The effects of thimerosal on calcium uptake and inositol 1,4,5-trisphosphate-induced calcium release in cerebellar microsomes

Lee G. SAYERS, Graham R. BROWN, Robert H. MICHELL and Francesco MICHELANGELI*
School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Thimerosal inhibits calcium uptake in skeletal muscle sarcoplasmic reticulum and rat cerebellar microsomes by inhibiting the Ca^{2+}-ATPase. In the presence of 5 mM dithiothreitol (DTT), Ca^{2+} uptake and ATPase activity were not inhibited by thimerosal, indicating that thimerosal modifies cysteine residues of the Ca^{2+}-ATPase. Low thimerosal concentrations (2 μM) sensitize the inositol 1,4,5-trisphosphate ($Ins P_3$)-sensitive Ca^{2+} channel, making it open at lower $Ins P_3$ concentrations. Higher concentrations of thimerosal, however, cause inhibition of $Ins P_3$-induced Ca^{2+} release. Both sensitization and inhibition of the $Ins P_3$ receptor by thimerosal can be prevented by DTT. The binding and metabolism of $Ins P_3$ by cerebellar microsomes is not affected by thimerosal. The amount of $Ins P_3$-induced Ca^{2+} release is co-operatively linked to the $Ins P_3$ concentration with a Hill coefficient of 2.0 ± 0.3. This is decreased to 1.0 ± 0.2 at inhibitory concentrations of thimerosal. Under our experimental conditions, we observed no dependence of quantal Ca^{2+} release on intraluminal Ca^{2+} concentration.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($Ins P_3$) is the second messenger responsible for the release of Ca^{2+} from intracellular Ca^{2+} stores following receptor activation. The $Ins P_3$-sensitive Ca^{2+} release channel has been purified [1] and the amino acid sequence determined from cDNA studies [2]. $Ins P_3$-induced Ca^{2+} release is quantal in nature, with the total amount of Ca^{2+} released regulated by the $Ins P_3$ concentration [3]. Most inhibitors of this channel, such as heparin, act competitively by displacing $Ins P_3$ from its receptor [4]. A notable exception is caffeine, which does not affect $Ins P_3$ binding but does inhibit $Ins P_3$-induced Ca^{2+} release [5]. Caffeine inhibits quantal release by increasing the $Ins P_3$ concentration required to cause half-maximal Ca^{2+} release (IC_{50}) from cerebellar microsomes and by abolishing the apparent co-operativity of the dependence of this process on $Ins P_3$ concentration [5].

It has been reported by Missiaen et al. [6] that the thiol-reactive compound thimerosal activates the $Ins P_3$-sensitive Ca^{2+} channel in permeabilized hepatocytes, such that in the presence of thimerosal normally suboptimal doses of $Ins P_3$ cause maximal levels of $Ins P_3$-induced Ca^{2+} release.

In this study we have characterized the effects of thimerosal on both ATP-dependent Ca^{2+} uptake and $Ins P_3$-induced Ca^{2+} release in rat cerebellum microsomes, in order to understand how thimerosal affects the biochemical properties of these intracellular Ca^{2+} pumps and channels.

MATERIALS AND METHODS

Fluo-3 was obtained from Molecular Probes, $Ins P_3$ from Calbiochem, [^{3}H]$Ins P_3$ from Amersham International and thimerosal from Sigma. All other reagents were of analytical grade.

Rat cerebellar microsomes were prepared as described in [7]. Rabbit skeletal muscle sarcoplasmic reticulum (SR) vesicles were prepared as described in [8].

