Interaction of Chromogranin B and the Near N-terminal Region of Chromogranin B with an Intraluminal Loop Peptide of the Inositol 1,4,5-Trisphosphate Receptor*

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Given the interaction of the inositol 1,4,5-trisphosphate receptor (IP3R) with chromogranins A (CGA) and B (CGB), two major Ca2+ storage proteins of secretory granules that have been shown to be IP3-sensitive intracellular Ca2+ store of neuroendocrine cells, we have investigated the potential interaction of the intraluminal loop regions of the IP3R with both intact CGB and the conserved near N-terminal region of CGB. The interaction studies carried out with CGB and glutathione S-transferase fusion proteins of intraluminal loop regions of bovine type 1 IP3R showed that CGB interacts with intraluminal loop 3-2 (the second loop formed between transmembrane regions 5 and 6) of the IP3R at both pH 5.5 and 7.5. Analytical ultracentrifugation studies also indicated that CGB interacts with the same intraluminal loop region of the IP3R and the interaction was much stronger than that between CGA and the loop. Moreover, the conserved near N-terminal region of CGB also interacted with the intraluminal loop region of the IP3R. The CGB interaction with the IP3R intraluminal loop peptide at pH 7.5 showed a ΔG° value of −8.1 kcal/mol at 37 °C for a 1:1 stoichiometry, indicating a Kd of 1.9 μM. These results give insight into the molecular organization of the IP3-sensitive Ca2+ store.

Since the initial finding that the secretory vesicles of the bovine adrenal medullary chromaffin cells release Ca2+ in response to inositol 1,4,5-trisphosphate (IP3)1 (1), the IP3-sensitive intracellular Ca2+ store role of the secretory granules has also been demonstrated in the zymogen granules of pancreatic acinar cells (2) and in the secretory granules of goblet cells (3). Recently, it was also shown that the IP3-mediated intracellular Ca2+ release in the pancreatic β-cells was severely reduced in the annexin 7 heterozygous (+/−) knockout mouse (4), thus resulting in the defect in insulin secretion from the secretory granules. The level of IP3R expression in the secretory granules of pancreatic β-cells in the annexin 7 heterozygous (+/−) knockout mouse was only one-tenth that of normal littermates (4), suggesting the importance of granular IP3R in insulin secretion.

The IP3-sensitive Ca2+ store role of secretory vesicles has been attributed to the presence of the high capacity, low affinity Ca2+ storage proteins chromogranin A and chromogranin B (5, 6), two major proteins of secretory vesicles (7–10). Chromogranin A binds 30–50 mol of Ca2+/mol with a dissociation constant (Kd) of 2–4 μmol (6). Both CGA and CGB bind to the secretory vesicle membrane (11, 12), but CGB binds much more strongly than CGA (12). Unlike CGA, which was dissociated from the secretory vesicle membrane by a simple pH change of the elution buffer from the intravesicular pH 5.5 to the near physiological pH 7.5 (11), CGB dissociation required both the pH change of the elution buffer and a high salt concentration (1 M KCl) (12). Moreover, CGA has been demonstrated to interact with several secretory vesicle integral membrane proteins, including the inositol 1,4,5-trisphosphate receptor (IP3R), at pH 5.5 and is dissociated from them at pH 7.5 (13). The IP3Rs exist in three subtypes (types 1–3), and these subtypes interact with each other to form a homo- or heterotetramer (14–18), which also functions as a Ca2+ channel (19).

Furthermore, unlike CGA, which exists in a tetrameric state at pH 5.5 and in a dimeric state at pH 7.5 (20, 21), CGB exists in a monomeric state at both pH values (22, 23). However, despite the monomeric existence of CGB and the dimeric or tetrameric existence of CGA, an equimolar mixture of CGB and CGA formed a heterodimer at pH 7.5 and a CGA2CGB2 heterotetramer at pH 5.5, leaving very little CGA homodimer or CGA homotetramer in the solution (23). A detailed study indicated that tetrameric CGA interacts with four molecules of the intraluminal loop region of the IP3R at pH 5.5 (24). The IP3Rs are supposed to have six transmembrane domains, resulting in four intraluminal loops (25, 26); two are very small, and two are moderate size loops (cf. Fig. 1). In view of the existence of homo- and/or heterotetrameric IP3R in the cell (14–18), the interaction of tetrameric CGA with four molecules of the intraluminal loop region of the IP3R at pH 5.5 (24) suggested the possibility of interaction between tetrameric IP3R and tetrameric CGA. Moreover, given that CGA can form a homotetramer and a CGA2CGB2 heterotetramer with CGB at pH 5.5 (20, 23) and that three subtypes (types 1–3) of IP3R can also form a homoo- or heterotetramer (14–18), it may be possible for homo- and/or heterotetrameric IP3Rs to interact with homo- and/or heterotetrameric chromogranins in the secretory vesicle.

