Effects of thimerosal on the transient kinetics of inositol 1,4,5-trisphosphate-induced Ca^{2+} release from cerebellar microsomes

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Thimerosal, a thiol-reactive reagent, has been shown to increase the cytosolic Ca^{2+} concentration in a variety of cells by sensitizing inositol 1,4,5-trisphosphate ($Ins P_3$) receptors. Thimerosal can have both sensitizing (at concentrations of $< 2\ \mu M$) and inhibitory (at concentrations of $> 2\ \mu M$) effects on $Ins P_3$-induced Ca^{2+} release (IICR) from cerebellar microsomes. Transient kinetic studies were performed by employing a fluorimetric stopped-flow approach using fluo-3. IICR was found to be a bi-exponential process with a fast and a slow component. At a maximal $Ins P_3$ concentration (20 μM), the fast phase had a rate constant of 0.9 s^{-1} and the slow phase had a rate constant of 0.4 s^{-1}. The amplitudes of the two phases were 60 % and 40 % respectively. When the rate constants for the two phases were plotted as Hill plots, the processes were found to be non-co-operative in both cases (Hill coefficient of 1.0), thus arguing for a simple mechanism linking $Ins P_3$ binding to channel opening. At a submaximal $Ins P_3$ concentration (0.2 μM), where the sensitizing effects of thimerosal are most pronounced, thimerosal increased the rate constants of both phases in a sigmoidal fashion, with a Hill coefficient of 4.0, suggesting that several cysteine residues (up to four) need to be modified in order for maximum sensitization to occur. The rate constants remained elevated even at thimerosal concentrations that inhibited IICR. The amplitude or extent of Ca^{2+} release was, however, elevated to a much greater extent in the slow phase, suggesting that the two phases respond differently. At maximal $Ins P_3$ concentrations, thimerosal has no effect upon the rate constants but inhibits the amplitude of Ca^{2+} release.

INTRODUCTION

It is now well established that thimerosal can evoke increases in intracellular Ca^{2+} concentrations in a variety of cell types [1]. The treatment of fura-2-loaded HeLa cells with low concentrations of thimerosal in Ca^{2+}-free medium caused Ca^{2+} spikes similar to those observed after treatment of the cells with the agonist histamine [2]. As no detectable increase in the phosphatidylinositol lipids above the resting levels was observed when the cells were treated with thimerosal, this result, in addition to other reports, has led to the conclusion that thimerosal exerts its effects on the inositol 1,4,5-trisphosphate ($Ins P_3$)-sensitive Ca^{2+} channel or $Ins P_3$ receptor [3–7]. Kaplin et al. [4], using the purified $Ins P_3$ receptor reconstituted into lipid vesicles, showed that low concentrations of thimerosal produced an approx. 30 % stimulation of $Ins P_3$-induced Ca^{2+} release (IICR), whereas a very high concentration (1 mM) abolished Ca^{2+} release. [^{3}H]$Ins P_3$ binding studies showed that thimerosal, at concentrations that would normally inhibit IICR, reduced the dissociation constant (K_d) for $Ins P_3$ binding by at most 2–3-fold, with little effect on the maximum amount of $Ins P_3$ bound [4,8]. These results thus confirm that thimerosal is acting directly upon the $Ins P_3$ receptor, possibly by modifying conserved cysteine residues located within the C-terminal region [9,10].

Using cerebellar microsomes, we have previously demonstrated that thimerosal causes a biphasic response that is dependent upon its concentration. At submaximal $Ins P_3$ concentrations, low concentrations of thimerosal (1–3 μM) caused an approx. 3-fold increase in the extent of IICR, while at higher thimerosal concentrations ($> 5\ \mu M$) inhibition was observed [7]. This increase in the extent of Ca^{2+} release was due to an increase in the efficacy of $Ins P_3$ in inducing Ca^{2+} release, changing the IC_{50} for release by approx. 3-fold without changing the maximal extent. At higher thimerosal concentrations the efficacy remained little affected, although the maximum extent of Ca^{2+} release decreased [7]. In addition, the effects of sensitization and inhibition by thimerosal could be protected by dithiothreitol. These results led us to propose that the $Ins P_3$ receptor has distinct classes of cysteine amino acids that have different sensitivities to thimerosal. The modification of highly reactive cysteines by low concentrations of thimerosal alters the conformational state of the receptor such that it becomes more sensitive to $Ins P_3$. At higher thimerosal concentrations, less reactive cysteines become modified, causing channel inhibition [7]. The fact that some thiol-reactive reagents such as Mersalyl [11], t-butyl hydroperoxide [12] and oxidized glutathione [3,13] also sensitize IICR, whereas other reagents such as *N*-ethylmaleimide [14], *p*-chloromercuriobenzoic acid [15] and Ag^+ [16] only appear to inhibit IICR, supports our proposal that different classes of cysteine residues that can be preferentially modified by different thiol-reactive reagents cause either sensitization or inhibition of the $Ins P_3$ receptor.