Ca^{2+} uptake and release were measured using fluo-3 as described elsewhere [5,9]. Briefly, rat cerebellular microsomes or SR were suspended in a buffer containing 10 mM potassium phosphate, 3.5 mM potassium pyrophosphate, 250 nM fluo-3, 10 μg/ml creatine kinase and 10 mM phosphocreatine, pH 7.2, and measurements were carried out at 37 °C. Cerebellar microsomes (0.2 mg/ml) or SR (0.01 mg/ml) were added to a stirred cuvette, Ca^{2+} uptake was initiated by the addition of 1.5 mM Mg-ATP and the change in fluorescence was monitored (excitation at 506 nm and detection at 526 nm). Following ATP-dependent Ca^{2+} loading, further Ca^{2+} uptake was inhibited by the addition of 0.5 mM sodium orthovanadate; this was prepared as an alkaline solution and therefore did not contain decavanadate, which is known to inhibit $Ins P_3$ binding to its receptor [10]. $Ins P_3$ (0.025–20 μM) was added and the maximum amount of Ca^{2+} release for each concentration was determined. Total Ca^{2+} accumulation within the microsomes or SR was measured by permeabilization with 12.5 μg/ml Ca^{2+} ionophore A23187. Fluorescence intensity was related to Ca^{2+} concentration using the equation described in [9] (the K_d for Ca^{2+} binding to fluo-3 is 900 nM, as determined at pH 7.2 and 37 °C [9]). The cerebellar microsomes or SR were preincubated with thimerosal for 10 min prior to the initiation of Ca^{2+} uptake. The Ca^{2+}-dependent ATPase activity was measured using the coupled enzyme assay described in [8].

The effect of thimerosal (up to 50 μM) on $Ins P_3$ binding was assessed by addition of thimerosal to cerebellar microsomes incubated with 40 nM [^{3}H]$Ins P_3$. The displacement binding assay was carried out as described in [5]. The effect of thimerosal on the metabolism of $Ins P_3$ when incubated with cerebellar microsomes was also investigated. The microsomes were preincubated with thimerosal (up to 20 μM) for 20 min in conditions identical to those used for Ca^{2+} uptake and release. $Ins P_3$ (10 μM) labelled with 0.003 μCi [^{3}H]$Ins P_3$ was added, and the reaction was quenched after 30 s with cold perchloric acid (final concentration 4%), neutralized with KOH and the products separated on gravity-fed anion-exchange columns as described in [11]. The $Ins P_3$ fraction was collected and the amount of $Ins P_3$ metabolized

Abbreviations used: SR, sarcoplasmic reticulum; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-nitrobenzoic acid.
* To whom correspondence should be addressed.

was determined. To assess 5-phosphatase activity under simulated 'intracellular' conditions, cerebellar microsomes were similarly incubated in 102 mM KCl, 10 mM NaCl, 0.1 mM EGTA, 5 mM $MgSO_4$, 50 mM Hepes, pH 7.2, and saponin (0.2 mg/ml) as described in [12].

Concentration–response curves were fitted using the non-linear curve-fitting computer programme Fig P, version 6.0, from Biosoft, U.K.

RESULTS

Figures 1(a) and 1(b) show the effects of thimerosal on ATP-dependent Ca^{2+} uptake in SR and cerebellar microsomes. Upon addition of ATP there was a rapid drop in [Ca^{2+}] due to Ca^{2+} complexing with ATP. This was also observed when ATP was added to the buffer containing fluo-3 but in the absence of microsomes. In the absence of thimerosal a decrease in extravesicular [Ca^{2+}] was observed as Ca^{2+} was accumulated into the vesicles. The rate of Ca^{2+} uptake progressively decreased as the thimerosal concentration was increased. Ca^{2+} uptake in SR was completely inhibited at thimerosal concentrations above 2 μM, and the thimerosal concentration which caused a half-maximal decrease in the rate of Ca^{2+} uptake was 0.35 ± 0.2 μM. Ca^{2+} uptake in rat cerebellar microsomes was also completely inhibited by thimerosal, but higher concentrations were required (75 μM for complete inhibition; the IC_{50} for the rate of Ca^{2+} uptake was 38 ± 18 μM). The SR Ca^{2+}-ATPase activity in the presence of the Ca^{2+} ionophore A23187 was also completely inhibited by thimerosal (IC_{50} 0.4 ± 0.1 μM), indicating that the inhibition of Ca^{2+} uptake by thimerosal was by a direct effect on the Ca^{2+}-ATPase rather than by non-specific effects on the leakiness of the vesicles (results not shown). Thimerosal inhibition of Ca^{2+}-ATPase activity and Ca^{2+} uptake was a time-dependent process, with maximal inhibition occurring after 10 min. When 5 mM DTT was included in the incubations, thimerosal inhibition of Ca^{2+} uptake in SR and cerebellar microsomes was largely prevented, giving rates of uptake between 70–80 % of the control values (Figures 1a and 1b).

