

Subtype-selective regulation of IP₃ receptors by thimerosal via cysteine residues within the IP₃-binding core and suppressor domain

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IP₃R (IP₃ [inositol 1,4,5-trisphosphate] receptors) and ryanodine receptors are the most widely expressed intracellular Ca²⁺ channels and both are regulated by thiol reagents. In DT40 cells stably expressing single subtypes of mammalian IP₃R, low concentrations of thimerosal (also known as thiomersal), which oxidizes thiols to form a thiomercurethyl complex, increased the sensitivity of IP₃-evoked Ca²⁺ release via IP₃R1 and IP₃R2, but inhibited IP₃R3. Activation of IP₃R is initiated by IP₃ binding to the IBC (IP₃-binding core; residues 224–604) and proceeds via re-arrangement of an interface between the IBC and SD (suppressor domain; residues 1–223). Thimerosal (100 μM) stimulated IP₃ binding to the isolated NT (N-terminal; residues 1–604) of IP₃R1 and IP₃R2, but not to that of IP₃R3. Binding of a competitive antagonist (heparin) or partial agonist (dimeric-IP₃) to NT1 was unaffected by thiomersal, suggesting that the effect of thimerosal is specifically related to IP₃R activation. IP₃ binding to NT1 in which all cysteine residues were replaced by

alanine was insensitive to thimerosal, so too were NT1 in which cysteine residues were replaced in either the SD or IBC. This demonstrates that thimerosal interacts directly with cysteine in both the SD and IBC. Chimaeric proteins in which the SD of the IP₃R was replaced by the structurally related A domain of a ryanodine receptor were functional, but thimerosal inhibited both IP₃ binding to the chimaeric NT and IP₃-evoked Ca²⁺ release from the chimaeric IP₃R. This is the first systematic analysis of the effects of a thiol reagent on each IP₃R subtype. We conclude that thimerosal selectively sensitizes IP₃R1 and IP₃R2 to IP₃ by modifying cysteine residues within both the SD and IBC and thereby stabilizing an active conformation of the receptor.

Key words: Ca²⁺ channel, IP₃ receptor, IP₃-binding core, redox regulation, ryanodine receptor, suppressor domain, thimerosal (thiomersal), thiol.

INTRODUCTION

IP₃Rs [IP₃ (inositol 1,4,5-trisphosphate) receptors] and RyRs (ryanodine receptors) are related families of intracellular Ca²⁺ channels (see Supplementary Figure S1 at <http://www.biochemj.org/bj/451/bj4510177add.htm>). IP₃Rs are expressed in most animal cells [1] and RyRs are expressed in many cells, but most abundantly in excitable tissues [2]. Most IP₃Rs and RyRs are expressed within the membranes of the endoplasmic or sarcoplasmic reticulum, where they mediate the release of Ca²⁺ stored within them. Vertebrate genomes have three genes for IP₃R subunits and most have three genes for RyR. Functional intracellular Ca²⁺ channels are tetrameric assemblies of these very large subunits, probably only homotetramers for RyR [2] (but see [3]), whereas IP₃R form both homo- and hetero-tetrameric channels [1]. Activation of IP₃R is initiated by binding of IP₃ to the IBC (IP₃-binding core; residues 224–604) of each IP₃R subunit [4] and then proceeds via re-arrangement of an interface between the IBC and SD (suppressor domain; residues 1–223) [5,6] (see Supplementary Figure S1). The links between these conformational changes within the NT (N-terminal residues 1–604) domains of the IP₃R and gating of the channel formed by transmembrane helices close to the C-terminal are poorly understood, but they are known to require the SD [7].

The two families of intracellular Ca²⁺ channels, IP₃Rs and RyRs, share many characteristics including related structures [6],

similar pores [8] and many aspects of their regulation. The latter includes regulation by cytosolic Ca²⁺ [9,10] and modulation by thiol-reactive reagents [11,12]. Indeed, modification of cysteinyl residues within ion channels is widespread [13–17], and contributes both to pathological responses to oxidative stress [18] and to physiological signals, including nitric oxide [19], hydrogen peroxide [20] and oxygen [21], which generate reactive oxygen or nitrogen species (see references in [22]).

Thimerosal (also known as thiomersal) was once widely used as an antiseptic and as a preservative, particularly in childhood vaccines, but after considerable controversy it is now rarely used [23]. However, as a membrane-permeable thiol-oxidizing agent, thimerosal remains a useful experimental tool that interacts with free thiol groups to form a thiomercurethyl complex. Many studies have reported thimerosal-evoked increases in cytosolic Ca²⁺ concentration and suggested that they, at least partly, result from its effects, whether direct or via oxidation of GSH, on IP₃Rs or RyRs (reviewed in [24]).

Many thiol reagents, including thimerosal [25], biphasically regulate RyR activity: low concentrations stimulate activity, whereas higher concentrations are inhibitory [12,22]. This pattern is reminiscent of the biphasic regulation of RyR by cytosolic Ca²⁺ [10], and consistent with evidence that oxidation of critical cysteine residues increases the sensitivity of RyRs to endogenous regulators [26] (reviewed in [12]). There are approximately 100 cysteine residues in each RyR subunit and within the reducing

Abbreviations used: CLM, cytosol-like medium; GST, glutathione transferase; IBC, inositol 1,4,5-trisphosphate-binding core; IP₃, inositol 1,4,5-trisphosphate; IP₃R, inositol 1,4,5-trisphosphate receptor; K_d, equilibrium dissociation constant; KO, knockout; NT, N-terminal; NT1^{CL}, cysteine-less NT1; NT1^{CL-IBC}, NT1 where cysteine residues within the IBC were replaced by alanine; NT1^{CL-SD}, NT1 where cysteine residues within the SD were replaced by alanine; RyR, ryanodine receptor; RyR1A, A domain of the type 1 RyR; RyR2A, A domain of the type 2 RyR; SD, suppressor domain.

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environment of the cytosol approximately half of these are in the reduced state [22]. A few of these residues are particularly susceptible to oxidation, and their susceptibility depends on whether the RyR is open or closed. But there is not yet any clearly defined relationship between oxidation of specific cysteine residues and channel activity (reviewed in [12]).

An abundant amount of evidence demonstrates that IP₃R activity is also modulated by thiol-oxidizing agents [27] (reviewed in [11]). In many cells, for example, the effects of thimerosal on IP₃-evoked Ca²⁺ release are rather similar to its biphasic effects on RyRs: potentiation of responses at low concentrations and inhibition of Ca²⁺ release at higher concentrations of thimerosal [11]. Although the responses to thimerosal in intact cells are undoubtedly made more complicated by its effects on additional Ca²⁺-handling proteins and perturbation of GSH/GSSG [28], there are clearly direct biphasic effects on IP₃R behaviour [29] (see references in [11]). There are 60 cysteine residues in each IP₃R1 subunit and most are likely to be in the reduced form in their native setting [11]. None of the 12 cysteine residues within the S1 splice variant of the IP₃R1 NT are required for effective gating of IP₃Rs by IP₃ [6], but two conserved cysteine residues within the C-terminal tail (Cys²⁶¹⁰ and Cys²⁶¹³) are essential [7], and three reduced cysteine residues within the third luminal loop of IP₃R1 (Cys²⁴⁹⁶, Cys²⁵⁰⁴ and Cys²⁵²⁷) are essential for its redox-sensitive interaction with a luminal thioredoxin-related protein, ERp44 [30]. The mechanisms whereby thimerosal or other thiol reagents modulate IP₃R behaviour have not been resolved, but they are relevant to understanding redox regulation of IP₃Rs under physiological and pathological conditions, and to defining further the mechanisms of IP₃R activation. The mechanism by which thimerosal regulates the three subtypes of IP₃Rs is addressed in the present study.

