The inositol 1,4,5-trisphosphate-gated Ca^{2+} channel: effect of the protein thiol reagent thimerosal on channel activity

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The solubilized partially purified Ins(1,4,5)P_3-sensitive Ca^{2+} channel from rat cerebellum has been reconstituted into planar lipid bilayer membranes by the 'tip-dip' method [Ehrlich (1992) Methods Enzymol. **207**, 463–471] allowing low noise current records. Single-channel events have been recorded. In the presence of 10 μM Ins(1,4,5)P_3, 50 μM ATP, and 0.2 μM Ca^{2+} the Ins(1,4,5)P_3 receptor channel opens to a conductance level of 53 pS. In the presence of 100 μM thimerosal (TMS), a sulphydryl-oxidizing agent, three subconductance levels (60 pS, 80 pS and 120 pS) were observed. More than one population of mean open times was found, both in the absence and presence of TMS, although TMS affected the length of the open time by decreasing the short opening significantly from 4.05 ms to 2.78 ms and increasing the longer open time from 27.8 ms to 94.8 ms. The results indicate that TMS enhances Ins(1,4,5)P_3-induced Ca^{2+} release by both altering the open times of the channel significantly and causing a shift to higher subconductance levels.

INTRODUCTION

Ins(1,4,5)P_3 is a second messenger of the phosphoinositide system which can mobilize Ca^{2+} from intracellular stores by interaction with its receptor/channel (InsP_3R), a process known as Ins(1,4,5)P_3-induced Ca^{2+} release (IICR). Sulphydryl-oxidizing reagents have been shown to sensitize IICR [1] and can initiate spontaneous Ca^{2+} release in permeabilized hepatocytes under conditions whereby IICR is the only Ca^{2+} release mechanism able to occur [2]. Ca^{2+} oscillations have also been induced by t-butyl hydroperoxide (TBHP) [1] in the absence of agonist-stimulated Ins(1,4,5)P_3 formation. TBHP is thought to increase the intracellular free Ca^{2+} concentration by oxidation of glutathione, leading to formation of mixed disulphides between protein thiol groups and oxidized glutathione (GSSG), subsequently increasing Ca^{2+} release. In support of this view, direct addition of GSSG to permeabilized hepatocytes has been shown to enhance the efficacy of Ins(1,4,5)P_3 in releasing Ca^{2+} as seen from analysis of dose–response curves [3].

A similar effect has been seen using thimerosal (TMS), another sulphydryl-oxidizing agent, in hamster eggs [4] and purified InsP_3Rs reconstituted into lipid vesicles [5]. The consensus of these findings is that the oxidative state of protein thiol groups present in the InsP_3R itself is able to influence IICR. It should be noted, however, that TMS is also able to affect the endoplasmic reticulum Ca^{2+} pump, providing an indirect means of affecting IICR [6,7].

All of these compounds that affect IICR can be used in studies in an attempt to elucidate the mechanism of the receptor. TMS, for example, provides a means for studying the sensitization of the InsP_3R to Ins(1,4,5)P_3. All of the studies involving TMS so far have explored its effect on populations of InsP_3Rs. This raises the question as to whether these reagents are simply causing more channels to open or whether they are modifying channels that have already been recruited. Using partially purified InsP_3R reconstituted into planar lipid bilayer membranes by the 'tip-dip' [8] technique, we show here that TMS modifies individual channel behaviour at saturating concentrations of Ins(1,4,5)P_3 and may thus have effects on Ca^{2+} release through this mechanism as well as any possible effects mediated via Ins(1,4,5)P_3 binding.

MATERIALS AND METHODS

Purification of the InsP_3R

The InsP_3R was solubilized in 1 % Triton X-100 and purified from rat cerebellum [9] using a two-step column purification procedure, the latter stage consisting of heparin affinity chromatography.

The heparin–agarose column was washed with 60 ml of 50 mM Tris/HCl, pH 8.3, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 M NaCl and 0.1 % octylglucoside in order to replace the Triton X-100 with octylglucoside, as Triton interferes with membranes formed by the 'tip-dip' method. This was followed by elution with a minimum of 20 ml of Tris/HCl, pH 7.7, 1 mM 2-mercaptoethanol, 0.1 % octylglucoside and 0.5 M NaCl. The purified receptor was collected as 0.5 ml fractions. Binding activity was determined by the poly(ethylene glycol) precipitation assay [9].

