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A sensitization of inositol 1,4,5-trisphosphate receptor (IP₃R)-mediated Ca²⁺ release is associated with oxidative stress in multiple cell types. These effects are thought to be mediated by alterations in the redox state of critical thiols in the IP₃R, but this has not been directly demonstrated in intact cells. Here, we utilized a combination of gel-shift assays with MPEG-maleimides and LC-MS/MS to monitor the redox state of recombinant IP₃R1 expressed in HEK293 cells. We found that under basal conditions, ~5 of the 60 cysteines are oxidized in IP₃R1. Cell treatment with 50 μM thimerosal altered gel shifts, indicating oxidation of ~20 cysteines. By contrast, the shifts induced by 0.5 mM H₂O₂ or other oxidants were much smaller. Monitoring of biotin-maleimide attachment to IP₃R1 by LC-MS/MS with 71% coverage of the receptor sequence revealed modification of two cytosolic (Cys-292 and Cys-1415) and two intraluminal cysteines (Cys-2496 and Cys-2533) under basal conditions. The thimerosal treatment modified an additional eleven cysteines, but only three (Cys-206, Cys-767, and Cys-1459) were consistently oxidized in multiple experiments. H₂O₂ also oxidized Cys-206 and additionally oxidized two residues not modified by thimerosal (Cys-214 and Cys-1397). Potentiation of IP₃R channel function by oxidants was measured with cysteine variants transfected into a HEK293 IP₃R triple-knockout cell line, indicating that the functionally relevant redox-sensitive cysteines are predominantly clustered within the N-terminal suppressor domain of IP₃R. To our knowledge, this study is the first that has used proteomic methods to assess the redox state of individual thiols in IP₃R in intact cells.

Inositol 1,4,5-trisphosphate receptor (IP₃R)² channels serve as the principal mechanism to mobilize Ca²⁺ from endoplasmic reticulum (ER) stores in response to cell stimulation by many hormones, growth factors, and neurotransmitters. The

three isoforms of the IP₃R family contain an N-terminal ligand-binding domain, a central regulatory domain, and a C-terminal channel domain. The ligand-binding domain contains a suppressor domain (SD; residues 1–223) that acts to inhibit IP₃ binding to the core binding domain (residues 224–604). Channel opening is stimulated when IP₃ levels are elevated during agonist stimulation. An alternative mode of channel opening results from regulatory modulation of IP₃Rs, which sensitize channels to basal or modest changes in IP₃ levels. A well-documented example of IP₃R sensitization results from channel oxidation produced by exogenous or endogenous redox modulators. The first redox agent reported to sensitize IP₃R channels was the organomercurial preservative thimerosal (reviewed in Ref. 1). Other compounds with similar effects include *t*-butylhydroperoxide (2), menadione (3), H₂O₂ (4), 2,2'-dithiodipyridine (5), diamide (6, 7), oxidized GSH (8, 9), and superoxide-generating systems such as xanthine/xanthine oxidase (10–13). In the case of thimerosal, the effects on purified reconstituted receptors suggest a direct reaction with thiol groups on the channel (14, 15). The recruitment of multiple IP₃R channels in isolated patches has been shown to be regulated by the redox state of the receptor (16). The effects of prolonged ER stress and enhanced translocation of Ca²⁺ into mitochondria has also been linked to altered IP₃R redox state as a result of hyperoxidation of the ER lumen promoting oxidation of critical intraluminal thiols of the IP₃R (17–19).

Although there is substantial evidence for the regulation of IP₃Rs by redox state, there is very little known regarding which IP₃R thiols are involved, the types of thiol modifications occurring in the intact cell, or how the channel is sensitized by redox modifications. Although changes in IP₃R redox state are presumed to occur, they have never been directly measured. The present study is the first to measure the redox state of IP₃R in intact cells treated with various oxidants and to apply proteomic methods to interrogate the redox state of individual thiols in the IP₃R1 isoform, which contains a total of 60 cysteines. These studies identify 4 oxidized thiols under basal conditions and an additional 11 or 4 thiols, which become oxidized in cells exposed to thimerosal or H₂O₂, respectively. The functional relevance of these oxidations was investigated using cysteine mutants transfected into a HEK293 IP₃R triple-knockout cell line. The results show that the redox-sensitive cysteines required for IP₃R1 channel

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² The abbreviations used are: IP₃R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; SD, suppressor domain; IAM, iodoacetamide; BSO, buthionine sulfoximine; 3KO, triple-knockout; DB, denaturing buffer.

potentiation are predominantly clustered within the N-terminal suppressor domain.

Results

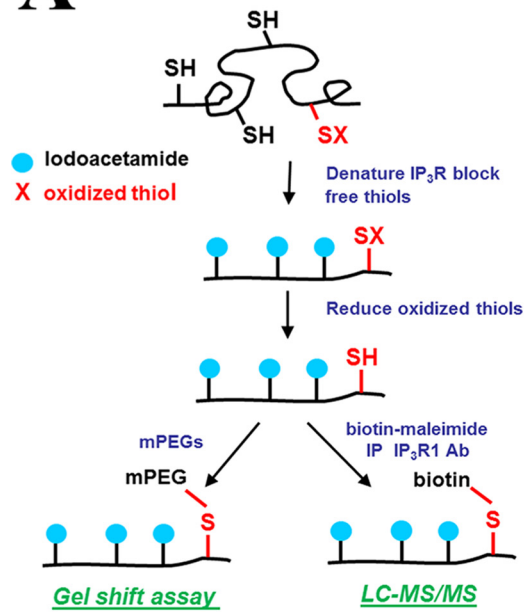
We have adapted a method originally described by Leichert and Jakob (20, 21) to measure the redox state of IP₃R_s *in situ* (Fig. 1A). The critical initial step is the lysis of cells directly in 10% TCA to prevent any alteration of thiol status. Precipitated proteins are solubilized under strongly denaturing conditions (0.5% SDS, 6 M urea), and free thiols are blocked irreversibly by reaction with 10 mM iodoacetamide (IAM). The samples are then reprecipitated with TCA, and any oxidized thiols are reduced with 10 mM DTT. A further TCA precipitation was used to remove the DTT, and free thiols on the receptor were reacted with PEG-maleimides of different sizes (2, 5, and 20 kDa). The resulting gel shifts can be measured on SDS-PAGE after immunoblotting for IP₃R. HEK293 cells were used as an experimental system. These cells contain very low levels of IP₃R1 and endogenously express IP₃R2 and IP₃R3 (22). Data from gel-shift assays are shown in Fig. 1 (B–E) for HEK293 cells transfected with IP₃R1. Reproducible, small shifts are observed with both MPEG-2 and MPEG-5 under basal conditions (Fig. 1B). These shifts are not artifactual because they are blocked by inclusion of DTT during the MPEG reactions (Fig. 1B) or by DTT pretreatment of the intact cells prior to lysate preparation (Fig. 1C, lanes 1–3). The addition of the oxidants H₂O₂ (0.5 mM; Fig. 1C, lanes 7–9) or thimerosal (50 μM; Fig. 1C, lanes 10–12) enhanced the shift observed with both MPEG-2 and MPEG-5. Thimerosal produced larger changes than H₂O₂, and the effect of both oxidants were not additive (Fig. 1C, lanes 13–15). There are 60 cysteines in the primary sequence of the rat IP₃R1. Lysates prepared in the absence of IAM treatment allow maximum reactivity to be measured, but shifted bands were retained on the gel only for the small MPEG-2 (Fig. 1D). In principle, given the molecular weight of the MPEGs, the total number of cysteines involved can be determined from the magnitude of the shift, but quantitation is subject to several inaccuracies and assumptions. One of these is the unusual hydrodynamic properties of MPEG-2 and MPEG-5 that produces gel shifts on SDS-PAGE corresponding to 5 and 15 kDa, respectively (23, 24). Taking this into account, the shifts with either MPEG-2 or MPEG-5 correspond to the presence of ~5 oxidized cysteines/IP₃R1 subunit under basal conditions and ~10 and ~20 oxidized cysteines/subunit in the presence of H₂O₂ and thimerosal, respectively (Fig. 1E). Previous studies have suggested that IP₃R_s in hepatocytes are stimulated by GSSG (25), and IP₃R_s are reported to be glutathionylated in response to diamide in endothelial cells (6, 7). Changes in the GSH redox status of HEK293 cells in response to H₂O₂ and thimerosal are shown in Fig. 2A. Both thimerosal and H₂O₂ caused similar increases in GSSG levels, but only thimerosal caused a large drop in total glutathione (GSH + GSSG). In contrast to H₂O₂, thimerosal forms an ethyl mercury adduct with reduced thiols and is known to deplete GSH in other systems (26). Preincubation of cells with buthionine sulfoximine (BSO, an inhibitor of GSH biosynthesis) reduced total GSH and GSSG levels but did not alter basal, H₂O₂, or thimerosal-induced gel shifts in IP₃R (Fig. 2B). Diamide selectively oxidizes GSH to GSSG (27). An IP₃R gel