MATERIALS AND METHODS

Materials

Thimerosal and heparin (sodium salt from bovine intestinal mucosa) were from Sigma–Aldrich. IP₃ was from Enzo Life Sciences (Exeter). [³H]IP₃ (18 Ci/mmol) was from PerkinElmer Life and Analytical Sciences. Dimeric IP₃ (structure shown in Figure 3A) was synthesized and characterized as reported previously [5]. Sources of other materials are specified in previous publications [31,32] or within the relevant methods sections.

Expression of NT fragments of IP₃ receptors

NT fragments of rat IP₃R1 (NT1 and IBC) (Supplementary Table S1 at <http://www.biochemj.org/bj/451/bj4510177add.htm>) were amplified by PCR from the full-length coding sequence of IP₃R1 lacking the S1 splice site (GenBank accession number: GQ233032.1) and ligated into pGEX-6P-2 vectors (GE Healthcare). The open reading frame encoding NT2 (residues 1–604 from mouse IP₃R2) (GenBank: GU980658.1) and NT3 (residues 1–604 from rat IP₃R3) (GenBank: GQ233031.1) were amplified by PCR using the primers listed in Supplementary Table S2 (at <http://www.biochemj.org/bj/451/bj4510177add.htm>). PCR products were digested with SmaI and XhoI and ligated into pGEX-6P-2 vectors for expression in *Escherichia coli*. The methods used to express an NT1^{CL} (cysteine-less NT1) in which all endogenous cysteine residues of the NT of IP₃R1 were replaced by alanine, and a chimaeric NT (RyR2A-IBC) in which the A domain of the type 2 RyR (RyR2; residues 1–210) (GenBank: GI164831) [33] was fused to the IBC of rat IP₃R1 were described recently [6].

The plasmid encoding NT1^{CL-IBC} (NT1 where cysteine residues within the IBC were replaced by alanine) (Supplementary Table S1) was generated from the NT1 construct using QuikChange multi-site-directed mutagenesis (Agilent). The NT1^{CL} construct was used as the template to prepare a plasmid encoding NT1^{CL-SD} (NT1 where all endogenous cysteine residues within the SD were replaced by alanine) (Supplementary Table S1). The sequence encoding the cysteine-less SD was PCR-amplified from the NT1^{CL} template using the primers listed in Supplementary Table S2. PCR products were then ligated into the pET41a(+) vector containing the open reading frame of the IBC of IP₃R1 as SpeI/EcoRV fragments for expression in *E. coli*. The coding sequences of all expression constructs were confirmed. Supplementary Table S1 lists the proteins used and their abbreviations.

All N-terminal fragments of IP₃R were expressed as fusion proteins linked to N-terminal GST (glutathione transferase) via a PreScission cleavage site in *E. coli* BL21 (DE3) exactly as described previously [32]. Bacteria were harvested (6000 g for 15 min at 4°C), washed twice with phosphate-buffered saline and stored at –80°C before purification of the fragments. Bacterial pellets were suspended (1 g/10 ml) in Tris-buffered medium [50 mM Tris and 1 mM EDTA (pH 8.3)] and lysed by incubation with 100 µg/ml lysozyme (Sigma–Aldrich), 5 units/ml DNase (Sigma–Aldrich) and 10 µg/ml RNase (Sigma–Aldrich) for 1 h on ice, followed by sonication (30 s). After centrifugation (30000 g for 60 min at 4°C), the supernatant was mixed with glutathione–Sepharose 4B beads [GE Healthcare; lysate/beads (v/v) 50:1] and incubated with gentle rotation for 1 h at 20°C. The beads were then washed with Tris-buffered medium supplemented with 1 mM dithiothreitol, centrifuged (500 g for 5 min at 4°C) and incubated in the same medium (1 ml) supplemented with 160 units/ml GST-tagged PreScission protease (GE Healthcare) for 16 h at 4°C with gentle rotation. After centrifugation (500 g for 5 min at 4°C), the NT fragments, cleaved from their GST tags, were recovered in the supernatant and rapidly frozen in liquid nitrogen before storage at –80°C. Immunoblots of the proteins used are shown in Supplementary Figure S3 (at <http://www.biochemj.org/bj/451/bj4510177add.htm>).

[³H]IP₃ binding

Equilibrium competition binding assays were performed in Tris-buffered medium (500 µl) containing purified protein (1–5 µg), [³H]IP₃ (0.25–0.75 nM) and appropriate concentrations of competing ligand. Where indicated, thimerosal was added 10 min before the addition of [³H]IP₃. Reactions were terminated after 5 min by addition of poly(ethylene glycol)-8000 [Sigma–Aldrich; 30% (w/v) 500 µl] and γ-globulin (Sigma–Aldrich; 750 µg in 30 µl of Tris-buffered medium). Bound and free [³H]IP₃ were then separated by centrifugation (20000 g for 5 min). Results were fitted to Hill equations using GraphPad Prism (version 5.0, GraphPad) from which IC₅₀, and thereby K_d (equilibrium dissociation constant) and pK_d, values were calculated.

Ca²⁺ release from the intracellular stores of DT40 cells expressing IP₃R subtypes

DT40 cells, in which the genes for all three endogenous IP₃R subtypes had been disrupted [DT40-KO (knockout) cells], were used to generate stable cell lines expressing rat IP₃R1, mouse IP₃R2 or rat IP₃R3 (DT40-IP₃R1–3 cells) or a chimaeric IP₃R (RyR1A-IP₃R1) in which the SD of IP₃R1 was replaced by the equivalent A domain of rabbit type 1 RyR (RyR1; residues

1–210; GenBank accession number: X15209) (DT40-RyR1A-IP₃R1 cells) (Supplementary Table S1). The methods used to establish these stable cell lines have been described previously [6,34]. All DT40 cells were grown in suspension at 37°C in an atmosphere of 95% air and 5% CO₂ in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 1% heat-inactivated chicken serum and 50 μM 2-mercaptoethanol. Cells were used or passaged when they reached a density of ~2 × 10⁶ cells/ml. Expression levels of IP₃R subtypes in the cell lines were compared by immunoblotting using antisera selective for each IP₃R subtype or an anti-peptide serum (AbC) that interacts equally with all three IP₃R subtypes. The immunoblotting methods were reported previously [34].

