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A Functional Interaction between Chromogranin B and the Inositol 1,4,5-Trisphosphate Receptor/Ca^{2+} Channel*

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Chromogranins A and B (CGA and CGB) are high capacity, low affinity calcium (Ca^{2+}) storage proteins found in many cell types most often associated with secretory granules of secretory cells but also with the endoplasmic reticulum (ER) lumen of these cells. Both CGA and CGB associate with inositol 1,4,5-trisphosphate receptor ($InsP_3R$) in a pH-dependent manner. At an intraluminal pH of 5.5, as found in secretory vesicles, both CGA and CGB bind to the $InsP_3R$. When the intraluminal pH is 7.5, as found in the ER, CGA totally dissociates from $InsP_3R$, whereas CGB only partially dissociates. To investigate the functional consequences of the interaction between the $InsP_3R$ and CGB monomers or CGA/CGB heteromers, purified mouse $InsP_3R$ type I were fused to planar lipid bilayers and activated by 2 μM $InsP_3$. In the presence of luminal CGB monomers or CGA/CGB heteromers the $InsP_3R/Ca^{2+}$ channel open probability and mean open time increased significantly. The channel activity remained elevated when the pH was changed to 7.5, a reflection of CGB binding to the $InsP_3R$ even at pH 7.5. These results suggest that CGB may play an important modulatory role in the control of Ca^{2+} release from the ER. Furthermore, the difference in the ability of CGA and CGB to regulate the $InsP_3R/Ca^{2+}$ channel and the variability of CGA/CGB ratios could influence the pattern of $InsP_3$-mediated Ca^{2+} release.

Chromogranin B (CGB)[1] belongs to the granin protein family, which also includes chromogranin A (CGA) and secretogranin II (chromogranin C). It is found in the large dense core secretory granules and in the endoplasmic reticulum (ER) lumen of most neurons, exo/endocrine cells, and neuroendocrine cells and shows a wide distribution in various areas of the brain (1). Levels of CGB expression in cells can be used as markers for a number of physiological and medically important pathophysiological conditions (2–4). In normal brain tissue CGB expression is enhanced after neuronal activation, providing a marker for stimulated neurons (3). In addition to the tissue-specific distribution, a regionally specific distribution of CGB has been found intracellularly in neuronally differentiated pheochromocytoma (PC12) cells (5). In these cells CGB levels are elevated in the neurites rather than in the soma, which correlates with the initiation site for intracellular calcium (Ca^{2+}) signals. The levels of CGB and chromogranin-derived peptides can be diagnostic markers for pathophysiological conditions. For example, levels of CGB are greatly reduced in the cerebrospinal fluid of chronic schizophrenia subjects (6, 7). Moreover, the levels of CGB and chromogranin-derived peptides are diagnostically significant as neuronal markers for synaptic degeneration in Alzheimer's disease (4).

At the cellular level CGB is believed to have many intra- and extracellular functions. CGB functions as a heparin binding extracellular matrix protein, mediating adhesion of cells and supporting neurite outgrowth (8). CGB is a prohormone with numerous di- and tribasic amino acid cleavage sites that act as targets for proteolytic enzymes such as the prohormone convertase (9, 10). Furthermore, chromogranin B is known to bind >90 mol of Ca^{2+}/mol with a dissociation constant (K_d) of 1.5 mM (11), distinguishing itself as a very efficient Ca^{2+} storage protein. Intracellularly, CGB has also been suggested to participate in packaging and sorting other proteins into the secretory vesicles of neuroendocrine cells, thus playing key roles in secretory granule biogenesis (12–14). Indeed, CGB has recently been shown to induce secretory granule biogenesis (15). CGB also localizes to the nucleus and controls transcription of many genes, including those for transcription factors (16).

