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Inositol 1,4,5-Trisphosphate Receptor/Ca^{2+} Channel Modulatory Role of Chromogranin A, a Ca^{2+} Storage Protein of Secretory Granules*

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The secretory granules of neuroendocrine cells, which contain large amounts of Ca^{2+} and chromogranins, have been demonstrated to release Ca^{2+} in response to inositol 1,4,5-trisphosphate (IP_3), indicating the IP_3-sensitive intracellular Ca^{2+} store role of secretory granules. In our previous study, chromogranin A (CGA) was shown to interact with several secretory granule membrane proteins, including the IP_3 receptor (IP_3R), 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 IP_3-mediated Ca^{2+} release property of the IP_3R reconstituted into liposomes in the presence and absence of CGA. Presence of CGA in the IP_3R-reconstituted liposome significantly enhanced the IP_3-mediated Ca^{2+} release from the liposomes. Moreover, the number of IP_3 bound to the reconstituted IP_3R increased. The fluorescence energy transfer and IP_3R Trp fluorescence quenching studies indicated that the structure of reconstituted IP_3R becomes more ordered and exposed in the presence of CGA, suggesting that the coupled CGA in the liposome caused structural changes of the IP_3R, changing it to a structure that is better suited to IP_3 binding and subsequent Ca^{2+} release. These results appear to underscore the physiological significance of IP_3R-CGA coupling in the secretory granules.

The secretory granules of adrenal medullary chromaffin cells have been shown to release Ca^{2+} in response to IP_3 [1] (1), and this observation has also been extended to the secretory granules of zymogen-secreting pancreatic acinar cells (2), further demonstrating the IP_3-sensitive intracellular Ca^{2+} store role of secretory granules. Recently, direct participation of secretory granule calcium in the control of cytoplasmic Ca^{2+} concentration has also been shown in the secretory granules of goblet cells (3); uptake of Ca^{2+} by secretory granules was temporally and spatially matched by simultaneous reduction of Ca^{2+} concentration in the surrounding cytoplasm, whereas IP_3-mediated release of Ca^{2+} by the secretory granules resulted in the simultaneous increase of cytoplasmic Ca^{2+} concentration in the immediate vicinity of the secretory granules, clearly indicating the participation of secretory granule calcium in the control of cytoplasmic Ca^{2+} concentration. Moreover, the IP_3-sensitive Ca^{2+} store role of secretory granules of bovine adrenal medullary chromaffin cells was attributed to the presence of high capacity, low affinity Ca^{2+} storage protein CGA, which binds 30–50 mol of Ca^{2+}/mol, inside the secretory granule (1, 4). IP_3 mediates release of Ca^{2+} from intracellular Ca^{2+} stores by binding to the IP_3R, which can also function as a Ca^{2+} channel (5). The IP_3R, 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 IP_3R (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 Ca^{2+}-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 IP_3R (19), suggesting the interaction of tetrameric CGA with tetrameric IP_3R in the cell.

In our recent study, it was shown that purified IP_3Rs 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 IP_3R and CGA into COS-7 cells followed by coimmunoprecipitation also demonstrated coimmunoprecipitation of these two proteins (20), indicating that IP_3R and CGA exist in a complexed state *in vivo*. These results strongly suggested that coupling of Ca^{2+} storage protein CGA to the IP_3R/Ca^{2+} channel might serve important physiological roles in the secretory vesicles not only during secretory vesicle biogenesis (14) but also in controlling IP_3-mediated Ca^{2+} mobilization in the cell.

Therefore, we have investigated in this report the physiological significance of CGA coupling to the IP_3R using IP_3R-reconstituted liposomes in the presence and absence of CGA, and found that CGA coupling to the IP_3R in the proteoliposomes causes structural changes of IP_3R so as to facilitate not only the IP_3 binding but also the Ca^{2+} release activity of the channel.

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[1] The abbreviations used are: IP_3, D-*myo*-inositol 1,4,5-triphosphate; 1,5-IAEDANS, (5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; IP_3R: inositol 1,4,5-triphosphate receptor; FITC, fluorescein 5-isothiocyanate; CGA, chromogranin A; F-mal, fluorescein 5-maleimide; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; Ab, antibody; PMSF, phenylmethylsulfonyl fluoride.

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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 IP_3 were obtained from Sigma. All radioactive reagents were from NEN Life Science Products. Other chemicals were of the highest grade commercially available.