shift was observed at concentrations of diamide (50 μM) that did not produce detectable elevations of GSSG (Fig. 2C). Higher diamide concentrations (250 μM), which caused large elevations of GSSG, only produced a small additional gel shift (Fig. 2C). Diamide and other oxidants such as menadione and *t*-butylhydroperoxide were no more effective than H₂O₂ (not shown). Overall, the data do not show a clear correlation of IP₃R oxidation with the GSH redox state. Rapid reversibility of both the total GSH levels (Fig. 2D) and the gel shifts were observed when DTT was added to the cells after the thimerosal (Fig. 2E).

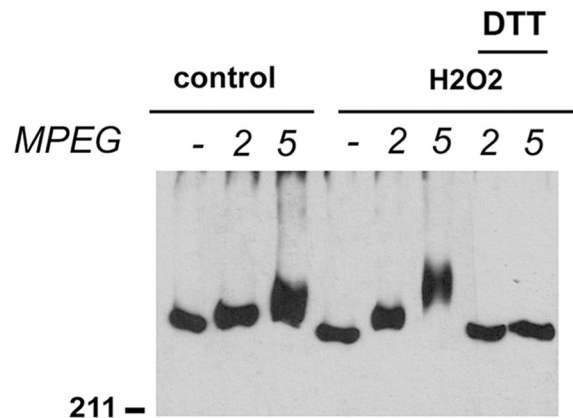
To get a comprehensive picture of the status of individual IP₃R cysteines, we adopted a proteomic approach in which the denatured cell lysates prepared after sequential iodoacetamide and DTT treatment were incubated with biotin-maleimide instead of PEG-maleimide (Fig. 1A). The modified IP₃R1 was then immunoprecipitated from the lysate, separated on SDS-PAGE, and silver-stained. The excised band was subjected to in-gel digestion with trypsin and processed for LC-MS/MS. The mass spectra were interrogated for cysteine containing peptides that were either modified with iodoacetamide (corresponding to reduced thiols) or modified with biotin-maleimide (corresponding to oxidized thiols). The compiled results from three independent experiments derived from control and thimerosal-treated cells are shown in Fig. 3 and Table 1. The mean sequence coverage was 71 ± 2% and resulted in the detection of 41 of 60 total cysteines when trypsin was used for cleavage. We observed four cysteines that were oxidized under basal conditions: Cys-292, Cys-1415, Cys-2496, and Cys-2533. Two of these cysteines are cytosol-facing (Cys-292 and Cys-1415), and two are intraluminal (Cys-2496 and Cys-2533). Thus of the five intraluminal cysteines, only two were oxidized. In the presence of thimerosal, the cysteines oxidized under basal conditions remained oxidized, and an additional 11 cytosol-facing cysteines were identified as being oxidized. These were Cys-37, Cys-206, Cys-638, Cys-767, Cys-897, Cys-995, Cys-1298, Cys-1459, Cys-1522, Cys-1647, and Cys-1976. It should be noted, however, that only six of these cysteines were consistently oxidized in all three trials (boxed in Fig. 3 and underlined in Table 1). In the case of Cys-206 and Cys-2533, we were able to identify only the oxidized form of the peptides in the spectra from thimerosal-treated cells. However, with the other redox-sensitive cysteines, both the oxidized and reduced forms of the peptides were evident (Table 1). For several cysteines the observed oxidation events were rare and in some cases observed only once in the three trials. However, the frequency of oxidation events was much higher (35–100%) for the six cysteines that were reproducibly oxidized. Lysates from H₂O₂-treated cells were analyzed in two experiments (Fig. 3, yellow boxes, and Table 1). A total of six cysteines were oxidized in both trials with three residues found under basal conditions (Cys-1415, Cys-2496, and Cys-2533), and an additional three residues oxidized upon H₂O₂ addition (Cys-206, Cys-214, and Cys-1397). The residues repeatedly oxidized by thimerosal and H₂O₂ were conserved in all three IP₃R isoforms, with the exception of the thimerosal-sensitive residue Cys-1459, which was present only in the IP₃R1 isoform (Fig. 3).

To use a mutagenesis approach to assess the functional role of individual cysteines in redox-modulation of the IP₃R chan-

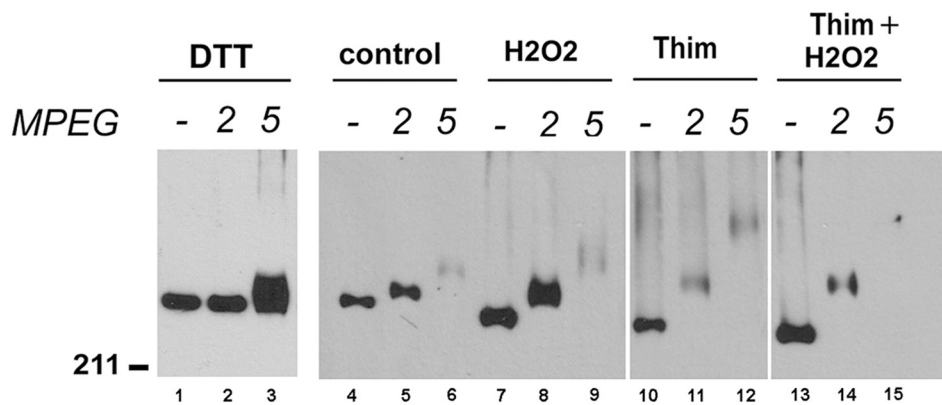
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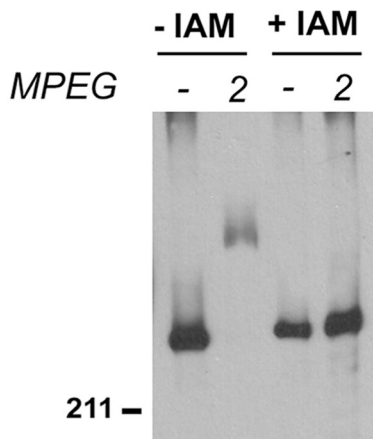
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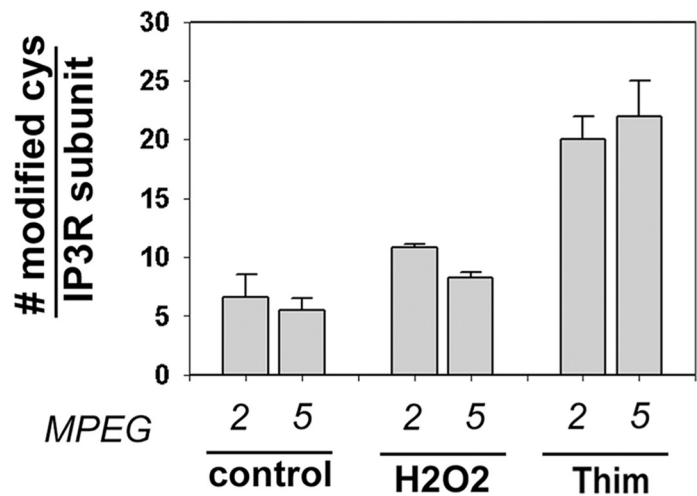
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E



