Purified Reconstituted Inositol 1,4,5-Trisphosphate Receptors

THIOL REAGENTS ACT DIRECTLY ON RECEPTOR PROTEIN*

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Thimerosal, a sulfhydryl oxidizing reagent, has been shown to induce Ca²⁺ mobilization in several cell types and to increase the sensitivity of intracellular Ca²⁺ stores to inositol 1,4,5-trisphosphate (IP₃). Using purified IP₃ receptor (IP₃R) protein reconstituted in vesicles, we demonstrate pronounced stimulation by thimerosal of its Ca²⁺ channel activity. Effects of thimerosal are dependent on the redox state of the receptor, implying an action of thimerosal on a critical sulfhydryl group(s) of IP₃R. Thimerosal enhances the affinity of IP₃R for IP₃ binding. The manner in which thimerosal modulates IP₃R responsiveness to IP₃ provides evidence for receptor heterogeneity with implications for mechanisms of quantal Ca²⁺ release. These results clarify regulation of IP₃R activity by redox modulation.

The dynamics of intracellular Ca²⁺ provide crucial signaling information in many aspects of cellular regulation. Intracellular Ca²⁺ flux is regulated by numerous processes, especially the release of Ca²⁺ from intracellular stores by inositol 1,4,5-trisphosphate (IP₃) (1,2) and a calcium-induced calcium release system involving channels labeled by the alkaloid ryanodine (3,4). Physiologic and pathologic changes in the oxidative state of cells influence Ca²⁺ disposition. Low levels of oxidants can stimulate cell proliferation and differentiation (5,6), while increased oxidative stress may exert cytotoxic effects, including death by apoptosis (7). Perturbations in the oxidative state of sulfhydryl groups can influence Ca²⁺ flux (7). Thimerosal (TMS), a sulfhydryl oxidizing agent, has been reported to stimulate (8–12) or inhibit (11,12) intracellular Ca²⁺ flux. TMS increases the potency of IP₃ in releasing Ca²⁺ (9,11,13–16). In some studies, TMS increased the affinity of IP₃ for receptor binding sites (13,16), while other studies showed no effect (11,12). These findings suggest that IP₃-induced Ca²⁺ flux is influenced by the oxidative state of sulfhydryl groups, perhaps those of the IP₃ receptor (IP₃R) itself. However, TMS can influence related Ca²⁺ regulating systems, such as the endoplasmic reticular Ca²⁺ pump (11,13), indirectly affecting IP₃-induced Ca²⁺ release.

We successfully reconstituted IP₃-induced Ca²⁺ flux in vesicles containing only purified IP₃R protein (17). IP₃-mediated Ca²⁺ flux in the reconstituted vesicles is regulated by phosphorylation (18,19) and adenine nucleotides (20). Using these reconstituted vesicles, we have characterized effects of thiol reagents on Ca²⁺ flux at the level of the IP₃R protein.

Intracellular release of Ca²⁺ is a discontinuous, quantal process in which successive increments of IP₃ transiently release precise amounts of Ca²⁺ (21,22). Utilizing IP₃R reconstituted into proteolipid vesicles (IP₃RV) we previously showed that quantal flux of Ca²⁺ elicited by IP₃ is a fundamental property of the IP₃R, suggesting that the receptors purified from rat cerebellum constitute a heterogeneous population with varying sensitivity to IP₃ (23). The effects of thiol reagents on IP₃-mediated flux in IP₃RV observed here provide additional evidence for functional receptor heterogeneity, which may help account for quantal Ca²⁺ release.

EXPERIMENTAL PROCEDURES

Materials—[³H]IP₃, Ca²⁺, and formula 963 scintillation mixture were obtained from DuPont NEN. n-my-o-Ins(1,4,5)P₃ hexapotassium salt was obtained from LC Laboratories (Woburn, MA). Concanavalin A-Sepharose and G-25, superfine, were obtained from Pharmacia LKB Biotechnology Inc. Phospholipids for reconstitution were obtained from Avanti Polar Lipids (Birmingham, AL). All other reagents were from Sigma.