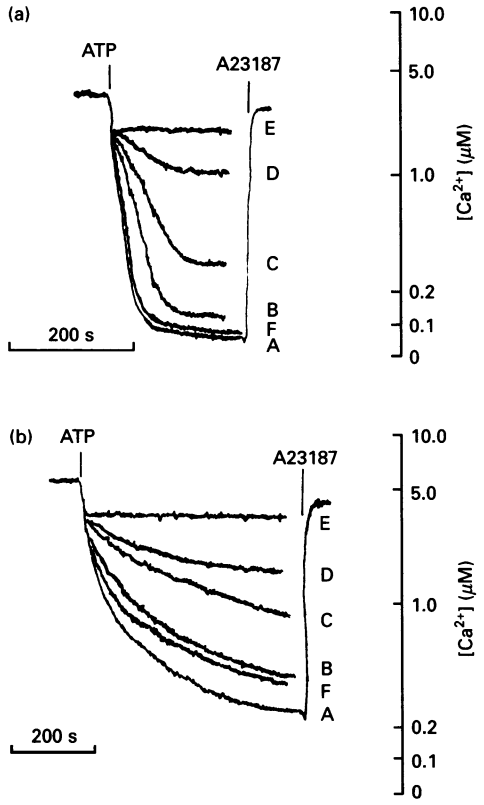

Figure 1 Effects of thimerosal on ATP-dependent Ca^{2+} uptake in SR and microsomes

Rabbit skeletal muscle SR (**a**) or rat cerebellar microsomes (**b**) were incubated for 10 min in the presence of thimerosal prior to the addition of 1.5 mM Mg-ATP. (**a**) Thimerosal concentrations were (in μM): A, 0; B, 0.25; C, 0.5; D, 1.0; E, 2.0. Trace F, 2.0 μM thimerosal plus 5 mM DTT. (**b**) Thimerosal concentrations were (in μM): A, 0; B, 10; C, 20; D, 50; E, 75. Trace F, 75 μM thimerosal plus 5 mM DTT. Ca^{2+} concentrations were measured as described in the Materials and methods section. The traces presented are representative examples of three separate experiments.

Figure 2 shows the effects of thimerosal on $InsP_3$-induced Ca^{2+} release from cerebellar microsomes at maximal (10 μM) and submaximal (0.5 μM) $InsP_3$ concentrations. In this microsomal preparation maximally effective $InsP_3$ concentrations released 14 ± 1.5 % of the total A23187-releasable Ca^{2+}. In other microsomal preparations used in this study, $InsP_3$ released 17–21 % of the Ca^{2+} that could be released by A23187. At maximal $InsP_3$ concentrations, 50 μM thimerosal almost completely inhibited $InsP_3$-induced Ca^{2+} release with an IC_{50} of 8 ± 1 μM. At 0.5 μM $InsP_3$, however, the $InsP_3$-induced Ca^{2+} release was increased by 54 ± 11 % at a thimerosal concentration of 2 μM, approaching the Ca^{2+} release observed at maximal $InsP_3$ concentrations. At higher thimerosal concentrations inhibition was again observed. Figure 3 shows that both the stimulatory and inhibitory effects of thimerosal were largely prevented by the presence of 5 mM DTT, with the degree of $InsP_3$-induced Ca^{2+} release returning to between 70 and 80 % of control values. Although there was some variation in the basal and intraluminal Ca^{2+} concentrations between the individual traces prior to $InsP_3$ addition, under our experimental conditions we observed no significant correlation between percentage $InsP_3$-induced Ca^{2+} release and these variations (see Figure 5).