Purification of CGA and IP_3R Antibody—Chromogranin A from bovine adrenal medulla was prepared from the secretory vesicle lysates of chromaffin cells as described previously (Fig. 1) (16). An IP_3R peptide (DEEEVWLFWRDSNKEI, in single-letter code) corresponding to residues 692–707 of the type I IP_3R (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 IP_3R from Cerebellum—Bovine cerebella were mixed with 3 volumes of buffer I (50 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 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 M NaCl, 1% Triton X-100, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin) and applied to an IP_3R antibody-coupled immunoaffinity column (0.35 × 1 cm) equilibrated with buffer C (see below). The IP_3R antibody-coupled column was prepared by coupling 0.6 mg of affinity purified anti-peptide IP_3R 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 IP_3R was eluted by 10 ml of elution buffer (0.1 M 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). The eluate was immediately neutralized by adding 1 M 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 M NaCl, and 1 mM β-mercaptoethanol), and then applied to a benzamidine-Sepharose column equilibrated with buffer D to remove any residual proteases from the IP_3R sample. The IP_3R containing flow-through was collected and stored at −70 °C until use (Fig. 1). The purified bovine cerebellum IP_3R bound approximately 320 pmol of IP_3/mg of protein, which is comparable to other purified IP_3Rs (9, 10), as determined according to the published method (10).

Reconstitution of IP_3R and Encapsulation of CGA into Liposomes—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 $CaCl_2$, and 1% CHAPS; B: 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$, and 1% CHAPS) containing 17 μg/ml IP_3R and 60 μg/ml CGA (Fig. 1). The mixtures were dialyzed for 72 h against excess volume of buffer C (buffer A or B without CHAPS) at 4 °C. The resulting proteoliposomes were pelleted by centrifugation at 100,000 × *g* for 30 min at 4 °C and washed with buffer D (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 M KCl, and 3 M urea) twice to remove unreconstituted proteins. The pellets were resuspended with buffer E (20 mM HEPES, pH 7.5, 100 mM NaCl) and then were dialyzed against buffer E for 48 h at 4 °C. The resulting proteoliposomes were passed through Chelex 100 to remove Ca^{2+}. The formation of proteoliposomes was monitored using light scattering during dialysis against buffer C and by electron microscopy; some portion of sample was collected as a function of time and, the emission values at 450 nm were measured with the same excitation wavelength (excitation band slit width: 1.5/emission band slit width: 5 nm). The average diameter of the liposomes was 280 ± 50 nm when assayed by light scattering (24). The proteoliposomes produced were estimated to contain ~300 μM Ca^{2+} and were stable for at least 3 days as determined by <10% deviation in light scattering values.

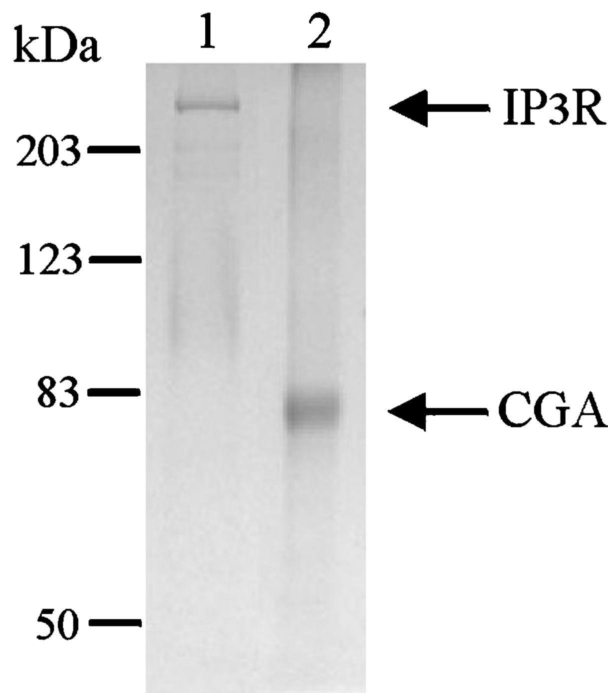

FIG. 1. **SDS-PAGE of purified bovine IP_3R and chromogranin A.** Coomassie Blue-stained purified bovine IP_3R (0.5 μg) and CGA (1.2 μg) on a 7.5% SDS gel.