Purification and Reconstitution of IP₃R—IP₃R was purified from adult male Sprague-Dawley rat cerebellum and reconstituted into lipid vesicles as described (17). Briefly, IP₃R was purified using a two-step affinity chromatography procedure employing sequential heparin-affine-rose and concanavalin A-Sepharose columns. Following purification to apparent homogeneity, detergent-solubilized receptor protein was mixed with sonicated lipids and the mixture was dialyzed at 4 °C against buffer A (50 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, pH 7.4), supplemented with 2.5 mM B-mercaptoethanol (BME) and 2 mM EDTA, to effect detergent removal and vesicle formation. The buffer was changed every 8 h for 48 h, and EDTA was omitted from the final buffer change. For experiments performed in the absence of reducing agent, 1 ml of IP₃RV was passed over a 5-ml G-25 desalting column equilibrated with buffer A to remove BME.

Ca²⁺ Flux—Reconstituted proteoliposomes were assayed for IP₃-stimulated Ca²⁺ flux as described (17). Following preincubation under various conditions vesicles were incubated (for either 10 or 15 s) in the presence of 2 µCi of ⁴⁰Ca²⁺ with or without IP₃. Under these conditions, tracer Ca²⁺ gained access to the lumen of vesicles when the IP₃R channels were opened by IP₃. The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin (200 µg/ml). Intravesicular Ca²⁺ content was isolated by immediately passing the vesicle/buffer mixture over a cation-exchange column (Dowex 50W, Sigma). The vesicles were collected and their intravesicular Ca²⁺ content was measured by scintillation spectrometry.

[³H]IP₃ Binding—Ligand binding was assayed by precipitation of IP₃RV with polyethylene glycol using γ-globulin as carrier protein, as described (20).

RESULTS

Several workers have shown TMS stimulation of Ca²⁺ flux in intact cells and platelets (14,15,24–29) and enhancement of
Effects of thiol modifying reagents on the Ca²⁺ flux in IP₃R vesicles

Reconstituted vesicles were preincubated for 2 min at room temperature under the conditions shown. Control vesicles were preincubated with buffer A. IAA and NEM refer to iodoacetamide and N-ethylmaleimide, respectively. All reagents were prepared fresh as stock solutions in buffer A. Following this preincubation, CA²⁺ flux was allowed to proceed for 15 s in the presence of tracer 45Ca²⁺ with or without 40 nM IP₃. Specific Ca²⁺ flux under each condition was calculated as cpm in the presence of IP₃ (total) minus the cpm in the absence of IP₃ (blank). None of the reagents shown altered the blank. Data is expressed as specific Ca²⁺ flux under each condition as a percentage of specific Ca²⁺ flux under control conditions. Measurements in duplicate or triplicate varied less than 10%. This experiment has been repeated three times with the same results.

<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Control</th>
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<tbody>
<tr>
<td>3 μM TMS</td>
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<tr>
<td>1 mM TMS</td>
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<tr>
<td>1 mM BME</td>
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<td>1 mM NEM</td>
<td>65</td>
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<td>1 mM NEM + 1 mM BME</td>
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IP₃-induced release of Ca²⁺ in permeabilized cells and microsomes (11–13, 15, 16). Pretreatment of IP₃RV with low micromolar concentrations of TMS produces a 30% stimulation in IP₃-mediated Ca²⁺ flux (Table I). Relatively high concentrations (1 mM) of TMS abolish flux activity. However, when combined with BME, which by itself has no effect, TMS pretreatment results in an 8-fold enhancement of Ca²⁺ flux induced by 40 nM IP₃. This effect is rapid with more than 50% maximal Ca²⁺ flux evident at 10 s pretreatment with TMS, reaching a plateau at 1–2 min (Fig. 1A). The enhancement of Ca²⁺ flux elicited by TMS in the presence of excess BME (2.5 mM) is concentration dependent, with half-maximal augmentation evident at 0.2 mM TMS and a plateau between 1 and 2.5 mM TMS (Fig. 1B).