The 'tip-dip' method

The micropipettes used in this technique were made from borosilicate glass capillaries (Clark Electromedical Instruments GC150-15) using a KOPF vertical pipette puller. The pipette acted as the *trans* (luminal) compartment, containing 25 mM KCl, 53 mM $Ba(OH)_2$/Hepes, pH 7.35. Ba^{2+} was used as the current carrier as it is not thought to regulate the receptor [9a] and single-channel currents have been shown to be 60 % larger with a 3-fold increase in mean open time of the channels compared

Abbreviations used: InsP_3R, Ins(1,4,5)P_3 receptor; IICR, Ins(1,4,5)P_3-induced Ca^{2+} release; TBHP, t-butyl hydroperoxide; TMS, thimerosal; p.d., potential difference.

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with experiments in which Ca^{2+} was the current carrier [10,11]. The buffer used in the *cis* (cytoplasmic) chamber was 25 mM KCl, 250 mM Hepes-KOH, 1 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA), pH 7.35.

The lipids used consisted of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in the ratio 7:3 (w/w) (Avanti Polar Lipids).

The apparatus was set up as described [8]. Briefly, the lipid monolayer was spread over the surface of the buffer in the *cis* chamber. The micropipette tip was immersed in the buffer, withdrawn, and then immersed again creating a bilayer. Upon introduction of 1 μg of Ins(1,4,5)P_3 channel protein and its subsequent incorporation into the bilayer, 50 μM ATP (which has been shown to increase the frequency of channel opening [11]) and 150 μM $CaCl_2$ {giving a free [Ca^{2+}] of 200 nM, i.e. within the range of free [Ca^{2+}] found to maximize activity of the Ins(1,4,5)P_3-gated channel [10]} to the *cis* (cytoplasmic) side, no channel events were seen. After addition of 10 μM Ins(1,4,5)P_3 single-channel events were recorded using a microcomputer (PC) together with an interface (CED 1401 Cambridge Electronic Design) and a software PAT V6.1 for single-channel analysis supplied by J. Dempster, University of Strathclyde, and Mathcad [12]. Since Ins(1,4,5)P_3 is only present on the *cis* side of the bilayer we assume that channel activity will only be seen from channel molecules incorporated with the Ins(1,4,5)P_3-binding domain orientated on that side of the bilayer. Channel activity was abolished by the addition of heparin (15 μg/ml), an InsP_3R-specific antagonist, to the *cis* side. Similar experiments were repeated, this time with the addition of 100 μM TMS, 2 min after channel incorporation into the bilayer. This concentration of TMS (100 μM) was found to be the optimal value for enhancement of the Ins(1,4,5)P_3 effect in previous kinetic studies with rat brain microsomal vesicles (A. P. Dawson, M. Bhogal and E. C. Thrower, unpublished work). The dose–response effect of TMS using the 'dip-tip' technique was technically too difficult to achieve.

RESULTS

Single-channel activity in the presence of Ins(1,4,5)P_3 and absence of TMS

The receptor used in these experiments was partially purified (25–30-fold) from rat cerebellar membranes. B_{max} of the original membranes was 8–10 pmol of Ins(1,4,5)P_3/mg and this increased to 200–400 pmol/mg for the partially purified receptor (typical of five similar purifications). Although this value is less than that for pure receptor, the preparation showed one major band on SDS/PAGE at a molecular mass of 230 kDa (results not shown), suggesting that the primary contaminant is inactive receptor protein. A 1 μg sample of partially pure Ins(1,4,5)P_3 channel protein was introduced to the *cis* chamber and subsequently incorporated into the bilayer. After addition of 10 μM Ins(1,4,5)P_3 and 50 μM ATP to the *cis* side single-channel events were recorded (see Figure 1, upper panel). The experiment shown is typical of five similar separate recordings. For each experiment the mean single-channel current step was 2.0 pA at zero applied potential difference (p.d.). The single-channel slope conductance, obtained from voltage–current analysis (see Figure 1, middle and lower panels), was approx. 53 pS. Inspection of the records and preliminary analysis did not suggest any significant voltage-dependence. The reversal potential was -31 mV. By plotting ln (N_t) (where N_t is the number of channels with a lifetime $\geqslant t$) against time (ms) the mean open time(s) of the channel can be determined. Where this plot is linear, the gradient, m, can be used to estimate mean open time τ by use of the relation $\tau = 1/m$. Figure 2 shows the data from five separate sets of records of channel openings at 0 mV p.d. plotted in this way. Analysis of the total population of channels gives a non-linear plot, suggesting that there is more than one channel lifetime. The linear portion of the curve shown in Figure 2 has a slope indicating a population of channels with a mean open time of 27.8 ± 0.8 ms ($\pm$S.E.M). When this population was stripped out of the data, the remaining channel lifetimes fitted on a single straight line and had a much shorter mean open time of 4.05 ± 0.62 ms ($\pm$S.E.M). Thus the channel can apparently open in two states with different mean open times. This behaviour can be seen by inspection of the channel records (e.g. Figure 1, upper and middle panels) where both long and very short events are present. However, also in Figure 1, it appears that the single-channel conductance is the same for both long- and short-lifetime channels.