The sensitization of Ca^{2+} release by thimerosal has also been observed with the ryanodine receptor [5,6]. As this channel has large sequence similarities with the $Ins P_3$ receptor at the C-terminal region [9,10], this would suggest that the mode of action of thimerosal might be similar in these related Ca^{2+} channels.

In the present study, we examine the effects of thimerosal on the transient kinetic properties of IICR in an attempt to explore further the process underlying the sensitizing and inhibitory properties of this reagent.

MATERIALS AND METHODS

Rat cerebellar microsomes were prepared as described previously [7]. Briefly, approx. 20 cerebella were homogenized in 10 vol. of buffer containing 0.32 M sucrose and 5 mM Hepes, pH 7.4, in the presence of 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A and 50 μM benzamidine, and then centrifuged for 10 min at

Abbreviation used: IICR, $Ins P_3$-induced Ca^{2+} release.

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500 ***g***. The pellet was resuspended in 5 vol. of the same buffer and centrifuged as above. The resulting supernatants were pooled and centrifuged for 20 min at 10000 ***g***. The supernatant from this stage was centrifuged for 1 h at 100000 ***g*** and the resulting pellet was finally resuspended in ~ 2 ml of the buffer, divided into 100 μl fractions, snap-frozen in liquid nitrogen and stored at -70 °C.

Calcium uptake and release experiments

Ca^{2+} uptake and release were measured as described in [17,18]. Typically, 300 μg of rat cerebellar microsomes were suspended in 2 ml of 40 mM Tris/phosphate buffer, 100 mM KCl, pH 7.2, at 37 °C in the presence of 10 mM phosphocreatine, 10 μg/ml creatine kinase and 1.25 μM fluo-3. The mixture was incubated for ~ 10 min at 37 °C to reach thermal equilibrium and then Ca^{2+} uptake was initiated by the addition of 1.5 mM MgATP. Changes in Ca^{2+} concentration were followed by measuring the fluorescence change of fluo-3 in a Perkin–Elmer LS-50B fluorimeter (excitation at 505 nm and emission at 525 nm). When sufficient Ca^{2+} had been taken up (i.e. no further Ca^{2+} uptake was observed), the pump was inhibited by the addition of between 75 and 150 μM orthovanadate (this was found to cause more than 90 % inhibition [19]). When the fluorescence change had reached a new steady state (i.e. where the rate of Ca^{2+} uptake was equal to the rate of Ca^{2+} efflux), InsP_3 was added, and the amount of Ca^{2+} release was expressed as a percentage of that released by 12.5 μg/ml ionophore A23187. The fluorescence intensity was related to $[Ca^{2+}]$ by using the following equation:

$$[Ca^{2+}] = K_d(F-F_{min})/(F_{max}-F) \quad (1)$$

where K_d is the dissociation constant for Ca^{2+} binding to fluo-3 (taken to be 900 nM under these conditions [17–19]), F is the fluorescence intensity of the sample and F_{min} and F_{max} are the fluorescence intensities of the sample in the presence of 1.5 mM EGTA and ~ 1.8 mM $CaCl_2$ respectively.