Like BME, two other reducing agents, dithiothreitol (DTT) and reduced glutathione (GSH), also markedly augment the TMS-mediated increase of Ca²⁺ flux, whereas DTT and GSH by themselves reduce IP₃-induced Ca²⁺ flux (Table I). DTT and GSH are less efficient than BME in facilitating the stimulation of Ca²⁺ flux mediated by TMS. The greater augmentation of the effects of TMS by BME might reflect its smaller size, permitting access to the relevant sites on the IP₃R. Thus, there is a nonspecific requirement for a reducing agent to achieve maximal stimulation by the oxidant TMS.

The TMS effect appears specific in that other thiol oxidizing reagents, iodoacetamide (IAA) and N-ethylmaleimide (NEM), fail to augment Ca²⁺ flux either by themselves or in the presence of BME. The presumption that modifying reagents tested, either alone or in combination, have no effect on Ca²⁺ flux in the absence of IP₃ (data not shown), demonstrating that these reagents do not exert nonspecific effects on Ca²⁺ flux in reconstituted vesicles.

To clarify interactions between BME and TMS, we evaluated effects on Ca²⁺ flux of variations in their order of addition (Table II). The pronounced facilitation of Ca²⁺ flux occurs only if BME is applied before TMS or simultaneously with it. When BME is applied after TMS, we observe only inhibition of IP₃-induced Ca²⁺ flux. This suggests that reducing agents, such as BME, DTT, and GSH, cause alterations in IP₃R that facilitate the TMS mediated augmentation of Ca²⁺ flux. These reducing agents might maintain crucial sulphydryl groups in a reduced form that can be modified by TMS. This is consistent with established actions of TMS involving alkylation only of reduced sulphydryl groups (30).

To investigate the mechanisms whereby TMS stimulates IP₃-induced Ca²⁺ flux, we evaluated concentration-response relationships for IP₃ (Fig. 2). In the absence of TMS pretreatment (i.e., 2.5 mM BME alone), IP₃ enhances Ca²⁺ flux in a concen-

**Fig. 1.** Time and concentration dependence of TMS stimulation. Reconstituted vesicles were preincubated for 2 min with TMS and BME followed by 45Ca²⁺ with and without 25 nM IP₃ for 10 s in A, vesicles were preincubated with 2.5 mM TMS and 2.5 mM BME for 4 min prior to Ca²⁺ flux measurements. Specific Ca²⁺ flux was calculated as described in Table I. Measurements in duplicate varied less than 10%. This experiment has been repeated two times with the same results.

**TABLE I**

Effects of thiol modifying reagents on the Ca²⁺ flux in IP₃R vesicles

<table>
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**TABLE II**

Effect of variations in the order of addition of TMS and BME on Ca²⁺ flux

Reconstituted vesicles were processed as described in Table I, except that two successive 2-min incubations were performed and 100 nM IP₃ was used to assess Ca²⁺ flux. Control vesicles were incubated in buffer A during both preincubations. As shown in Fig. 2, relative to control values the stimulation of Ca²⁺ flux by TMS + BME seen with 100 nM IP₃ is less than that achieved with 40 mM IP₃ (compare Tables I and II). Measurements in duplicate or triplicate varied less than 10%. This experiment has been repeated three times with the same results.
treated samples, a single component of IP, binding is evident. TMS treatment reduces the dissociation constant with TMS (1.61, binding to IP,RV (Fig. 3). This experiment has been repeated three times with the same results. Might compete with each other so that pre-exposure of the IP,R would sterically inhibit the effects of TMS. Accordingly, we compared effects of TMS on Ca2+ flux in samples receiving IP,R (40 nM) either before or after TMS exposure (Table II). When IP,R produces respective 2-fold from 13.6 to 2.5, similar to values for TMS-treated samples (17). TMS (2.5 mM) in the presence of BME for 10 s. Specific Ca2+ flux was calculated as described in Table I. Measurements in duplicate varied less than 10%. This experiment has been repeated three times with the same results.