$InsP_3$-induced Ca^{2+} release has been shown to occur in a quantal manner [3], with the amount of Ca^{2+} released co-operatively dependent on the $InsP_3$ concentration [5,9,13]. From five separate preparations of rat cerebellar microsomes we have obtained an IC_{50} for $InsP_3$-induced Ca^{2+} release of 0.55 ± 0.15 μM and an apparent Hill coefficient of 1.98 ± 0.09. Figure 4(a) compares the dependence of quantal Ca^{2+} release upon $InsP_3$ concentration in the presence of stimulatory (2 μM) or inhibitory (10 μM) concentrations of thimerosal. At 2 μM thimerosal the dose–response curve of $InsP_3$-induced Ca^{2+} release was shifted from the control such that the $InsP_3$ concentration required to cause half-maximal calcium release was reduced from 0.55 ± 0.06 μM to 0.22 ± 0.04 μM; the maximum amount of Ca^{2+} released was unaffected. The Hill coefficient was essentially unchanged from the control value (from 2.0 ± 0.3 to 1.7 ± 0.2) (Figure 4b). In the presence of an inhibitory concentration of thimerosal (10 μM), the total amount of Ca^{2+} released by high levels of $InsP_3$ was much less than in the control (7 % compared with 12 %). The $InsP_3$ concentration required to cause half-maximal release under these conditions remained low (0.35 ± 0.08 μM), but the Hill coefficient was reduced to 1.0 ± 0.2.

As thimerosal affects the rate and extent of Ca^{2+} accumulation within cerebellar microsomes, and since intraluminal Ca^{2+} concentrations have been suggested to play a role in the regulation of $InsP_3$-induced quantal Ca^{2+} release [14], the amount of Ca^{2+} release at maximal and submaximal $InsP_3$ concentrations was measured in cerebellar microsomes which had taken up variable

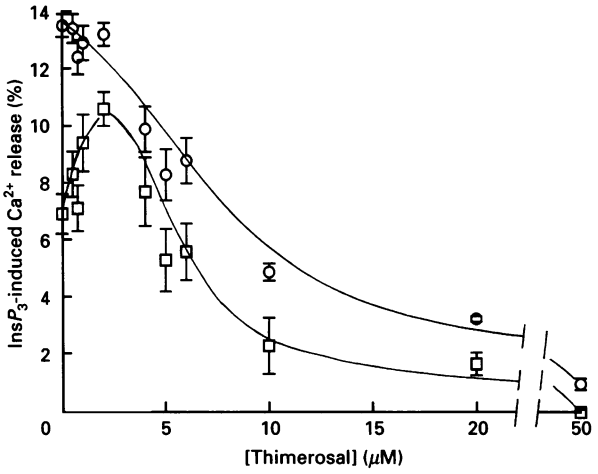

Figure 2 Effects of thimerosal on $InsP_3$-induced Ca^{2+} release in cerebellar microsomes

$InsP_3$-induced Ca^{2+} release was calculated as the percentage of Ca^{2+} released by $InsP_3$ compared with that released by A23187. The effect of thimerosal on Ca^{2+} release was measured at 0.5 μM (□) and 10 μM (○) $InsP_3$. The values are expressed as the mean ± S.E.M. of three determinations.

amounts of Ca^{2+} (Figure 5b). This was achieved by allowing microsomes to accumulate Ca^{2+} over different time periods, prior to the addition of 0.5 mM orthovanadate in order to inhibit Ca^{2+} accumulation. Figure 5 shows Ca^{2+} released by 0.5 and 10 μM $InsP_3$ expressed as a percentage of that which could be released by A23187. This Ca^{2+} release by submaximal concentrations of $InsP_3$ remained unaffected over a large range of Ca^{2+} concentrations in the microsomes.

Table 1 shows that thimerosal concentrations up to 50 μM had no effect on $InsP_3$ binding or on $InsP_3$ metabolism when incubated with cerebellar microsomes.

DISCUSSION

It has been well established that mercurial-containing organic compounds, of which thimerosal is a typical example, react specifically with the thiol groups of cysteine amino acids [15]. A number of SH-reactive reagents (e.g. the metal ions Ag^+ and Zn^{2+}) inhibit the Ca^{2+}-ATPase in muscle SR [16,17]. It is likely that the inhibition of both SR and cerebellar Ca^{2+}-ATPases by thimerosal is by a similar mechanism, and this can also be protected by DTT. The Ca^{2+}-ATPase of SR has been shown to possess cysteines of different reactivities with the thiol-reactive reagent 5,5′-dithiobis-nitrobenzoic acid (DTNB). One class of cysteines was found to be slow-reacting, taking several minutes to become modified by DTNB [18,19]. Since thimerosal inhibition of ATPase activity and Ca^{2+} uptake in SR or microsomes requires 10 min for completion, one possibility is that thimerosal may be interacting with these less reactive cysteine residues. The finding that thimerosal affects both the Ca^{2+} pump and $InsP_3$-sensitive Ca^{2+} channels must be taken into account when interpreting intracellular Ca^{2+} changes upon addition of thiol-reactive reagents to cell preparations.