To test the encapsulation of CGA into liposomes, we used the fluorescence of FITC which is amine-reactive and non-permeable to membranes. 50 μl of proteoliposome was incubated with 5 mM of FITC for 2 h at room temperature in the presence or absence of 2% Triton X-100, and then 10 μl of 500 mM Tris-HCl, pH 8.0 was added to the mixture to stop the reaction. To remove free FITC, the sample was applied to Sephadex G-25 column (10 × 200 mm) and then was extensively dialyzed against buffer E at 4 °C. After labeling the proteoliposomes with the probe, the emission intensity at 519 nm was measured at the excitation wavelength of 494 nm. In addition, the encapsulation of CGA was also confirmed by lysing the CGA-containing and non-CGA-containing proteoliposomes with Triton X-100 and separating on SDS-polyacrylamide gels, followed by quantitation of the proteins. These procedures not only confirmed the encapsulation of CGA in the proteoliposomes but also indicated that the presence of CGA did not affect the the amount of IP_3R inserted into the liposomes (data not shown).

Removal of Ca^{2+} Contamination—Removal of Ca^{2+} contamination was conducted according to the method described previously (25). Ca^{2+} contamination during all experiments was checked using the fluorescence of Ca^{2+} indicator, indo-1, before measurements.

IP_3-induced Ca^{2+} Release Measurement—Ca^{2+} 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 spectrofluorometer 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 IP_3-induced Ca^{2+} release, the data were acquired every 20 ms after addition of indicated concentration of IP_3 to 1.7 ml of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca^{2+} concentrations using Ca^{2+}-EGTA buffering system (26).

In experiments designed to determine the effect of IP_3R antibody (Ab) on the Ca^{2+} release from the IP_3R-reconstituted liposomes, the IP_3R Ab was mixed with the reaction solution at the ratio of 1 μg of IP_3R/10 or 50 μg of IP_3R Ab. After preincubating the sample at 35 °C for 30 min, the time course of IP_3-induced Ca^{2+} release was monitored with the same method described above.

IP_3 dose-dependent release of Ca^{2+} was also measured by the fluorescence intensity of indo-1 after addition of IP_3 and compared with the fluorescence intensity after addition of Triton X-100 instead of IP_3. To

exclude the possibility of Ca^{2+} regulation of IP_3-induced Ca^{2+} release, 10 μM 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_3R—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, 0.1 volume of each sample was filtered through a spun concentrator (Microcon from Amicon) with molecular weight cutoff of 100,000 at 5000 × *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_3R by iodide, a varying amount of KI, up to 0.16 M final, 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_3R with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) and fluorescein 5-maleimide (F-mal), respectively, CPM or F-mal, both from Molecular Probes, was added dropwise to 2 μg of IP_3R, 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_3R 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 CGA, 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 $^{45}Ca^{2+}$ to include ~50,000 cpm of $^{45}Ca^{2+}$ in buffer E (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 × 00 mm) equilibrated with buffer E and, after collection of the vesicle fractions, the liposomes were pelleted by centrifugation (100,000 × *g*, 30 min, 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 μM of IP_3 in the reaction mixtures, the sample was diluted with buffer F (buffer E plus 1.5 M KCl). The liposomes were pelleted by centrifugation at 100,000 × *g* for 30 min at 35 °C followed by washing in buffer D twice. The pellet was 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} M^{-1} at 336 nm for 1,5-IAEDANS, 77,000 cm^{-1} M^{-1} at 494 nm and 83,000 cm^{-1} M^{-1} at 492 nm for F-mal, and 33,000 cm^{-1} M^{-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 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

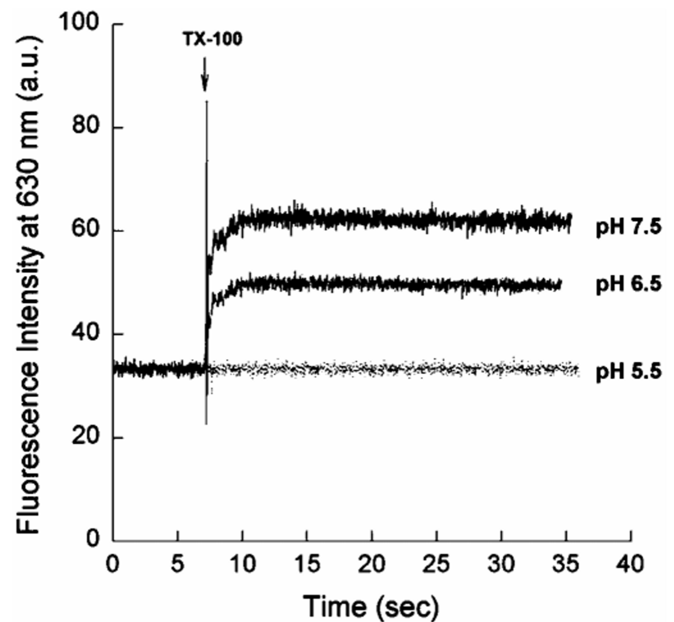

FIG. 2. **Maintenance of pH gradient inside the lipid vesicles.** Oxonol V, a pH-sensitive fluorescent probe, was encapsulated into the liposomes (pH 5.5) in the absence of proteins. After removal of free oxonol V, the emission fluorescence at 630 nm (excitation at 610 nm) was measured as a function of time. Each indicated pH value indicates the pH outside the liposomes.

in the presence of 1% Triton X-100.