To explore factors accounting for the increased responsiveness to IP,R following TMS treatment, we monitored [3H]IP3 binding to IP,RV (Fig. 3). In both control and TMS/BME-treated samples, a single component of IP3 binding is evident. TMS/BME treatment reduces the dissociation constant (Kd) by 2-fold from 13.6 to 6.4 nM with no effect on Bmax.

We wondered whether TMS acts directly at the recognition site for IP3 binding on the receptor. If so, the two substances might compete with each other so that pre-exposure of the IP,R to IP3 would sterically inhibit the effects of TMS. Accordingly, we compared effects of TMS on Ca2+ flux in samples receiving IP3 (40 nM) either before or after TMS exposure (Table III). Because of the quantal nature of the response of IP3RV to IP3 (23), preincubation with IP3 does not augment the measured Ca2+ flux relative to controls. Similar to earlier experiments, exposure to 3 mM TMS alone or TMS/BME (1 mM each) prior to IP3 produces respective 30 and 670% increases in Ca2+ flux compared to samples treated with IP3 alone (Table III). When these samples are exposed to IP3 prior to and during treatment with TMS (3 mM) or TMS/BME (1 mM each), the increase in Ca2+ flux is twice as great as in samples in which IP3 stimulation follows TMS treatment (Table III). The augmentation of the TMS responses elicited by pre-exposure to IP3 indicates that TMS and IP3 act at different sites which presumably interact allosterically. The greater effect of pretreatment with IP3 suggests that IP3 binding elicits a conformational alteration of IP3R in which critical sulfhydryl groups are made more accessible to TMS.

As described above, whereas millimolar concentrations of TMS in the absence of BME inhibit IP3-induced Ca2+ flux, low micromolar concentrations have a stimulatory effect (Table I). Preincubation with 0.1–3 mM TMS leads to a progressively greater Ca2+ flux elicited by 80 nM IP3, relative to control vesicles incubated in the absence of TMS (Fig. 4A). The maximal stimulation, seen with 3 mM TMS, is greater than 30%. At concentrations of TMS between 3 and 10 mM, there is a decline in the stimulation of Ca2+ flux seen with 80 nM IP3. At even greater TMS concentrations, flux activity decreases below control levels, with 100 mM TMS being sufficient to abolish flux activity.

Surprisingly, when vesicles are preincubated exactly as described above with varying concentrations of TMS, and subsequently stimulated with a maximal concentration of IP3 (1 mM), TMS does not stimulate but rather only inhibits Ca2+ flux relative to controls (Fig. 4A). When TMS concentrations are plotted on a logarithmic scale and Ca2+ flux is normalized relative to control values (as shown in Fig. 4B), the following can be seen: 1) the effects of TMS preincubation on Ca2+ flux elicited by a submaximal concentration of IP3 are biphasic, with TMS concentrations up to 3 mM stimulating flux and between 3 and 100 µM inhibiting flux; 2) the effects of TMS on Ca2+ flux elicited by a maximal concentration of IP3 are monophasic and inhibitory.

Having explored the effects of millimolar concentrations of TMS with BME and micromolar concentrations of TMS alone, we examined the effect of variable amounts of BME added to several fixed concentrations of TMS. The relationship between
BME, TMS, and Ca2+ flux is complex (Fig. 5). Maximal stimulation of Ca2+ flux occurs with a 2-fold excess of BME over TMS in the case of 3 and 100 μM TMS. At higher BME concentrations, the stimulation produced by TMS decreases gradually to control levels, presumably because excess BME covalently reacts with TMS reducing its availability to modify the IP3R. Experiments using DTT and TMS yield essentially the same results (data not shown) except that: 1) DTT alone inhibits flux at higher concentrations (see Table I), and 2) maximal stimulation is obtained with equimolar DTT and TMS. Thus, to achieve maximal stimulation of IP3R activity there is an optimal reducing agent concentration range for each TMS concentration.