Compounds such as oxidized glutathione and *t*-butyl hydroperoxide at millimolar concentrations have been shown to sensitize $InsP_3$-induced Ca^{2+} release [6,20]. Thimerosal has been shown to sensitize the $InsP_3$-induced Ca^{2+} release response in permeabilized hepatocytes at lower concentrations, of the order of 50 μM [6]. We have also observed this phenomenon in

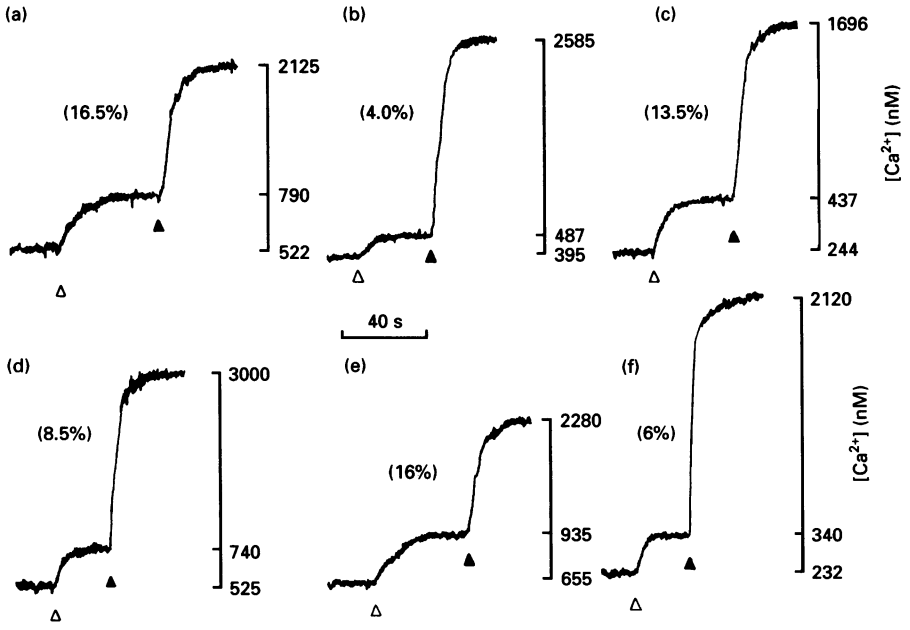

Figure 3 Effects of thimerosal and DTT on $InsP_3$-induced Ca^{2+} release

(**a**) Effect of addition of 10 μM $InsP_3$ (open arrowhead), followed by the addition of 12.5 μg/ml A23187 (solid arrowhead) on Ca^{2+} release. (**b**) Effect of 10 μM thimerosal on 10 μM $InsP_3$-induced Ca^{2+} release. (**c**) Effect of 10 μM thimerosal plus 5 mM DTT on 10 μM $InsP_3$-induced Ca^{2+} release. (**d**) Effect of 0.5 μM $InsP_3$ on Ca^{2+} release (open arrowhead). (**e**) Effect of 2 μM thimerosal on 0.5 μM $InsP_3$-induced Ca^{2+} release. (**f**) Effects of 2 μM thimerosal plus 5 mM DTT on 0.5 μM $InsP_3$-induced Ca^{2+} release. The numbers in parentheses give the percentage $InsP_3$-induced Ca^{2+} release compared with that released by A23187.