Time Course of IP_3-induced Ca^{2+} Release—Fig. 3*A* shows the time course of IP_3-induced Ca^{2+} efflux from the IP_3R-reconstituted liposomes, as assayed by the fluorescence change of indo-1 (1 μM) 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 μM IP_3 (Fig. 3*A*). 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 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. 3*A*).

We also tested the specificity of Ca^{2+} release through the IP_3R using the IP_3R Ab (Fig. 3, *A* and *B*). Addition of IP_3R Ab at the ratio of 1 μg of IP_3R/10 μg of IP_3R Ab inhibited about 70% of the IP_3-induced Ca^{2+} release. Addition of more IP_3R Ab, at the IP_3R/IP_3R 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 biexponential with the fast and the slow rate (Fig. 3*C*). Interestingly, 1 μM IP_3 released almost a maximumal amount of Ca^{2+} in the presence of CGA (Fig. 3*C*), whereas the same amount of IP_3 released only a half-maximum in the absence of CGA (Fig. 3*A*). These results indicate that the Ca^{2+} channel activity of IP_3R becomes far more efficient as a result of CGA encapsulation. However, when the pH value of inside the liposome was kept at 7.5 (Fig. 3*C*), the kinetic profile of IP_3-induced Ca^{2+} release was very similar to that obtained in the absence of CGA (Fig. 3*A*), confirming our previous result, which indicated CGA interaction with the IP_3R at pH 5.5 but not at pH 7.5 (14, 19).

As a control experiment, IP_3R was labeled with FITC or 1,5-IAEDANS after completion of the reconstitution in the

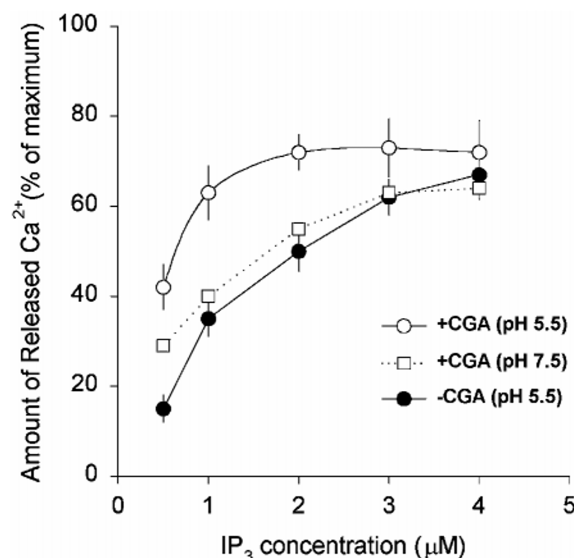

FIG. 4. **IP_3 dose-dependent Ca^{2+} release from the IP_3R-reconstituted liposomes.** The amount of Ca^{2+} released as a result of one-time addition of indicated concentrations of IP_3 to the proteoliposome solution containing 10 μM of indo-1 is expressed as percentage of maximum releasable Ca^{2+}. IP_3-induced fluorescent changes were compared with the fluorescent changes caused by 1% Triton X-100 (this intensity was set as the maximal fluorescent change).

presence or absence of CGA, and the fluorescence intensity was measured to determine the effect of CGA on the amount of reconstituted IP_3R. In both cases, we could not find any difference in the fluorescence intensities of the reconstituted samples, indicating that the amount of IP_3R reconstituted was not affected by the presence of CGA (data not shown).

IP_3 Dose-dependent Ca^{2+} Release—Since it has also been shown that Ca^{2+} release from the IP_3R can be regulated by cytosolic Ca^{2+} concentrations (29), there was a possibility that the IP_3-induced Ca^{2+} release might not represent net IP_3-mediated Ca^{2+} release. To address this possibility, we determined the amount of Ca^{2+} released after one-time dose of each indicated concentration of IP_3 in the presence of 10 μM of indo-1, which is a high enough concentration to buffer the released Ca^{2+} (Fig. 4). As shown in Fig. 4, presence of CGA at pH 5.5 significantly increased the amounts of Ca^{2+} released, compared with those in the absence of CGA, although the difference in the amounts of Ca^{2+} released in both cases diminished as IP_3 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 IP_3 Binding to IP_3R—To determine whether the increased release of Ca^{2+} in the presence of CGA is due to increased binding of IP_3 to the proteoliposome, the amount of IP_3 bound to the IP_3R was determined as a function of increasing IP_3 concentrations (Fig. 5). As shown in Fig. 5, the presence of CGA enhanced the IP_3 binding 2-fold over that in the absence of CGA. When the IP_3R alone was reconstituted, a maximum of 0.4 mol of IP_3 appeared to bind 1 mol of IP_3R. However, when CGA was also present inside the liposomes at the