Stopped-flow measurements of IICR

Rapid kinetic measurements of IICR were carried out as described in [17,18], Ca^{2+} uptake by the microsomes was followed in a conventional fluorimeter as described above. After the addition of orthovanadate to inhibit any further uptake, the mixture was transferred to syringe A of a stopped-flow spectrofluorimeter (Applied Photophysics, Model SX17 MV); syringe B was filled with InsP_3 at a concentration 10 times the concentration in the mixing chamber, as the mixing ratio of syringe A to syringe B is 10:1 (v/v). The temperature of the syringe compartment was maintained at 37 °C by a circulating water bath. The fluorescence change of fluo-3 was then monitored by exciting the sample at 505 nm and measuring the emission above 515 nm using a cut-off filter. The data were then collected and the fluorescence intensities were correlated to $[Ca^{2+}]$ by comparing the traces with those from identical experiments carried out in a conventional fluorimeter. The traces were analysed using non-linear regression analysis programs supplied by Applied Photophysics and Biosoft. IICR in the microsomal preparation under study was shown to be biphasic and, as previously described [18,20,21], fitted well to a bi-exponential process using the following equation:

$$Ca^{2+} \text{ release} = A_1(1-\exp^{-k_1 t}) + A_2(1-\exp^{-k_2 t}) \quad (2)$$

where A_1, A_2, k_1 and k_2 are the amplitudes and rate constants of Ca^{2+} release for the fast and slow phases respectively, and t is the time (s).

Over the Ca^{2+} concentration range for which IICR was monitored, the fluorescence changes when related to $[Ca^{2+}]$ were around the K_d value for Ca^{2+} binding to fluo-3. Over this range, the fluorescence was linearly related to $[Ca^{2+}]$ ($r > 0.99$) [19]. We have also calculated that any underestimation in the rate constant for Ca^{2+} release due to the small amount of residual Ca^{2+} pump activity (i.e. < 10 % of the total pump activity) would affect the release rate constants by less than 0.01 s^{-1} [19].

RESULTS

With the rat cerebellar microsomes used in the present study, maximal Ca^{2+} release occurred at about 1–3 μM InsP_3, with approx. 18 % of stored Ca^{2+} being mobilized compared with that released by A23187. The EC_{50} for IICR was found to be 0.24 ± 0.05 μM InsP_3, which is in the typical range of values that we have obtained with other cerebellar microsomal preparations (Table 1). Figure 1 shows the effects of thimerosal on IICR at maximal (20 μM) and submaximal (0.2 μM) InsP_3 concentra-

Table 1 Comparison of the kinetic parameters of IICR obtained with different cerebellar microsomal preparations

The kinetic parameters shown are results obtained with various cerebellar microsomal preparations in a number of studies; Ca^{2+} release by 20 μM InsP_3 was analysed in each case. Results are from: *, the present study, †, [18]; ‡, [20]; §, [17] (fitted to a mono-exponential equation); ‖, [19] (fitted to a mono-exponential equation). Preparations 2–4 are unpublished work (M. Mezna and F. Michelangeli).

Microsomal preparation	EC_{50} (μM)	Rate constant (s^{-1})		Amplitude (fraction of Ca^{2+} released)	
		Fast phase	Slow phase	Fast phase	Slow phase
1*	0.24	0.90	0.40	0.60	0.40
2	0.18	1.54	0.31	0.51	0.49
3	0.20	1.20	0.30	0.55	0.45
4	0.21	1.70	0.30	0.56	0.44
5†	0.14	1.60	0.50	0.45	0.55
6†	0.20	1.45	0.32	0.58	0.42
7‡	0.40	1.69	0.35	0.51	0.49
8§	1.30	0.50	–	1.00	–
9‖	0.50	0.93	–	1.00	–

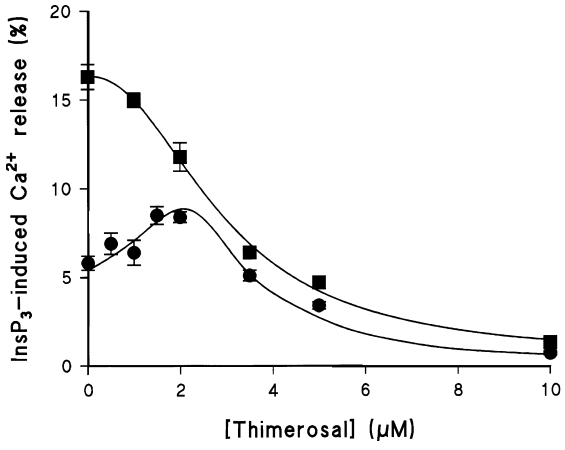

Figure 1 Effects of thimerosal on IICR

The effects of thimerosal on IICR were monitored at both maximal (20 μM; ■) and submaximal (0.2 μM; ●) InsP_3 concentrations. The values are expressed as means $\pm$ S.D. of three or more determinations.

