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\textsuperscript{2+} mobilization in several cell types and to increase the sensitivity of intracellular Ca\textsuperscript{2+} stores to inositol 1,4,5-trisphosphate (IP\textsubscript{3}). Using purified IP\textsubscript{3} receptor (IP\textsubscript{R}) protein reconstituted in vesicles, we demonstrate pronounced stimulation by thimerosal of its Ca\textsuperscript{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\textsubscript{R}. Thimerosal enhances the affinity of IP\textsubscript{R} for IP\textsubscript{3}, binding. The manner in which thimerosal modulates IP\textsubscript{R} responsiveness to IP\textsubscript{3} provides evidence for receptor heterogeneity with implications for mechanisms of quantal Ca\textsuperscript{2+} release. These results clarify regulation of IP\textsubscript{R} activity by redox modulation.

The dynamics of intracellular Ca\textsuperscript{2+} provide crucial signaling information in many aspects of cellular regulation. Intracellular Ca\textsuperscript{2+} flux is regulated by numerous processes, especially the release of Ca\textsuperscript{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP\textsubscript{3}). Utilizing IP\textsubscript{3} receptor (IP\textsubscript{R}) reconstituted in proteoliposomes, we previously showed that quantal flux of Ca\textsuperscript{2+} elicited by IP\textsubscript{3} is a fundamental property of the IP\textsubscript{R}, suggesting that the receptors purified from rat cerebellum constitute a heterogeneous population with varying sensitivity to IP\textsubscript{3}. These effects of thiol reagents on IP\textsubscript{3} mediated flux in IP\textsubscript{R} vesicles observed here provide additional evidence for functional receptor heterogeneity, which may help account for quantal Ca\textsuperscript{2+} release.

**EXPERIMENTAL PROCEDURES**

Materials—[\(^3\)H]IP\textsubscript{3}, Ca\textsuperscript{2+}, and formula 963 scintillation mixture were obtained from DuPont NEN. D-myoinositol, 1,4,5-trisphosphate; TMS, 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\textsubscript{R}—IP\textsubscript{R} was purified from adult male Sprague-Dawley rat cerebellum and reconstituted into lipid vesicles as described (17). Briefly, IP\textsubscript{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-activated receptor protein was mixed with sonicated lipids and the mixture was dialyzed 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 5 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 mM IP\textsubscript{R} was passed over a 5 ml G-25 desalting column equilibrated with buffer A to remove BME.

[\(^6\)Ca\textsuperscript{2+}] Flux—Reconstituted proteoliposomes were assayed for IP\textsubscript{3} stimulated [\(^6\)Ca\textsuperscript{2+}] flux as described (17). Following preincubation under various conditions vesicles were incubated (for either 10 or 15 s) in the presence of [\(^6\)Ca\textsuperscript{2+}] with or without IP\textsubscript{R}. Under these conditions, tracer [\(^6\)Ca\textsuperscript{2+}] gained access to the lumen of vesicles when the IP\textsubscript{R} channels were opened by IP\textsubscript{3}. The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin (200 μg/ml). Intravesicular [\(^6\)Ca\textsuperscript{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 [\(^6\)Ca\textsuperscript{2+}] content was measured by scintillation spectrometry.

**RESULTS**

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