,5)\text{P}_3$ binding and inhibit Ca^{2+} release in microsomal preparations from a variety of tissues [13,14,16]. Although in the present study DTT (2 mM) had no effect on its own, other workers have reported that high concentrations (10–200 mM) can decrease $\text{Ins}(1,4,5)\text{P}_3$ affinity in adrenal-cortex microsomes [14]. Thus it appears that Ca^{2+} release mediated by the $\text{Ins}(1,4,5)\text{P}_3$ receptor is dependent on the chemical status of one or more thiol groups; alkylation blocks $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release, under reducing conditions the receptor has a low affinity for $\text{Ins}(1,4,5)\text{P}_3$, and when it reacts with oxidized thiols the sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ is enhanced.

The effect of GSSG observed in the present study is reminiscent of the recently reported cyclic-AMP-dependent enhancement of $\text{Ins}(1,4,5)\text{P}_3$ -sensitivity for Ca^{2+} release in hepatocytes [22]. In our studies the effects of GSSG were not affected by incubation with cyclic-AMP-dependent protein kinase inhibitor (results not shown). However, it is possible that GSSG and the cyclic-AMP-mediated effects on the hepatocyte $\text{Ins}(1,4,5)\text{P}_3$ receptor could bring about similar conformational changes, resulting in similar

effects on Ca^{2+} release. It is also of interest that the binding affinity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor can be modulated by extraluminal Ca^{2+} [23–25]. However, the Ca^{2+} -induced enhancement of $\text{Ins}(1,4,5)\text{P}_3$ binding is associated with a desensitization of the Ca^{2+} -release process that has been postulated to involve the conversion of the $\text{Ins}(1,4,5)\text{P}_3$ -activated channel into an inactive form [24,26,27]. This suggests that the effects of Ca^{2+} are distinct from those of GSSG. This conclusion is supported by our finding that GSSG still enhanced $\text{Ins}(1,4,5)\text{P}_3$ sensitivity when the free Ca^{2+} in the medium was buffered to less than 10 nM. The data presented here suggest that the locus of GSSG action on the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} -release system may represent an additional site at which the magnitude of hormone-induced $[\text{Ca}^{2+}]_i$ changes can be modulated independently of the dose of the hormone.

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