On the basis of all-point histograms (which can be used to determine the percentage time of the recording the channel spends in a particular state, or the probability of the channel opening to a particular state), the InsP_3R was found to be in the closed state 70% of the recording time and at the 53 pS conductance level for 30% of the recording time at 0 mV p.d. (see Figure 3). A plot of channel lifetime against current for individual channel events (Figure 4) showed that there was no apparent correlation between lifetime and conductance. Channel activity was abolished by the addition of the antagonist heparin (15 μg/ml).

Single-channel activity in the presence of Ins(1,4,5)P_3 and TMS

After treatment with TMS, single-channel characteristics were found to be changed. A typical record of channel activity [again in the presence of 10 μM Ins(1,4,5)P_3 and 50 μM ATP at 0 mV p.d.] following incubation with 100 μM TMS is shown in Figure 5. Some very long-lived channel events are present, together with some short ones. Once again, channel open times were analysed by plotting ln (N_t) against time to determine the mean open time(s) (Figure 6). The linear region describing the long open time events had a slope yielding a mean open time of 94.8 ± 26.8 ms ($\pm$S.E.M.). Stripping out the long open time events left another straight line, giving a much shorter mean open time of 2.78 ± 0.26 ms. By comparing long open time in the presence of TMS with long open time in the absence of TMS and doing the same for the short open times, we found $P < 0.01$ in both cases.

The all-points histogram of single-channel current in the presence of TMS is shown in Figure 7. About 3 min after TMS additions, three conductance states were observed in addition to the closed state. Assuming the reversal potential to be -31 mV, as obtained from the data of Figure 1(lower panel), these were calculated to be 60 pS, 80 pS (seen as a shoulder to the left of the 60 pS peak but not very obvious) and approx. 120 pS conductance levels. The channel was closed for 60% of the recording time and spent 35% of the time in the 60/80 pS conductance state and 5% of the recording time in the 120 pS state. The data shown are typical of four similar separate experiments. Plotting channel current against open time for individual opening events showed no correlation between the subconductance state and the open time (see Figure 8). This result also indicates that the higher subconductance state is very unlikely to be due to simultaneous opening of two channels in the same bilayer, since such an event would be much more probable during a long channel opening and hence there would be some correlation between open time and conductance.

