Inositol 1,4,5-Trisphosphate Receptor/Ca2+ Channel Modulatory Role of Chromogranin A, a Ca2+ Storage Protein of Secretory Granules*

Seung Hyun Yoo† and Choon Ju Jeon

From the National Creative Research Initiative Center for Secretory Granule Research, Biomedical Research Center, Korea Advanced Institute of Science and Technology, Yu Sung Gu, 305-701 Dae Jeon, Korea

The secretory granules of neuroendocrine cells, which contain large amounts of Ca2+ and chromogranins, have been demonstrated to release Ca2+ in response to inositol 1,4,5-trisphosphate (IP3), indicating the IP3-sensitive intracellular Ca2+ store role of secretory granules. In our previous study, chromogranin A (CGA) was shown to interact with several secretory granule membrane proteins, including the IP3 receptor (IP3R), at the intravesicular pH 5.5 (Yoo, S. H. (1994) J. Biol. Chem. 269, 12001–12006). To examine the functional aspect of this coupling, we measured the IP3-mediated Ca2+ release property of the IP3R reconstituted into liposomes in the presence and absence of CGA. Presence of CGA in the IP3R-reconstituted liposome significantly enhanced the IP3-mediated Ca2+ release from the liposomes. Moreover, the number of IP3 bound to the reconstituted IP3R increased. The fluorescence energy transfer and IP3R Trp fluorescence quenching studies indicated that the structure of reconstituted IP3R becomes more ordered and exposed in the presence of CGA, suggesting that the coupled CGA in the liposome caused structural changes of the IP3R, changing it to a structure that is better suited to IP3 binding and subsequent Ca2+ release. These results appear to underscore the physiological significance of IP3R-CGA coupling in the secretory granules.

The secretory granules of adrenal medullary chromaffin cells have been shown to release Ca2+ in response to IP3 (1), and this observation has also been extended to the secretory granules of zymogen-secreting pancreatic acinar cells (2), further demonstrating the IP3-sensitive intracellular Ca2+ store role of secretory granules. Recently, participation of secretory granule calcium in the control of cytoplasmic Ca2+ concentration has also been shown in the secretory granules of goblet cells (3); uptake of Ca2+ by secretory granules was temporally and spatially matched by simultaneous reduction of Ca2+ concentration in the surrounding cytoplasm, whereas IP3-mediated release of Ca2+ by the secretory granules resulted in the simultaneous increase of cytoplasmic Ca2+ concentration in the immediate vicinity of the secretory granules, clearly indicating the participation of secretory granule calcium in the control of cytoplasmic Ca2+ concentration. Moreover, the IP3-sensitive Ca2+ store role of secretory granules of bovine adrenal medullary chromaffin cells was attributed to the presence of high capacity, low affinity Ca2+ storage protein CGA, which binds 30–50 mol of Ca2+/mol, inside the secretory granule (1, 4). IP3 mediates release of Ca2+ from intracellular Ca2+ stores by binding to the IP3R, which can also function as a Ca2+ channel (5). The IP3R, which has been found in the endoplasmic reticulum, nuclei, and plasma membrane (6–8), is known to exist in at least three types, i.e. type I, II, and III, and to form homo- or heterotetrameric structures (9–13). In our previous study, chromogranin A was shown to interact with several integral membrane proteins of secretory granules of bovine adrenal medullary chromaffin cells, including the IP3R (14). This was the first time an ion channel protein was shown to be physically linked to a cognate ion storage protein.

Chromogranin A, which is the major secretory granule matrix protein of bovine adrenal chromaffin cells, interacts with the secretory granule membrane at the intravesicular pH of 5.5 but dissociates from it at the near physiological pH of 7.5 (15). It also undergoes pH- and Ca2+-dependent conformational changes (16) and forms a homodimer at pH 7.5 and a homotetramer at pH 5.5 (17, 18). Furthermore, a tetrameric CGA has been shown to bind four molecules of an intraluminal loop peptide of the IP3R (19), suggesting the interaction of tetrameric CGA with tetrameric IP3R in the cell.