The conserved near N-terminal region of CGA and CGB has previously been suggested as the membrane-binding anchor region (11, 27). The disulfide-bonded loop form of the same region has also been reported to be important in CGA dimer-
ization (21) and in CGB sorting (28). Further, the conserved near N-terminal region of CGB was shown to interact with an intraluminal loop region of the IP$_3$R (29), probably revealing the interacting domains of two proteins. In our recent study, we have demonstrated direct interaction between purified IP$_3$R and chromogranins A and B (30). Not only did cotransfection and communoprecipitation experiments indicate the existence of IP$_3$R-CGA or IP$_3$R-CGB complex in the cell, but also the immunogold electron microscopic study showed the colocalization of IP$_3$R and CGA in the secretory granules of bovine adrenal medullary chromaffin cells (30).

In light of the interaction of CGB with purified IP$_3$R (30), it was of immediate interest to determine not only the interacting regions of CGB and IP$_3$R but the interaction strengths as well. Therefore, we have investigated the potential interaction of the intraluminal loop region of the IP$_3$R with both intact CGB and the conserved near N-terminal CGB region in the present study and found that intact CGB as well as the conserved near N-terminal region of CGB interact with the intraluminal loop region of the IP$_3$R.

**EXPERIMENTAL PROCEDURES**

Glutathione S-Transferase (GST) Fusion Proteins of the Intraluminal Loop Regions of IP$_3$R1 and Interaction Study with CGB—To construct GST fusion proteins, DNA sequences corresponding to three intraluminal loops of bovine type I IP$_3$R (Fig. 1), L1 (between transmembrane region 1 (TM1) and TM2), L2 (between TM3 and TM4), L3-1 (the first loop between TM5 and TM6), and L3-2 (the second loop between TM5 and TM6) were amplified by polymerase chain reaction and subcloned into expression vector pGEX-5X (Amersham Pharmacia Biotech). The GST fusion proteins were expressed in BL21 cells and purified on glutathione-agarose beads. The interaction of these proteins with CGB was carried out by incubating 10 μg each of GST fusion proteins with 2.5 μg of purified recombinant CGB prepared as described before (31) in a pH 5.5 buffer (20 mM sodium acetate, pH 5.5, 0.1 M KCl, 4 mM EGTA, 1 mM phenylmethanesulfonyl fluoride) or in a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 4 mM EGTA, 1 mM phenylmethanesulfonyl fluoride) for 1 h at 4 °C. The mixture was then washed five times with the same buffer containing 0.1% Triton X-100 to remove the unbound proteins. The remaining proteins, including the bound CGB, were visualized by SDS-polyacrylamide gel electrophoresis followed by Coomassie staining.

**Purification of Native CGB**—Chromogranin B was purified from bovine adrenal chromaffin granules in native form according to the method described previously (12).

**CGB Peptide and the IP$_3$R Intraluminal Loop Peptide Synthesis**—The conserved near N-terminal CGB peptide (32–34) with the sequence of IIEVLSNALLKSSAPPITPE (residues 17–36) was synthesized with the addition of Trp at the N terminus as a chromophore, and the peptide was confirmed by amino acid composition analysis. The purity of this peptide was in excess of 98%.

**Determination of the Molar Extinction Coefficient**—The molecular masses of intact chromogranin B, the CGB peptide, and the IP$_3$R peptide, calculated from the amino acid sequences (31, 32, 35), were 71,425 daltons, 2,279 daltons, and 2,549 daltons, respectively, and the molar extinction coefficient used for purified bovine chromogranin B was determined to be 4.80 × 10$^4$ μm$^{-1}$ cm$^{-1}$ at 280 nm (25).

