

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 Ca^{2+} mobilization in several cell types and to increase the sensitivity of intracellular Ca^{2+} stores to inositol 1,4,5-trisphosphate (IP_3). Using purified IP_3 receptor (IP_3R) protein reconstituted in vesicles, we demonstrate pronounced stimulation by thimerosal of its 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 IP_3R . Thimerosal enhances the affinity of IP_3R for IP_3 binding. The manner in which thimerosal modulates IP_3R responsiveness to IP_3 provides evidence for receptor heterogeneity with implications for mechanisms of quantal Ca^{2+} release. These results clarify regulation of IP_3R activity by redox modulation.

The dynamics of intracellular Ca^{2+} provide crucial signaling information in many aspects of cellular regulation. Intracellular Ca^{2+} flux is regulated by numerous processes, especially the release of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP_3)¹ (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 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 Ca^{2+} flux (7). Thimerosal (TMS), a sulfhydryl oxidizing agent, has been reported to stimulate (8–12) or inhibit (11, 12) intracellular Ca^{2+} flux. TMS increases the potency of IP_3 in releasing Ca^{2+} (9, 11, 13–16). In some studies, TMS increased the affinity of IP_3 for receptor binding sites (13, 16), while other studies showed no effect (11, 12). These findings suggest that IP_3 -induced Ca^{2+} flux is influenced by the oxidative state of sulfhydryl groups, perhaps those of the IP_3 receptor (IP_3R) itself. However, TMS can influence related Ca^{2+} regulating systems, such as the endoplasmic reticular Ca^{2+} pump (11, 13), indirectly affecting IP_3 induced Ca^{2+} release.

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¹ The abbreviations used are: IP_3 , inositol 1,4,5-trisphosphate; TMS, thimerosal; IP_3R , IP_3 receptor; IP_3RV , IP_3 reconstituted vesicles; BME, B-mercaptoethanol; DTT, dithiothreitol; IAA, iodoacetamide; NEM, N-ethylmaleimide.

We successfully reconstituted IP_3 induced Ca^{2+} flux in vesicles containing only purified IP_3R protein (17). IP_3 -mediated 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 Ca^{2+} flux at the level of the IP_3R protein.

Intracellular release of Ca^{2+} is a discontinuous, quantal process in which successive increments of IP_3 transiently release precise amounts of Ca^{2+} (21, 22). Utilizing IP_3R reconstituted into proteolipid vesicles (IP_3RV) we previously showed that quantal flux of Ca^{2+} elicited by IP_3 is a fundamental property of the IP_3R , suggesting that the receptors purified from rat cerebellum constitute a heterogeneous population with varying sensitivity to IP_3 (23). The effects of thiol reagents on IP_3 -mediated flux in IP_3RV observed here provide additional evidence for functional receptor heterogeneity, which may help account for quantal Ca^{2+} release.

EXPERIMENTAL PROCEDURES

Materials— $^3\text{H}\text{IP}_3$, $^{45}\text{Ca}^{2+}$, and formula 963 scintillation mixture were obtained from DuPont NEN. *D-myo*-Ins(1,4,5) 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 IP_3R — IP_3R was purified from adult male Sprague-Dawley rat cerebellum and reconstituted into lipid vesicles as described (17). Briefly, IP_3R 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 IP_3RV 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 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 μCi of $^{45}\text{Ca}^{2+}$ with or without IP_3 . Under these conditions, tracer $^{45}\text{Ca}^{2+}$ gained access to the lumen of vesicles when the IP_3R channels were opened by 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.

$^3\text{H}\text{IP}_3$ Binding—Ligand binding was assayed by precipitation of IP_3RV with polyethylene glycol using γ -globulin as carrier protein, as described (20).

RESULTS

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