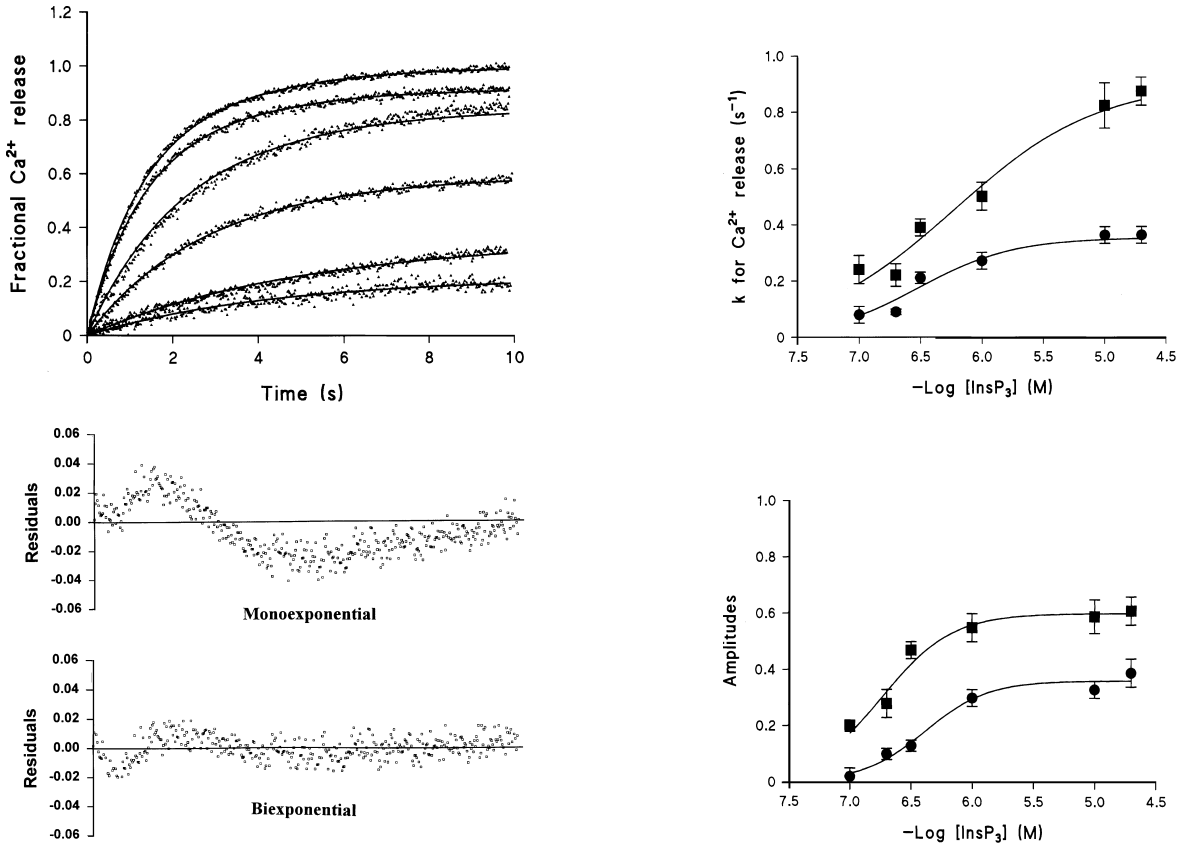

Figure 2 Transient kinetics of IICR

Top left panel: the time course of IICR was resolved on a stopped-flow spectrofluorimeter as described in the Materials and methods section. Each trace represents the average of at least six acquisitions. InsP_3 concentrations used were, from bottom to top, 0.1, 0.2, 0.3, 1.0, 10 and 20 μM. The solid lines through the data points represent the best bi-exponential fits, with χ^2 values of less than 0.1. The lower panels show the residual plots of Ca^{2+} release traces induced by 20 μM InsP_3, fitted to mono-exponential and bi-exponential equations. The right-hand panels show the rate constants (top) and amplitudes (or relative extent) of Ca^{2+} release (bottom) for the fast (■) and slow (●) phases of IICR determined from the fits described above. An amplitude of 1.0 is equal to the extent of Ca^{2+} release obtained by 20 μM InsP_3.