In secretory granules both CGA and CGB have been shown to interact with the $InsP_3R$ at the intravesicular pH of 5.5 (11, 17). Purified $InsP_3R$ interacts directly with CGA and CGB at pH 5.5. CGA dissociates from the $InsP_3R$ at pH 7.5, whereas CGB remains partially associated (18). Both chromogranin proteins form a complex with the $InsP_3R$ *in vivo* (11). The functional aspect of this coupling has been investigated for CGA alone using single channel experiments and Ca^{2+} flux studies (19). In the presence of CGA the open probability and mean open time of the $InsP_3R$ channel increases 10-fold.

Despite the role of secretory granules of secretory cells and ER as major $InsP_3$-sensitive intracellular Ca^{2+} stores (13, 20, 21) and the abundance of CGB in these (10) and a variety of other cell types (1), the functional interaction of $InsP_3R$ and CGB is less well characterized. Given also the tendency of CGA/CGB mixture to spontaneously form a CGA/CGB heterodimer at a near physiological pH 7.5 and a CGA_2CGB_2

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[1] The abbreviations used are: CGB and CGA, chromogranin B and A, respectively; ER, endoplasmic reticulum; $InsP_3R$, inositol 1,4,5-trisphosphate ($InsP_3$) receptor; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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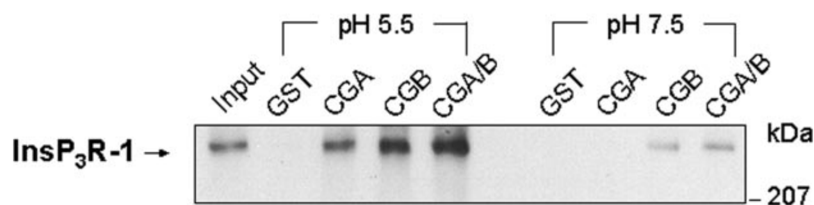

FIG. 1. **pH-dependent interaction of $InsP_3R$ type I with CGA, CGB, and CGA/CGB.** Purified $InsP_3R$ type I (0.5–0.7 μg) was reacted with the GST fusion proteins of CGA, CGB, and an equimolar mixture of GST-CGA and GST-CGB at both pH 5.5 and 7.5. The bound $InsP_3R$ type I was separated on 7.5% SDS-gels and analyzed by immunoblot using a type 1-specific $InsP_3R$ antibody specific for the type I isoforms (11).