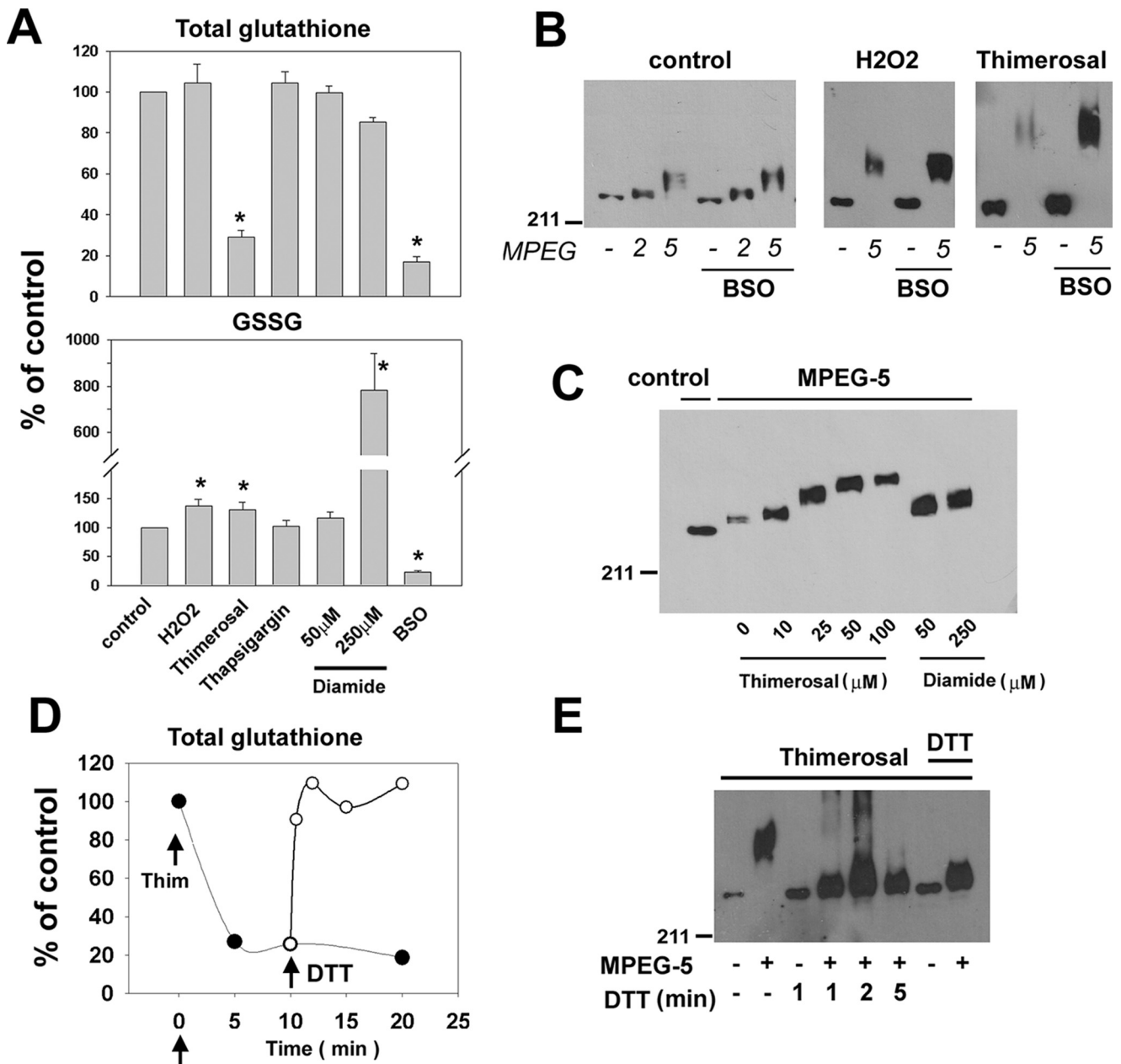


Figure 2. Additional characterization of IP₃R redox responses. *A*, total GSH and oxidized GSH was measured after treatment for 10 min with H₂O₂ (0.5 mM), thimerosal (50 μM), thapsigargin (1 μM), the indicated concentrations of diamide, and 16 h preincubation with 0.25 mM BSO. *B*, gel shift assays after BSO treatment. *C*, gel-shift dose responses for thimerosal and diamide. *D* and *E*, rapid DTT (10 mM) reversibility of thimerosal-induced changes in total GSH (*D*) and IP₃R (*E*) gel shifts. In the last two lanes, the DTT was added before the thimerosal.

nel, we chose to employ a recently created human HEK293 cell line from which all three IP₃R isoforms have been deleted by CRISPR (28). These 3KO cells do not elicit agonist or IP₃-mediated cytosolic Ca²⁺ signals (28, 29) as illustrated in Fig. 4*A* for stimulation with carbachol in the presence of extracellular Ca²⁺. By contrast, robust carbachol responses are observed in WT cells, and Ca²⁺ signals can be restored in 3KO cells by

transfection with IP₃R1 DNA (Fig. 4*B*). Comparable gel shifts in response to thimerosal (50 μM) were observed in IP₃R1 transfected WT or 3KO cells (not shown). To assay for redox potentiation of IP₃R1, we pretreated the cells for 2 min with thimerosal. In initial trials, we observed that the use of concentrations of thimerosal of >20 μM induced by itself a delayed increase of cytosolic Ca²⁺ (Fig. 4*C*). This presumably reflects sensitization

Figure 1. Gel-shift assays to measure IP₃R redox state. *A*, cartoon showing the sequential treatments employed in the two main assays used in this study. *B* and *C*, TCA precipitates solubilized from HEK293 cells transfected with IP₃R1 were sequentially treated with IAM and DTT and then reacted for 1 h with 0.5 mM MPEG-2 or MPEG-5. Gel shifts in IP₃R were assessed by immunoblotting. Treatment of cells was for 10 min with H₂O₂ (0.5 mM) and thimerosal (Thim, 50 μM). In *B*, DTT (10 mM) was added during the MPEG reaction, and in *C* (lanes 1–3), the cells were pretreated for 30 min with DTT before lysis. Lanes 4–15 are from the same blot, but a higher exposure of lanes 10–12 is shown for better visualization of the bands. *D*, IAM (10 mM) treatment of the lysates was omitted as a control. *E*, the number of reactive cysteines was calculated from the magnitude of the gel shift relative to molecular weight markers as described under “Materials and methods.” The data shown are the means ± S.E. for three to five measurements. For additional details see text.