In our recent study, it was shown that purified IP3Rs interact directly with CGA at the intravesicular pH 5.5 and dissociate from it at a near physiological pH 7.5 (20). Further, cotransfection of IP3R and CGA into COS-7 cells followed by immunoprecipitation also demonstrated coimmunoprecipitation of these two proteins (20), indicating that IP3R and CGA exist in a complexed state in vivo. These results strongly suggested that coupling of Ca2+ storage protein CGA to the IP3R/Ca2+ channel might serve important physiological roles in the secretory vesicles not only during secretory vesicle biogenesis (14) but also in controlling IP3-mediated Ca2+ mobilization in the cell.

Therefore, we have investigated in this report the physiological significance of CGA coupling to the IP3R using IP3R-reconstituted liposomes in the presence and absence of CGA, and found that CGA coupling to the IP3R in the proteoliposomes causes structural changes of IP3R so as to facilitate not only the IP3 binding but also the Ca2+ release activity of the channel.
**EXPERIMENTAL PROCEDURES**

**Materials—**Phospholipids were purchased from Avanti Polar Lipids (Albaster, AL) and were used without further purification. Fluorescence probes were from Molecular Probes (Eugene, OR). Chloroform solutions of lipids were stored in sealed ampules under argon gas at -20 °C. Cholesterol and IP3 were obtained from Sigma. All radioactive reagents were from NEN Life Science Products. Other chemicals were of the highest grade commercially available.

**IP3R Antibody—**Chromogranin A from bovine adrenal medulla was prepared from the secretory vesicle lysates of chromaffin cells as described previously (Fig. 1) (16). An IP3R peptide (DEEVEWLFWRDSNKEL, in single-letter code) corresponding to residues 692-707 of the type I IP3R (22, 23) was synthesized with a carboxyl-terminal cysteine. A polyclonal antibody was raised in rabbits against the peptide coupled to keyhole limpet hemocyanin and affinity-purified on the immobilized peptide following the procedure described (14).

**Purification of IP3R from Cerebellum—**Bovine cerebella were mixed with 3 volumes of buffer I (50 mM Tris-HCl, pH 7.4, 0.3 mM succrose, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin) and were broken into small pieces in a blender, followed by homogenization in a glass-Teflon homogenizer. The homogenates were then centrifuged at 2,000 × g for 10 min at 4 °C, and the supernatants were recentrifuged at 105,000 × g for 1 h to precipitate the membrane pellet. The pellet was resuspended in buffer II (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin) containing 1% Triton X-100 to give the membrane protein concentration of approximately 2 mg/ml. The membrane solution was stirred for 1 h and then centrifuged at 32,000 × g for 1 h at 4 °C. The supernatant obtained was mixed with an equal volume of buffer III (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1% Triton X-100, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin) and applied to an IP3R antibody-coupled immunoaffinity column (0.35 × 1 cm) equilibrated with buffer C (see below). The IP3R antibody-coupled column was prepared by coupling 0.6 mg of affinity-purified IP3R antibody (14) either to 1.2 ml of the immobilized protein A resin from the Immunopure protein A IgG orientation kit (Pierce) or to 0.2 g of CNBr-activated Sepharose 4B according to the method described previously (8), and stored in 20 mM Tris-HCl, pH 7.5, containing 0.02% sodium azide until use. The protein-loaded column was washed with 20 bed volumes of buffer C to remove unbound proteins, and the IP3R was eluted by 10 ml of elution buffer (0.1 mM glycine, pH 2.8, 0.2% Triton X-100, 0.5 M NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin). After the eluate was immediately neutralized by adding 10 mM Tris-HCl, pH 9.5, and mixed with an equal volume of buffer IV (50 mM Tris-HCl, pH 8.0, 0.2% Triton X-100, 0.5 mM NaCl, and 1 mM β-mercaptoethanol), and then applied to a benzenamine-Sepharose column equilibrated with buffer D to remove any residual proteases from the IP3R sample. The IP3R containing fraction was collected and stored at -70 °C until use (Fig. 1). The purified bovine cerebel IP3R bound approximately 320 pmol of IP3/mg of protein, which is comparable to other purified IP3Rs (9, 10), as determined according to the published method (10).