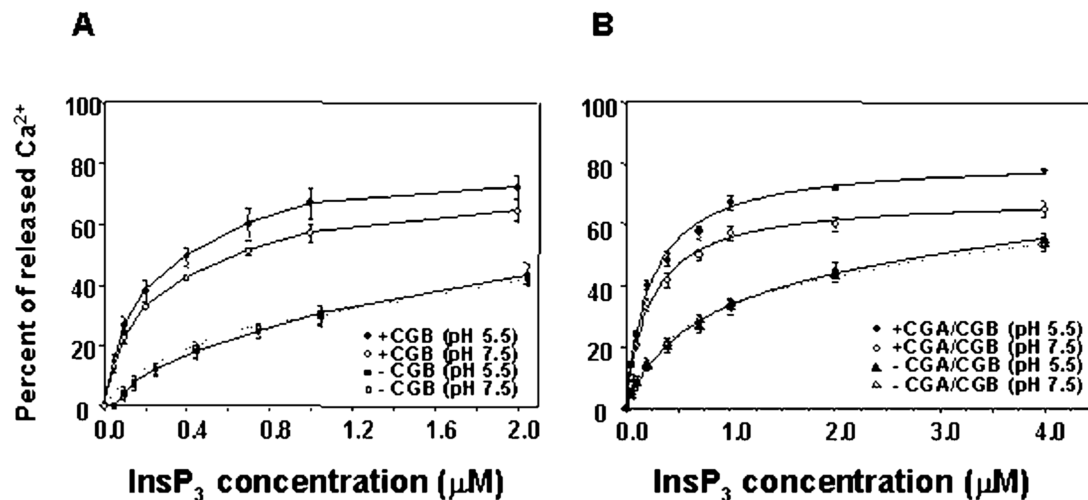

FIG. 2. **Effects of CGB and CGA/CGB on $InsP_3$-induced Ca^{2+} release from $InsP_3R$-reconstituted liposomes.** $InsP_3$-induced Ca^{2+} efflux through the proteoliposomes (300 μM Ca^{2+} inside) was determined by the fluorescence change of 10 μM indo-I at 393 nm after a series of incremental additions of $InsP_3$ (2.0 μM final) to the proteoliposome solution. *A*, the $InsP_3$-induced Ca^{2+} release was measured both in the presence of encapsulated CGB at intraliposomal pH of 5.5 (●) and 7.5 (○) and in the absence at pH 5.5 (■) and 7.5 (□). The figure shows the amount of released Ca^{2+} expressed as percentage of maximum releasable Ca^{2+}. $InsP_3$-induced fluorescent changes were compared with that obtained by the addition of 1% Triton X-100 (this value was set at 100%). *B*, the $InsP_3$-induced Ca^{2+} release was measured in the presence of an equimolar mixture of CGA and CGB at an intraliposomal pH of 5.5 (●) and 7.5 (○) and in the absence at pH 5.5 (▲) and 7.5 (△). The figure shows the amount of released Ca^{2+} expressed as percentage of maximum releasable Ca^{2+}. $InsP_3$-induced fluorescent changes were compared with that obtained by the addition of 1% Triton X-100 (this value was set at 100%).

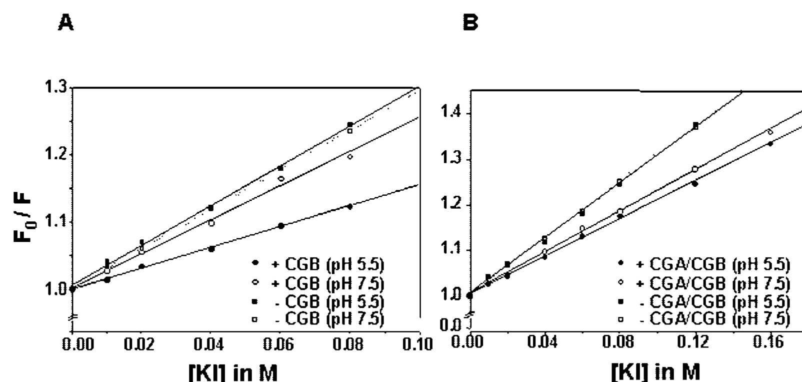

FIG. 3. **Quenching of $InsP_3R$ fluorescence by iodide in the presence of CGB and CGA/CGB.** *A*, the changes in tryptophan fluorescence from the proteoliposomes containing $InsP_3R$ only at pH 5.5 (■) or pH 7.5 (□) or $InsP_3R$ and CGB either at pH 5.5 (●) or pH 7.5 (○) were measured as a function of increasing concentrations of KI. F_o and F represent the fluorescence intensities in the presence (F) and absence (F_o) of KI. *B*, the changes in tryptophan fluorescence from the proteoliposomes containing $InsP_3R$ only at pH 5.5 (■) or pH 7.5 (□) or $InsP_3R$ and CGA/CGB mixture either at pH 5.5 (●) or pH 7.5 (○) 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.

heterotetramer at the intragranular pH 5.5 (22), it is important to understand the effects of CGB and CGA/CGB heteromers. In this study, we examined the effect of CGB and CGA/CGB heteromers on the channel gating properties of the $InsP_3R$ type I. CGB increased the open probability and mean open time of the channel by almost 20-fold. However, in contrast to CGA, this functional effect was less sensitive to changes in pH when compared with the effect of CGA alone. These results show the functional interaction of $InsP_3R$ with CGB monomers and CGA/CGB heteromers and suggest that regulation of these interactions plays a physiologically important role in determining the pattern of $InsP_3$-mediated Ca^{2+} release.