A low-affinity Ca²⁺ indicator, Mag-fluo-4, trapped within the endoplasmic reticulum, was used to measure luminal free Ca²⁺ concentration [31]. The endoplasmic reticulum of DT40 cells (~4 × 10⁶ cells/ml) was loaded with indicator by incubating cells in the dark (for 1 h at 20°C) with 20 μM Mag-fluo-4/AM (acetoxymethyl ester) in HEPES-buffered saline [135 mM NaCl, 5.9 mM KCl, 11.6 mM HEPES, 1.5 mM CaCl₂, 11.5 mM glucose and 1.2 mM MgCl₂ (pH 7.3)], supplemented with Pluronic F127 (1 mg/ml) and BSA (0.4 mg/ml). After centrifugation (650 g for 2 min), cells (~4 × 10⁶ cells/ml) were suspended in Ca²⁺-free CLM [cytosol-like medium; 140 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 1 mM EGTA and 20 mM Pipes (pH 7.0)], supplemented with saponin (final concentration 20 μg/ml) to allow for selective permeabilization of the plasma membrane. After ~4 min at 37°C, when all cells had become permeable to 0.1% Trypan Blue, the cells (5 × 10⁵ cells/ml) were recovered (650 g for 2 min) and suspended at 20°C in Mg²⁺-free CLM, supplemented with CaCl₂ (375 μM, to provide a final free Ca²⁺ concentration of 220 nM after addition of 1.5 mM MgATP) and 10 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). The cells were then distributed into poly-L-lysine-coated 96-well plates (50 μl/well), centrifuged (300 g for 2 min) and used for fluorescence measurements. Fluorescence (excitation 485 nm, emission 520 nm, measured at 1.5 s intervals) was recorded at 20°C using a FlexStation III plate-reader (Molecular Devices) [31]. MgATP (1.5 mM) was added to allow intracellular stores to sequester Ca²⁺. After the stores had loaded to steady state (~150 s), IP₃ was added with thapsigargin (1 μM to inhibit Ca²⁺ re-uptake). Ca²⁺ release was measured after a further 60 s. The Ca²⁺ release evoked by IP₃ is expressed as a fraction of the ATP-dependent Ca²⁺ uptake [31]. Concentration–effect relationships were fitted to Hill equations using non-linear curve fitting (version 5.0, GraphPad Prism).

Statistical analyses of IP₃R sensitivity to IP₃ used pEC₅₀ and pK_d values (negative logarithm of the EC₅₀ and K_d respectively). Results are presented as means ± S.E.M. For clarity, mean EC₅₀ and K_d values (derived from pEC₅₀ or pK_d) are shown alongside. In paired comparisons of the effects of thimerosal, ΔpEC₅₀ (or ΔpK_d) values are shown, where ΔpEC₅₀ = EC₅₀^{thimerosal} – pEC₅₀^{control}. Statistical analyses used ANOVA followed by paired Student's *t* test. *P* < 0.05 was considered significant.

RESULTS AND DISCUSSION

Thimerosal potentiates IP₃-evoked Ca²⁺ release via IP₃R1 and IP₃R2

In DT40 cells stably expressing only IP₃R1 (DT40-IP₃R1 cells) (Figure 1A), IP₃ caused a concentration-dependent release of Ca²⁺ from intracellular stores (pEC₅₀ = 7.95 ± 0.04) (Figure 1B). Pre-incubation with thimerosal (10 μM for 120 s) increased the sensitivity to IP₃ (pEC₅₀ = 8.95 ± 0.31, ΔpEC₅₀ = 1.00 ± 0.32)

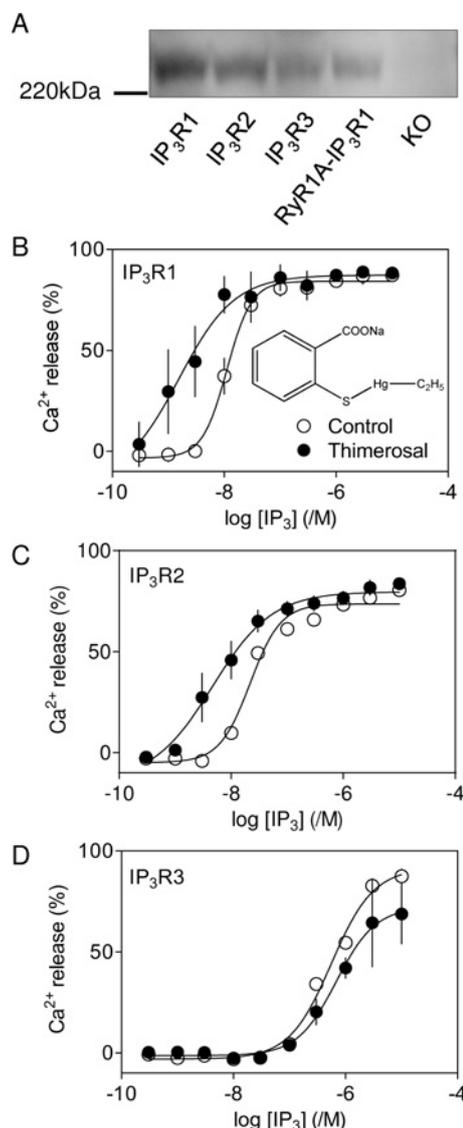


Figure 1 IP₃-evoked Ca²⁺ release via IP₃R1 and IP₃R2 is sensitized by thimerosal

(A) Immunoblot (4 × 10⁴ cells/lane) using an antiserum (AbC) that recognizes a conserved NT sequence in all IP₃R subtypes demonstrates that expression levels of the three IP₃R subtypes and chimeric IP₃R are broadly similar in each of the four cell lines. There are no detectable IP₃R in the DT40-KO cells. (B–D) Concentration-dependent effects of IP₃ on Ca²⁺ release are shown for control cells and after treatment with thimerosal (10 μM, 2 min) for DT40 cells expressing each of the three IP₃R subtypes. Results are means ± S.E.M. for at least three independent experiments. The structure of thimerosal is shown within (B). ○, control; ●, thimerosal.

without affecting loading of the Ca²⁺ stores or the maximal response to IP₃ (Table 1 and Figure 1B). Similar results were obtained with DT40 cells expressing only IP₃R2 (DT40-IP₃R2 cells) (Figure 1A), where 10 μM thimerosal also increased the sensitivity to IP₃ (ΔpEC₅₀ = 0.60 ± 0.20) (Figure 1C). For both IP₃R1 and IP₃R2, higher concentrations of thimerosal inhibited responses to IP₃ (Table 1). However, in DT40 cells expressing IP₃R3 (DT40-IP₃R3 cells) (Figure 1A), 10 μM thimerosal inhibited IP₃-evoked Ca²⁺ release (ΔpEC₅₀ = –0.17 ± 0.01) (Figure 1D), without significantly affecting loading of the intracellular Ca²⁺ stores (Table 1). The latter demonstrates that the diminished responses to IP₃ were not due to thimerosal activating IP₃R3 and thereby draining the Ca²⁺ stores before

Table 1 Effects of thimerosal on IP₃-evoked Ca²⁺ release by different IP₃Rs

DT40 cells expressing each of the IP₃R subtypes were incubated with the indicated concentrations of thimerosal during loading of the intracellular stores with Ca²⁺ (2 min) before addition of IP₃. Results show Ca²⁺ uptake (as a percentage of its value in the absence of thimerosal), maximal IP₃-evoked Ca²⁺ release (as a percentage of the Ca²⁺ content of the stores before addition of IP₃) and the Hill coefficient (*h*), pEC₅₀ and EC₅₀ values for the IP₃-evoked Ca²⁺ release. All results (except EC₅₀, which shows only the mean, see the Materials and methods section) are shown as means ± S.E.M. for three to four independent experiments. ND, not determined.