If IP3 pretreatment alters the conformation of IP3R to affect TMS stimulation of Ca2+ flux, as suggested in Table III, perhaps IP3 exposure would also influence the inhibitory actions of TMS on Ca2+ flux that are observed when high concentrations of TMS are applied to IP3RV in the absence of BME. Accordingly, we evaluated Ca2+ flux in IP3RV exposed to 0, 40, 200, or 500 nM IP3 followed by treatment with TMS, and then by application of BME (Fig. 6A). In samples preincubated without IP3, no flux activity is detected after treatment with TMS followed by BME, despite subsequent stimulation with up to 500 nM IP3. In the samples pretreated with 200 nM IP3, Ca2+ flux is 2.5 times that of samples pre-exposed to 40 nM IP3. At 500 nM IP3 pre-exposure, Ca2+ flux is only modestly greater than at 200 nM. Thus, preincubation with IP3 preserves, in a concentration-dependent fashion, between one-half to one-third of the flux activity of the samples following TMS and BME treatment, when compared to samples for which the TMS treatment is omitted (Fig. 6B). This suggests that IP3 elicits conformational changes in the IP3R which render it resistant to subsequent degradation by TMS. Dramatically, however, addition of greater amounts of IP3 after treatment with TMS and BME produces no further augmentation of Ca2+ flux, regardless of the amount of IP3 employed during preincubation (Fig. 6A). This suggests that this treatment regimen dramatically alters the IP3R concentration-response relationship of the IP3R which retained activity.

Cells contain substantial basal levels of IP3, often about 1 μM (31), which should suffice to open a substantial proportion of IP3R channels. Physiologic and pathologic stimulation of cells alters the oxidative-reductive status of the intracellular milieu with associated influences on sulfhydryl groups of proteins. Such sulfhydryl alterations of IP3R may evoke Ca2+ release in the absence of receptor-mediated generation of intracellular IP3. To approximate this situation, we initially exposed IP3RV to IP3 and Ca2+ and then added TMS (2.5 mM) in the continuous presence of BME (2.5 mM) (Fig. 7). In the absence of TMS, IP3 stimulates Ca2+ flux with effects first detectable at 10 nM IP3. At 10 and 20 nM IP3, TMS increases Ca2+ flux about 6-fold compared to values obtained without TMS addition. These findings suggest that intracellular Ca2+ release can be regulated by changes in oxidative states of IP3R sulfhydryl groups in the absence of receptor-mediated signal transduction.

DISCUSSION

Earlier studies reported that TMS, a sulfhydryl oxidizing reagent, enhances IP3-induced Ca2+ release (9, 11, 13–16). These effects could involve numerous mechanisms, such as inhibiting the Ca2+ pump of the endoplasmic reticulum, inactivating a negative modulator of IP3R activity, or augmenting the effects of a protein that acts on the IP3R to promote its release of Ca2+. By utilizing IP3R purified to apparent homogeneity and reconstituted into proteolipid vesicles, this study establishes that the stimulatory effects of TMS take place at the level of the IP3R protein itself.

Whereas pretreatment of IP3RV with micromolar concentrations of TMS provides a modest stimulation of IP3-mediated...
Ca\textsuperscript{2+} flux, and millimolar concentrations of TMS abolish flux activity, pretreatment with TMS in the presence of BME enhances IP\textsubscript{3}-induced Ca\textsuperscript{2+} flux up to 8-fold. Although the stimulatory effects of TMS are specific, there appears to be a non-specific requirement for the presence of a reducing agent to maximize the stimulation by TMS. TMS itself does not gate the IP\textsubscript{3} receptor; rather, TMS pretreatment enhances the response of IP\textsubscript{3} to IP\textsubscript{3}. Pretreatment of IP\textsubscript{3}RV with TMS in the presence of BME produces a leftward shift of the concentration-response relationship with a 7-fold increase in the potency of IP\textsubscript{3}. TMS/BME treatment of IP\textsubscript{3}RV reduces the dissociation constant (K\textsubscript{d}) for [\textsuperscript{3}H]IP\textsubscript{3} binding 2-fold without affecting the B\textsubscript{max}. Recently, two groups (13, 16) also observed a TMS elicited increase in IP\textsubscript{3} binding affinity to permeabilized cells and microsomes with no change in B\textsubscript{max}.