FIG. 3. **IP_3-induced Ca^{2+} release from IP_3R-reconstituted lipid vesicles.** The fluorescence changes were recorded after a series of incremental additions of 0.5–1.0 μM IP_3 to the proteoliposome solution containing 1 μM of indo-I at 35 °C. The inside pH of the proteoliposome was 5.5 unless stated otherwise. *A*, IP_3-induced Ca^{2+} release from the proteoliposomes and its inhibition by IP_3R Ab. Two different concentrations of IP_3R Ab, at a ratio of 1 μg of IP_3R/10 or 50 μg of IP_3R Ab, were added. *TX-100*, Triton X-100. *B*, percent changes of released Ca^{2+} from the liposomes described in *A*. Addition of preimmune serum at the IP_3R:preimmune ratio of 1:50 (w/w) did not affect the Ca^{2+} release. Each indicated concentration of IP_3 represents the amount of cumulative IP_3 added. *C*, IP_3-induced Ca^{2+} release in the presence of encapsulated CGA at the inside pH of 5.5 and 7.5.

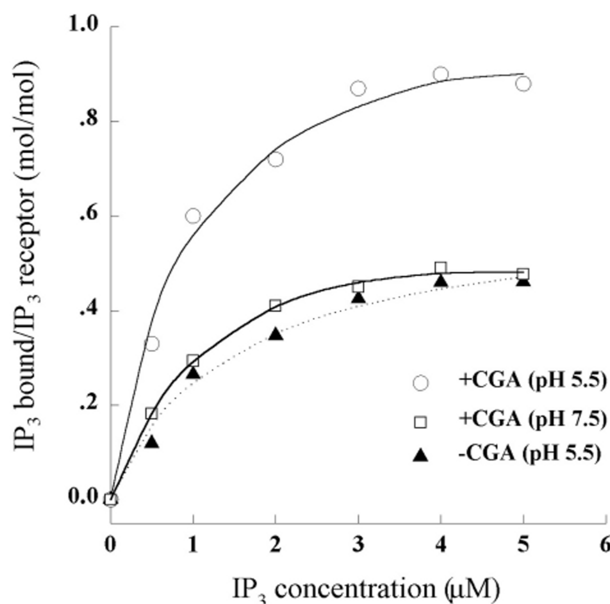

FIG. 5. **IP_3 binding to the reconstituted IP_3R.** The IP_3R-reconstituted liposomes were incubated with various concentrations of [^{3}H]IP_3 for 10 min at 35 °C. Each sample was then filtered through a spun concentrator, and the bound IP_3 was determined by counting the radioactivity of filtrates.

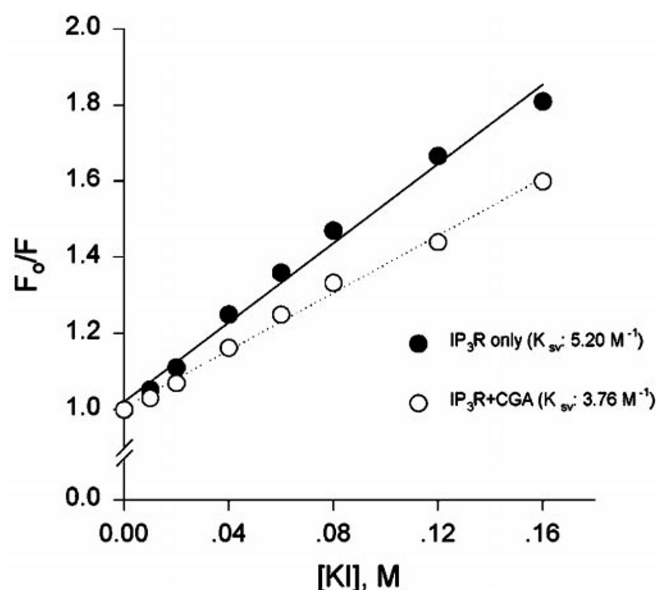

FIG. 6. **Quenching of IP_3R Trp fluorescence by iodide.** The changes in Trp fluorescence from the proteoliposomes containing IP_3R only or IP_3R and CGA were measured as a function of increasing concentrations of KI. F_o and F represent the fluorescence intensities in the presence (F) and absence (F_o) of KI.

intravesicular pH of 5.5, the IP_3 binding increased to about double the value shown with the IP_3R alone. But when the pH of the liposomes was 7.5, CGA showed no effect on the IP_3 binding to the IP_3R. Nevertheless, the half-maximal value was almost the same for both cases regardless of the presence of CGA, indicating that the affinity of IP_3R for IP_3 has not changed.