**Reconstitution of IP3R and Encapsulation of CGA into Lipo- somes—**Phosphatidylcholine (from bovine brain), phosphatidylserine (from bovine brain), and cholesterol dissolved in chloroform were mixed to give a molar ratio of 60%, 20%, and 20%, respectively. The final lipid concentration was 5 mM in a total volume of 500 μl. The solvent was evaporated under a stream of argon gas, and the residual chloroform was removed by speed vacuuming. The dry lipids were hydrated in buffer A or B solution: A: 20 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM CaCl2, and 1% CHAPS; B: 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM CaCl2, and 1% CHAPS containing 17 μg/ml IP3R and 60 μg/ml CGA (Fig. 1). The mixtures were dialyzed for 72 h against excess buffer C (buffer A or B without CHAPS) solution (A: 20 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM CaCl2, and 1% CHAPS) containing flow-through was collected and stored at 4 °C. The resulting proteoliposomes were then separated on a G-25 column (10 × 200 mm) twice to remove unreconstituted proteins. The final proteoliposomes had a molar ratio of 60%, 20%, and 20%, respectively. The final lipid concentration was 5 mM in a total volume of 500 μl when assayed by light scattering (24). The proteoliposomes produced were estimated to contain ~300 μM Ca2+ and were stable for at least 3 days as determined by <10% deviation in light scattering values. The proteoliposomes were dialyzed for 30 min, the time course of IP3-induced Ca2+ efflux from the proteoliposomes was observed by measuring the fluorescence changes of indo-1. Fluorometric measurements were performed at 35 °C by using a Shimadzu RF-5301 PC spectrophotometer equipped with a temperature-controlled cuvette holder. The fluorescence intensity was measured at the emission wavelength of 393 nm (excitation of 355 nm) with 1.5 nm of excitation band slit width and 10 nm of emission band slit width. For the kinetic analysis of IP3-induced Ca2+ release, the data were acquired every 20 ms after addition of indicated concentration of IP3, to 1.7 mM of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca2+ concentrations using Ca2+-EGTA buffering system (26).

In experiments designed to determine the effect of IP3R antibody (Ab) on the Ca2+ release from the IP3R-con-taing lipidosomes, the IP3R Ab was mixed with the reaction solution at the ratio of 1 μg of IP3R/10 or 50 μg of IP3R Ab. After preincubating the sample at 35 °C for 30 min, the time course of IP3-induced Ca2+ release was monitored with the same method described above. IP3 dose-dependent release of Ca2+ was also measured by the fluorescence intensity of indo-1 after addition of IP3 and compared with the control. The data were acquired every 20 ms after addition of indicated concentration of IP3 to 1.7 mM of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca2+ concentrations using Ca2+-EGTA buffering system (26).

**Removal of Ca2+ Contamination—**Removal of Ca2+ contamination was conducted according to the method described previously (25). Ca2+ contamination during all experiments was checked using the fluorescent dye for Ca2+ indicator, indo-1, before measurements.

**IP3-induced Ca2+ Release Measurement—**Fluorometric measurements were performed at 35 °C by using a Shimadzu RF-5301 PC spectrophotometer equipped with a temperature-controlled cuvette holder. The fluorescence intensity was measured at the emission wavelength of 393 nm (excitation of 355 nm) with 1.5 nm of excitation band slit width and 10 nm of emission band slit width. For the kinetic analysis of IP3-induced Ca2+ release, the data were acquired every 20 ms after addition of indicated concentration of IP3, to 1.7 mM of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca2+ concentrations using Ca2+-EGTA buffering system (26).

In experiments designed to determine the effect of IP3R antibody (Ab) on the Ca2+ release from the IP3R-containing liposomes, the IP3R Ab was mixed with the reaction solution at the ratio of 1 μg of IP3R/10 or 50 μg of IP3R Ab. After preincubating the sample at 35 °C for 30 min, the time course of IP3-induced Ca2+ release was monitored with the same method described above. IP3 dose-dependent release of Ca2+ was also measured by the fluorescence intensity of indo-1 after addition of IP3 and compared with the control. The data were acquired every 20 ms after addition of indicated concentration of IP3 to 1.7 mM of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca2+ concentrations using Ca2+-EGTA buffering system (26).
exclude the possibility of Ca\(^{2+}\) regulation of IP\(_3\)-induced Ca\(^{2+}\) release, 10 \(\mu\)M of indo-1 was used in these experiments, which was a high enough concentration to buffer the released Ca\(^{2+}\).