tions. At the maximal InsP_3 concentration, thimerosal caused a dose-dependent decrease in IICR, with 10 μM thimerosal almost completely inhibiting Ca^{2+} release (IC_{50} 3.0 ± 0.5 μM). In contrast, at the submaximal InsP_3 concentration, thimerosal produced a biphasic response, with low concentrations (2 μM) causing $\sim 60\%$ stimulation in the extent of Ca^{2+} release and higher concentrations (> 3 μM) inhibiting IICR.

Figure 2 (top left panel) shows the time-resolved IICR obtained using stopped-flow spectrofluorimetry. The traces obtained were analysed using non-linear regression analysis and shown to fit extremely well to the bi-exponential equation, assuming two independent processes (the χ^2 values for all traces were between 0.03 and 0.10, and were consistently lower than the corresponding χ^2 values gained when fitting the traces to a mono-exponential equation). In addition, Figure 2 also shows the residual plots of the data for IICR by 20 μM InsP_3, and illustrates that the data approximate better to a bi-exponential process than a mono-exponential one, as in the latter case the residuals show less of a random distribution. The rate constants and amplitudes of Ca^{2+} release generated from these data are shown in Figure 2 (right panels). It is clear that the rate constants for both the fast and slow phases increased in response to increasing InsP_3 concentration; the rate constant for the fast phase increased from 0.22 s^{-1} at 0.1 μM InsP_3 to 0.9 s^{-1} at 20 μM InsP_3, while the rate constant for the slow phase increased from 0.06 s^{-1} to 0.37 s^{-1} at 0.1 and 20 μM InsP_3 respectively. The Hill coefficients of the rate constants for both the fast and slow phases were identical and non-co-operative ($h = 1.0 \pm 0.1$; see Figure 5). The amplitude (or fractional release) also increased with InsP_3 concentration, with the amplitude of the fast phase increasing from 0.18 to 0.6 and that of the slow phase increasing from 0.02 to 0.4 at InsP_3 concentrations between 0.1 and 20 μM. A slightly larger amount of Ca^{2+} is therefore released by the fast-phase component (60%) than by the slow-phase component (40%). Table 1 also shows that the kinetic parameters, i.e. EC_{50} values, rate constants and amplitudes, of this microsomal preparation are reasonably typical of other cerebellar microsomal preparations so far characterized (mean values for all preparations in Table 1 are: EC_{50}, 0.37 ± 0.3 μM; fast-phase k, 1.28 ± 0.40 s^{-1}; slow-phase k, 0.35 ± 0.07 s^{-1}; fast-phase fractional amplitude, 0.54 ± 0.06; slow-phase fractional amplitude, 0.46 ± 0.06).

The effects of different concentrations of thimerosal on the time course of IICR at a low InsP_3 concentration (0.2 μM) were studied. The analysis of these traces showed that the rate constant for the fast phase increased from 0.2 s^{-1} in the absence of thimerosal to 0.65 s^{-1} at 2 μM thimerosal, and then remained constant even at 10 μM thimerosal. The rate constant for the slow phase of Ca^{2+} release also increased, giving a maximum rate