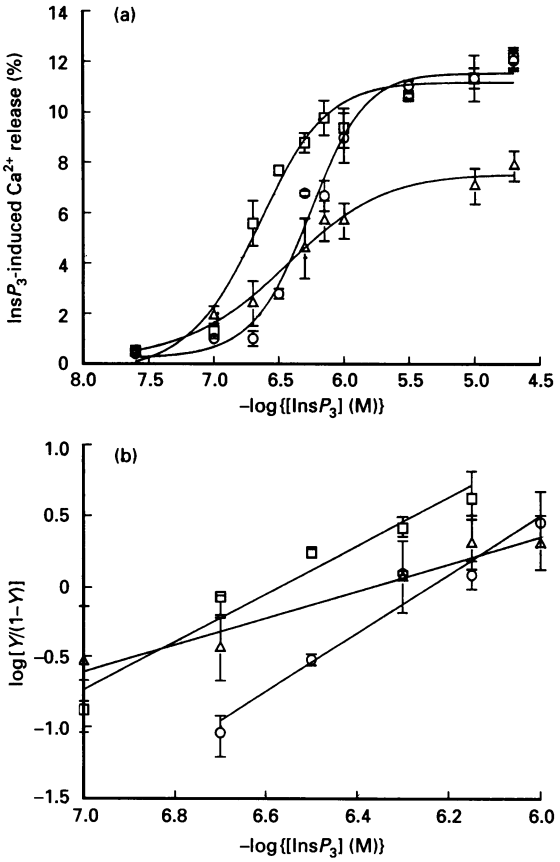

Figure 4 Effects of thimerosal on quantal Ca^{2+} release

(**a**) Effect of $InsP_3$ on the amount of Ca^{2+} release in the absence (○) or in the presence of 2 μM (□) or 10 μM (△) thimerosal. Data presented are means ± S.E.M. of at least three determinations from one microsomal preparation. (**b**) The data in (**a**) are re-expressed in the form of a Hill plot, where Y is the fractional response.

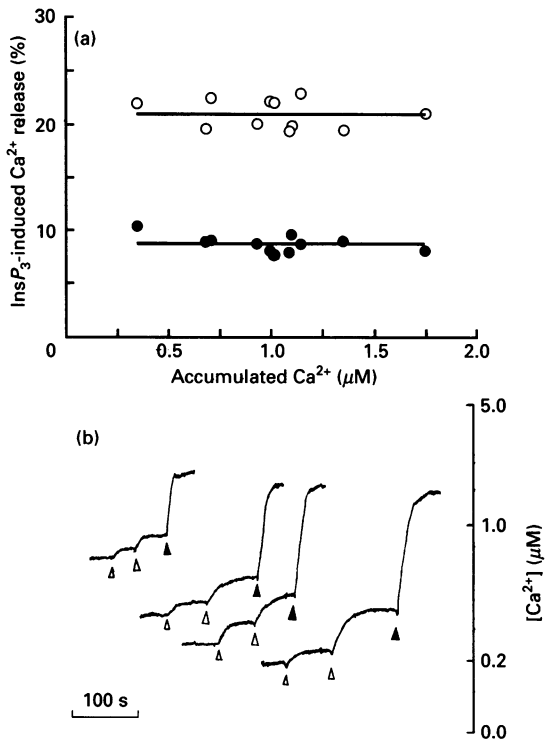

Figure 5 Effects of Ca^{2+} accumulation upon $InsP_3$-induced Ca^{2+} release at maximal and submaximal $InsP_3$ concentrations

(**a**) Ca^{2+} uptake was initiated by the addition of 1.5 mM MgATP. After different time periods of Ca^{2+} accumulation, uptake was inhibited and $InsP_3$-induced Ca^{2+} release was measured at 0.5 μM (●) and 10 μM (○) $InsP_3$ concentrations. The total Ca^{2+} accumulated was determined as the amount of Ca^{2+} released by $InsP_3$ and A23187 together, and Ca^{2+} release is expressed as a percentage of that released by $InsP_3$ compared with the total amount of releasable calcium. (**b**) Levels of Ca^{2+} accumulated with time; the effects of 0.5 μM $InsP_3$ (1), 10 μM $InsP_3$ (2) and A23187 (3) on Ca^{2+} release are shown.

Table 1 Effects of thimerosal on $InsP_3$ binding and $InsP_3$ metabolism in cerebellar microsomes

Values are means ± the S.E.M. of 3–6 determinations. N.D., not determined.