**Analytical Ultracentrifugation**—Analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge at 20,000 rpm and over the temperature range of 2–32 °C with 3 °C increments for intact CGB and the IP$_3$R peptide interaction study and at 42,000 rpm and over the temperature range of 6–30 °C with 4 °C increments for the CGB peptide and the IP$_3$R peptide interaction study. Three cells with six-channel centrepieces were used, one cell for each type of buffer. In each cell, the upper compartment contained intact CGB or the CGB peptide, the lower compartment contained the IP$_3$R peptide, and the center compartment contained a mixture of intact CGB or the CGB peptide and the IP$_3$R peptide having the same concentrations that they had in their respective compartments. The compartment adjoining each sample contained the reference buffer. Sample volumes were 0.12 ml, giving column heights of approximately 3 mm. The buffer used was 20 mM MOPS, pH 7.5, 0.1 M KCl, and 1 mM EGTA to ensure the absence of any Ca$^{2+}$ effect.

The compositional partial specific volume, $\bar{v}$, were calculated for 25 °C from the amino acid sequences using the consensus values of Perkins (36). A value of $\bar{v}$ was 0.00425 cm$^3$ g$^{-1}$ degrees$^{-1}$ was used to calculate the values of $\bar{v}$ at other temperatures. The extinction coefficients of CGB and the peptides at 280 nm were determined spectrophotometrically, and the extinction coefficients at 295, 300, and 310 nm were calculated from the ratios of the concentration gradients at equilibrium measured at that wavelength to the concentration gradients measured at 290 nm. Scans were taken at 280, 295, and 310 nm when the CGB peptide-IP$_3$R peptide interaction was studied and at 280 and 300 nm when the CGB-IP$_3$R peptide interaction was studied in order to obtain more rigorous analyses. The data were analyzed by a simplified version of the multiwavelength procedure (37).

**RESULTS**

**Interaction of the Intraluminal Loop Region of the IP$_3$R with CGB**—Given that the intravesicular protein CGB or CGB can come in contact with the integral vesicle membrane protein IP$_3$R only through the intraluminal loop regions of the IP$_3$R and that CGB has already been shown to interact with one of the intraluminal loop regions of the IP$_3$R (24), we have constructed GST fusion proteins of the intraluminal loop regions of bovine IP$_3$R1 (Fig. 1) and examined the potential interaction of these loop regions with CGB (Fig. 2). As shown in Fig. 2, the loop L3-2 (Fig. 1), which contains the conserved sequence DLKPSKEELPFAARVYD (residues 2510–2529 of bovine IP$_3$R1), was shown to interact with purified CGB both at pH 5.5 (Fig. 2A) and pH 7.5 (Fig. 2B). Interaction of L3-2 with CGB is in accord with the interaction of the identical loop region of rat type II IP$_3$R with CGA (24), which showed interaction at the intravesicular pH of 5.5 but not at the near physiological pH of 7.5. Nevertheless, unlike the interaction that has been demonstrated between L3-2 and CGB (Fig. 2), the interaction study between the GST fusion proteins of the intraluminal loop regions of IP$_3$R1 and CGB failed to show interaction between the L3-2 region and CGA (not shown). The inability of CGA to interact with GST-L3-2 fusion protein at pH 5.5 appears to
reflect the significantly weaker affinity of CGA for the L3-2 region than that of CGB. The affinity of CGB for the secretory vesicle membrane has previously been demonstrated to be much greater than that of CGA that the vesicle-bound CGB was not released by a change of pH alone from 5.5 to 7.5 (12), the condition that has been proved to be sufficient to release bound CGA from the secretory vesicle membrane (11). Rather, the release of bound CGB required the combination of a pH change from 5.5 to 7.5 and high salt (1M KCl) (12). Judging from these results, the interaction of CGB with L3-2 of IP3R even at pH 7.5 seemed to reflect the greater affinity of CGB for L3-2 at pH 7.5 than that of CGA at pH 5.5. This prediction has indeed been proved to be the case, since the ΔG⁰ value of interaction between CGA and the L3-2 region of IP3Ra at pH 5.5 was 26.9 kcal/mol (24), whereas the ΔG⁰ value of the CGB-L3-2 interaction at pH 7.5 was 28.1 kcal/mol (Table I).