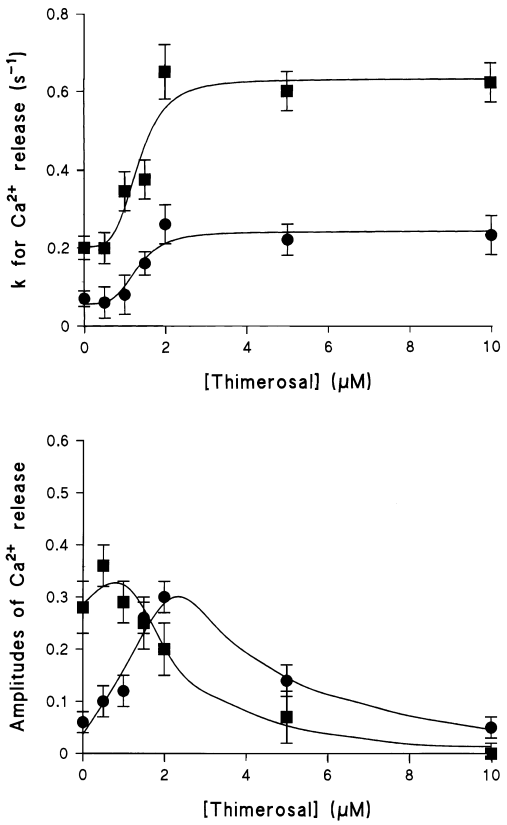

Figure 3 Effects of thimerosal on the transient kinetics of IICR at a submaximal InsP_3 concentration (0.2 μM)

The kinetic parameters were determined for the effects of thimerosal on IICR at 0.2 μM InsP_3 and at thimerosal concentrations of 0, 0.5, 1.0, 1.5, 2.0, 5.0 and 10 μM. Upper panel: rate constants for the fast (■) and slow (●) phases of IICR (0.2 μM InsP_3) at different concentrations of thimerosal. The solid lines represent the best fits to the Hill equation, assuming a Hill coefficient of 4.0. Lower panel: amplitude (extent) of Ca^{2+} release for the fast (■) and slow (●) phases of IICR (0.2 μM InsP_3) at different thimerosal concentrations. The values were determined from bi-exponential fits of the Ca^{2+}-release traces.

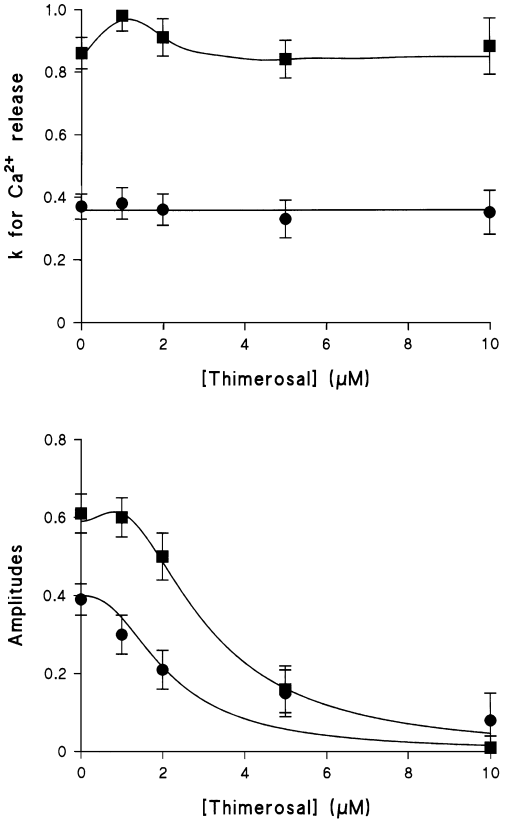

Figure 4 Effects of thimerosal on the transient kinetics of IICR at a maximal InsP_3 concentration (20 μM)

The kinetic parameters were determined for the effects of thimerosal on IICR using 20 μM InsP_3 at thimerosal concentrations of 0, 1, 2, 5 and 10 μM. Upper panel: rate constants for the fast (■) and slow (●) phases of IICR at 20 μM InsP_3, determined from the bi-exponential fits to the Ca^{2+}-release traces. Lower panel: amplitude (or extent of Ca^{+} release) for the fast (■) and slow (●) phases of IICR determined from the bi-exponential fits to the Ca^{2+}-release traces.

constant of $\sim 0.2\ s^{-1}$ at 2 μM thimerosal, which again remained unchanged at up to 10 μM thimerosal (Figure 3, upper panel). In addition, both the rate constants for the fast and slow components increased by a similar proportion ($\sim$ 3.5-fold). The apparent dissociation constants for the sensitization of the two components were also identical (1.2 ± 0.2 μM). Figure 3 (upper panel) also shows that the increases in the rate constants for both phases were sigmoidal in appearance. The data were best fitted to the Hill equation assuming $h = 4.0$ (shown as the solid line). Figure 3 (lower panel) shows the changes in the amplitude (or fractional extent) of Ca^{2+} release for the two components. The amplitude of the fast phase, after a slight increase at low thimerosal concentrations, decreased at thimerosal concentrations above 0.5 μM, whereas the amplitude of the slow component increased up to 2 μM thimerosal and then decreased at higher concentrations. The total increase in the slow-phase amplitude was 5-fold (0.06 to 0.30), which is approaching the maximal extent of Ca^{2+} release for this component observed with saturating InsP_3 concentrations (the maximum fractional release at 20 μM InsP_3 being 0.4).