IP ₃ R subtypes	Thimerosal (μM)	Ca ²⁺ uptake (%)	EC ₅₀ (nM)	pEC ₅₀	<i>h</i>	Maximal Ca ²⁺ release (%)
IP ₃ R1	0	100	11	7.95 ± 0.04	2.0 ± 0.1	84 ± 2
	1	98 ± 5	7	8.16 ± 0.13	1.2 ± 0.2	89 ± 1*
	10	97 ± 7	1	8.95 ± 0.31*	2.7 ± 0.8	85 ± 2
	100	93 ± 6	77	7.11 ± 0.18*	1.0 ± 0.4	42 ± 11*
IP ₃ R2	0	100	23	7.64 ± 0.01	1.6 ± 0.1	74 ± 2
	1	99 ± 2	14	7.87 ± 0.22	1.3 ± 0.2	78 ± 3
	10	96 ± 7	6	8.24 ± 0.26*	2.2 ± 0.3	77 ± 4
	100	93 ± 3	35	7.46 ± 0.28	0.9 ± 0.2	67 ± 2*
IP ₃ R3	0	100	576	6.24 ± 0.04	1.2 ± 0.1	88 ± 2
	1	99 ± 3	687	6.16 ± 0.08	1.0 ± 0.1	90 ± 5
	10	97 ± 8	860	6.07 ± 0.06*	1.3 ± 0.1	78 ± 8
	100	86 ± 6	2185	5.66 ± 0.02*	3.0 ± 0.5	54 ± 2*
RyR1A-IP ₃ R1	0	100	65	7.19 ± 0.01	2.4 ± 0.1	81 ± 4
	1	97 ± 4	78	7.11 ± 0.04	2.0 ± 0.2	76 ± 3
	10	89 ± 4	238	6.62 ± 0.18*	1.6 ± 0.2	80 ± 6
	100	66 ± 8*	ND	ND	ND	12 ± 1#

**P* < 0.05 relative to its control. #Response to 1 μM IP₃.

addition of IP₃. Functional effects of thimerosal were reported previously for DT40 cells expressing IP₃R1 or IP₃R3 [29], but use of the same single sub-threshold concentration of IP₃ for both IP₃R subtypes when IP₃R3 is much less sensitive to IP₃ (Figure 1 and Table 1) [35] compromised the conclusion that thimerosal sensitizes only IP₃R1. Our results, demonstrating that low concentrations of thimerosal sensitize IP₃R1 and IP₃R2 to IP₃ while inhibiting IP₃R3, are consistent with previous studies in which thimerosal [29,36–42] or other thiol reagents [40,43,44] potentiated IP₃-evoked Ca²⁺ signals in cells expressing predominantly IP₃R1 [29,37,42] or IP₃R2 [38,40,43,44], but not in cells where IP₃R3 predominates [29,39,41]. We conclude that within homotetrameric populations of IP₃R, thimerosal potentiates responses from IP₃R1 and IP₃R2, but inhibits IP₃R3.

Thimerosal stimulates IP₃ binding to the NTs of IP₃R1 and IP₃R2

Thimerosal stimulates IP₃-evoked Ca²⁺ release via purified IP₃R1 [37] and it stimulates [³H]IP₃ binding to purified IP₃R1 [37] and its isolated NT [29]. This confirms that thimerosal directly affects IP₃R1. Activation of IP₃R is initiated by IP₃ binding to the IBC and proceeds via re-arrangement of interactions between the IBC and SD [6]. We therefore examined [³H]IP₃ binding to the NTs of IP₃R1–IP₃R3, a region that comprises the critical SD and IBC (Supplementary Figure S1A).

Incubation (10 min) of purified NT from IP₃R1 (NT1) with thimerosal caused a concentration-dependent stimulation of [³H]IP₃ binding that was maximal with 100 μM thimerosal and then reversed at higher concentrations (Figure 2A). A biphasic effect of thimerosal on [³H]IP₃ binding to full-length IP₃R1 [45] and an NT region of IP₃R1 (residues 1–581) was reported previously [46]. The maximally effective concentration of thimerosal (100 μM) caused a 12-fold increase in the affinity of NT1 for IP₃ ($\Delta pK_d = 1.07 \pm 0.28$) (Figure 2B and Table 2). The effect of maximally effective concentrations of thimerosal

(10–100 μM) on the sensitivities of functional responses of IP₃R1 to IP₃ ($\Delta pEC_{50} = 1.00 \pm 0.32$) and IP₃ binding to NT1 ($\Delta pK_d = 1.07 \pm 0.28$) are similar: both are increased ~10-fold. The results were similar with IP₃R2: 100 μM thimerosal caused a ~10-fold increase in the affinity of NT2 for IP₃ ($\Delta pK_d = 1.05 \pm 0.19$) (Figure 2C and Table 2). There was no significant effect of thimerosal on IP₃ binding to the NT of IP₃R3 (Figure 2D and Table 2).

Previous work suggested that thimerosal stimulates [³H]IP₃ binding to full-length IP₃R1, but not to IP₃R3, whereas it stimulated binding to the NT region (residues 1–581) of IP₃R1 and IP₃R3, but not to that of IP₃R2 [29,46]. The previous conclusions are neither internally consistent nor consistent with our observations using NT1–NT3 (residues 1–604) (Figure 2). The residues missing in the shorter NT fragments are reasonably conserved between IP₃R subtypes and they lack cysteine residues (Supplementary Figure S1B). However, from the high-resolution structure of the IBC [4], an NT region truncated at residue 581 is likely to disrupt an α -helix (α_9 in [4]), the NT of which forms essential interactions with IP₃. Furthermore, the published affinities for IP₃ of these shorter fragments from IP₃R1 and IP₃R3 are indistinguishable, which is inconsistent with evidence that in both NT and full-length proteins, IP₃R3 has lower affinity than IP₃R1 [35] (Table 2). It seems likely that the shorter fragments used in the previous studies [29,46] may not retain the native structure of the IBC.

Our results with [³H]IP₃ binding to the NT from all three IP₃R subtypes (Figure 2), evidence from [³H]IP₃ binding to full-length IP₃R1 and IP₃R3 expressed in Sf9 insect cells [29], our functional analyses of DT40 cells expressing homogenous populations of IP₃R subtypes (Figure 1) and substantial evidence from native cells expressing different IP₃R subtypes (discussed above) are consistent with the conclusion that low concentrations of thimerosal stimulate IP₃ binding and IP₃-evoked Ca²⁺ release via IP₃R1 and IP₃R2, but not via IP₃R3. These observations support the hypothesis that cysteine residues within the NT of IP₃R1 and IP₃R2 mediate the sensitizing effects of thimerosal on

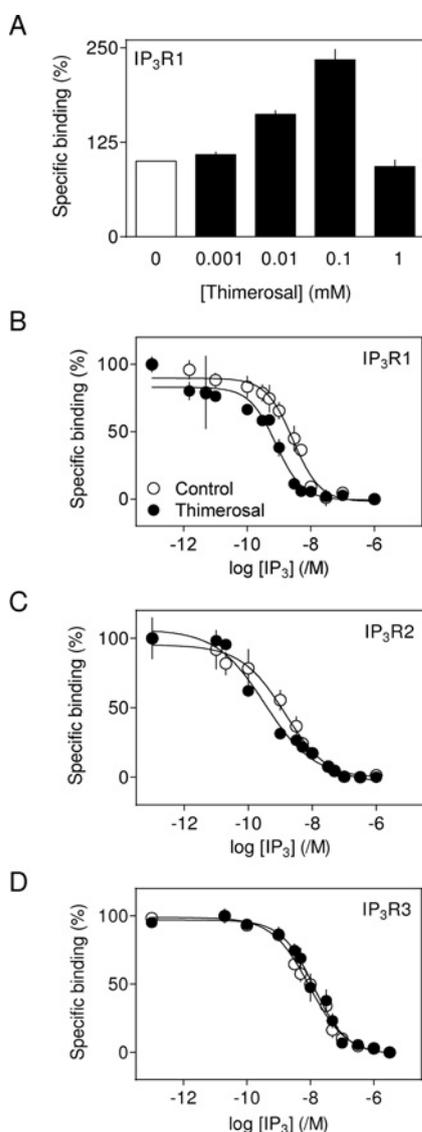


Figure 2 Thimerosal stimulates [³H]IP₃ binding to the NT of IP₃R1 and IP₃R2

(A) Specific binding of [³H]IP₃ (0.5 nM) to the NT of IP₃R1 is shown after pre-incubation (10 min) with the indicated concentrations of thimerosal. Results are shown as the percentage of specific [³H]IP₃ binding measured in the absence of thimerosal. (B–D) Concentration-dependent effects of IP₃ on specific [³H]IP₃ binding (0.5 nM) to the NT from each of the three IP₃R subtypes alone or after incubation with thimerosal (100 μM, 10 min). (A–D) Results are means ± S.E.M. for four independent experiments. ○, control; ●, thimerosal.