Analysis of Ultracentrifuge Data—Chromogranin B at pH 7.5 in the absence of Ca²⁺ was found only as an ideal monomer, the concentration distribution of which could be appropriately described by the mathematical model,

\[ c_r = c_{b,1}\exp(AM(r_r^2 - r_b^2)) + \varepsilon \]  

(Eq. 1)

where \( c_r \) is the concentration, expressed as absorbance at 280 nm, as a function of radial position; \( c_{b,1} \) is the concentration of monomer at the cell bottom (\( r_b \)); \( A = (1 - \psi)\omega^2/2RT \), where \( \psi \) is the compositional partial specific volume, \( \rho \) is the solvent density, \( \omega \) is the rotor angular velocity, \( R \) is the gas constant, and \( T \) is the absolute temperature; \( M \) is the molar mass of CGB monomer, which has the value of 71,425 daltons (32); \( \varepsilon \) is a small base-line error correction term. Likewise, both the CGB peptide and the IP₃R peptide alone at pH 7.5 in the absence of Ca²⁺ were found to be ideal monomers, the concentration of which could be appropriately described by the same mathematical model using appropriate values of \( A \) and \( M \) for each of these peptides. The \( M \) value used for the IP₃R was 2,549 daltons, and that for the CGB peptide was 2,279 daltons.

In light of the previous observation that CGA interacts with the IP₃R peptide and CGB interacts with the secretory vesicle membrane even at pH 7.5 (12), we explored the possibility of interaction between either intact CGB or the conserved near N-terminal CGB peptide at pH 7.5. Analysis of the concentration distribution data of mixtures of either CGB peptide (Fig. 3) or intact CGB (Fig. 5) with the IP₃R peptide indicated that both CGB and the

<table>
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<th>CGB-IP₃R peptide</th>
<th>CGB peptide-IP₃R peptide</th>
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<tr>
<td>ΔG⁰ (kcal/mol⁻¹)</td>
<td>-8.10</td>
<td>-4.70</td>
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<tr>
<td>ΔH² (kcal/mol⁻¹)</td>
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<td>-0.41</td>
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<td>ΔS² (kcal/mol⁻¹ K⁻¹)</td>
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<td>-0.09</td>
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<td>TDΔS⁰ (kcal/mol⁻¹)</td>
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In light of the previous observation that CGA interacts with the IP₃R peptide and CGB interacts with the secretory vesicle membrane even at pH 7.5 (12), we explored the possibility of interaction between either intact CGB or the conserved near N-terminal CGB peptide and the intraluminal loop peptide of the IP₃R at pH 7.5. Analysis of the concentration distribution data of mixtures of either CGB peptide (Fig. 3) or intact CGB (Fig. 5) with the IP₃R peptide indicated that both CGB and the

![Fig. 2. Interaction of GST fusion proteins of intraluminal loop regions of IP₃R with CGB. Interaction of GST fusion proteins of each of the intraluminal loop regions of bovine IP₃R with CGB at pH 5.5 (A) and pH 7.5 (B). Lane 1, a size marker; lane 2, purified recombinant CGB. GST indicates GST alone, while L1, L2, L3-1, and L3-2 indicate GST fusion proteins of loop 1, loop 2, loop 3-1, and loop 3-2 of bovine IP₃R, respectively, in the absence (−) and presence (+) of CGB.](http://www.jbc.org)
CGB peptide interacted with the IP₃R peptide at pH 7.5. A 1:1 stoichiometry association appeared to be the only viable model of association. The mathematical model for this association is given by the equation,

\[ c_i = c_{B,B}' \exp(\Delta M_B (r_i - r_0^B)) + c_{B,P}' \exp(\Delta M_P (r_i - r_0^P)) + c_{B,P}' \exp(\ln K_{BP} - \ln(E_B + E_P)) + (A_m E_B + A_m E_P (r_i - r_0^M)) + \epsilon \]  