The effects of thimerosal at the maximal InsP_3 concentration (20 μM) on the transient kinetics of IICR were investigated. As observed in Figure 1, at this high InsP_3 concentration thimerosal mainly inhibited the extent of IICR. However, Figure 4 (upper panel) shows that the rate constants for the fast and slow phases of Ca^{2+} release remained little affected over a thimerosal concentration range of 0–10 μM. The amplitude (or extent of fractional release) for both the fast and slow phases decreased with increasing thimerosal concentration (Figure 4, lower panel), with IC_{50} values of 3.3 ± 0.5 and 2.7 ± 0.5 μM thimerosal respectively.

DISCUSSION

The kinetics of IICR from microsomal vesicles are highly complex, and are likely to be dependent upon a multitude of factors, including the distribution of isoforms and spliced variants, the phosphorylation state of the receptor, the concentration of luminal Ca^{2+} and other ions within individual Ca^{2+} stores, the receptor density per vesicle and the presence of modulatory proteins [1]. However, attempts to simplify the system by using the purified receptor from the cerebellum (which contains 99 % type I isoform), reconstituted into sealed membrane vesicles, have not significantly resolved the complex kinetic behaviour of this channel [22]. The transient release of Ca^{2+} in this purified

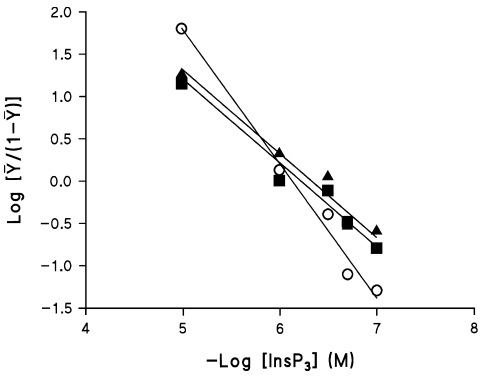

Figure 5 Hill plots of the rates of IICR

Ca^{2+}-release rates are presented as Hill plots, where the fractional rates ($\bar{Y}$) were determined from the initial rate (○) or from the fractional rate constants ($\bar{Y}$) of the fast (■) and slow (▲) phases.

system still appears to be biphasic, with a fast and a slow component similar to those observed with crude cerebellar microsomes. Analysis of the initial rates of release as a function of InsP_3 concentration, in the purified system, showed the process to be apparently co-operative with a Hill coefficient of ~ 1.8. This type of co-operativity, with a similar value for the Hill coefficient, was also observed by Champeil et al. [23] using permeabilized rat hepatocytes, which have a different distribution of InsP_3 receptor isoforms [24].

Figure 5 shows that the Hill coefficient in our system, calculated by taking the initial rates, is also similar (1.7 ± 0.2). However, the Hill equation assumes that the initial rates being measured are due to a single process. It is clear from our studies showing variations in the k_{fast}/k_{slow} ratio for Ca^{2+} release with InsP_3 concentration [20] that IICR consists of two or possibly more independent components that contribute to this process. As such, calculating the Hill coefficient from the initial rates introduces bias depending upon the contributions that each of the components make during the initial phase of release. In order to gain a more reliable value for the Hill coefficient for Ca^{2+} release, it would be more accurate to first resolve the contribution that each component makes to the release process and then calculate initial rates for each component. Alternatively, since the rate constants measured for the two phases plateau at maximal InsP_3 concentrations, the Hill coefficient can also be calculated from the changes in the rate constants for each phase with InsP_3 concentration. As can be seen in Figure 5, when the rate constants for each phase are plotted, the calculated Hill coefficients for the two phases appear to be identical and non-co-operative ($h = 1.0$). Although the receptor, in its native state, is believed to exist as a tetramer [25,26], these findings show that IICR is non-co-operative. This would suggest that there is no beneficial interaction between receptor monomers in influencing channel gating, and argues for a simple mechanism relating InsP_3 binding to Ca^{2+} release.