IP₃-evoked Ca²⁺ release. Subsequent experiments address this hypothesis.

Thimerosal stimulates agonist, but not antagonist, binding to the NT of IP₃R1

Heparin is a competitive antagonist of IP₃R [47], and dimeric IP₃ (see Figure 3A for structure) is a high-affinity partial agonist: the IP₃ dimer binds to the IP₃R, but causes lesser activation than IP₃ such that the channel opens less frequently [5]. Both heparin and IP₃ dimer completely displaced specifically bound [³H]IP₃ from NT1, but thimerosal (100 μM, 10 min) affected the affinity of neither: ΔpK_d was 0.11 ± 0.09 and −0.06 ± 0.19 for heparin and IP₃ dimer respectively (Figure 3 and Table 3). These

Table 2 Effects of thimerosal on [³H]IP₃ binding to the NT of each IP₃R subtype

Equilibrium-competition binding experiments using [³H]IP₃ (0.5 nM) and NT1–NT3 show the effects of thimerosal (100 μM, 10 min) on the K_d (mean), pK_d (mean ± S.E.M.) and Hill coefficient (*h*, mean ± S.E.M.) for IP₃. ΔpK_d = pK_d^{thimerosal} − pK_d^{control}. Results are from at least four independent experiments.

NT subtype	pK _d (K _d ; nM) <i>h</i>		ΔpK _d
	Control	Thimerosal	
NT1	8.62 ± 0.10 (2.39)	9.69 ± 0.26* (0.20)	1.07 ± 0.28
	1.2 ± 0.2	1.2 ± 0.3	
NT2	9.12 ± 0.07 (0.76)	10.17 ± 0.15* (0.07)	1.05 ± 0.19
	0.6 ± 0.04	0.6 ± 0.02	
NT3	8.06 ± 0.06 (8.72)	7.91 ± 0.17 (12.4)	0.15 ± 0.22
	0.7 ± 0.06	1.0 ± 0.05	

**P* < 0.05 relative to its control.

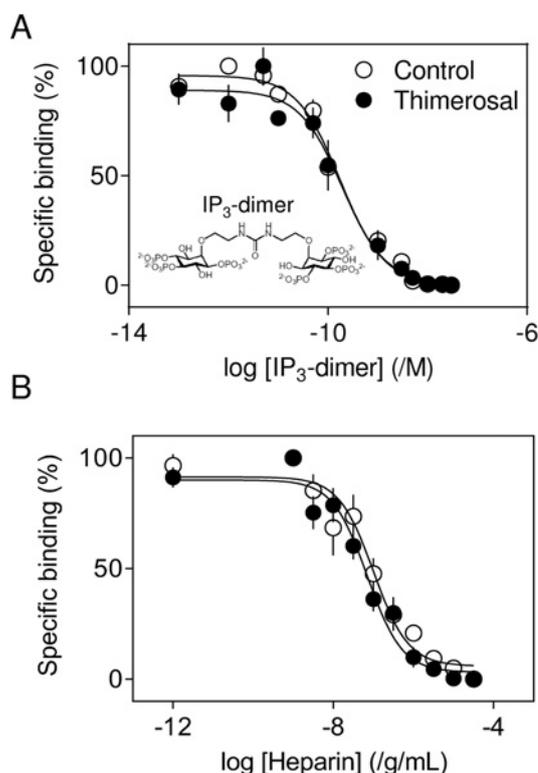


Figure 3 Thimerosal does not affect binding of an antagonist or partial agonist

(A and B) Concentration-dependent effects of the IP₃ dimer (A) or heparin (B) on specific [³H]IP₃ binding (0.25 nM) to the NT from IP₃R1 alone or after incubation with thimerosal (100 μM, 10 min). Results are means ± S.E.M. for four independent experiments. The structure of the IP₃ dimer is shown within (A). ○, control; ●, thimerosal.

results are important because they demonstrate that thimerosal selectively affects the binding of ligands that activate IP₃R. It is, however, noteworthy that thimerosal alone does not activate IP₃R. Concentrations of thimerosal (≤ 10 μM) that cause substantial sensitization of IP₃R1 and IP₃R2 do not affect the Ca²⁺ content of the stores in the absence of IP₃ (Table 1). These results suggest

Table 3 Effects of thimerosal on binding of heparin and IP₃ dimer to the NT from IP₃R1

Equilibrium-competition binding experiments using [³H]IP₃ (0.25 nM) and NT1 show the effects of thimerosal (100 μM, 10 min) on the K_d (mean), pK_d (mean ± S.E.M.) and Hill coefficient (*h*, mean ± S.E.M.) for IP₃ dimer and heparin. ΔpK_d = pK_d^{thimerosal} – pK_d^{control}. Results are from ≥four independent experiments.

	pK _d (K _d) <i>h</i>		ΔpK _d
	Control	Thimerosal	
IP ₃ dimer	9.81 ± 0.07 (0.15 nM)	9.75 ± 0.14 (0.18 nM)	–0.06 ± 0.19
Heparin	1.2 ± 0.1 7.06 ± 0.09 (87 ng/ml)	1.7 ± 0.4 7.17 ± 0.03 (68 ng/ml)	0.11 ± 0.09
	0.82 ± 0.17	0.73 ± 0.09	

that thimerosal might selectively stabilize an agonist-bound active conformation of IP₃R1.

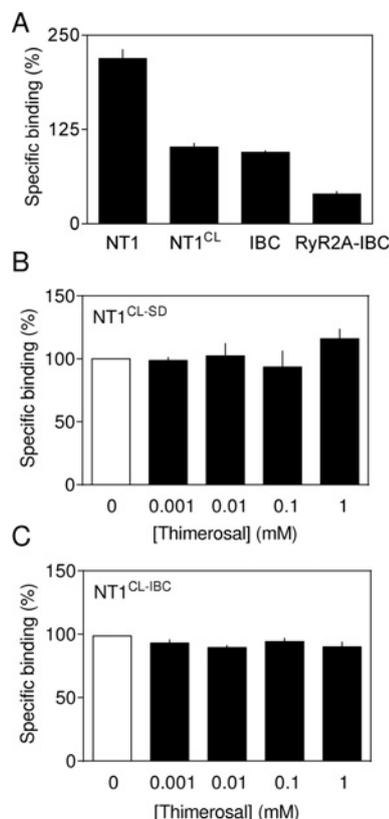
Cysteine residues within both the SD and IBC are required for thimerosal to stimulate IP₃ binding

Previous work demonstrated that thimerosal stimulates [³H]IP₃ binding to NT1, but not to the IBC [29]. Results shown in Figure 4 confirm that conclusion. We have shown that NT1^{CL} is structurally and functionally indistinguishable from native NT1 [6]. The lack of effect of thimerosal on [³H]IP₃ binding to NT1^{CL} (Figure 4A) confirms that cysteine residues mediate its effects. We had anticipated, in keeping with earlier evidence [29], that cysteine residues within the SD were likely to mediate the effects of thimerosal. However, thimerosal had no effect on [³H]IP₃ binding to NT lacking cysteine in either the SD (NT1^{CL-SD}) or the IBC (NT1^{CL-IBC}) (Figures 4B and 4C). These results demonstrate that stimulation of IP₃ binding by thimerosal requires cysteine residues within both the SD and IBC.

