

# 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  $\text{Ca}^{2+}$  mobilization in several cell types and to increase the sensitivity of intracellular  $\text{Ca}^{2+}$  stores to inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ). Using purified  $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ) protein reconstituted in vesicles, we demonstrate pronounced stimulation by thimerosal of its  $\text{Ca}^{2+}$  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  $\text{IP}_3\text{R}$ . Thimerosal enhances the affinity of  $\text{IP}_3\text{R}$  for  $\text{IP}_3$  binding. The manner in which thimerosal modulates  $\text{IP}_3\text{R}$  responsiveness to  $\text{IP}_3$  provides evidence for receptor heterogeneity with implications for mechanisms of quantal  $\text{Ca}^{2+}$  release. These results clarify regulation of  $\text{IP}_3\text{R}$  activity by redox modulation.**

The dynamics of intracellular  $\text{Ca}^{2+}$  provide crucial signaling information in many aspects of cellular regulation. Intracellular  $\text{Ca}^{2+}$  flux is regulated by numerous processes, especially the release of  $\text{Ca}^{2+}$  from intracellular stores by inositol 1,4,5-trisphosphate ( $\text{IP}_3$ )<sup>1</sup> (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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  flux (7). Thimerosal (TMS), a sulfhydryl oxidizing agent, has been reported to stimulate (8–12) or inhibit (11, 12) intracellular  $\text{Ca}^{2+}$  flux. TMS increases the potency of  $\text{IP}_3$  in releasing  $\text{Ca}^{2+}$  (9, 11, 13–16). In some studies, TMS increased the affinity of  $\text{IP}_3$  for receptor binding sites (13, 16), while other studies showed no effect (11, 12). These findings suggest that  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  flux is influenced by the oxidative state of sulfhydryl groups, perhaps those of the  $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ) itself. However, TMS can influence related  $\text{Ca}^{2+}$  regulating systems, such as the endoplasmic reticular  $\text{Ca}^{2+}$  pump (11, 13), indirectly affecting  $\text{IP}_3$  induced  $\text{Ca}^{2+}$  release.

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<sup>1</sup> The abbreviations used are:  $\text{IP}_3$ , inositol 1,4,5-trisphosphate; TMS, thimerosal;  $\text{IP}_3\text{R}$ ,  $\text{IP}_3$  receptor;  $\text{IP}_3\text{RV}$ ,  $\text{IP}_3$  reconstituted vesicles; BME, B-mercaptoethanol; DTT, dithiothreitol; IAA, iodoacetamide; NEM, N-ethylmaleimide.

We successfully reconstituted  $\text{IP}_3$  induced  $\text{Ca}^{2+}$  flux in vesicles containing only purified  $\text{IP}_3\text{R}$  protein (17).  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  flux at the level of the  $\text{IP}_3\text{R}$  protein.

Intracellular release of  $\text{Ca}^{2+}$  is a discontinuous, quantal process in which successive increments of  $\text{IP}_3$  transiently release precise amounts of  $\text{Ca}^{2+}$  (21, 22). Utilizing  $\text{IP}_3\text{R}$  reconstituted into proteolipid vesicles ( $\text{IP}_3\text{RV}$ ) we previously showed that quantal flux of  $\text{Ca}^{2+}$  elicited by  $\text{IP}_3$  is a fundamental property of the  $\text{IP}_3\text{R}$ , suggesting that the receptors purified from rat cerebellum constitute a heterogeneous population with varying sensitivity to  $\text{IP}_3$  (23). The effects of thiol reagents on  $\text{IP}_3$ -mediated flux in  $\text{IP}_3\text{RV}$  observed here provide additional evidence for functional receptor heterogeneity, which may help account for quantal  $\text{Ca}^{2+}$  release.

## EXPERIMENTAL PROCEDURES

**Materials**— $[\text{H}]\text{IP}_3$ ,  $^{45}\text{Ca}^{2+}$ , and formula 963 scintillation mixture were obtained from DuPont NEN. *D-myo*-Ins(1,4,5) $\text{P}_3$ , 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  $\text{IP}_3\text{R}$** — $\text{IP}_3\text{R}$  was purified from adult male Sprague-Dawley rat cerebellum and reconstituted into lipid vesicles as described (17). Briefly,  $\text{IP}_3\text{R}$  was purified using a two-step affinity chromatography procedure employing sequential heparin-agarose 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  $\text{IP}_3\text{RV}$  was passed over a 5-ml G-25 desalting column equilibrated with buffer A to remove BME.

**$^{45}\text{Ca}^{2+}$  Flux**—Reconstituted proteoliposomes were assayed for  $\text{IP}_3$ -stimulated  $^{45}\text{Ca}^{2+}$  flux as described (17). Following preincubation under various conditions vesicles were incubated (for either 10 or 15 s) in the presence of 2  $\mu\text{Ci}$  of  $^{45}\text{Ca}^{2+}$  with or without  $\text{IP}_3$ . Under these conditions, tracer  $^{45}\text{Ca}^{2+}$  gained access to the lumen of vesicles when the  $\text{IP}_3\text{R}$  channels were opened by  $\text{IP}_3$ . The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin (200  $\mu\text{g}/\text{ml}$ ). Intravesicular  $^{45}\text{Ca}^{2+}$  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  $^{45}\text{Ca}^{2+}$  content was measured by scintillation spectrometry.

**$[\text{H}]\text{IP}_3$  Binding**—Ligand binding was assayed by precipitation of  $\text{IP}_3\text{RV}$  with polyethylene glycol using  $\gamma$ -globulin as carrier protein, as described (20).

## RESULTS

Several workers have shown TMS stimulation of  $\text{Ca}^{2+}$  flux in intact cells and platelets (14, 15, 24–29) and enhancement of