(Eq. 2)

where \( \ln K_{BP} \) is the logarithm of the molar equilibrium constant for the formation of the heterodimer, where \( E_B \) is the molar extinction coefficient of either CGB or the CGB peptide, depending on the experiment. \( E_P \) is the molar extinction coefficient of the IP₃R peptide; the values of \( A \) and \( M \) are similarly denoted, and the other terms have their usual meaning as described above. The logarithmic term of the extinction coefficient is needed to convert the molar equilibrium constant to an equilibrium constant on an absorbancy scale, since this is the unit of concentration actually measured. This equation was used to generate three models for globally fitting the peptide-peptide interaction using scans at 280, 295, and 310 nm with appropriate values of \( E_B \) and \( E_P \), obtaining \( \ln K_{BP} \) as a global fitting parameter and the \( c_{B,P}' \) values and \( \epsilon \) as local fitting parameters. Similarly, two models were used for global fitting of the CGB-IP₃R peptide interaction at 280 and 300 nm.

The analysis of the interaction of the CGB peptide and the IP₃R peptide was quite straightforward (Fig. 3). Both the molar masses and the molar extinction coefficients at 280 nm were quite similar. Global analysis at three wavelengths enhanced the precision, since at 280 nm, both peptides contributed approximately equally to the observed absorbances; at 310 nm, the absorbance was due virtually entirely to the 5-OH tryptophan-labeled IP₃R peptide, either free or complexed with the CGB peptide; at 295 nm, an intermediate absorbance was observed. This can be clearly seen in Fig. 3.

On the other hand, the analysis of the CGB protein with the IP₃R peptide was considerably more complex (Figs. 4 and 5). Because of the large difference of their respective molar masses and molar extinction coefficients, it was found necessary to load the cells with a significant molar excess of the IP₃R peptide so that the initial absorbance of each species was approximately equal before centrifugal redistribution. The result of this was the creation of an abnormally shaped sum of squares surface in parameter space. Fig. 4 illustrates the sum of squares of a global fit of absorbances at 280 and 300 nm as a function of \( \ln K \) and demonstrates a sigmoid distribution instead of the usual approximately parabolic distribution. Accordingly, it was difficult to obtain a clear minimum in the sum of squares and impossible to obtain a meaningful parameter error estimate. Obtaining the best value for the minimum sum of squares was aided by invoking conservation of mass within the centrifuge cell.

For any component in the centrifuge cell, conservation of mass of that component is defined by the equation,

\[ \int_{r_i}^{r_0} c_i dr^2 = \int_{r_0}^{r_i} c_i dr^2 = c_i (r_i^2 - r_0^2) dr^2 = c_i \Delta r^2 \]  

(Eq. 3)

Integration of the first term and rearrangement then gives the following:

\[ c_0 = c_i (1 - \exp(-AM \Delta r^2))/AM \Delta r^2 \]  

(Eq. 4)
This may now be applied to Equation 2, and the resulting equation can be used to calculate the apparent total initial absorbance at 280 nm as a function of the value of ln $K$. Since the total initial absorbance was measured, the calculated values of initial absorbance, taking errors of the cell bottom concentrations of the reactants and the base-line offset into account, permitted calculation of a range of values of ln $K$ consistent with those values. The fitting procedure then facilitated optimization within these limits. However, statistical theory does not justify using these limits as error estimates for the values of ln $K$. An example of a global fit at 280 and 300 nm is illustrated in Fig. 5.

**FIG. 5.** Distribution of concentration of chromogranin B and the intraluminal loop peptide of IP$_3$R at pH 7.5. Distribution of chromogranin B (0.1 mg/ml) and the intraluminal loop peptide of the IP$_3$R (35) in 20 mM MOPS, pH 7.5, 0.1 M KCl, and 1 mM EGTA is shown at ultracentrifugal equilibrium at 10 °C. The line shows the best fitting curve for the CGB-IP$_3$R intraluminal loop peptide interaction model.