It is widely believed from electron microscopy evidence [26] that one functional channel exists per InsP_3 receptor tetramer. To reconcile the presence of a single channel per tetramer with the non-co-operativity of Ca^{2+} release, elaborate models (other than those previously proposed, which have assumed some co-operativity [27]) need to be developed. One such model has been proposed by Watras et al. [28] to explain how the rates of release vary with InsP_3 concentration. However, in order to fit their experimental data obtained with permeabilized basophilic leukaemia cells, they also had to assume that there are two distinct types of InsP_3 receptors with different affinities, forming mixed populations of heterotetramers, and that three out of the four receptors within the tetramer need to be occupied for channel opening to occur. Such a model could also explain our data since, although 99% of the InsP_3 receptors in the cerebellum are of the type I isoform [24], both types of the splice variant within the InsP_3 binding domain (S1) exist (25% $S1^+$ and 75% $S1^-$ [29]), allowing for some heterogeneity in InsP_3 sensitivity in our system.

The initial observation that the thiol-reactive reagent thimerosal was able to sensitize IICR in permeabilized hepatocytes [3] has provoked much interest in the effects of cysteine modification on InsP_3 receptor function. There is some controversy at present as to whether the stimulatory effects observed with thimerosal are due solely to an increase in receptor affinity. Studies by Poitras et al. [30] in bovine adrenal chromaffin microsomes, Hilly et al. [8] in cerebellar membranes and Kaplin et al. [4] with the purified receptor have demonstrated that thimerosal increases the affinity of InsP_3 binding to these membranes by at most 2–3-fold, and then only at 100 μM thimerosal, a concentration at which we would normally see complete inhibition of IICR. Conversely, Parys et al. [31] and ourselves [7] have detected little or no change in InsP_3 affinity in the presence of thimerosal. From Figure 3 (upper panel), it can be seen that the rate constants for Ca^{2+} release for the two phases increased by > 3-fold, which, if related to the rate constants for Ca^{2+} release as a function of InsP_3 concentration (Figure 2), would mean that 0.2 μM InsP_3 is acting on the release process as if it were ~ 2 μM. This would suggest that the change in affinity would need to be ~ 10-fold, i.e. much greater than any changes in affinity previously reported. This implies that thimerosal must be exerting its effect upon the channel opening process, in addition to any changes in affinity. This proposal is also supported by the very recent observation of the effects of thimerosal on the InsP_3 receptor at the single-channel level [32]. By reconstituting the InsP_3 receptor into planar lipid bilayer membranes and using electro-physiological means to record activity, it was revealed that thimerosal created additional higher-conductance states of the channel that were not observed in the presence of maximal InsP_3 concentrations alone [32]. In addition, the fact that the cysteine residues believed to be modified by thimerosal are located at the C-terminal region of the receptor, close to the channel domain [10], is also consistent with additional effects upon the channel pore.

Furthermore, Figure 3 (upper panel) shows a pronounced sigmoidal dependence of the rate constants of both the fast and slow phases on thimerosal concentration at low InsP_3 concentrations. Both profiles could be fitted well to a Hill equation, assuming a Hill coefficient of 4.0. Such an observation might imply that up to four cysteine residues, either on individual subunits or one on each of the four subunits of the tetramer, are required to be modified by thimerosal before full sensitization of Ca^{2+} release can be achieved.

Our results are consistent with the idea that thimerosal modifies highly sensitive cysteine residues on the channel, inducing higher-conductance states and thus resulting in faster kinetics of Ca^{2+} release. Moreover, our study also suggests that these higher-conductance states are likely to remain even at high thimerosal concentrations that inhibit IICR. In the case of the thimerosal inhibition of IICR, it appears that the rate constants for release are essentially unaffected; rather, it is the extent of Ca^{2+} release that is progressively decreased. This decrease could be due to progressively more Ca^{2+} stores having their InsP_3 receptors modified on lower-sensitivity cysteines, which, when modified,

inhibit IICR. Assuming either that the receptor density per Ca^{2+} store ratio is low, or that each Ca^{2+} store has $InsP_3$ receptors showing similar sensitivities to $InsP_3$ and thimerosal, then these Ca^{2+} stores would play no further role in IICR.

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