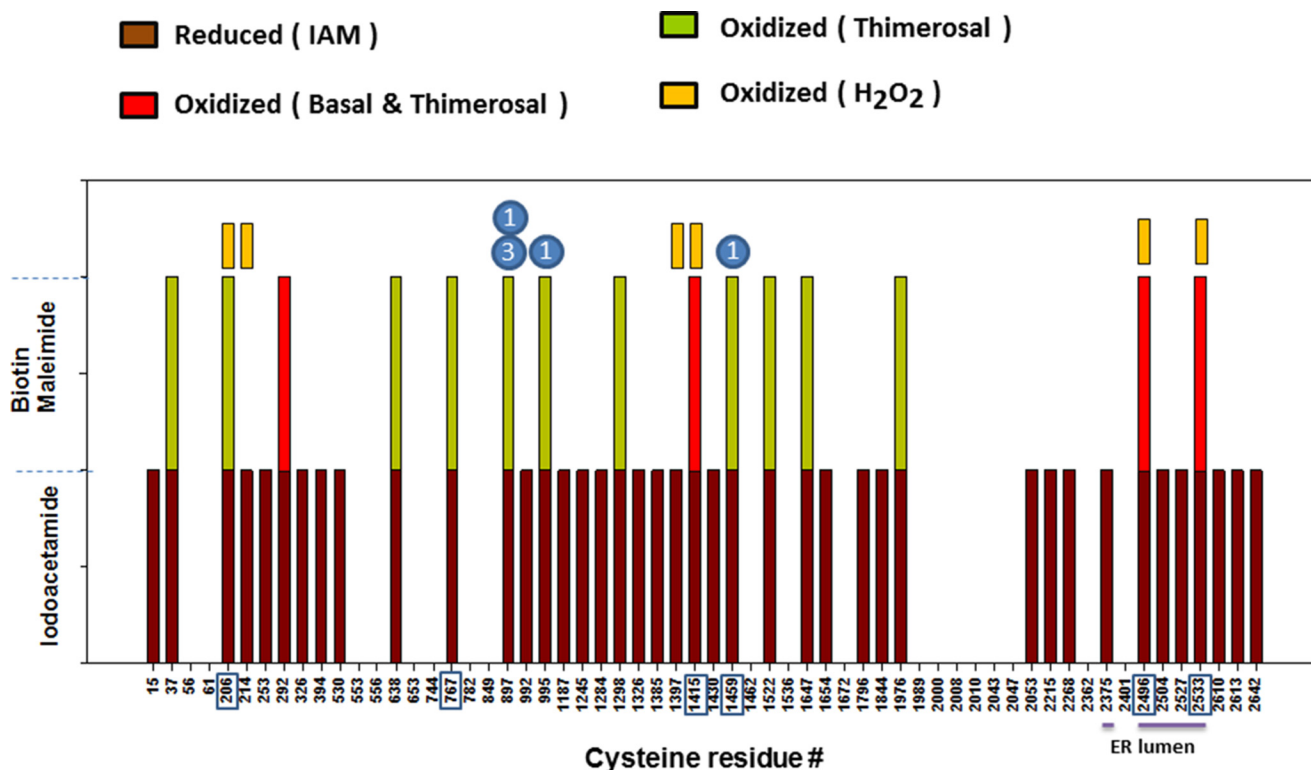


Figure 3. Identification of redox-sensitive thiols in IP₃R1 using LC-MS/MS. Redox lysates were prepared from transfected HEK293 cells treated in the presence and absence of thimerosal (50 μ M) and processed with IAM to block free thiols. Oxidized residues were liberated with DTT, and the lysate was TCA-precipitated again and reacted with biotin-maleimide (0.5 mM, 5 h). The lysates were processed on a Sephadex G25 column to exchange the sample into a buffer suitable for immunoprecipitation, which was carried out with a C-terminal Ab. The immunoprecipitates were processed on 5% SDS-PAGE, and the silver-stained IP₃R was excised and processed for LC-MS/MS. The spectra were analyzed with the SEQUEST search engine for tryptic peptides containing cysteines modified with IAM (reduced) or biotin-maleimide (oxidized). The boxed residues were identified in each of three independent trials. Oxidized residues observed in H₂O₂ (0.5 mM)-treated cells are indicated by yellow rectangles. Cysteine residues located in the ER lumen are underlined. All oxidized residues are conserved in all three isoforms with the exceptions shown in filled blue circles.

of IP₃R1 to endogenous IP₃ because the response to thimerosal was greatly blunted in untransfected 3KO cells (Fig. 4D). To be able to measure redox potentiation of carbachol responses, we chose to use a low concentration of thimerosal (10 μ M), which allowed a stable baseline to be maintained during thimerosal pretreatment prior to stimulation by carbachol. The carbachol addition was combined with EGTA to chelate extracellular Ca²⁺ and ensured that only intracellular Ca²⁺ mobilization was being measured. Under these conditions a thimerosal-induced potentiation of the Ca²⁺ signal was observed for WT IP₃R1 at low subsaturating concentrations of carbachol (Fig. 4E) but not at a maximal dose of carbachol (Fig. 4F). IP₃R1 in which the N-terminal 12 cysteines have been mutated to alanine (Cys-less) has been shown to be a functional channel that is insensitive to a stimulatory effect of thimerosal on [³H]IP₃ binding (30, 31). In agreement with these studies, the Cys-less mutant failed to show thimerosal-induced potentiation of IP₃R function in 3KO cells (Fig. 4, G and H). Thimerosal at higher concentrations has been found to inhibit IP₃-mediated Ca²⁺ release (32). It should be noted that in our studies, using 10–50 μ M thimerosal, only stimulatory effects were observed.

A selected group of cysteine mutants were further analyzed for redox potentiation. These mutants included the individual residues within the N-terminal Cys-less construct, as well as three residues that were reproducibly oxidized in the MS assays (C767S, C1415S, and C1459S), and a cysteine found only in the

SI(+) alternatively spliced form of IP₃R1 (C326S). Several of these mutants have been used in our previous studies (33). The expression of new mutants in 3KO cells was verified by Western blotting (Fig. 5A). To quantitate potentiation we measured the ratio of Ca²⁺ responses in the presence and absence of thimerosal pretreatment using 0.25 μ M carbachol as in Fig. 4C. The maximal response to 20 μ M carbachol was also measured and normalized to the maximal response observed with WT IP₃R1 DNA. The potentiation ratio and maximal responses for each of the mutants are shown in Fig. 5 (B and C), respectively. Five of the eleven mutants showed diminished potentiation by thimerosal. Of these, four showed a partial inhibition (C37S, C56S/C61S, C214S, and C326S), whereas C15S was almost as effective as the Cys-less in removing the potentiating effect of thimerosal. The majority of the mutants showed maximal carbachol responses that were comparable with the WT IP₃R1, although several had significantly lower (e.g. C37S) or higher (e.g. C15S) responses (Fig. 5C). The variation in maximal responses may reflect different levels of functional receptors in the ER stores that may not be evident at the level of immunoblots. It should be noted, however, that the maximal responses did not correlate with diminished redox potentiation.

The residues having no effects (colored orange in Fig. 5) include Cys-767, Cys-1415, and Cys-1459, which were all residues that were reproducibly oxidized in MS assays after thimerosal treatment, with Cys-1459 being constitutively oxidized

Table 1

The cysteine residues oxidized by thimerosal in three independent trials are shown in green

The numbers of occurrences of biotin maleimide-modified peptides (oxidized), carbidomethylated peptides (reduced), or unmodified peptides in these samples are tabulated with the number of experiments shown in parentheses. The underlined residues are where at least one occurrence of oxidized peptide was observed in all three trials. In three trials the cysteine residues that were oxidized under basal conditions were Cys-292, Cys-1415, Cys-2496, and Cys-2533. The numbers of occurrences of peptides containing these oxidized residues were 10 (2), 16 (3), 18 (3), and 6 (3) with the number of experiments shown in parentheses. The oxidized peptides in H₂O₂-treated cells were also tabulated for two independent trials.

Cys residue	#occurrences of oxidized peptide (thimerosal)	#occurrences of reduced peptide (thimerosal)	#occurrences of unmodified peptide (thimerosal)	#occurrences of oxidized peptide (H ₂ O ₂)
15	0	15 (2)	2 (1)	0
37	1 (1)	24 (3)	0	0
206	3 (3)	0	0	7 (2)
214	0	9 (2)	0	6 (2)
253	0	42 (3)	0	0
292	1 (1)	13	0	0
326	0	66 (3)	0	0
394	0	49 (3)	2 (1)	0
530	0	137 (3)	0	0
638	3 (2)	16 (3)	0	0
767	15 (3)	18 (3)	0	0
897	1 (1)	7 (3)	0	0
992	0	41 (2)	2 (1)	0
995	1 (1)	9 (2)	0	0
1187	0	122 (3)	0	0
1245	0	55 (3)	0	0
1284	0	35 (3)	0	0
1298	3 (2)	16 (2)	0	0
1326	0	53 (3)	0	0
1385	0	62 (3)	0	0
1397	0	69 (2)	0	2 (1)
1415	5 (3)	9 (2)	0	3 (2)
1430	0	42 (3)	0	0
1459	5 (3)	14 (3)	0	0
1522	1 (1)	8 (3)	0	0
1647	1 (1)	3 (2)	0	0
1654	0	37 (3)	0	0
1796	0	141 (3)	0	0
1844	0	24 (2)	0	0
1976	2 (1)	24 (3)	0	0
2053	0	6 (1)	0	0
2215	0	121 (3)	0	0
2268	0	17 (3)	1 (1)	0
2375	0	21 (3)	0	0
2496	6 (3)	4 (2)	0	14(2)
2504	0	12 (2)	0	0
2527	0	3 (2)	1 (1)	0
2533	6 (3)	0	0	3 (2)
2610	0	10 (3)	0	0
2613	0	3 (2)	0	0
2642	0	6 (2)	0	0

even under basal conditions (Fig. 3). Three residues that are included in the Cys-less mutant but did not appear in the mass spectra (Cys-530, Cys-553, and Cys-556) were also without effect on redox potentiation. The location of the redox-sensitive and -insensitive residues, color-coded as in Fig. 5B, have been mapped to the cryo-EM structure of IP₃R1 (34) in Fig. 5D. The main conclusion is that the redox-sensitive residues are clustered within the first 225 residues. This region has variously been referred to as the suppressor domain (or β -trefoil domain-1) and has been shown to be critical for channel gating (35–37). The structural implications of these findings are discussed in more detail below.