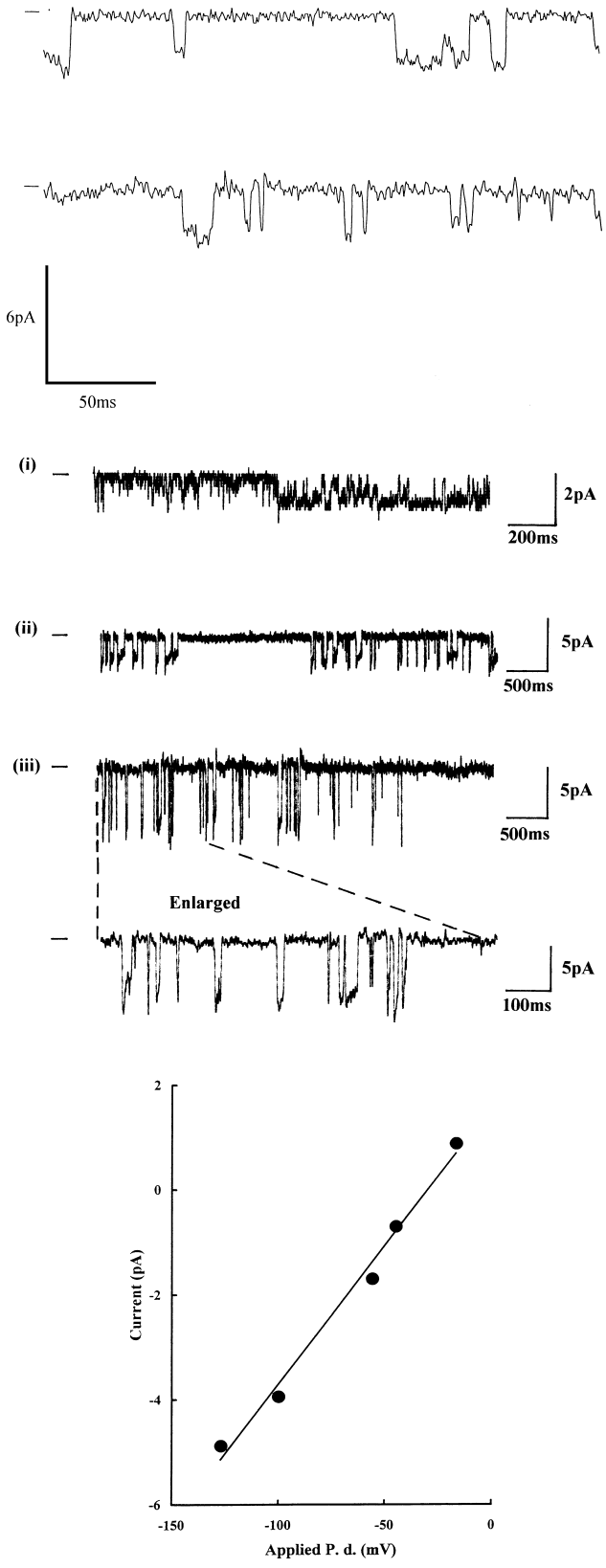

Figure 1 Typical single-channel recordings of InsP_3R

Upper panel: single-channel recordings of InsP_3R in the presence of 0.2 μM Ca^{2+}, 10 μM Ins(1,4,5)P_3 and 50 μM ATP at 0 mV applied p.d. Openings are defined as a downward deflection from the baseline. Both long and short openings are shown. Middle panel: single-channel recordings in the presence of 0.2 μM Ca^{2+}, 10 μM Ins(1,4,5)P_3 and 50 μM ATP at different potential differences: (i) −25 mV; (ii) −70 mV; (iii) −100 mV. Again, channel openings are defined as a downward deflection from the baseline. Lower panel: current–voltage curve. Channel current was measured at different applied p.d.s (−17 mV; −45 mV; −56 mV; −100 mV; −127 mV).

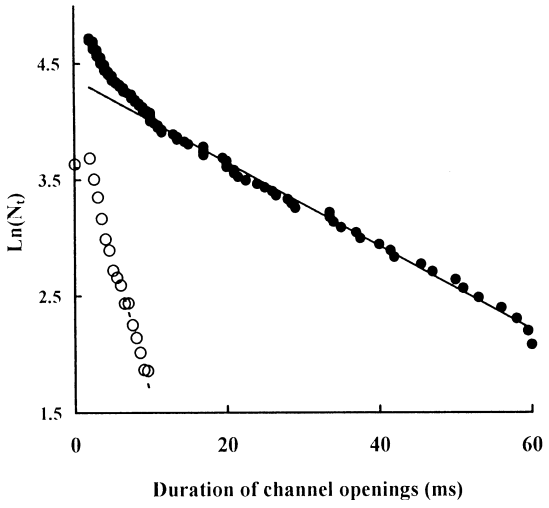

Figure 2 Determination of mean open time(s) for InsP_3R

Current records of the type shown in Figure 1(upper panel) at 0 mV applied p.d. were analysed. Plotted against time t is ln (N_t) where N_t is the number of channels with open time $\geqslant t$ (denoted by ●). Where the plot was linear, the gradient m was used to estimate mean open time τ by the relation $\tau = 1/m$. The line fitted is indicated by the continuous line. A linear fit was also applied to the initial few points on the curve, after removal of the longer open times, to yield another population of mean open times. This portion of the curve has been plotted separately and a line fitted (denoted by ○ and the broken line).