The enhancement of IP\textsubscript{3}-induced Ca\textsuperscript{2+} flux by TMS in the presence of BME in our experiments is more pronounced than similar effects observed by others (9, 11, 13–16). However, none of the earlier studies examined a broad range of concentrations of reducing agents and TMS. In previous studies, IP\textsubscript{3} activity was assessed either in intact cells, which retain their reducing cytoplasmic environments, or in microsomes derived from tissues homogenized in the presence of reducing agents, so that substantial concentrations would likely have been present during the Ca\textsuperscript{2+} release experiments.

The requirement for a reducing agent to achieve maximal stimulation by TMS is unexpected because IP\textsubscript{3}R used in these experiments were purified, reconstituted, and stored until use in the continual presence of BME. Nevertheless, the pronounced facilitation of IP\textsubscript{3}-induced Ca\textsuperscript{2+} flux by TMS occurs only if BME is applied before TMS or simultaneously with it. IP\textsubscript{3}RV were desalted over a gel filtration column to remove BME for use in experiments to assess the effects of TMS on IP\textsubscript{3} activity in the absence of a reducing agent (see "Experimental Procedures"). One reason for the requirement to add back BME to achieve maximal stimulation by TMS might be that IP\textsubscript{3}R is capable of autooxidation, such as through the formation of disulfide bonds, which prevents TMS from gaining access to the relevant sulfhydryl groups. Another possibility is that the redox reaction between TMS and sulfhydryl group(s) on the IP\textsubscript{3}R is complex, possibly involving intermediate steps, and requires the presence of BME at specific stages to achieve the proper products which confer enhanced receptor activity.

The fact that both the concentration and order of addition of TMS and BME to IP\textsubscript{3}RV can dramatically effect IP\textsubscript{3}R activity suggests that the oxidative state of critical sulfhydryl residue(s) on the IP\textsubscript{3}R protein can have a profound impact on receptor activity and responsiveness to redox modulation. Which sulfhydryl groups on IP\textsubscript{3}R might be uniquely responsive to oxidative-reductive changes? IP\textsubscript{3}R is a large protein comprising 4 protomer subunits (18, 32). Mutagenesis (33, 34) and chemical modification (35) studies have localized the IP\textsubscript{3} recognition site to the first 400 amino acids at the NH\textsubscript{2} terminus. The Ca\textsuperscript{2+} ion channel resides in the carboxyl-terminal portion of the protein, where there is high sequence homology to the Ca\textsuperscript{2+} channel in the ryanodine receptor (36, 37). The large cytoplasmic portion of the receptor between the NH\textsubscript{2}-terminal IP\textsubscript{3} binding domain and the COOH-terminal transmembrane domain has been designated the coupling domain (2). Since our studies indicate that TMS does not act at the IP\textsubscript{3} recognition site (Table III), alternative possibilities are the carboxyl-terminal Ca\textsuperscript{2+} channel domain or the large coupling domain in the interior of the molecule.

Antibodies directed against the carboxyl-terminal cytoplasmic tail of the IP\textsubscript{3}R, like TMS treatment, both augment or inhibit Ca\textsuperscript{2+} release (38, 39). This area of the type 1 IP\textsubscript{3}R contains two cysteines at positions 2610 and 2613 which are
Effects of Thimerosal on Purified IP_3 Receptor Activity

Present in a TXCFCG sequence motif highly conserved in all subtypes of IP_R and ryanodine receptors (1). Interestingly, TMS stimulates Ca^{2+} flux through the ryanodine receptor in much the same manner that it stimulates flux through IP_R (27). Accordingly, these cysteines are strong candidates to be the targets for the regulation of IP_R by TMS and possibly by endogenous oxidative-reductive processes.