In the final series of experiments, we have examined the potentiating effects of H₂O₂ and also tested the redox sensitivity of the IP₃R2 and IP₃R3 isoforms (Fig. 6). A maximal dose of carbachol (25 μ M) elicited similar Ca²⁺ responses from IP₃R1 and IP₃R2 transfected cells and a 20% smaller response in IP₃R3 transfected cells (Fig. 6A). The dose response to carbachol was examined for all three isoforms (Fig. 6B). The EC₅₀ for carba-

chol for IP₃R1 (0.62 \pm 0.05 μ M) and IP₃R2 (0.66 \pm 0.07 μ M) were similar, although IP₃R3 (1.7 \pm 0.12 μ M) was somewhat less sensitive. This can be compared with the rank order of sensitivity IP₃R2 > IP₃R1 > IP₃R3 found in direct measurements with IP₃ in permeabilized DT40 3KO cells stably expressing different receptor isoforms (13, 38, 39). All of the cysteine mutants also showed similar IP₃ sensitivities as the WT IP₃R1 (not shown). This is illustrated for the C15S mutant (EC₅₀ = 0.76 \pm 0.02 μ M) in Fig. 6B. An N-terminal fusion protein containing the Cys-less mutant has also been shown to have a comparable IP₃-binding affinity as WT receptors (30). Thus the pronounced loss of redox potentiation seen with the C15S and Cys-less mutants (Fig. 5B) is unlikely to be related to an intrinsically different IP₃ sensitivity of the channel.

Thimerosal potentiated the carbachol response approximately equally with IP₃R1 and IP₃R2 isoforms but was much less effective with IP₃R3 (Fig. 6C). A similar isoform preference was observed when H₂O₂ (100 μ M) was used to potentiate carbachol responses. The lack of redox sensitivity of the IP₃R3 isoform is in agreement with previous findings in DT40 cells (13, 31). As observed with thimerosal, the C15S IP₃R1 mutant was insensitive to potentiation by H₂O₂.

Discussion

Although IP₃Rs have been established to be redox-sensitive channels, the present study is the first to directly measure changes in the redox state of the IP₃R protein in intact cells. To do this, we have utilized gel-shift assays and MS. Although the gel-shift assay is convenient and provides qualitative information, the method is also insensitive, because heterogeneous small shifts in a large protein such as IP₃Rs can be easily missed. Many membrane proteins are known to retain folded structures even in the presence of SDS and urea (40–42). It is therefore possible that not all thiols are freely accessible to the MPEG gel-shift reagent even in the “denaturing” conditions used in our experiments. The MS approach is more sensitive and provides more information. However, this method is limited by incomplete sequence coverage, which resulted in 19 cysteines not being observed in the mass spectrum when the sample was digested with trypsin. Interestingly, the same cysteines were missing in each attempt, including Cys-556 and a cluster of six cysteines located prior to the first transmembrane domain (Fig. 3). To increase coverage, a single trial was conducted with chymotrypsin as the cleavage enzyme, but the same cysteines were also absent in this analysis (not shown). Several recent studies have shown that both ryanodine receptors (43) and IP₃Rs (44) are subject to S-palmitoylation. The attachment of palmitic acid is known to interfere with the chromatography of trypsin peptides, making it difficult to identify modified residues by LC-MS/MS (45). Using MALDI-TOF, Cys-56 and Cys-849 were identified as sites of S-palmitoylation (44), providing a possible explanation for why peptides containing these sites were absent from the LC-MS/MS analysis. Overall, the limitations in coverage make it likely that the number of redox-sensitive thiols identified by MS is underestimated.

The redox state of the ER lumen is considerably more oxidized than the cytosol, based on the GSH redox couple (46). Therefore, it may be anticipated that the intraluminal thiols of

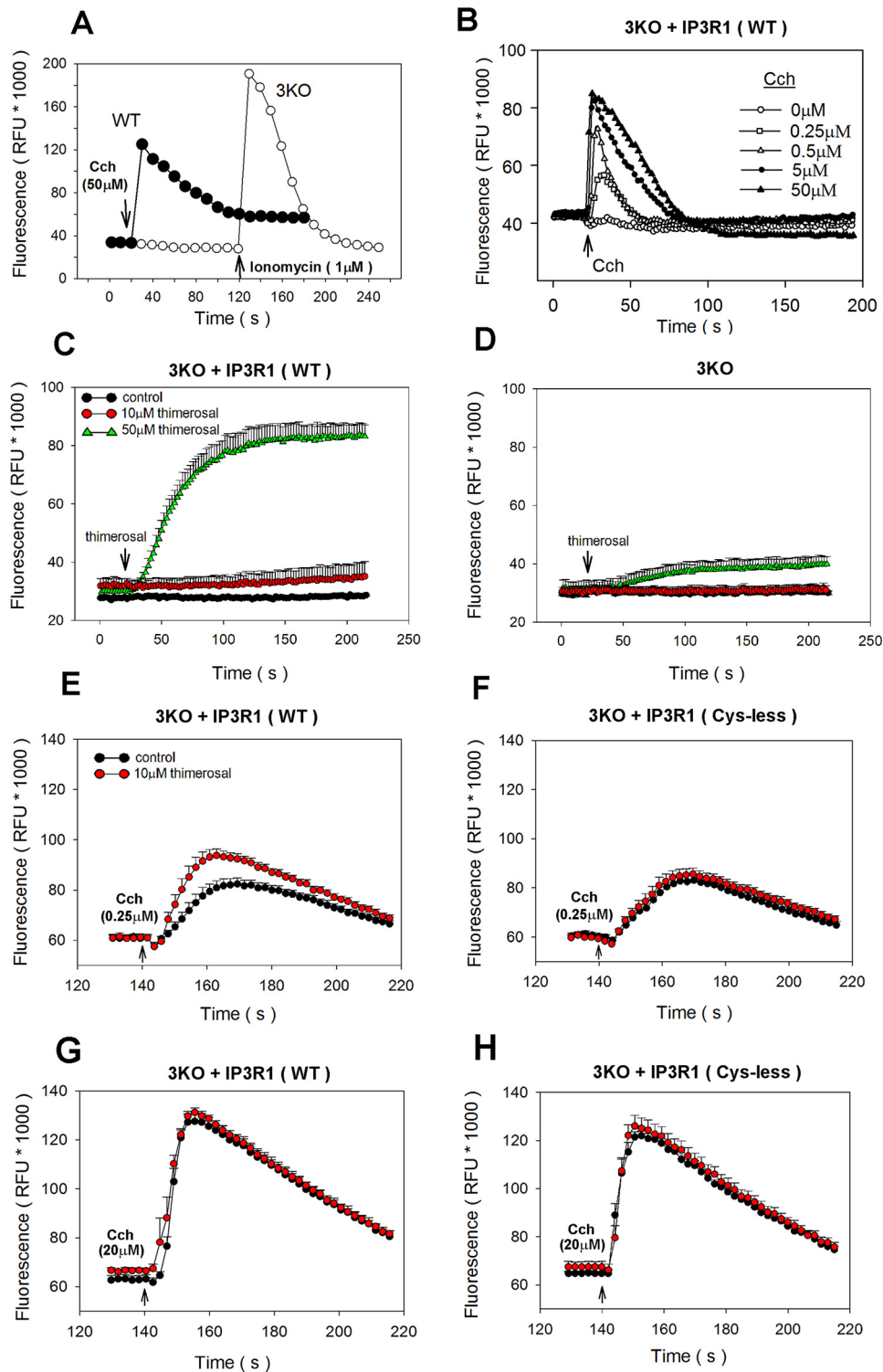


Figure 4. Measurements of carbachol-mediated cytosolic [Ca²⁺] in HEK293 IP₃R 3KO cells transfected with IP₃R1. Cytosolic free [Ca²⁺] changes were measured with Fluo-8 in 96-well plates on a Flex station apparatus as described under "Materials and methods." *A*, representative traces showing responses of WT and IP₃R 3KO HEK293 cells to a maximal dose of carbachol (Cch) in the presence of extracellular Ca²⁺. *B*, changes in cytosolic Ca²⁺ of 3KO cells transfected with IP₃R1 and stimulated with the indicated concentrations of carbachol. EGTA (1.3 mM) was added together with the carbachol to chelate extracellular Ca²⁺. *C*, thimerosal (10 or 50 μM) was added at 18 s to 3KO cells transfected with IP₃R1. Cytosolic Ca²⁺ changes are shown as the means ± S.D. of Fluo-8 fluorescence for four wells of a representative experiment. *D*, as in *C* but using untransfected 3KO cells. *E* and *G*, 3KO cells transfected with IP₃R1 were stimulated with a submaximal (*E*) or maximal dose (*G*) of carbachol in the absence of extracellular Ca²⁺ under control conditions (*black symbols*) or after pretreatment with 10 μM thimerosal (*red symbols*) for 2 min. *F* and *H*, as in *E* and *G* but with 3KO cells transfected with a cysteine-less IP₃R1 construct in which the first 12 N-terminal cysteines are mutated to alanine (31). The data shown for *E-H* are the mean ± S.D. of four wells from a representative experiment. Pooled data from independent experiments are shown in Fig. 5.