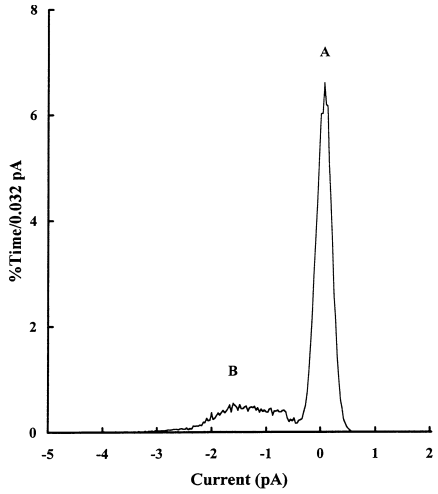

Figure 3 All-points histogram from single-channel recordings

Data were from records similar to those shown in Figure 1(upper panel) at 0 mV. The histogram was based on percentage time spent in bin sizes of 0.032 pA increments. Peak A represents the percentage amount of time spent in the closed state. Peak B is the percentage amount of time spent in the open state and corresponds to a single-channel slope conductance of 53 pS. Total percentage times spent in the two states (given in the text) were calculated from the area under the peaks.

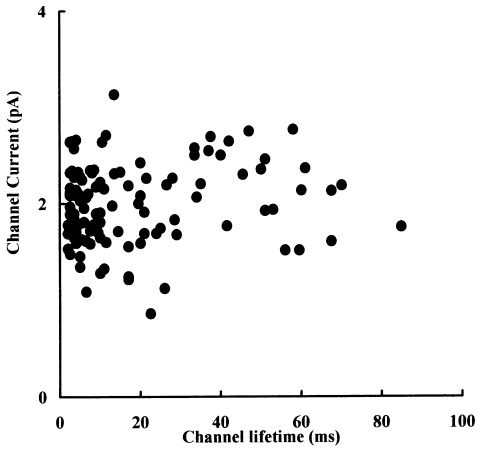

Figure 4 Lack of correlation between channel open time and current (pA) for InsP_3R

Individual values of channel current are plotted against their respective lifetimes. The data were taken from the experimental records shown in Figure 1(upper panel), in the absence of TMS and analysed for channel current in Figure 3.

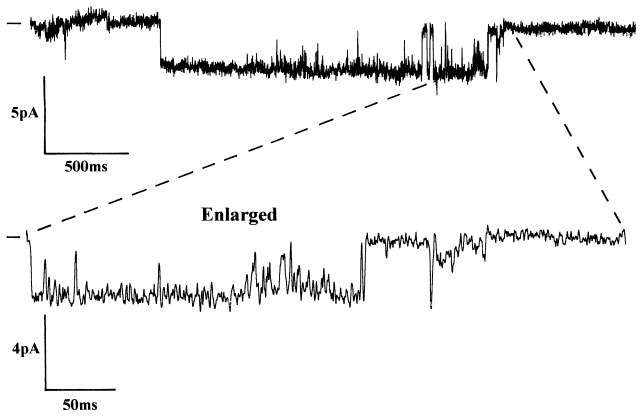

Figure 5 Single-channel recordings in the presence of TMS

Channels were recorded under the same conditions as for Figure 1 (upper panel) at 0 mV applied p.d. except that 100 μM TMS was present for 3 min prior to the start of recording. Openings are defined as downward deflections from baseline. Again, long and short openings can be seen.

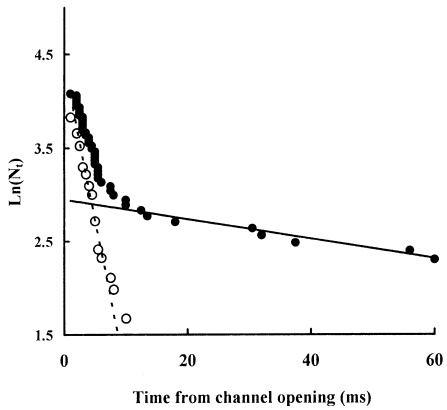

Figure 6 Determination of mean open time(s) of InsP_3R in the presence of 100 μM TMS

Data were derived from traces similar to those shown in Figure 5. Analysis was as for Figure 2 with, as before, the symbol (●) representing the complete data set and the continuous line the fit to the linear portion of the data, and the symbol (○) representing the open events with the long open times removed and the dotted line the linear fit to these data points. The very short closure events (> 50% change from the open state) seen in Figure 5 were treated as channel closures in this analysis.