**FIG. 6.** Thermodynamic parameters as a function of temperature for the formation of the conserved near N-terminal CGB peptide-IP$_3$R intraluminal loop peptide heterodimer at pH 7.5 in the absence of Ca$^{2+}$. A, the values of $\Delta G^0$ were calculated from the values of ln $K$, using Equation 1, and the data were fit using Equation 6. Since the S.E. values whose magnitudes were obtained by the Monte Carlo simulations give error bars approximately the same size as the squares indicating the data points, the error bars have been omitted for simplicity. B, the values of $\Delta G^0$, $\Delta H^0$, and $\Delta S^0$ were calculated using Equation 10, and the integrated forms of Equations 7 and 8, respectively, with the values of $\Delta H^0_{T_{RN}}$, $\Delta S^0_{T_{RN}}$, and $\Delta C^0_{P,T_{RN}}$ obtained when fitting the data illustrated in A.

\[ \Delta G^0 = -RT\ln K_T \]  
(Eq. 5)

and fitting the values of $\Delta G^0$ as a function of temperature using a mathematical model derived from standard definitions of the various thermodynamic parameters as follows.

\[ \Delta G^0_{P,STR} = \Delta H^0_{P,STR} - T \Delta S^0_{P,STR} \]  
(Eq. 6)

\[ \Delta H^0_{P,STR} = \Delta H^0_{P} + \int_{T_0}^{T} \Delta C^0_{P,T}dT \]  
(Eq. 7)

\[ \Delta S^0_{P,STR} = \Delta S^0_{P} + \int_{T_0}^{T} (\Delta C^0_{P,T})dT \]  
(Eq. 8)

\[ \Delta C^0_{P,T} = \Delta C^0_{P,T_{RN}} + \int_{T_0}^{T} (d\Delta C^0_{P,T})dT \]  
(Eq. 9)

where $T_{RN}$ is a reference temperature, here taken to be 273.15 K, and where it is assumed that d$\Delta C^0_{P,T}$ is constant with respect to temperature. Carrying out the integration indicated in Equations 7–9 and substituting the results in Equation 6 and simplifying, one then obtains the following.

\[ \Delta G^0 = \Delta H^0 - T \Delta S^0 + \Delta C^0_{P,T_{RN}}(1 - T_{RN}/T - \ln(T/T_{RN})) + TT_0(d\Delta C^0_{P,T})dT(T_{RN}/T - T_{RN}/T_0 + 2\ln(T/T_{RN}) + 2\ln(T/T_0))/2 \]  
(Eq. 10)

When all of the data obtained were fit with Equation 10, it was found that the use of the d$\Delta C^0_{P,T}$ term did not improve the quality of the fits and gave significantly larger standard errors for the parameters. Accordingly, it was assumed that $\Delta C^0_{P}$ was constant with temperature, and Equation 10 was terminated after the $\Delta C^0_{P,T}$ term and only $\Delta H^0_{T_{RN}}$, $\Delta S^0_{T_{RN}}$, and $\Delta G^0_{P,T_{RN}}$ were used as fitting parameters. In order to perform weighted fits for the
CGB-IP₃R peptide interaction, each value of $\Delta G^0$ was weighted with the reciprocal of its variance, which was calculated from the S.E. value of ln $K_r$ obtained by Monte Carlo simulations when fitting the concentration distribution data for a given temperature. The fits of the CGB-IP₃R peptide interaction were unweighted.

The plots illustrating the fitting of $\Delta G^0$ as a function of temperature using Equation 10 for either interaction are shown in Figs. 6A and 7A, respectively. The plots illustrate the distributions of the calculated values of $\Delta G^0$, $\Delta H^0$, and $T \Delta S^0$ as functions of temperature. The values of the thermodynamic parameters for both associations at 37°C (310.15 K) are given in Table I.

**DISCUSSION**

The initial finding regarding the IP₃-sensitive intracellular Ca²⁺ store role of secretory vesicles has been made with the chromaffin granules of bovine adrenal medullary chromaffin cells in which the chromaffin granules rapidly released Ca²⁺ in response to IP₃ (1). The IP₃-mediated Ca²⁺ release has also been observed in the zymogen-containing secretory granules of pancreatic acinar cells (2). Further, this observation has been extended to the secretory granules of goblet cells that direct participation of secretory granule calcium in the control of cytoplasmic Ca²⁺ concentration has been shown (3); the uptake of Ca²⁺ by the secretory granules of goblet cells 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 demonstrating the participation of secretory granule calcium in the control of cytoplasmic Ca²⁺ concentration. The IP₃-mediated Ca²⁺ release from the secretory granules of adrenal medullary chromaffin cells (1), pancreatic acinar cells (2), and goblet cells (3) underscores the role of the secretory granules as the IP₃-sensitive intracellular Ca²⁺ store of neuroendocrine cells. Although the IP₃-sensitive Ca²⁺ store role of secretory granules has implied the existence of the IP₃R on the secretory granule membrane, the presence of IP₃R on the secretory granule membrane has not been addressed until the intragranular Ca²⁺ storage protein chromogranin A was shown to interact with several secretory vesicle integral membrane proteins including the IP₃R at pH 5.5 (13). The existence of IP₃R in the secretory granules has since been shown in the insulin-containing secretory granules of pancreatic β-cells (4, 38).