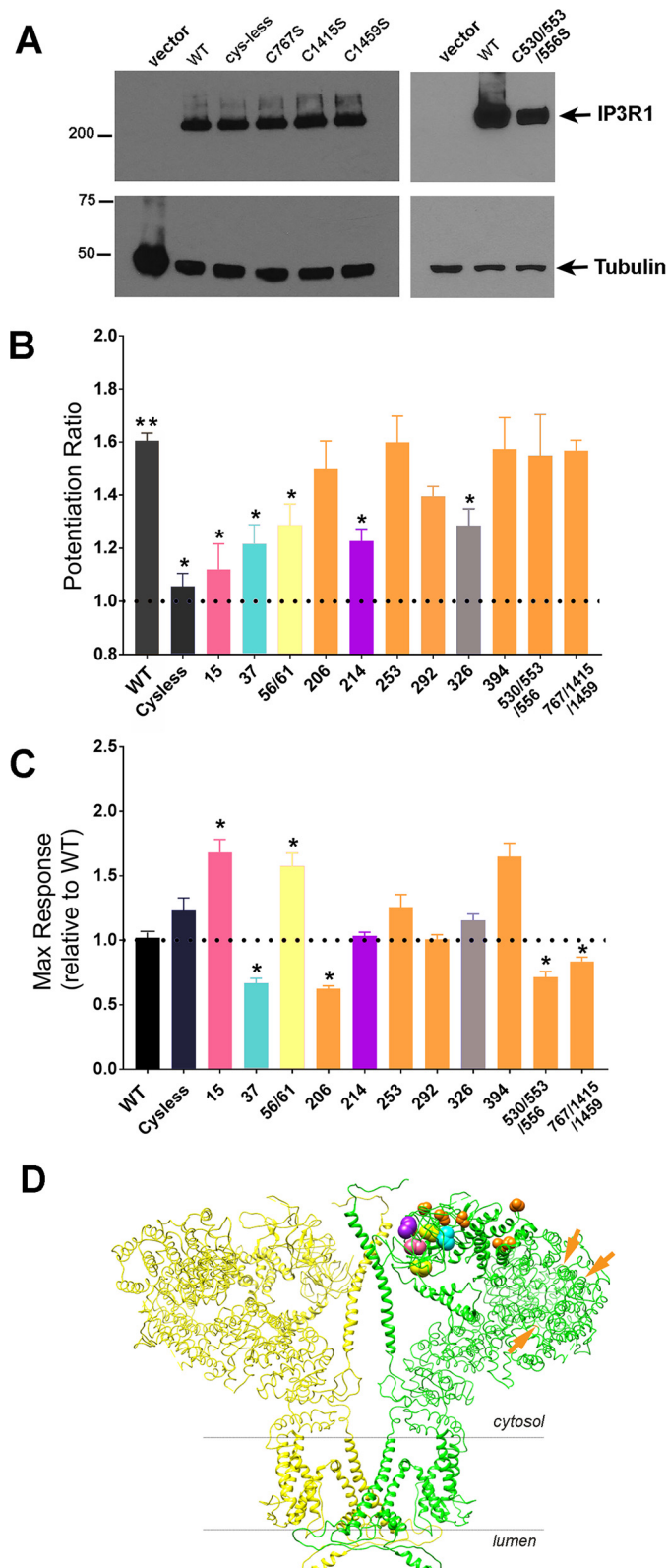


Figure 5. Quantitation of thimerosal potentiation of carbachol-mediated intracellular Ca²⁺ release for specific cysteine mutants of IP₃R1. *A*, expression of selected cysteine mutants in 3KO cells detected by immunoblotting with IP₃R1 Ab. The expression of the remaining cysteine mutants used in this study has been verified previously (33). *B*, the amplitude of the Ca²⁺ response to 0.25 μM carbachol after thimerosal treatment was expressed as a ratio to the control response using data obtained as shown in Fig. 4E. *C*, the response to a maximal dose of carbachol (20 μM) was quantitated for each mutant and expressed as a ratio of WT IP₃R1 responses. All data

the IP₃R would be predominantly oxidized. However, it is now understood that conditions favoring oxidative protein folding can occur under more reducing conditions and that the reactive thiols of key ER luminal oxidoreductases are predominantly in the reduced state (47–49). Indeed, under basal conditions only Cys-2496 and Cys-2533 of the five intraluminal IP₃R1 cysteines were found to be oxidized. The pair of intraluminal cysteines at Cys-2496 and Cys-2504 have previously been suggested to form a disulfide bond based on NMR studies of a fusion protein encoding the intraluminal loop (50). However, in our experiments Cys-2504 was consistently reduced, and therefore a disulfide bond between these residues is unlikely to occur in an intact cell under basal conditions. Both Cys-2496 and Cys-2504 have been implicated as important for association with ERp44, an intraluminal chaperone of the thioredoxin family, which selectively associates with the reduced form of IP₃R1 and has an inhibitory effect on channel function (51). Our results would suggest that ERp44 would preferentially associate with the Cys-2504 residue under basal conditions. The Cys-2533 residue is located at the intraluminal end of a pore helix, and mutation of this residue has previously been shown to inactivate channel function (52). A constitutively oxidized Cys-2533 residue may stabilize the intraluminal structure of the pore, possibly by formation of disulfide bonds with other ER luminal proteins. Neither thimerosal nor H₂O₂ changed the redox state of IP₃R intraluminal thiols.

Despite the reducing environment in the cytosol, a significant number of cysteines in key cytosolic signaling proteins are in the oxidized state (53). In the case of IP₃R1, two cytosol-facing cysteines (Cys-292 and Cys-1415) were found to be oxidized under basal conditions. This could indicate that these thiols have a sufficiently low pK_a to allow the formation of thiolate anions that can form disulfides with GSH or with other redox-sensitive proteins. It is also possible that IP₃Rs are present in a local environment that is more oxidizing than the bulk redox potential of the cytosol (54).

Mass spectroscopy indicated a total of 11 cytosolic cysteines that were modified in the presence of thimerosal. However, there was considerable variability in the results with some of the cysteines being modified in only one of three experiments. The reason for this variability is not clear but could be related to the experimental methodology and/or heterogeneity in the redox state of specific cysteines in the IP₃R. Only three cysteines were consistently oxidized by thimerosal in all three experiments, and they were Cys-206, Cys-767, and Cys-1459. In the case of H₂O₂, there were also three cysteines that were oxidized in each of two experiments. They were Cys-206, Cys-214, and Cys-1397. Cys-206 and Cys-214 are located in the SD, whereas Cys-767, Cys-1397, and Cys-1459 are located in the regulatory domain. To assess the functional relevance of these

are the means ± S.E. of *n* = 3–6 independent experiments. *, *p* < 0.001 significantly different from WT; **, *p* < 0.0001 significantly different from 1 (one sample *t* test). *D*, two opposing subunits of the tetrameric structure of apo-IP₃R1 determined by cryo-EM (Protein Data Bank (PDB) ID 3JAV) are shown in a side view. The redox-sensitive cysteine residues are color coded according to the data in *B*. Redox-insensitive residues are colored orange. Side chains for Cys-767, Cys-1415, and Cys-1459 were not resolved in the structure and are indicated by orange arrows. Cys-326, which is located in the SI splice site, was also not present in the structure.