Structural and Motional Changes of IP_3R by Interaction with CGA—To investigate possible conformational changes of the IP_3R by its interaction with CGA, we utilized collisional quenching of the IP_3R Trp fluorescence by iodide (Fig. 6). As shown in Fig. 6, the IP_3R 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).

$$F_o/F = K_{sv}[I^-] + 1 \quad \text{(Eq. 1)}$$

F_o 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 $[I^-]$ is the molar concentration of iodide. The K_{sv} value estimated from the slope was 5.20 M^{-1} for the reconstituted IP_3R 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 IP_3R are less exposed to the solvent when CGA is present and that CGA induced conformational changes of IP_3R.

In order to further examine the effect of CGA on the molecular property of IP_3R in the reconstituted vesicles, we carried out the fluorescence resonance energy transfer study using the CPM- and F-mal-labeled IP_3Rs (Fig. 7). As shown in Fig. 7, the reconstituted IP_3Rs 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 IP_3R 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 IP_3Rs was enhanced by their interaction with CGA.

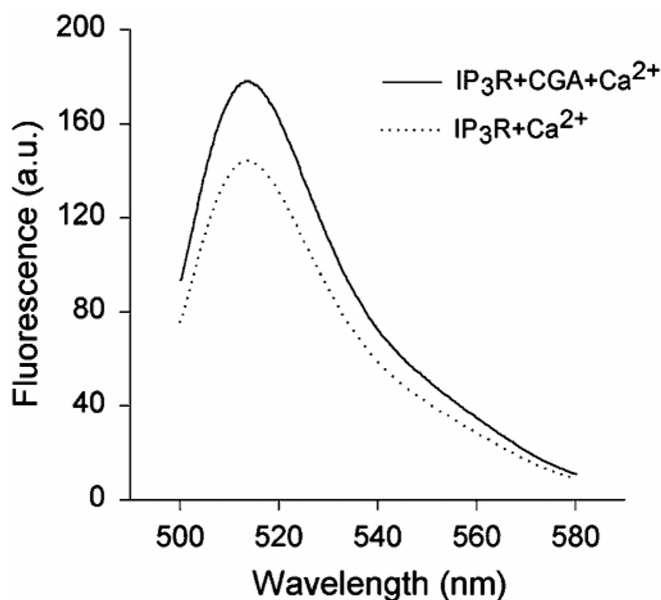

FIG. 7. **The resonance energy transfer from CPM to F-mal.** CPM- and F-mal-labeled IP_3R were mixed (1:1 ratio) and were reconstituted into the liposomes in the presence and absence of CGA. The emission fluorescence of F-mal was measured at 35 °C at the excitation wavelength of 384 nm (instead of 492 nm), the excitation wavelength of CPM.

Effect of Ca^{2+} on IP_3-induced Ca^{2+} Release from the Proteoliposomes—In view of the potential inhibitory effect of increasing Ca^{2+} concentrations on the IP_3-induced Ca^{2+} release activity of IP_3R (31), we examined the Ca^{2+} release activity of the proteoliposome in response to a fixed amount of IP_3 in the presence of increasing concentrations of Ca^{2+} (Fig. 8). As shown in Fig. 8, 0.2 μM IP_3 induced far greater Ca^{2+} releases in the presence of CGA than in the absence in the Ca^{2+} concentration range of 0.1–1.0 μM. However, in the higher Ca^{2+} concentration range, the differences in the amount of Ca^{2+} released disappeared, indicating the inhibitory effect of Ca^{2+} on the Ca^{2+} release property of IP_3R. These results clearly show