The IP₃R exists in three subtypes (types 1–3), and they are known to interact with each other to form a homo- and/or heterotetramer (14–18), functioning also as a Ca²⁺ channel (19). Interestingly, CGA has also been demonstrated to exist in a tetrameric state at the intravesicular pH 5.5 and in a dimeric...
state at the near physiological pH 7.5 (20). Moreover, the tetrameric CGA bound four molecules of the intraluminal loop peptide (L3-2 region) of the IP$_3$R at pH 5.5 (24), suggesting the possibility of interaction between tetrameric CGA and tetrameric IP$_3$R. The intraluminal loop peptide (L3-2 region) of the IP$_3$R has been shown to interact with the conserved near N-terminal region of CGA (29). The present results clearly demonstrate that CGB interacts with the intraluminal loop region of the IP$_3$R. The fact that CGB interacts with the intraluminal loop region of the IP$_3$R even at pH 7.5 (Figs. 5 and 7 and Table I) appears to indicate a strong interaction of CGB with the IP$_3$R (30), thus reflecting the strong interaction of CGB with the secretory vesicle membrane (12). This interaction at pH 7.5 contrasts with the dissociation of CGA from the same intraluminal loop region of the IP$_3$R at pH 7.5 (24), indicating a weaker interaction of CGA with the secretory vesicle membrane. Indeed, it was shown in our recent study that the purified IP$_3$R appeared to dissociate from CGA completely at pH 7.5 but substantial amounts of the same IP$_3$R still remained bound to CGB even at pH 7.5 (30); according to the band thickness of the IP$_3$R immunoblot result, approximately one-third of IP$_3$R that bound to CGB at pH 5.5 still remained bound to CGB at pH 7.5 (30).

The present results show that the interaction of CGB with the IP$_3$R intraluminal loop peptide at pH 7.5 is 1 order of magnitude stronger than that between CGA and the IP$_3$R intraluminal peptide at pH 5.5 (Table I and Ref. 24). The CGB interaction with the intraluminal loop region of the IP$_3$R at pH 5.5 is characterized by $\Delta G^0$ values of $-6$ to $-6.9$ kcal/mol ($K_d$ of 11–17 $\mu$M) for a 1:1 stoichiometry (24). However, the apparently much stronger interaction of CGB with the IP$_3$R intraluminal loop peptide at pH 5.5 could not be measured in the present experimental conditions due to aggregation of CGB at pH 5.5. Nevertheless, measurement of the CGB interaction with the IP$_3$R intraluminal loop peptide at pH 7.5 showed a $\Delta G^0$ value of $-8.1$ kcal/mol at 37 °C for a 1:1 interaction stoichiometry (Table I), indicating a strong interaction with a $K_d$ of $-1.9$ $\mu$M. This means that the CGB-IP$_3$R intraluminal loop peptide interaction at pH 7.5 is 1 order of magnitude stronger than that between CGA and the IP$_3$R loop peptide at pH 5.5, thus explaining the observed interaction of the L3-2 of the IP$_3$R with CGB even at pH 7.5 (Fig. 2), while there was no detectable interaction between the L3-2 of the IP$_3$R with CGA at pH 5.5, in the experiments that had been carried out with the GST fusion forms of intraluminal loop proteins of the IP$_3$R and CGA and CGB (Fig. 2).