**IP\(_3\) Binding to IP\(_3\)-R—**Reconstituted proteoliposomes were incubated with various concentrations of IP\(_3\) containing 1/1000 as much \(^{3H}\)IP\(_3\). After 10 min of incubation at 35 °C, the sample was filtered through a spin concentrator (Microcon from Amicon) with a molecular weight cutoff of 100,000 at 5000 \(\times \text{g}\). The radioactivity of each filtrate was determined by a liquid scintillation counter (Beckman LS6000LL) and compared with the control values without proteoliposomes.

**Fluorescence Quenching—**For the collisional fluorescence quenching of Trp residues in IP\(_3\)-R by iodide, a varying amount of KI, up to 0.16 M KI plus KCl, was added to the reaction mixtures while maintaining the total concentration of KI plus KCl constant, and the fluorescence intensity at the emission wavelength of 340 nm was measured with the excitation at 295 nm at 35 °C.

**Fluorescence Resonance Energy Transfer—**To label the IP\(_3\)-R with 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM) and fluorescent 5-maleimide (F-mal), both from Molecular Probes, was added dropwise to 2 \(\mu\)g of IP\(_3\)-R, and the labeling was allowed to proceed overnight at 4 °C. Upon completion of the labeling, an excess of glutathione and mercaptoethanol was added to stop the reaction. CPM- or F-mal-labeled IP\(_3\)-R molecules were separated by Sephadex G-25 and dialyzed extensively against buffer E. The labeled receptor molecules were reconstituted into the liposomes along with Ca\(^{2+}\) in the presence and absence of CCM, and the fluorescence resonance energy transfer was determined at 35 °C by measuring the emission spectra of F-mal in the range of 500–580 nm at the excitation wavelength of 384 nm, the excitation wavelength of CPM.

**Effect of Ca\(^{2+}\) on the IP\(_3\)-mediated Ca\(^{2+}\) Release from the Proteoliposomes—**The proteoliposomes were produced in the presence of \(^{45}\text{Ca}\) to include \(50,000 \text{ cpm of } ^{45}\text{Ca}^{2+}\) in buffer F (20 mM HEPES, pH 7.5, 100 mM NaCl) according to the procedure described. To remove residual Ca\(^{2+}\) bound to the vesicle surface, the sample was applied to Sephadex G25 column (10 × 05 mm) equilibrated with buffer E and, after collection of the vesicle fractions, the liposomes were pelleted by centrifugation for 10 min at 4 °C. The pellet was then redissolved and dialyzed against excess volume of buffer E for 12 h at 4 °C. The proteoliposomes were mixed with each indicated concentration of CaCl\(_2\) and incubated for 10 min at 35 °C. After further incubation of the sample for 10 min in the presence of 0.2 \(\mu\)M of IP\(_3\)-R in the reaction mixtures, the sample was diluted with buffer F (buffer E plus 1.5 \(\times\) KCl). The liposomes were pelleted by centrifugation at 100,000 \(\times\) g for 30 min at 35 °C followed by washing in buffer D twice. The pellet then dissolved with 1% Triton X-100, and the radioactivity of each fraction (pellet and supernatant) was determined by scintillation counting.

**Other Procedures—**Protein concentrations were determined using bicinchoninic acid according to the manufacturer’s instruction (Sigma). The concentrations of non-fluorescent phospholipids were determined by phosphorus assay (28). The concentrations of fluorescent probes were determined spectrophotometrically using the following values as the molar extinction coefficients: 5700 cm\(^{-1}\) at 338 nm for F-mal, and 33,000 cm\(^{-1}\) at 384 nm for CPM.