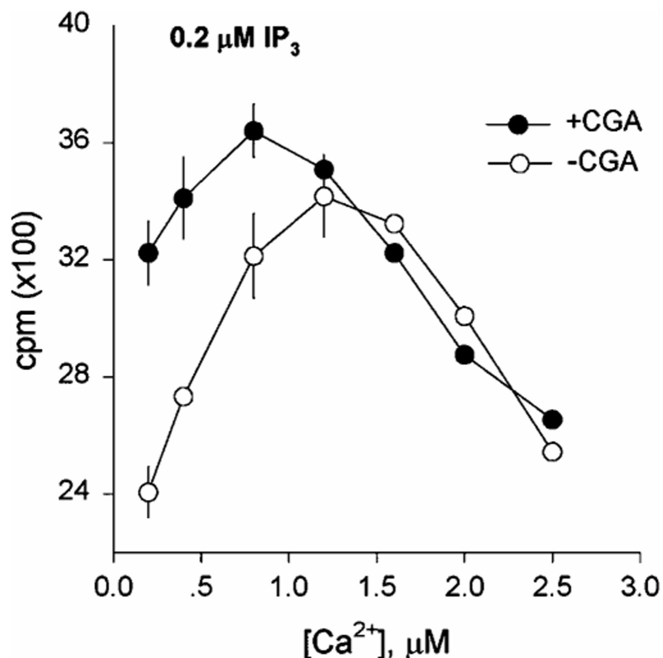

FIG. 8. **Effect of external Ca^{2+} on IP_3-mediated Ca^{2+} release.** The release of $^{45}Ca^{2+}$ by 0.2 μM IP_3 is shown as a function of free Ca^{2+} concentrations present outside the lipid membranes in the presence or absence of CGA. Data shown are mean ± standard error of three independent experiments.

that IP_3 releases far greater amounts of Ca^{2+} from the proteoliposome in the presence of CGA when there is no inhibitory amount of Ca^{2+} in the liposome solution. As a control experiment, we measured Ca^{2+}-induced Ca^{2+} release without IP_3. However, no appreciable release of stored Ca^{2+} could be observed in the presence of up to 2 μM free Ca^{2+} (data not shown).

DISCUSSION

Since the original study that demonstrated the IP_3-induced Ca^{2+} release from chromaffin granules (1), Petersen and colleagues (2) have shown the IP_3-mediated Ca^{2+} release from the zymogen granules, thus supporting the concept of secretory granules of endocrine and neuroendocrine cells serving as the IP_3-sensitive intracellular Ca^{2+} store (1, 32). Recently, additional evidence indicating direct participation of secretory granule calcium in the control of cytoplasmic Ca^{2+} has been obtained in the secretory granules of goblet cells (3); the uptake of Ca^{2+} by secretory granules was temporally and spatially matched by simultaneous reduction of Ca^{2+} concentration in the surrounding cytoplasm, whereas IP_3-mediated release of Ca^{2+} by secretory granules resulted in the simultaneous increase of cytoplasmic Ca^{2+} concentration in the immediate vicinity of the secretory granules, clearly demonstrating the active participation of secretory granules in the control of cytoplasmic Ca^{2+} 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 Ca^{2+} (40, 41). Chromogranin A binds 32 mol of Ca^{2+}/mol with dissociation constant of 2.7 mM at pH 7.5, and 55 mol of Ca^{2+}/mol with dissociation constant of 4 mM at pH 5.5 (4). Due to the high capacity Ca^{2+} binding of chromogranins, most (>99.9%) of 40 mM intravesicular calcium stay bound to chromogranins, resulting in the free Ca^{2+} 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 Ca^{2+} binding property of chromogranins proves to be essential, enabling the secretory granules to store an exceptionally large amount of Ca^{2+}. It appears therefore very natural that the secretory granules play pivotal roles in controlling the intracellular Ca^{2+} concentrations.

Although the discovery that the secretory granule of adrenal medullary chromaffin cells is a major IP_3-sensitive intracellular Ca^{2+} store (1) implied the presence of IP_3R on the secretory granule membrane, the presence of IP_3R 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 IP_3R (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 IP_3R/Ca^{2+} channel.