Residues within the NT are required for thimerosal to potentiate IP₃-evoked Ca²⁺ release

It would be instructive to examine the functional effects of thimerosal on full-length IP₃R1 in which cysteine residues within the NT are replaced by alanine and so verify whether the NT mediates the effects of thimerosal on IP₃-evoked Ca²⁺ release. We have demonstrated that IP₃R1 with a cysteine-less NT is functional, but only in transiently transfected DT40-KO cells [6], in which it is impracticable to complete the concentration–effect relationships needed to define the effects of thimerosal. We have not yet succeeded in establishing stable DT40 cell lines expressing IP₃R1 with a cysteine-less NT. We therefore adopted another approach and used a stable DT40 cell line expressing a chimaeric IP₃R1 in which the native SD is replaced by the equivalent region (the A domain) from a RyR (RyR1A-IP₃R1) [6] (Figure 5A, and Supplementary Table S1).

The A domain of RyR, which is structurally similar to the SD of IP₃R [6,48–50] (Supplementary Figure S1C and S1D), can functionally replace the SD of IP₃R1 to allow both IP₃-evoked Ca²⁺ release from a chimaeric IP₃R and appropriate regulation of IP₃ binding to a chimaeric NT [6]. All effective concentrations of thimerosal (1 μM–1 mM) inhibited [³H]IP₃ binding to the chimaeric NT (RyR2A-IBC) (Figures 5A and 5B), although the effect appeared biphasic. Thimerosal (10 μM, 2 min)

**Figure 4** Stimulation of IP₃ binding by thimerosal requires cysteine residues within the SD and IBC

(A) Effects of thimerosal (100 μM, 10 min) on specific binding of [³H]IP₃ (0.5 nM) to each of the indicated proteins. Results are expressed as a percentage of the binding measured in the absence of thimerosal. (B and C) Concentration-dependent effects of thimerosal on specific [³H]IP₃ binding to NT1^{CL-SD} (B) and NT1^{CL-IBC} (C). All results are means ± S.E.M. for four independent experiments.

also inhibited IP₃-evoked Ca²⁺ release from the chimaeric IP₃R (RyR1A-IP₃R1) (Figure 5C and Table 1). These results suggest that these cysteine residues present within the SD of IP₃R1, but absent from the A domain of RyR, are required for thimerosal to potentiate IP₃-evoked Ca²⁺ release.

Conclusions

Thimerosal potentiates IP₃-evoked Ca²⁺ release via IP₃R1 and IP₃R2, but not via IP₃R3 (Figure 1). The parallel behaviour of NTs from each of the three IP₃R subtypes (Figure 2), our demonstration that cysteine residues are required within both the IBC and SD for thimerosal to stimulate IP₃ binding (Figure 4) and evidence that thimerosal does not potentiate responses from a chimaeric IP₃R1 in which the SD is replaced by the equivalent domain of a RyR (Figure 5) provide persuasive evidence that cysteine residues within the SD and IBC mediate functional responses to thimerosal. Furthermore, thimerosal selectively stimulates agonist, but not antagonist, binding to NT1 (Figure 3), suggesting that its interactions with critical cysteine residues selectively stabilize an active conformation of the NT. Because IP₃ binding rearranges interactions between the SD and IBC [6], this conclusion aligns with a previous suggestion that thimerosal stabilizes an interaction between the IBC and SD of IP₃R1 [29].

We do not know which specific cysteine residues are modified by thimerosal. Despite its subtype-selective actions (Figures 1 and 2) and evidence that sensitization by thimerosal is mediated

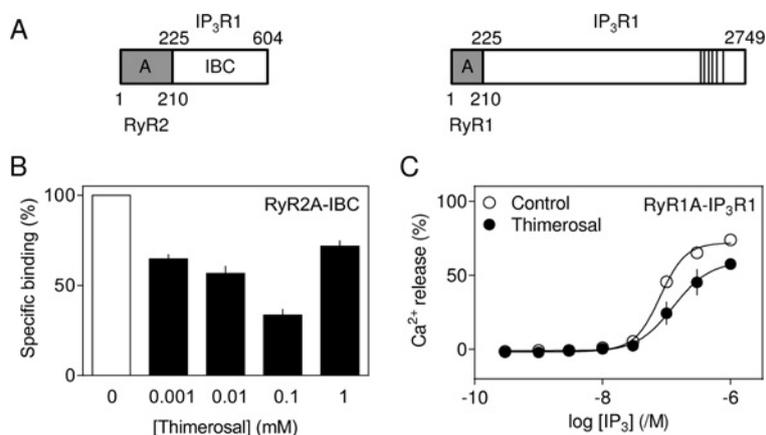


Figure 5 A chimaeric IP₃R1 is not stimulated by thimerosal

(A) The proteins used. (B) Concentration-dependent effects of thimerosal on specific [³H]IP₃ (0.5 nM) binding to RyR2A-IBC. Results are expressed as percentages of the binding measured in the absence of thimerosal. (C) Concentration-dependent effects of IP₃ on Ca²⁺ release from DT40-RyR1A-IP₃R1 cells are shown for control cells and after treatment with thimerosal (10 μM, 2 min). Results in (B) and (C) are means ± S.E.M. from four independent experiments.

by cysteine residues within the NT, we have been unable to identify candidate cysteine residues from sequence data alone (see legend to Supplementary Figure S2 at <http://www.biochemj.org/bj/451/bj4510177add.htm>). Preliminary MALDI-TOF (matrix-assisted laser desorption ionization–time-of-flight) analyses were uninformative, and it is impracticable to assay NT in which every pair of cysteine residues are mutated. It is perhaps surprising that cysteine residues in both the SD and IBC are required for thimerosal to sensitize IP₃R1. This requirement is unlikely to result from cross-linking of a pair of cysteine residues by thimerosal because available evidence suggests that thimerosal forms a mercuryethyl adduct with a single cysteine [24]. An alternative possibility is that thimerosal disrupts a disulfide bridge between cysteine residues in the SD and IBC that constrains IP₃R activation. This too seems unlikely. There is no evidence from the high-resolution structure of the NT for such a disulfide bridge [6]. Furthermore, IP₃R with cysteine-less NT are not constitutively active and respond normally to IP₃ [6]. We also used algorithms that predict the accessibility [51] and reactivity [52] of cysteine residues together with modelled structures of NT2–NT3 [53] to identify cysteine residues that might account for the differential sensitivities of IP₃R1–IP₃R3 to thimerosal. The results suggest that within the SD, there are two accessible cysteine residues (Cys²⁰⁶ and Cys²¹⁴ in IP₃R1) and they are predicted to be equally accessible in all three subtypes, but the residue equivalent to Cys²¹⁴ in IP₃R1 is less reactive in IP₃R3 (Supplementary Figure S2). Within the IBC, Cys⁵⁵⁶ is predicted to be accessible in IP₃R1 and IP₃R2, but it is buried in IP₃R3. Cys²¹⁴ in the SD and Cys⁵⁵⁶ in the IBC of IP₃R1 therefore have the properties expected to explain the sensitization of IP₃R1 and IP₃R2, but not IP₃R3, by thimerosal. Further experimental work would be needed to assess this directly. We have provided the first systematic analysis of the effects of thiol reagents on each IP₃R subtype. We conclude that thimerosal sensitizes IP₃R1 and IP₃R2, but not IP₃R3, to IP₃. It does so by modifying cysteine residues within both the SD and IBC and thereby stabilizing an active conformation of the IP₃R.