**RESULTS**

**Maintenance of pH Gradient in the Liposomes—**To mimic an in vivo physiological pH environment, the inside pH of the liposomes was maintained at 5.5 while that of outside was kept at 7.5. To determine whether the inside pH of the proteoliposome is stably maintained at pH 5.5, oxonol V (Molecular Probes) was encapsulated in the proteoliposome and its fluorescence increase upon exposure to higher pH environment was measured (Fig. 2). As shown in Fig. 2, exposure of the internalized oxonol V to the pH 5.5 environment upon lysis of the proteoliposome by 1% Triton X-100 treatment did not change the fluorescence of oxonol V. However, exposure of the internalized oxonol V to pH 6.5 or 7.5 environment upon lysis of the liposome increased the fluorescence of oxonol V, indicating the maintenance of pH 5.5 inside the proteoliposome. This result indicated that the inside pH of the proteoliposome was well maintained, suggesting the suitability of these proteoliposomes for subsequent experiments. The emission fluorescence was measured at 630 nm with the excitation wavelength of 610 nm in the presence of 1% Triton X-100.

**Time Course of IP\(_3\)-induced Ca\(^{2+}\) Release—**Fig. 3A shows the time course of IP\(_3\)-induced Ca\(^{2+}\) efflux from the IP\(_3\)-R-reconstituted liposomes, as assayed by the fluorescence change of indo-1 (1 \(\mu\)M) at 393 nm. Indo-1 is a fluorescent Ca\(^{2+}\) indicator, which is non-permeable to membrane and has a dual emission character on Ca\(^{2+}\) binding; the fluorescence at 390 nm increases, while that at 465 nm decreases. When increasing the IP\(_3\) concentrations successively, Ca\(^{2+}\) was released from the proteoliposomes with only the fast rate, and the fluorescence changes virtually reached a plateau after addition of 4 \(\mu\)M IP\(_3\) (Fig. 3A). No further increase of the emission fluorescence was observed by adding more IP\(_3\) (data not shown). The total amount of Ca\(^{2+}\) released by IP\(_3\)-R was estimated to be approximately 50% of the total encapsulated Ca\(^{2+}\) concentration considering the maximal fluorescence signal obtained when the liposomes were lysed by 1% Triton X-100 (Fig. 3A).

We also tested the specificity of Ca\(^{2+}\) release through the IP\(_3\)-R using the IP\(_3\)-R Ab (Fig. 3, A and B). Addition of IP\(_3\)-R Ab at the ratio of 1 \(\mu\)g of IP\(_3\)-R/10 \(\mu\)g of IP\(_3\)-R Ab inhibited about 70% of the IP\(_3\)-induced Ca\(^{2+}\) release. Addition of more IP\(_3\)-R Ab, at the IP\(_3\)/IP\(_3\)-R Ab ratio of 1:50 (w/w), inhibited the Ca\(^{2+}\) release only slightly more (Fig. 3, A and B). When CGA was present inside the vesicle, the IP\(_3\)-induced Ca\(^{2+}\) release was biphasic with the fast and the slow rate (Fig. 3C). Interestingly, 1 \(\mu\)M IP\(_3\) released almost a maximal amount of Ca\(^{2+}\) in the presence of CGA (Fig. 3C), whereas the same amount of IP\(_3\) released only a half-maximum in the absence of CGA (Fig. 3A). These results indicate that the Ca\(^{2+}\) channel activity of IP\(_3\)-R became more sensitive as a result of CGA encapsulation. However, when the pH value of inside the liposome was kept at 7.5 (Fig. 3C), the kinetic profile of IP\(_3\)-induced Ca\(^{2+}\) release was very similar to that obtained in the absence of CGA (Fig. 3A), confirming our previous result, which indicated CCGA interaction with the IP\(_3\)-R at pH 5.5 but not at pH 7.5 (14, 19).

As a control experiment, IP\(_3\)-R was labeled with FITC or 1,5-IAEDANS after completion of the reconstitution in the
presence or absence of CGA, and the fluorescence intensity was measured to determine the effect of CGA on the amount of reconstituted IP3R. In both cases, we could not find any difference in the fluorescence intensities of the reconstituted samples, indicating that the amount of IP3R reconstituted was not affected by the presence of CGA (data not shown).