The values of the enthalpy and entropy changes decreased as the temperature was increased from 0 to 37 °C (Figs. 6B and 7B and Table I). Nevertheless, the entropy changes still showed positive values even at 37 °C, indicating that the interaction of the IP$_3$R intraluminal loop region with intact CGB and with the conserved near N-terminal peptide of CGB is entropically dominated. The decreasing entropy suggested that the interacting structures become more ordered with increasing temperatures, which was also the case in the CGA-CGB heterodimer formation at pH 7.5 (23). Further, the interaction of the intraluminal loop region of the IP$_3$R with the conserved near N-terminal region of CGA at pH 7.5 showed a $\Delta G^0$ value of $-4.7$ kcal/mol at 37 °C with a $K_d$ of 0.48 $\mu$M (Table I). Although this is a much weaker interaction than that between intact CGB and the IP$_3$R intraluminal peptide (Table I), the interaction is significant in the sense that the vesicle membrane binding region of CGB is shown to be the site interacting with the IP$_3$R intraluminal peptide. Therefore, the interaction of the intraluminal loop region of the IP$_3$R with the conserved near N-terminal regions of CGB and CGA (29) accords with the identified anchor role of the conserved near N-terminal regions of CGB (27, 31) and CGA (11, 39), further suggesting the vesicle membrane binding region of chromogranins and the intraluminal loop region of the IP$_3$R as the interacting domains.

Although intact CGB interacted with the intraluminal loop region of the IP$_3$R at pH 7.5 in vitro, it is probable that CGB will dissociate from the secretory vesicle membrane during exocytosis. Moreover, given that CGB interacts with CGA to form either a heterodimer at pH 7.5 or a CGB$_4$ heterotetramer at pH 5.5 and that CGB interacts with the vesicle membrane only at the intravesicular pH 5.5 and dissociates from it at the near physiological pH 7.5, the CGB-CGB heterodimer is likely to dissociate from the vesicle membrane at pH 7.5 during exocytosis. Unlike the interaction observed between CGB and purified IP$_3$R at pH 7.5 in vitro (30), the CGB interaction with the secretory vesicle membrane in vivo would be markedly weaker due to potential interaction between CGB and other vesicular matrix proteins in the secretory vesicle, which may significantly weaken CGB interaction with the secretory vesicle membrane, thus contributing to the dissociation of CGB from the vesicle membrane at pH 7.5 during exocytosis. This concept is supported by the fact that the secretory vesicle matrix proteins, including CGB and CGA, were shown to dissociate from the secretory vesicle membrane at pH 7.5 (27). In this regard, CGB will also be released from the secretory vesicle into the extracellular space and then into the bloodstream along with other hormones and ions during exocytosis.

In the past, we and others have proposed the essential roles of CGB and CGB in secretory vesicle biogenesis (9, 10, 27, 40, 41). The chromogranins were shown to interact with many secretory matrix proteins at pH 5.5 (41), thereby selectively sorting the vesicle matrix proteins in the trans-Golgi network.
and these chromogranin-matrix protein complexes were proposed to bind to the secretory vesicle integral membrane proteins (41), leading to selective segregation of secretory vesicle membrane proteins from non-vesicle membrane proteins. In this regard, the interaction of the membrane-binding region of CGB with the intraluminal loop region of the IP₃R gives further insight into the selective sorting and segregation of vesicle matrix proteins and vesicle membrane proteins in the trans-Golgi network. A recent report that indicated the disulfide-bonded loop form of the membrane-binding region of CGB to be essential in sorting of CGB into secretory granule (28) underscores the importance of the conserved near N-terminal regions of chromogranins A and B in their interaction with the secretory vesicle membrane. In particular, the strong binding of CGB, along with the formation of a CGA-CGB₂ heterotetramer suggests the possibility of the interaction between the IP₃-sensitive intracellular Ca²⁺ stores to IP3.  

In addition to the observation that tetrameric CGA interacts with four molecules of the intraluminal loop region of the IP₃R (24) and that the IP₃Rs are also known to exist in a heterotetrameric state (14–18), the formation of a CGA₂CGB₂ heterotetramer suggests that at least some Ca²⁺-bound loop form of the membrane-binding region of CGB to be essential in sorting of CGB into secretory granule (9, 10, 27, 40, 41).  

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Interaction of Chromogranin B and the Near N-terminal Region of Chromogranin B with an Intraluminal Loop Peptide of the Inositol 1,4,5-Trisphosphate Receptor

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