Redox regulation of IP₃ receptors

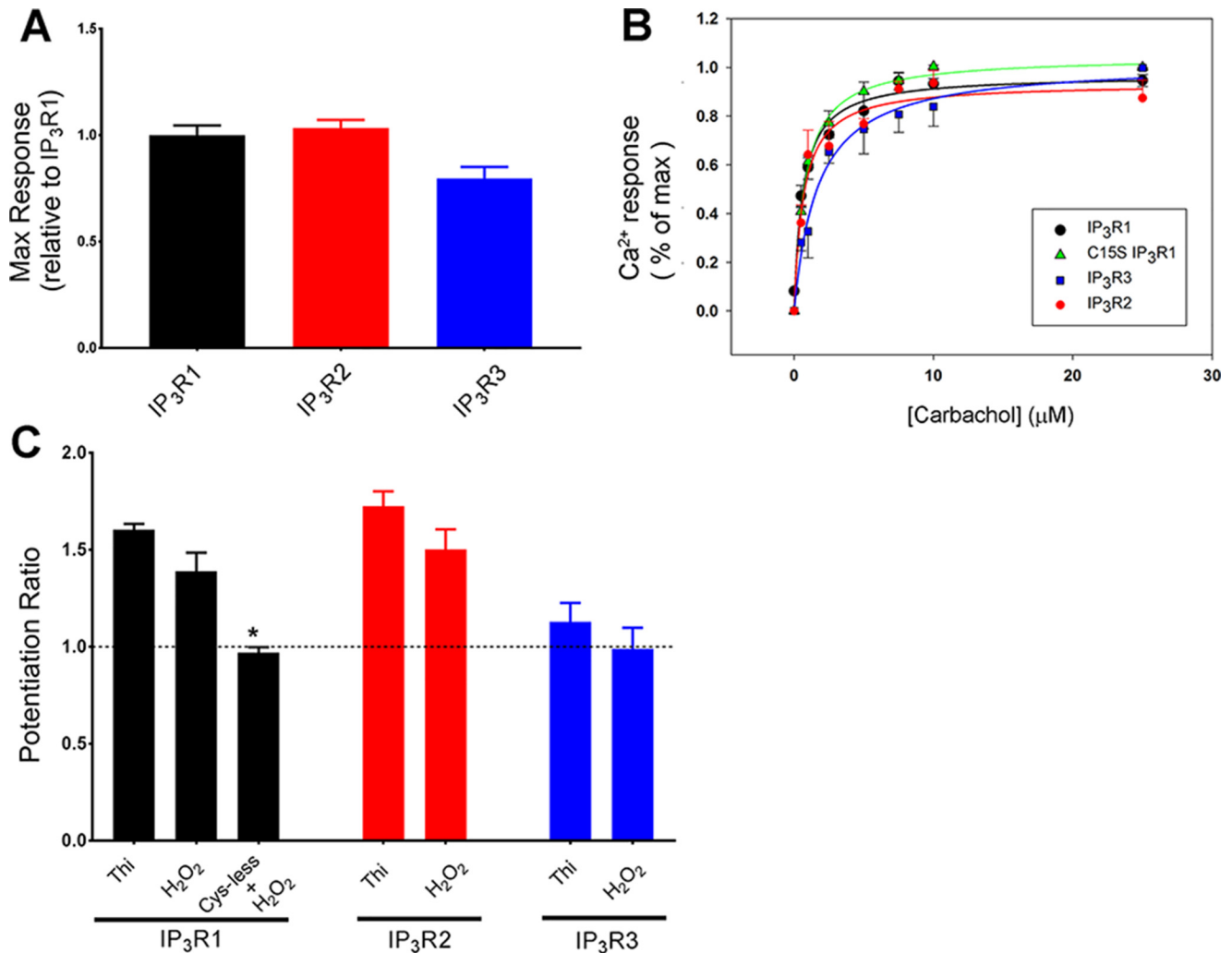


Figure 6. Redox potentiation by thimerosal and H₂O₂ of IP₃R1, IP₃R2, and IP₃R3. A, the three different IP₃R isoforms were transfected into HEK293 3KO cells (10 μg of DNA/60-cm plate) and the maximal Ca²⁺ response obtained with 25 μM carbachol in each experiment was quantitated relative to the maximal response for IP₃R1. B, intracellular Ca²⁺ release was measured using different concentrations of carbachol. Also shown are the responses of the C15S IP₃R1 mutant. The amplitude of the Ca²⁺ response was normalized to the maximum and is shown as means ± S.E. (n = 3). The lines drawn through the data points are fits to the equation $y = ax/(b + x)$, where b corresponds to the EC₅₀ values (given in the text). C, the 3KO cells transfected with the three different isoforms were pretreated for 2 min with either 10 μM thimerosal (Thi) or 100 μM H₂O₂, and the potentiation of responses to 0.25 μM carbachol or 0.5 μM carbachol (IP₃R3) was measured as described in Fig. 5B. The data shown are means ± S.E. of three to five independent experiments. *, $p < 0.05$ statistical significant from thimerosal for each isoform.

residues, we have used an assay in which cysteine mutants were transfected into HEK293 IP₃R 3KO cells. Redox potentiation was measured by preincubating cells for 2 min with oxidant prior to stimulation of intracellular Ca²⁺ mobilization with a subsaturating concentration of carbachol. These studies revealed that redox sensitivity was lost in an IP₃R1 mutant lacking the N-terminal 12 cysteines. Further mutagenesis of the individual cysteines indicates that multiple cysteines confer redox sensitivity to thimerosal. With the exception of Cys-326, all the redox-sensitive residues are clustered in the SD (Fig. 5D).

The SD (also referred to as the β-trefoil domain 1 (34)) plays a critical role in channel gating (35–37). This domain makes contacts with two adjacent downstream domains called the β-trefoil domain-2 and the armadillo solenoid fold-1 domain, which together constitute the inner binding core that specifically binds IP₃ (30). The cryo-EM structures of apo-IP₃R1 (34) and multiple liganded form of IP₃R3 (55) have been reported. Long-range conformational changes resulting from IP₃ binding

can be transmitted to the channel domain by several mechanisms (34, 55). This includes changes in the relative position of a loop variously referred to as a “hot spot” or “gating loop” within the SD that contains the Tyr-167 residue. Mutations within the Tyr-167 loop disrupt the coupling between IP₃ binding and channel opening (56). We suggest that the oxidation of specific cysteines in the SD may enhance the coupling efficiency of the mechanisms by which the SD transduces the ligand-binding signal to the channel domain. This is consistent with earlier studies that found an interaction between recombinant SD and inner binding core proteins that was strengthened by adding thimerosal (32). A more detailed understanding of the molecular mechanism of redox potentiation of the IP₃R channel awaits high resolution structures of the open state of the IP₃R1 channel. Oxidation of key thiols could also influence channel activity indirectly by modifying interactions of the receptor with its many binding partners. For example, one of the interaction sites of Bcl-2 encompasses the H₂O₂-sensitive

Cys-1397 (57). Interestingly, an R36C mutation within the SD has been identified in three affected individuals with spinocerebellar ataxia (58). This mutation would place a cysteine immediately adjacent to the redox-sensitive Cys-37 residue. Unlike other inactivating IP₃R1 mutations linked to inherited neurodegenerative diseases, this mutation results in a gain of channel function. The redox regulation properties of the R36C mutant remain to be investigated.

An additional finding in the present study was that the results from MS and functional assays were not always well-correlated. For example, several residues were reproducibly oxidized by thimerosal in the MS measurements but played no role when tested in functional assays (e.g. Cys-206, Cys-767, Cys-1415, and Cys-1459). Cys-206 has previously been shown to be a highly accessible cysteine (33) and was oxidized by both thimerosal and H₂O₂. However, robust oxidation of specific thiols may simply reflect their higher accessibility/reactivity and may not necessarily result in altered channel function. There were also several cysteine residues that contributed to redox potentiation in functional measurements that were not detected as being oxidized in the MS assay (e.g. Cys-15 and Cys-326). A functional effect obtained from mutating a particular cysteine residue does not prove that this residue is the physiological target of oxidants in the WT receptor. Although there may be limitations regarding sensitivity and coverage, the MS analysis does accurately identify the redox-sensitive thiols of the IP₃R *in situ*.