[Thimerosal] (μM)	$InsP_3$ bound (pmol/mg)	$InsP_3$ metabolized (%)
0	7.2±1.3	5±3, 73.0±7*
1	7.5±1.5	4±3
2	7.1±0.7	6±4
5	6.5±1.5	8±4
20	7.9±1.1	7±5
50	6.2±1.0	N.D.

*Measured in 'intracellular' buffer (see Materials and methods section).

cerebellar microsomes, although at much lower concentrations (2 μM), and show that high thimerosal concentrations completely inhibit $InsP_3$-induced Ca^{2+} release. Both thimerosal sensitization and inhibition of the $InsP_3$-induced Ca^{2+} release response can be protected by DTT. We therefore propose that this channel has distinct classes of cysteine residues, which have different sensitivities to thimerosal. The modification of highly reactive cysteines by thimerosal alters the conformational state of the channel so that it becomes more sensitive to $InsP_3$. At higher thimerosal concentrations, lower-reactivity cysteines also become modified, inhibiting the channel. Neither stimulation nor inhibition of the $InsP_3$-sensitive channel by thimerosal can be due to changes in the affinity for $InsP_3$, since [^{3}H]$InsP_3$ binding remains unaffected.

Here we have shown that the quantal Ca^{2+} release properties are affected in the presence of stimulatory concentrations of thimerosal, with a change in the $InsP_3$ concentration required to cause half-maximal release from 0.55±0.06 to 0.22±0.04 μM, but with little change in co-operativity. Recent studies have shown quantal release to occur and behave in an apparently co-operative fashion with the purified cerebellar $InsP_3$ receptor reconstituted into sealed vesicles [21]. This observation therefore indicates that co-operative quantal Ca^{2+} release is an intrinsic property of the receptor. Since $InsP_3$-sensitive Ca^{2+} channels have been shown by cross-linking experiments to exist mainly as tetramers [22], one can speculate that this co-operativity may, in part, be due to allosteric interactions between adjacent channels [5,9]. As no co-operativity has been reported for $InsP_3$ binding to this channel [1], a complex mechanism must exist to link ligand binding with channel opening.

At present, two models have been proposed to account for the phenomenon of quantal Ca^{2+} release. One model postulates that intraluminal Ca^{2+} regulates the opening of the $InsP_3$-sensitive channel [14]. The other model suggests that Ca^{2+} is released in an 'all-or-nothing' manner, with different $InsP_3$-sensitive Ca^{2+} stores having different sensitivities to $InsP_3$ [23]. In order to predict the apparent co-operativity of $InsP_3$-induced calcium release using this second model, within a heterogeneous popu-

lation of Ca^{2+} stores there must be a greater proportion with lower sensitivity to $InsP_3$.

Our findings that $InsP_3$-induced Ca^{2+} release at submaximal $InsP_3$ concentrations is independent of the Ca^{2+} accumulated within cerebellar microsomes would indicate that intraluminal Ca^{2+} does not influence quantal Ca^{2+} release under our experimental conditions. This is consistent with some studies [24,25] in which manipulation of the intraluminal Ca^{2+} concentration had no effect upon quantal Ca^{2+} release. However, other studies in smooth muscle cells and hepatocytes have shown some dependence upon intraluminal Ca^{2+} [26,27].

The finding that half-maximal inhibition of $InsP_3$-induced Ca^{2+} release by thimerosal also affects the apparent co-operativity of quantal Ca^{2+} release, such that the Hill coefficient changes from 2 to 1, is very difficult to explain solely in terms of the 'all-or nothing' model unless the $InsP_3$-sensitive Ca^{2+} stores have different sensitivities to both $InsP_3$ and thimerosal. However, since other unrelated inhibitors such as caffeine [5] and tetrahexyl ammonium chloride (L. Sayers and F. Michelangeli, unpublished work) increase the concentration of $InsP_3$ required to cause half-maximal Ca^{2+} release and decreases the Hill coefficient, this proposal would seem highly unlikely.