The present results obtained in the reconstitution experiments using the purified IP_3R and CGA are considered to reflect the important roles these molecules play in the cytoplasm, which enable the secretory granules to control the intracellular Ca^{2+} levels in neuroendocrine cells. The Ca^{2+} flux experiments in Figs. 3*C* and 4 show that the amount of Ca^{2+} released in response to submaximal levels of IP_3 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 IP_3R/Ca^{2+} 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 IP_3-mediated Ca^{2+} release (Figs. 3*C* and 4), underscoring the importance of the intravesicular acidic pH environment in the coupling of CGA to the IP_3R. Although IP_3R monomers can bind IP_3, they do not exist in a monomeric state in the cell; rather, four molecules of same or different types of IP_3R interact with each other to form either a homotetrameric or a heterotetrameric IP_3R (9–13), forming a Ca^{2+} 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 IP_3R (19), thus suggesting the potential interaction between a tetrameric CGA and a tetrameric IP_3R. Therefore, the results showing the direct interaction between the purified IP_3R and CGA (20), combined with the co-immunoprecipitation of transfected IP_3R and CGA (20), strongly suggest the importance of this coupling in fine-tuning the intracellular Ca^{2+} control mechanisms in the cell. In view of the fact that CGA and IP_3R 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 IP_3-mediated Ca^{2+} release at pH 7.5 is probably due to the failure of CGA to couple with the IP_3R. This is also reflected in the fact that the number of IP_3 bound to the IP_3R 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 IP_3 releases almost the maximal amount of Ca^{2+} that can be released. Given that approximately 2 molecules of IP_3 bound/tetrameric IP_3R at 1 μM IP_3 (Fig. 5), the result in Fig. 4 suggests that almost a maximal amount of Ca^{2+} can be released when 2 molecules of IP_3 are bound per tetrameric IP_3R, although a maximum of 4 molecules of IP_3 can be bound per tetrameric IP_3R at 4 μM IP_3 (Fig. 5). This result is in general agreement with previous results, which indicated that one molecule of IP_3 opens the IP_3R/Ca^{2+} channel, which exhibits four conductance states (42). Although it has also been sug-

gested that three or more IP_3 molecules are needed to open the IP_3R/Ca^{2+} channel (43), the general consensus is that only one IP_3 molecule is necessary to open the IP_3R/Ca^{2+} channel (42, 44, 45).

Since the pH of intravesicular milieu of secretory vesicles of neuroendocrine cells is 5.5 (46, 47) and that of the extravesicular or extracellular environment is 7.4, the coupling of IP_3R, an integral membrane protein, and CGA, an intravesicular matrix protein, at pH 5.5 and dissociation at pH 7.5 are in line with the physiological demands of the environments these molecules experience as they travel from the *trans*-Golgi network where the secretory vesicles are formed to the bloodstream. During exocytosis, the secretory vesicle contents will be exposed to a new pH environment of the extracellular space, which maintains a pH of 7.4. This change of pH from the intravesicular pH of 5.5 to 7.4 will automatically cause dissociation of the vesicular contents from the vesicle membrane, thus allowing the secretory cargo to move into the extracellular space and then on to the bloodstream.

The fluorescence resonance energy transfer (Fig. 7) measurements suggested that the enhanced rate of Ca^{2+} release is due to the structural stabilization of the IP_3R by coupled CGA. This result suggested that the presence of CGA in the liposome changes the conformation of the IP_3R, probably changing it to a structure that is better suited to IP_3 binding (Fig. 5) and subsequent Ca^{2+} release. Furthermore, quenching experiments designed to measure the Trp fluorescence of the IP_3R also indicated that at least some Trp residues of the IP_3R are less exposed when CGA is present (Fig. 6). Therefore, it appears that CGA play pivotal roles in controlling the Ca^{2+} release property of the IP_3R in neuroendocrine cells, not only by binding and freeing of the intravesicular Ca^{2+} but also by modulating the channel activity of the IP_3Rs.

IP_3 binding to the IP_3R has been known to cause conformational changes of the IP_3R (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_3R that occur as a result of IP_3 binding will be transmitted instantly to the coupled CGA in the secretory granules, causing their conformational changes. It may then be assumed that the resulting conformational changes in CGA will concomitantly result in lowering of CGA's affinity for Ca^{2+}, thus freeing some Ca^{2+} from the protein and making them available for release through the IP_3R channel to the cytoplasm.

In view of the fact that the secretory granules contain up to 40 mM Ca^{2+} and most (>99.9%) of it remains bound to chromogranins in the secretory granules (40), the coupling of calcium storage proteins to the IP_3R/Ca^{2+} channel appears to reflect the efficient and intricate structural organization of an intracellular Ca^{2+} store whose Ca^{2+} storage/release function should be strictly and subtly controlled for the Ca^{2+} store to respond appropriately to the changing cytoplasmic Ca^{2+} concentrations. Moreover, the insight into the molecular organization of Ca^{2+} control mechanism of secretory granules is certain to shed further light on elucidation of Ca^{2+} storage/release mechanisms of other intracellular Ca^{2+} stores. Given the physiological needs of cells to tightly control the cellular Ca^{2+}, the physical coupling of Ca^{2+} storage proteins to Ca^{2+} channels seems to be a natural consequence.

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