What might be the endogenous regulator of the TMS-sensitive sulfhydryl groups on the IP_R? Besides TMS, oxidized glutathione (GSSG) can stimulate Ca^{2+} flux in permeabilized cells (13, 40). Presumably, the GSSG reacts with free sulfhydryl groups of IP_R to form a mixed disulfide bond (Protein-SSG). Glutathione is the most abundant reducing agent in cells, and the relative proportion of oxidized and reduced glutathione changes markedly in response to alterations in cellular physiology and pathology (41). Our findings with TMS (Fig. 7) suggest that even in the presence of low ambient levels of intracellular IP, and in a reducing environment, cellular alterations that increase levels of GSSG could alter the conformation of the IP_R and thereby stimulate Ca^{2+} release.

One puzzling observation was the enhancement by low concentrations of TMS (in the absence of reducing agents) of Ca^{2+} flux at 80 nM IP, and inhibition at 1 µM IP (Fig. 4). Conceivably the higher IP, concentration exposes IP_R to an inhibitory action of TMS masking the stimulatory effect of TMS seen at lower (IP,). This seems unlikely, as IP, occupancy of IP_R appears to enhance rather than inhibit stimulation by TMS of receptor activity (Table III and Fig. 6).

Another explanation is that TMS pretreatment might cause IP_R to lock in a subconductive state following the addition of IP,. The IP_R conductance in this state could be greater than the conductance normally elicited by 80 nM IP, but less than the conductance elicited by a saturating concentration of IP,. This would be similar to the effects of low concentrations of ryanodine on the ryanodine receptor (42). This seems unlikely because the specific Ca^{2+} flux elicited by 80 nM IP, is considerably less than the flux obtained by 1 µM IP, following TMS treatment, suggesting that under these conditions IP_R are not in the same conductance state.

A third explanation invokes heterogeneity of IP_R with only a fraction of receptors sensitive to 80 nM IP, by increasing IP, binding affinity (Fig. 3), low micromolar concentrations of TMS lower the threshold for receptor responsiveness. Under these conditions TMS would stimulate Ca^{2+} flux by recruiting receptors that normally respond only to higher IP, concentrations. With saturating concentrations of IP, such that all receptors present are occupied by their ligand, a shift in the binding affinity of the IP, receptors present would be inconsequential and no stimulation would be observed. Perhaps the inhibitory effects of TMS are the result of modification of a different sulfhydryl group on the IP_R than the site which accounts for IP_R stimulation.

Successive treatment of IP_R with IP, TMS, and BME provides another example of the way in which redox modulation of IP_R can dramatically alter its responsiveness to IP, (Fig. 6). Treatment with 2 mM TMS followed by 2 mM BME abolishes IP, flux activity. Incubation of IP_R with increasing concentrations of IP, prior to treatment with TMS and BME preserves flux activity. Dramatically, no further increase is seen in flux activity following addition of increasing amounts of IP, during the flux assay, regardless of the initial IP, concentration used during preincubation. It appears that the receptors which remain active following the treatment protocol achieve a maximal response over a very narrow range of IP, concentrations. Unfortunately, we were not able to characterize this range directly because in samples pretreated with IP, the levels of the ligand could only be partially diluted following incubations with TMS and BME. It was not possible, therefore, to assess Ca^{2+} flux activity at extremely low levels of IP,.

An alternative explanation for these results is that successive treatment of IP_R with IP, TMS, and BME lock the IP_R in subconductive states with Ca^{2+} conductances proportional to the amount of IP, initially present during preincubation. This seems unlikely because it would necessitate a very large number of possible subconductive states, whereas no more than four have been identified (43), or an unusual coincidence that we happened to have selected concentrations of IP, that led to the induction of distinct states. Moreover, these states would have to be at least partially irreversible, in that they are no longer responsive to changes in the concentration of IP,. The design of the assay we employ to measure Ca^{2+} flux necessitates that the channel activation we measure is reversible. Flux assays are terminated by the addition of heparin, a competitive IP, antagonist, and divalent cations to close IP_R channels which have become activated (see “Experimental Procedures”). If the channels were to remain open, all of the "Ca^{2+}" which had entered the vesicles during the flux assay would be free to diffuse out and be lost during passage over the Dowex column we employ to chelate extrasynaptic Ca^{2+}.