**IP3 Dose-dependent Ca²⁺ Release**—Since it has also been shown that Ca²⁺ release from the IP3R can be regulated by cytosolic Ca²⁺ concentrations (29), there was a possibility that the IP3-induced Ca²⁺ release might not represent net IP3-mediated Ca²⁺ release. To address this possibility, we determined the amount of Ca²⁺ released after one-time dose of each indicated concentration of IP3 in the presence of 10 μM of indo-1, which is a high enough concentration to buffer the released Ca²⁺ (Fig. 4). As shown in Fig. 4, presence of CGA at pH 5.5 significantly increased the amounts of Ca²⁺ released, compared with those in the absence of CGA, although the difference in the amounts of Ca²⁺ released in both cases diminished as IP3 concentrations increased. However, even the presence of CGA failed to exert this effect when the pH of the liposomes was 7.5.

**CGA Effect on IP3 Binding to IP3R**—To determine whether the increased release of Ca²⁺ in the presence of CGA is due to increased binding of IP3 to the proteoliposome, the amount of IP3 bound to the IP3R was determined as a function of increasing IP3 concentrations (Fig. 5). As shown in Fig. 5, the presence of CGA enhanced the IP3 binding 2-fold over that in the absence of CGA. When the IP3R alone was reconstituted, a maximum of 0.4 mol of IP3 appeared to bind 1 mol of IP3R. However, when CGA was also present inside the liposomes at the containing 1 μM of indo-1 at 35 °C. The inside pH of the proteoliposome was 5.5 unless stated otherwise. A, IP3-induced Ca²⁺ release from the proteoliposomes and its inhibition by IP3R Ab. Two different concentrations of IP3R Ab, at a ratio of 1 μg of IP3R/10 or 50 μg of IP3R Ab, were added. TX-100, Triton X-100. B, percent changes of released Ca²⁺ from the liposomes described in A. Addition of preimmune serum at the IP3R:preimmune ratio of 1:50 (w/w) did not affect the Ca²⁺ release. Each indicated concentration of IP3 represents the amount of cumulative IP3 added. C, IP3-induced Ca²⁺ release in the presence of encapsulated CGA at the inside pH of 5.5 and 7.5.
intravesicular pH of 5.5, the IP₃ binding increased to about double the value shown with the IP₃R alone. But when the pH of the liposomes was 7.5, CGA showed no effect on the IP₃ binding to the IP₃R. Nevertheless, the half-maximal value was almost the same for both cases regardless of the presence of CGA, indicating that the affinity of IP₃R for IP₃ has not changed.

**Structural and Motional Changes of IP₃R by Interaction with CGA**—To investigate possible conformational changes of the IP₃R by its interaction with CGA, we utilized collisional quenching of the IP₃R Trp fluorescence by iodide (Fig. 6). As shown in Fig. 6, the IP₃R Trp fluorescence was quenched by iodide regardless of the presence of CGA. The emission fluorescence at 340 nm was measured, and the results were plotted according to the Stern-Volmer equation (30).

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\frac{F_0}{F} = K_{sv}[\text{I}] + 1 \quad \text{(Eq. 1)}
\]

\(F_0\) is the emission intensity in the absence of iodide, \(F\) is the intensity in the presence of iodide, \(K_{sv}\) is the Stern-Volmer quenching constant, and \([\text{I}]\) is the molar concentration of iodide. The \(K_{sv}\) value estimated from the slope was 5.20 M⁻¹M⁻¹ for the reconstituted IP₃R in the absence of CGA. This value decreased to 3.76 M⁻¹ when CGA was present. From this experiment, it is clear that at least some Trp residues of the IP₃R are less exposed to the solvent when CGA is present and that CGA induced conformational changes of IP₃R.

In order to further examine the effect of CGA on the molecular property of IP₃R in the reconstituted vesicles, we carried out the fluorescence resonance energy transfer study using the CPM- and F-mal-labeled IP₃Rs (Fig. 7). As shown in Fig. 7, the reconstituted IP₃Rs exhibited efficient fluorescence resonance energy transfer as evidenced by the increase of F-mal emission fluorescence under the excitation wavelength for CPM. This result indicated that the IP₃R molecules have a strong tendency to exist as oligomers (tetramers) in the membranes. When CGA was present in the liposomes, the emission intensity increased by approximately 30%, suggesting that the organizational order of IP₃Rs was enhanced by their interaction with CGA.