It is clear from this study that the opening of the $InsP_3$-sensitive Ca^{2+} channel is complex, and that present models to explain $InsP_3$-induced Ca^{2+} release in cerebellar $InsP_3$ receptors are inadequate. Since modification of specific cysteine amino acids by thimerosal can alter quantal Ca^{2+} release, this thiol-reactive compound may prove to be a useful tool in elucidating this phenomenon.

The MRC, the Royal Society and the SmithKline (1982) foundation are thanked for financial support. The SERC is also thanked for a studentship to L. G. S. Dr. C. J. Kirk and Dr. L. Missiaen are thanked for discussions, as is A. DaSilva for preliminary experiments.

REFERENCES

1 Suppattapone, S., Worley, P. F., Baraban, J. M. and Snyder, S. H. (1988) J. Biol. Chem. **263**, 1530–1534
2 Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Nature (London) **342**, 32–38
3 Muallem, S., Pandol, S. J. and Beeker, T. G. (1989) J. Biol. Chem. **264**, 205–212
4 Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. and Gill, D. L. (1988) J. Biol. Chem. **263**, 11075–11079
5 Brown, G. R., Sayers, L. G., Kirk, C. J., Michell, R. H. and Michelangeli, F. (1992) Biochem. J. **282**, 309–312
6 Missiaen, L., Taylor, C. W. and Berridge, M. J. (1991) Nature (London) **352**, 241–244
7 Michelangeli, F., Di Virgilio, F., Villa, A., Podini, P., Meldolesi, J. and Pozzan, T. (1991) Biochem. J. **275**, 555–561
8 Michelangeli, F. and Munkonge, F. M. (1991) Anal. Biochem. **194**, 231–236
9 Michelangeli, F. (1991) J. Fluoresc. **1**, 203–206
10 Strupish, J., Wojcikiewicz, R. J. H., Challiss, R. A. J., Safrany, S. T., Willcocks, A. L., Potter, B. V. L. and Nahorski, S. R. (1991) Biochem. J. **277**, 294
11 Kirk, C. J., Morris, A. J. and Shears, S. B. (1990) in Peptide Hormone Action: A Practical Approach (Siddle, K. and Hutton, J. C., eds.), pp. 151–184, IRL Press, Oxford
12 Shears, S. B., Parry, J. B., Tang, E. K. Y., Irvine, R. F., Michell, R. H. and Kirk, C. J. (1987) Biochem. J. **246**, 139–147
13 Volpe, P. and Alderson-Lang, B. H. (1990) Am. J. Physiol. **258**, C1086–C1091
14 Irvine, R. F. (1990) FEBS Lett. **263**, 5–9
15 Means, G. E. and Feeney, R. E. (1971) in Chemical Modification of Proteins, pp. 198–204, Holden-Day Inc., San Francisco
16 Gould, G. W., Colyer, J., East, J. M. and Lee, A. G. (1987) J. Biol. Chem. **262**, 7676–7679
17 Henao, F. and Gutierrez-Merino, C. (1989) Biochim. Biophys. Acta **984**, 135–142
18 Gutierrez-Merino, C. (1987) Arch. Biochem. Biophys. **252**, 303–314
19 Andersen, J. P., LeMaire, M. and Moller, J. V. (1980) Biochim. Biophys. Acta **603**, 84–100
20 Rooney, T. A., Renard, D. C., Sass, E. J. and Thomas, A. P. (1991) J. Biol. Chem. **266**, 12272–12282
21 Ferris, C. D., Cameron, A. M., Huganir, R. L. and Snyder, S. H. (1992) Nature (London) **356**, 350–352
22 Miyawaki, A., Furuichi, T., Ryou, Y., Yoshikawa, S., Nakagawa, T., Saitoh, T. and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4911–4915
23 Oldersaw, K. A., Nunn, D. L. and Taylor, C. W. (1991) Biochem. J. **278**, 705–708
24 Shuttleworth, T. J. (1992) J. Biol. Chem. **267**, 3573–3576
25 Combettes, L., Claret, M. and Champeil, P. (1992) FEBS Lett. **301**, 287–290
26 Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992) Nature (London) **357**, 599–602
27 Nunn, D. L. and Taylor, C. W. (1992) Mol. Pharmacol. **41**, 115–119

Received 3 June 1992/7 August 1992; accepted 19 August 1992