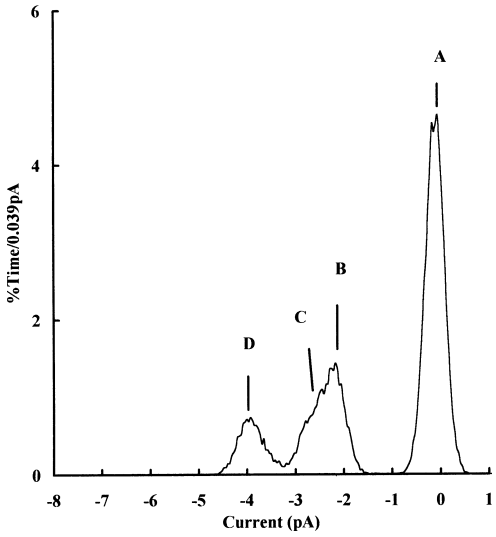

Figure 7 All-points histogram of single-channel recordings in the presence of 100 μM TMS

Data were from records similar to those shown in Figure 5. The histogram was based on percentage time spent in bin sizes of 0.039 pA increments. Peak A represents percentage amount of time in the closed state. Peaks B and C represent percentage amount of time at open states with conductance levels of 60 pS and 80 pS respectively, and peak D has a conductance of 120 pS. Total percentage times spent in all the states (given in the text) were calculated from the area under the peaks, but the 60 and 80 pS peaks were not resolved.

DISCUSSION

IICR, as seen from earlier studies, is enhanced by TMS, a sulphydryl-oxidizing agent [5–7,13–15]. In the presence of TMS, at the single-channel level, mean open times were altered and three conductance states were seen. The lower conductance state (60 pS) is very similar to that observed in the absence of TMS (53 pS). The presence of the other two levels (80 pS and 120 pS), as well as the changes in mean open times, suggests a more definite modification of the Ca^{2+}-conducting properties of the channel, as well as any effects there might be on Ins(1,4,5)P_3 affinity [5] (which in intact or permeabilized cells would result in recruitment of more channels).

A feature of this study has been the observation of more than one population of mean open times for the receptor. The short and long openings can be seen in both the control experiments and in the presence of TMS [see Figures 1(upper panel) and 5] in a minimum of five different experiments in each case. The short opening time of 4.05 ms seen in the control experiment [10 μM Ins(1,4,5)P_3, 50 μM ATP and 0.2 μM free Ca^{2+}] is similar to that observed by Bezprozvanny and Ehrlich [11] of 3.3 ms [in the presence of 2 μM Ins(1,4,5)P_3, 100 μM ATP and 0.2 μM Ca^{2+}]. Short open times have generally been observed at the single-channel level using Ba^{2+} as the current carrier [11]. The longer open time of the channel, however, has not been reported

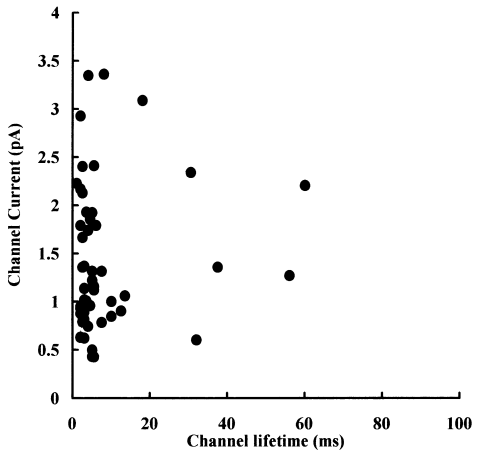

Figure 8 Lack of correlation between channel open time and current (pA) for InsP_3R in the presence of 100 μM TMS

This plot illustrates the absence of any correlation between single-channel open times and their respective channel currents in the presence of TMS. The data are taken from experimental records similar to those shown in Figure 5 and analysed in Figure 7 for channel currents. Note that the different channel current peaks seen in Figure 7 are reflected in the clusters of points up the vertical axis.

previously. A possible explanation of the long open times is that more than one channel is present in the bilayer, the longer open time being the result of an additive effect, although this is unlikely. Indeed, a plot of channel open time against current indicated the presence of only one channel and there was also no correlation between conductance and open time (see Figure 4). The presence of TMS accentuates the difference between the two mean open times, decreasing the shorter open time from 4.05 ms to 2.78 ms and increasing the longer open time from 27.8 ms to 94.8 ms.