**Effect of Ca²⁺ on IP₃-induced Ca²⁺ Release from the Proteoliposomes**—In view of the potential inhibitory effect of increasing Ca²⁺ concentrations on the IP₃-induced Ca²⁺ release activity of IP₃R (31), we examined the Ca²⁺ release activity of the proteoliposome in response to a fixed amount of IP₃ in the presence of increasing concentrations of Ca²⁺ (Fig. 8). As shown in Fig. 8, 0.2 μM IP₃ induced far greater Ca²⁺ releases in the presence of CGA than in the absence of CGA. The emission fluorescence of F-mal was measured at 35 °C at the excitation wavelength of 384 nm (instead of 492 nm), the excitation wavelength of CPM.
that IP₃ releases far greater amounts of Ca²⁺ from the proteoliposome in the presence of CGA when there is no inhibitory amount of Ca²⁺ in the liposome solution. As a control experiment, we measured Ca²⁺-induced Ca²⁺ release without IP₃. However, no appreciable release of stored Ca²⁺ could be observed in the presence of up to 2 μM free Ca²⁺ (data not shown).

DISCUSSION

Since the original study that demonstrated the IP₃-induced Ca²⁺ release from chromaffin granules (1), Petersen and colleagues (2) have shown the IP₃-mediated Ca²⁺ release from the zymogen granules, thus supporting the concept of secretory granules of endocrine and neuroendocrine cells serving as the IP₃-sensitive intracellular Ca²⁺ store (1, 32). Recently, additional evidence indicating direct participation of secretory granule calcium in the control of cytoplasmic Ca²⁺ has been obtained in the secretory granules of goblet cells (3); the uptake of Ca²⁺ by secretory granules was temporally and spatially matched by simultaneous reduction of Ca²⁺ concentration in the surrounding cytoplasm, whereas IP₃-mediated release of Ca²⁺ by secretory granules resulted in the simultaneous increase of cytoplasmic Ca²⁺ concentration in the immediate vicinity of the secretory granules, clearly demonstrating the active participation of secretory granules in the control of cytoplasmic Ca²⁺ concentration.

Chromogranins are widely distributed in neurons, endocrine cells, and neuroendocrine cells, and they are known to be marker proteins of neuroendocrine cells (33–36). In the secretory granules of bovine adrenal chromaffin cells, chromogranins are present in 1–2 mM range (35, 37), and bind ATP (38), catecholamine (39), in addition to Ca²⁺ (40, 41). Chromogranin A binds 32 mol of Ca²⁺/mol with dissociation constant of 2.7 mM at pH 7.5, and 55 mol of Ca²⁺/mol with dissociation constant of 4 mM at pH 5.5 (4). Due to the high capacity Ca²⁺ binding of chromogranins, most (>99.9%) of 40 mM intravesicular calcium stay bound to chromogranins, resulting in the free Ca²⁺ concentration of only 24 μM (40). In view of the fact that the secretory granules occupy approximately 10% of bovine chromaffin cell volume (37), the calcium storage capacity of the secretory granules must be playing key roles in the overall control of calcium in the cells. In this regard, the high capacity, low affinity Ca²⁺ binding property of chromogranins proves to be essential, enabling the secretory granules to store an exceptionally large amount of Ca²⁺. It appears therefore very natural that the secretory granules play pivotal roles in controlling the intracellular Ca²⁺ concentrations.

Although the discovery that the secretory granule of adrenal medullary chromaffin cells is a major IP₃-sensitive intracellular Ca²⁺ store (1) implied the presence of IP₃R on the secretory granule membrane, the presence of IP₃R on the secretory granule membrane was not addressed until the intravesicular matrix protein CGA was shown to interact with several integral membrane proteins of secretory granules including the IP₃R (14), which was the first example demonstrating a physical coupling between an ion channel and a cognate ion storage protein. Nevertheless, it was not known what effect the coupled CGA exert on the IP₃R/Ca²⁺ channel.