It thus appears that successive treatment with IP, TMS, and BME results in a population of functional receptors that are maximally activated by very low levels of IP,. How is it that increasing concentrations of IP, during preincubation result in the preservation of maximal flux activity? Again, we believe that functional receptor heterogeneity can best account for this result. Low concentrations of IP, would enhance Ca^{2+} flux selectively from more sensitive populations of receptors while progressively increased IP, concentrations would influence less sensitive receptors. With IP_R, where each vesicle contains on average one or fewer receptors (33), we propose that 40 nM IP, protects a more sensitive population of IP_R from the degradative effects of TMS while 200 and 500 nM protect less sensitive receptor populations. Treatment with TMS in the presence of 40 nM IP, would inactivate the less sensitive receptors enabling us to selectively examine IP_R of greater sensitivity.

These findings provide insight into quantal aspects of IP, induced Ca^{2+} release. In intact and permeabilized cells, IP, releases Ca^{2+} in a discontinuous quantal process in which successive increments of IP, transiently release precise amounts of Ca^{2+} (21, 22). It has been well documented that this discrete release of Ca^{2+} takes place in the absence of receptor desensitization (44). This novel phenomenon has so far only been seen in IP, and ryanodine receptors (44). Although there has been considerable speculation regarding the mechanisms underlying quantal Ca^{2+} release, two alternative models are currently believed to best account for this phenomenon: the all-or-none model and the steady-state model (44). The central difference between these two models relates to their assumptions about the differences in the sensitivity of IP, to IP,. The all-or-none model proposes that quantal Ca^{2+} release is the result of complete emptying of distinct stores that are heterogeneous in their sensitivity to IP,. The steady-state model proposes that quantal release occurs because decreasing luminal Ca^{2+} allosterically attenuates the channel activity of IP_R that are homogenous in their sensitivity to IP,. There exists evidence in support of both models (44). Data have been reported supporting heterogeneity in the sensitivity of different intracellular stores to IP, (45, 46), but few studies have directly addressed heterogeneous sensitivity of IP_R themselves.

We provided the novel evidence indicating that IP_R are intrinsically heterogeneous in their sensitivity to IP, by showing...
that purified and reconstituted receptors display quantal Ca\textsuperscript{2+} release (23). As discussed above, the effects of TMS on IP\textsubscript{3}R response to various concentrations of IP\textsubscript{3} can best be explained as a result of intrinsic variations in receptor sensitivity to its ligand. In order for heterogeneously sensitive receptors to manifest quantal Ca\textsuperscript{2+} release, they must respond maximally over a very narrow range of IP\textsubscript{3} concentrations. This is one of the predictions of the all-or-none model, and it accounts for how a highly sensitive subpopulation of receptors can release a fraction of the total releasable Ca\textsuperscript{2+} in response to low levels of IP\textsubscript{3} without desensitizing (44). This behavior is implied by the response of IP\textsubscript{3}R to successive treatment with IP\textsubscript{3}, TMS, and BME (Fig. 6). The molecular basis of the functional receptor heterogeneity could arise from variations in receptor subtypes, splice forms, and phosphorylation or redox states.

In summary, our findings document that TMS directly modifies critical sulphhydril group(s) present on the IP\textsubscript{3}R to markedly increase or abolish its activity. The stimulation of IP\textsubscript{3}R activity by TMS, which occurs at a location remote from the IP\textsubscript{3} binding domain, doubles the affinity of the receptor for its ligand without increasing the number of available sites. In the presence of a fixed concentration of IP\textsubscript{3} and Ca\textsuperscript{2+}, addition of TMS can stimulate IP\textsubscript{3}R activity, with implications for physiologic and pathophysiologic redox modulation of intracellular Ca\textsuperscript{2+}. Finally, we have used TMS mediated perturbations of IP\textsubscript{3}R activity to provide evidence for functional IP\textsubscript{3}R heterogeneity. These findings are consistent with our previous observations (23) and with the original hypothesis regarding the mechanism of quantal Ca\textsuperscript{2+} release by IP\textsubscript{3}R (21).

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