AUTHOR CONTRIBUTION

Samir Khan completed most experimental analyses, with additional input from Ana Rossi. Andrew Riley and Barry Potter provided the IP₃ dimer. Colin Taylor designed the study, contributed to analysis of data and, with Ana Rossi, wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Subtype-selective regulation of IP₃ receptors by thimerosal via cysteine residues within the IP₃-binding core and suppressor domain

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Table S1 Proteins used and their abbreviations

Abbreviations	Protein
IP ₃ R1	Full-length rat IP ₃ R1 lacking S1 splice region (GenBank: GQ233032.1)
IP ₃ R2	Full-length mouse IP ₃ R2 (GenBank: GU980658.1)
IP ₃ R3	Full-length rat IP ₃ R3 (GenBank: GQ233031.1)
RyR1A-IP ₃ R1	Full-length rat IP ₃ R1 (GenBank: GQ233032.1) with residues 1–224 replaced by residues 1–210 from rabbit RyR1 (GenBank: X15209)
NT1	Residues 1–604 from rat IP ₃ R1
NT2	Residues 1–604 from mouse IP ₃ R2
NT3	Residues 1–604 from rat IP ₃ R3
IBC	Residues 224–604 from rat IP ₃ R1
NT1 ^{CL}	Residues 1–604 from rat IP ₃ R1 with all cysteine residues replaced by alanine
NT1 ^{CL} –SD	Residues 1–604 from rat IP ₃ R1 with all cysteine residues within the SD (residues 1–223) replaced by alanine
NT1 ^{CL} –IBC	Residues 1–604 from rat IP ₃ R1 with all cysteine residues within the IBC (residues 224–604) replaced by alanine
RyR2A-IBC	Residues 1–604 from rat IP ₃ R1 with residues 1–224 replaced by residues 1–210 from rabbit RyR2 (GenBank: G1164831)

Table S2 Primers used

Sequences of the forward (F) and reverse (R) primers used to amplify by PCR the sequences encoding the constructs used. Other primers have been specified in earlier publications [1,2].

Construct	Direction	Sequence (5'–3')
NT2	F	GGCCCCGGGATGCTGACAAAATGTCCAGCTTCTCTACATTGGG
	R	GGCCCTCGAGTTATTTGCGGTTGTTGTGTAACAAGGCTGTAATCGT
NT3	F	GGCCCCGGGATGAATGAAATGTCCAGCTTTCTTACATCGGGGAC
	R	GGCCCTCGAGTTACTTCCGGTTGTTGTGCAGCAGGGC CGTGATGGT
NT1 ^{CL} –SD	F	ACTAGTATGCTGACAAAATGTCTAGTTTCCT
	R	GATATCGAAAAGCACTATTTTCCAGCTTG

¹ These authors contributed equally to this work.² To whom correspondence should be addressed (email cwt1000@cam.ac.uk).

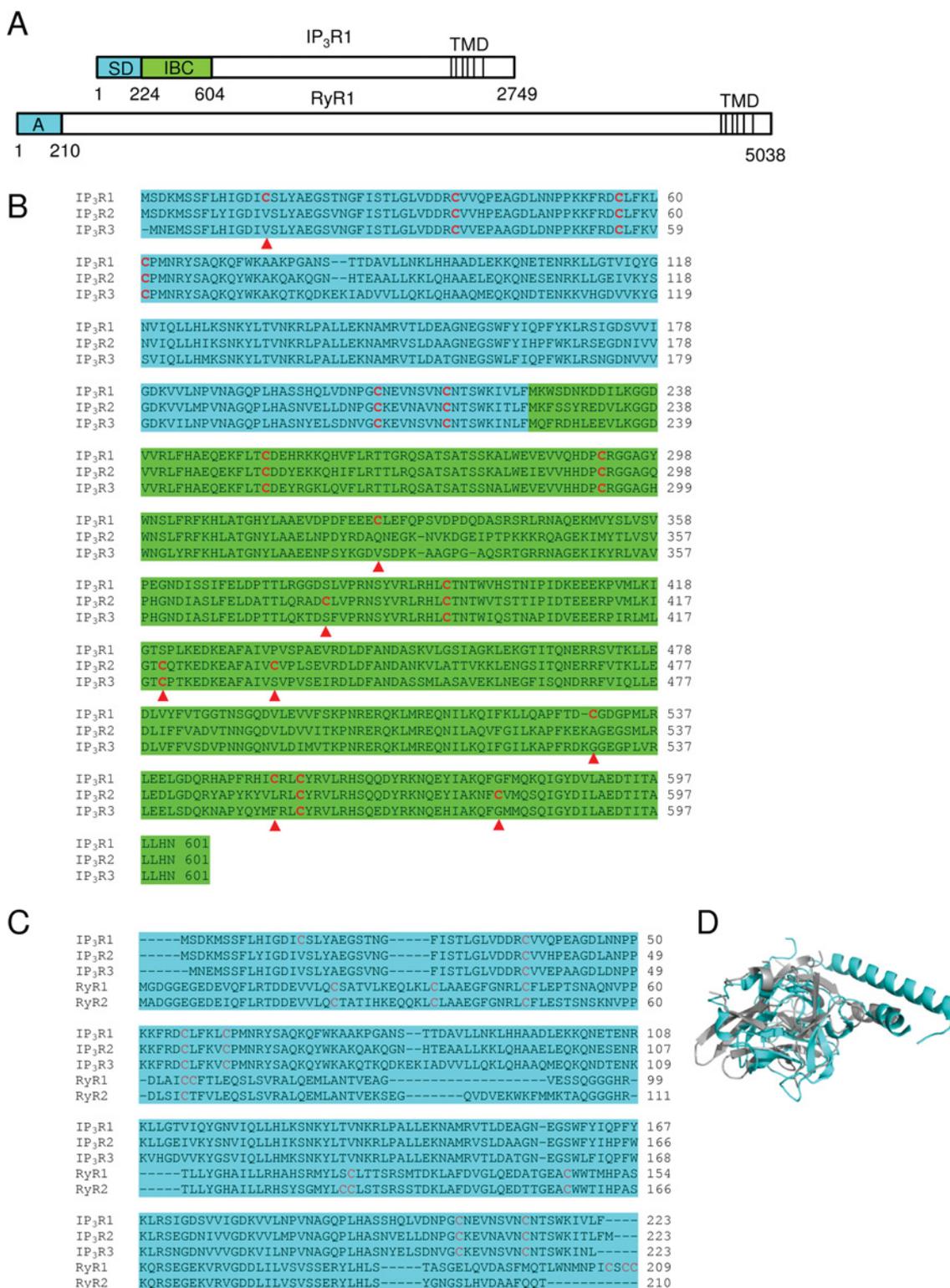


Figure S1 Comparison of key sequences between IP₃R subtypes and RyR

(A) Key domains of a single subunit of rat IP₃R1 and rabbit RyR1 shown to highlight locations of the SD and A domain. (B) Sequence alignment (Clustal) of the three IP₃R subtypes. Cyan shows the SD and green shows the IBC. Red arrowheads highlight cysteine residues that differ between IP₃R subtypes. (C) Sequence alignment (Clustal) of the SD of the three IP₃R subtypes and A domains of RyR1 and RyR2. (D) Structural alignments (Pymol) of the SD of IP₃R1 (cyan; PDB 1XZ) and A domain of RyR2 (grey; PDB 3IM5).