The present results obtained in the reconstitution experiments using the purified IP₃R and CGA are considered to reflect the important roles these molecules play in the cytoplasm, which enable the secretory granules to control the intracellular Ca²⁺ levels in neuroendocrine cells. The Ca²⁺ flux experiments in Figs. 3C and 4 show that the amount of Ca²⁺ released in response to submaximal levels of IP₃ was significantly enhanced as a result of coupled CGA. These results suggest not only a direct physical coupling but also a functional coupling between the IP₃R/Ca²⁺ channel and CGA at the intravesicular pH of 5.5. Nevertheless, at a near physiological pH of 7.5, CGA failed to exert any effect on the IP₃-mediated Ca²⁺ release (Figs. 3C and 4), underscoring the importance of the intravesicular acidic pH environment in the coupling of CGA to the IP₃R. Although IP₃R monomers can bind CGA, they do not exist in a monomeric state in the cell; rather, four molecules of same or different types of IP₃R interact with each other to form either a homotetrameric or a heterotetrameric IP₃R (9–13), forming a Ca²⁺ channel. Interestingly, CGA is also known to form a dimer at the near physiological pH 7.5 and a tetramer at the intravesicular pH 5.5 (17). Further, a tetrameric CGA has also been shown to bind four molecules of intraluminal loop peptide of IP₃R (19), thus suggesting the potential interaction between a tetrameric CGA and a tetrameric IP₃R. Therefore, the results showing the direct interaction between the purified IP₃R and CGA (20), combined with the co-immunoprecipitation of transfected IP₃R and CGA (20), strongly suggest the importance of this coupling in fine-tuning the intracellular Ca²⁺ control mechanisms in the cell. In view of the fact that CGA and IP₃R interact with each other at pH 5.5 and dissociate at pH 7.5 (14, 20), the lack of any effect of CGA on the IP₃-mediated Ca²⁺ release at pH 7.5 is probably due to the failure of CGA to couple with the IP₃R. This is also reflected in the fact that the number of IP₃ bound to the IP₃R remained virtually the same when the liposome pH was maintained at 7.5, regardless of the presence of CGA (Fig. 5).

Furthermore, the result in Fig. 4 shows that 1 μM IP₃ releases almost the maximal amount of Ca²⁺ that can be released. Given that approximately 2 molecules of IP₃ bound/tetrameric IP₃R at 1 μM IP₃ (Fig. 5), the result in Fig. 4 suggests that almost a maximal amount of Ca²⁺ can be released when 2 molecules of IP₃ are bound per tetrameric IP₃R, although a maximum of 4 molecules of IP₃ can be bound per tetrameric IP₃R at 4 μM IP₃ (Fig. 5). This result is in general agreement with previous results, which indicated that one molecule of IP₃ opens the IP₃R/Ca²⁺ channel, which exhibits four conductance states (42). Although it has also been sug-
responded appropriately to the changing cytoplasmic Ca\(^{2+}\) pears that CGA play pivotal roles in controlling the Ca\(^{2+}\) less exposed when CGA is present (Fig. 6). Therefore, it ap- these observations, it is natural to think that the conforma-

The fluorescence resonance energy transfer (Fig. 7) measurements suggested that the enhanced rate of Ca\(^{2+}\) release from a structure that is better suited to IP\(_3\) binding (Fig. 5) and subsequent Ca\(^{2+}\) release property of the IP\(_3\)R in neuroendocrine cells, not only by binding and freeing of the intravesicular Ca\(^{2+}\) but also by modulating the channel activity of the IP\(_3\)Rs.

IP\(_3\) binding to the IP\(_3\)R has been known to cause conformational changes of the IP\(_3\)R (48). Further, it has been known that CGA assumes different conformations in different pH environment (16) and that CGA in different conformations exhibits different Ca\(^{2+}\)-binding capacity and affinity (4). In light of these observations, it is natural to think that the conformational changes of the IP\(_3\)R that occur as a result of IP\(_3\) binding will be transmitted instantly to the coupled CGA in the secre-

In view of the fact that the secretory granules contain up to 40 m\(\text{mol} \text{Ca}^{2+}\) and most (>99.9%) of it remains bound to chromo-

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