Although in the presence of TMS different subconductance states were observed, again there is no correlation between conductance state and channel open time (see Figure 8). Since the channel events observed were due to a single InsP_3R in the bilayer, the different channel open times cannot be ascribed to receptor heterogeneity, but must reflect two different states of the open channel, albeit with identical channel conductance properties. In other studies [5], TMS was found to enhance Ca^{2+} efflux through InsP_3Rs reconstituted into lipid vesicles at low Ins(1,4,5)P_3 concentrations but to inhibit Ca^{2+} flux at saturating concentrations of Ins(1,4,5)P_3. At saturating concentrations of Ins(1,4,5)P_3, all receptors would be occupied by their ligand and a shift in their binding affinity would be of no consequence. The changes in single-channel properties which we see at 10 μM Ins(1,4,5)P_3 would be more in line with an increase in channel activity, since both channel lifetime and conductance are observed. However, it may be that the concentration and nature of the bivalent cation on the *trans* side may influence the response to TMS. It is possible that channel opening may be regulated by factors such as bivalent cation binding sites which determine the time of channel opening, depending on whether they are occupied or empty. Such a possibility has been invoked to explain the biphasic kinetics of Ca^{2+} release from intracellular stores [2].

The change in channel lifetime and subconductance states brought about by TMS implies that TMS causes a change in the conformational state of the channel. The effects of the increased conductance and open time together would be to increase the amount of cation flow through the channel and hence, in a more intact system, to increase the Ca^{2+} release from intracellular stores. This raises the question as to where TMS interacts with the receptor to produce this effect. The receptor contains three different domains which could be targets: (1) the Ins(1,4,5)P_3 recognition site; (2) the Ca^{2+} ion channel in the C-terminal portion of the receptor; and (3) the large coupling domain in the interior of the molecule.

Recent studies [5] in which TMS has been shown not to interact with the Ins(1,4,5)P_3 binding site provides evidence ruling out the first possibility. InsP_3Rs, reconstituted into lipid vesicles, were exposed to Ins(1,4,5)P_3 before and during treatment with TMS (3 mM) and Ca^{2+} flux was shown to double, compared with samples in which Ins(1,4,5)P_3 stimulation followed TMS treatment. This indicates that Ins(1,4,5)P_3 and TMS are binding at different sites. Evidence favouring the second and third options involves antibodies directed against the C-terminal cytoplasmic tail of the InsP_3R [4,17,18]. The monoclonal antibody 18A10 has been shown to block TMS Ca^{2+} oscillations, TMS-enhanced Ca^{2+}-induced Ca^{2+} release and IICR, and reacts with the receptor close to the Ca^{2+}-channel region [4,17,18] which may indicate that 18A10 interacts at a similar region to TMS (but might also block a conformational change induced by TMS at a remote site). An effect of TMS has also been seen using ryanodine receptors [19,20], which do not respond to Ins(1,4,5)P_3 and therefore have major differences in ligand recognition and coupling domains, strongly indicating that the modification is in the Ca^{2+}-channel region. Two cysteines at positions 2610 and 2613 are located in this area of the type-1 InsP_3R, present in a TXCFICG sequence motif, which is highly conserved in all InsP_3R subtypes and ryanodine receptors [21]. These cysteine groups are thus the likely target sites for channel modification by TMS and, indeed, any other sulphydryl-oxidizing agents involved in endogenous oxidative–reductive processes.

In conclusion, our findings have shown that TMS modifies individual channel behaviour of the InsP_3R possibly by interacting with critical sulphydryl groups present on the InsP_3R itself. Since very high concentrations of Ins(1,4,5)P_3 were used in these experiments, it is unlikely that the effects of TMS on channel characteristics can be attributed to alterations in receptor occupancy by Ins(1,4,5)P_3. It seems likely that the modifications we observe at the single-channel level may contribute to TMS-induced sensitization of IICR and this may have implications for other Ca^{2+} release mechanisms, such as those that mediate Ca^{2+} release via ryanodine receptors [20].

E.C.T. was in receipt of a BBSRC studentship.

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Received 19 February 1996/12 April 1996; accepted 26 April 1996

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