Although this study focused primarily on IP₃R1, we have also made some functional measurements on IP₃R2 and IP₃R3 expressed in HEK293 3KO cells. In line with previous studies on DT40 cells (31, 32), we observed reduced potentiation of IP₃R3 by thimerosal and H₂O₂ relative to IP₃R1 or IP₃R2 (Fig. 6). Five of the six cysteines present in the SD of IP₃R1 are highly conserved in all three isoforms; only Cys-15 is unique to IP₃R1, and its mutation markedly reduces redox potentiation of the IP₃R1 channel by either H₂O₂ or thimerosal. Although the remaining five cysteines are conserved, it is also apparent from IP₃-binding studies with chimeric receptors that the SD of IP₃R3 is unique and is not interchangeable with the other two isoforms (35, 59). Thus differences in the precise intra- and intermolecular contacts made by the SD may determine differences in redox regulation of the three isoforms. We also cannot exclude the possibility that other nonconserved cysteine residues outside of the SD may play a role in redox regulation of IP₃R2 and IP₃R3. Clearly other cell and species-specific regulatory factors can also play a role because previous studies on DT40 cells have noted that the endogenous chicken IP₃R3 and stably expressed rat IP₃R3 differ in their redox sensitivity (13). Different redox microenvironments (54) or variable antioxidant defenses could also contribute to differential sensitivity of IP₃R isoforms in different cell types.

Redox regulation of IP₃R-mediated Ca²⁺ signaling has been implicated in ER stress and apoptosis (19, 60–63). The redox-insensitive mutants identified in this study may be potentially useful in examining the contribution of IP₃R redox regulation in different models of cellular injury.

Materials and methods

Gel-shift assays for measurements of IP₃R redox state

The method used was a modification of the procedure described by Leichert and Jacob (20, 21). The key step is the quenching of the cellular redox state by rapid acidification of the cells with 10% TCA (Fig. 1A). The cells were scraped from the plates and centrifuged. The protein precipitate was dissolved in a strongly denaturing buffer (DB) containing 200 mM Tris-HCl (pH 8.5), 10 mM EDTA, 0.5% SDS, and 6 M urea. The lysate was incubated in this buffer supplemented with 10 mM iodoacetamide for 45 min to block all available free thiols. The lysate was then reprecipitated with 10% TCA and resuspended in DB buffer containing 10 mM DTT and incubated for a further 45 min. After reprecipitation with 10% TCA, the pellet was resuspended in 0.25 ml of DB buffer and adjusted to pH 7.0 with NaOH. Lysate protein (15 μg) was incubated with 0.5 mM PEG-maleimides of various sizes (2, 5, and 20 kDa) for 1 h. After SDS-PAGE on 5% gels for 2.5 h, the samples were transferred to nitrocellulose and immunoblotted with a C-terminal Ab specific for IP₃R1 raised to the C-terminal 18 amino acids (64). The size of the MPEG-shifted bands was estimated by comparing the relative mobility of the bands to standards retained on the 5% gels including the unmodified IP₃R1 subunit (320 kDa), a lysate from a stable HEK293 cell expressing a concatenated dimer of IP₃R1 (640 kDa) (65), prestained myosin (211 kDa), and prestained β-gal (114 kDa).

GSH assays

Total GSH was measured in the supernatant of TCA-quenched cell lysates using an enzymatic recycling assay utilizing 5,5'-dithiobis(nitrobenzoic acid), NADPH, and GSH reductase as originally described (66). Oxidized GSH was measured with a lumiglo kit obtained from Promega (Madison, WI). HEK293 cells (5 × 10⁴) were grown for 24 h in 96-well plates and treated with various oxidants. The cells were lysed on the plate using a supplied buffer containing NEM to block GSH. Residual GSSG was converted to GSH and measured using a luciferin probe as described by the manufacturer.

Mass spectroscopy

HEK293 cells were transfected with plasmid encoding rat IP₃R1 for 48 h. The cells were treated in the presence or absence of 50 μM thimerosal for 10 min. For each condition two 150-mm plates were used. After removal of the medium, each plate was quenched in 2.5 ml of 10% TCA. The precipitated protein was sequentially treated with iodoacetamide and DTT as described above for the gel-shift assays. The final DTT-treated lysate was solubilized in DB buffer and adjusted to a pH of 7.0. The lysate (1 mg of protein) was incubated with 0.5 mM biotin-maleimide (Sigma) for 16 h at room temperature in the dark. The reaction was terminated with 10 mM DTT, and the entire reaction was loaded on a PD-10 desalting column (GE Healthcare) equilibrated in a buffer containing 150 mM NaCl, 20 mM NaMOPS (pH 7.0), 0.5 mM EDTA, 0.2 mM DTT, 0.1% deoxycholate, and 0.2% Triton X-100. This buffer exchange step was carried out to reduce the SDS and urea concentrations to allow more efficient immunoprecipitation. The eluted pro-

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tein was incubated with Ab to the C terminus of IP₃R1 and protein A–Sepharose overnight. Immunocomplexes were processed on 5% SDS–PAGE and stained with silver (SilverSNAP stain; Thermo Fisher Scientific) or with colloidal blue (Novex). The excised band above the myosin marker was processed for in-gel trypsin digestion and LC–MS/MS using a Thermo Q Exactive Plus mass spectrometer at the Wistar Proteomics facility (Wistar Institute, Philadelphia, PA). Peptide identification was achieved by searching against the rat IP₃R1 (NP_001007236.2) and the nonredundant human database from the National Center for Biotechnology Information. Mass accuracy was limited to 10 ppm for precursor ions and 0.6 Da for product ions, with tryptic enzyme specificity and up to two missed cleavages. Variable modifications included cysteine alkylation by iodoacetamide (+57.02 Da) and reaction with biotin–maleimide (+451.54 Da) (67).

Cytosolic [Ca²⁺] measurement in HEK293 cells

The cells were grown in 60-cm plates to ~70% confluence and were transfected with 10 μg of IP₃R DNA using Lipofectamine 3000 according to the manufacturer's instructions (Thermo Fisher Scientific). After 24 h, the cells were removed with trypsin and seeded at 50,000 cells/well in a 96-well transparent black plate. The culture medium was removed after a further 24 h and replaced with 90 μl of Hanks' buffered salt solution, 10 mM HEPES (pH 7.2) (HBSS–HEPES) for 30 min. An additional 100 μl of HBSS–HEPES containing 5 μM Fluo-8 AM, 0.1 mM sulfinpyrazone, and 500 μM Brilliant Black (to quench extracellular dye fluorescence) was added to the plates, which were incubated for 1 h at 37 °C. Changes in cytosolic Ca²⁺ were monitored using a Flex Station II plate reader in the fluorescence mode. The plate was maintained at 37 °C, and measurements were made using excitation and emission wavelengths of 485 and 525 nm, respectively. The various additions of thimerosal, H₂O₂, and carbachol were made utilizing the fluidics addition system of the Flex station II. Data acquisition and analysis was carried out with SoftMax Pro and Excel.

DNA constructs and cysteine mutagenesis

The cDNA encoding the IP₃R1 isoform was a gift of Dr. Greg Mignery (Loyola University, Chicago, IL) and encoded the splice variant SI(+), SII(+), SIII(–). All amino acid numbering is with reference to the rat IP₃R1 (68). The cDNA encoding mouse IP₃R2 was a gift of Dr. Katsuhiko Mikoshiba (69), and rat IP₃R3 was a gift of Dr. Graeme Bell (70). The Cys-less construct was a kind gift of Dr. Colin Taylor and contains mutations of the first 12 N-terminal cysteines modified to alanine in IP₃R1 (SI(–)). The Cys-less construct has previously been shown to retain channel function (30, 31). Individual mutation of each of the 12 N-terminal cysteines to serine was described previously and included an additional cysteine located within the SI splice site (C326S) (33). The C530A/C553A/C556A mutant was produced by excising the BamHI/KpnI fragment from the Cys-less mutant and replacing it with the BamHI/KpnI PCR-amplified product derived from WT IP₃R1. The three amino acids Cys-767, Cys-1415, and Cys-1459 were changed to serine using the QuikChange II XL site-directed mutagenesis kit (Agilent Tech-

nologies; catalog no. 200521) and the primer design method developed by Zheng *et al.* (71).

Antibodies and reagents

MPEG-2 and MPEG-20 was purchased from Nektar (Huntsville, AL). MPEG-5, sulfinpyrazone, and Brilliant Black were obtained from Sigma. Fluo-8AM was from AbCam (Cambridge, MA).

Author contributions—S. K. J. conceptualization; S. K. J. funding acquisition; S. K. J. writing-original draft; S. K. J. project administration; S. K. J. writing-review and editing; M. Y., D. I. Y., M. A., D. M. B., and G. H. investigation; K. A. methodology.

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