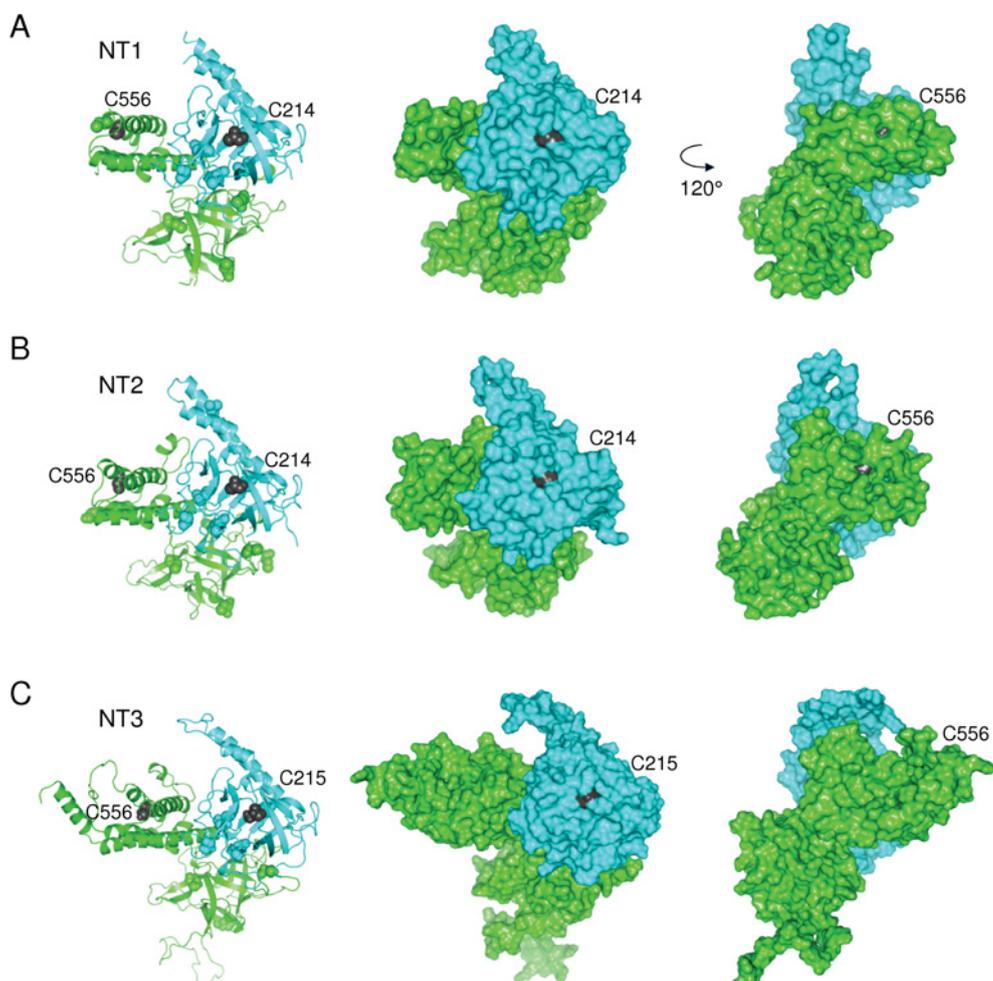


Figure S2 Differences in the accessibility and reactivity of cysteine residues within the NT of IP₃R subtypes may account for their differential regulation by thimerosal

(A–C) Structural homology models [3] of NT2 and NT3 on the basis of the known structure of NT1^{CL} (PDB 3UJ4) [2] with the native cysteine restored (using Pymol). The structures show the SD (cyan) and IBC (green) and the positions of all cysteine residues. Thimerosal increases the sensitivity to IP₃ of IP₃R1 and IP₃R2, but not of IP₃R3 (Figure 1 of the main text), and its actions require cysteine residues within the NT (Figures 4 and 5 of the main text). We therefore considered whether residues conserved within the SD and IBC of IP₃R1 and IP₃R2, but absent from IP₃R3, might be responsible. There are six cysteine residues within the SD of IP₃R1, two of these (Cys⁵⁶ and Cys⁶¹) are not required for thimerosal to stimulate IP₃ binding [4], and five of the residues are conserved in all three IP₃R subtypes. The remaining cysteine residue (Cys¹⁵) is unique to IP₃R1. The IBC of the IP₃R1 used in the present study (where the S1 splice site, which includes Cys³²⁶, is removed) has six cysteine residues, four of which are conserved, but the remaining two (Cys⁵³⁰ and Cys⁵⁵³) are unique to IP₃R1. There are therefore no cysteine residues in the NT that are uniquely conserved in IP₃R1 and IP₃R2. Replacing the SD of IP₃R1 with the A domain of RyR1 caused thimerosal to become inhibitory, suggesting that cysteine residues present in the SD, but absent from the A domain, might contribute to the stimulatory effect of thimerosal. Two of the six cysteine residues within the SD (Cys³⁶ and Cys⁵⁶) are conserved in all three IP₃R subtypes, RyR1 and RyR2, and are therefore unlikely to mediate stimulation of IP₃R1 by thimerosal. None of the remaining four cysteine residues in the SD are present in the A domain, but neither are they uniquely shared with IP₃R2. We were therefore unable to identify from these sequence comparisons alone the cysteine residues within the NT that are likely to mediate the stimulatory effect of thimerosal. The reactivity of a cysteine residue depends on its accessibility and whether it is deprotonated [5,6]. Using the NT1–NT3 structures, two cysteine residues were identified that would be expected to be reactive in NT1 and NT2, but non-reactive in NT3. These residues are highlighted (grey) in the structures and their surface accessibility is shown in the right-hand panels.

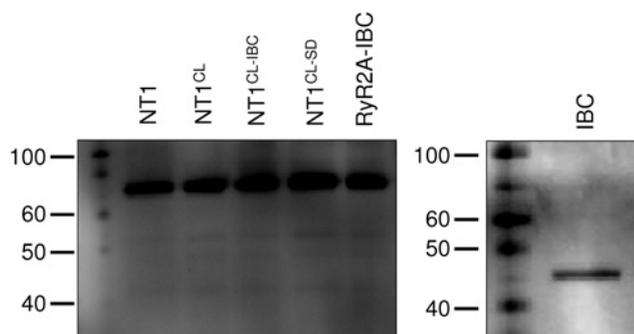


Figure S3 Expression of NT fragments of IP₃R

Immunoblots of the indicated purified fragments (3 μ g/lane), using AbC, which recognizes a sequence conserved in all IP₃R subtypes (residues 240–253 of rat IP₃R1). Molecular mass markers are shown on the left in kDa.

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