MATERIALS AND METHODS

Antibody—An $InsP_3R$ peptide specific to terminal 10–13 amino acids of type 1 (HPPHMNVNPQQPA) was synthesized with a carboxyl-ter-

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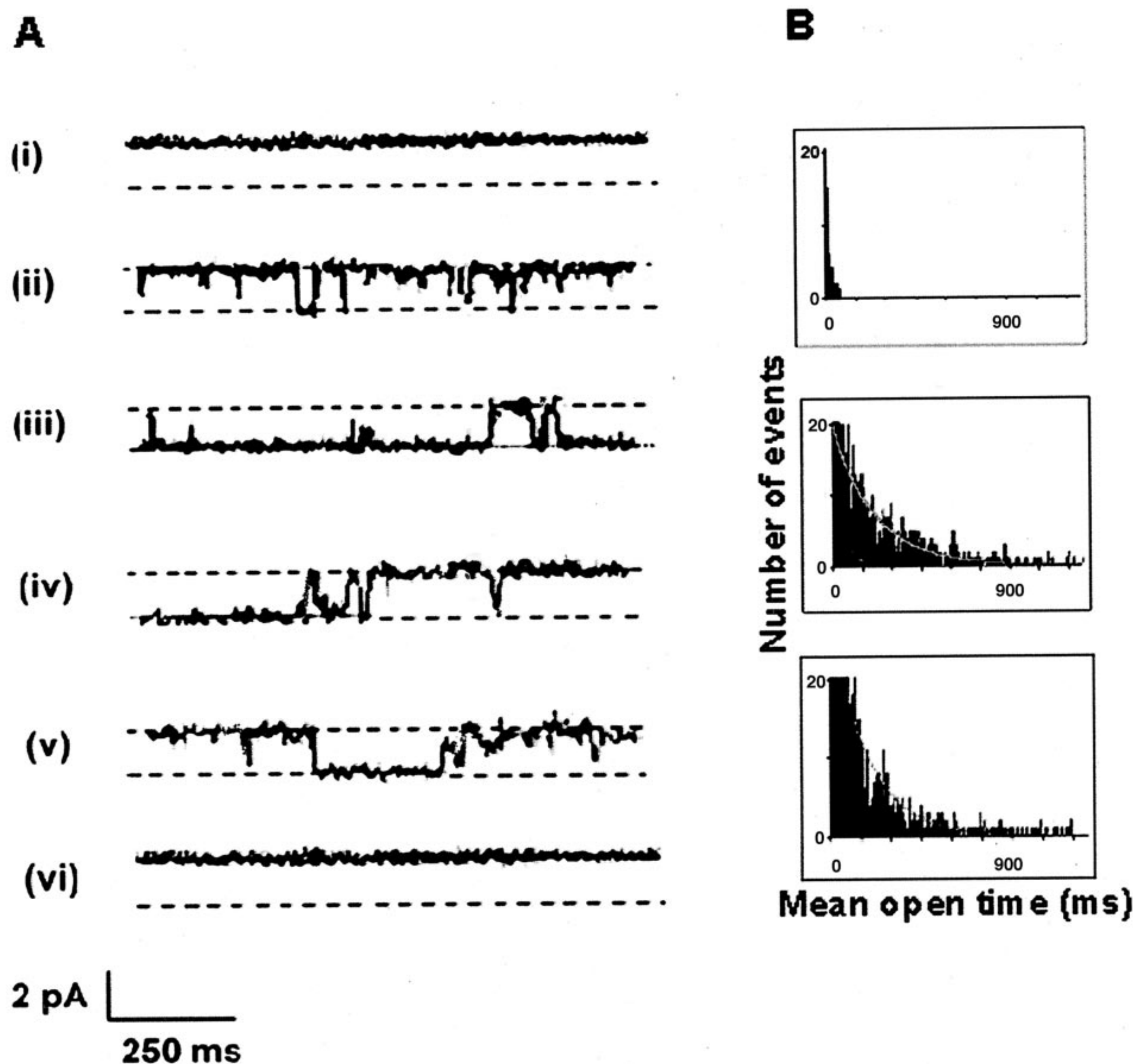

FIG. 4. **Single channel activity and mean open time in the presence and absence of CGB.** *A*, *i* and *ii*, $InsP_3R$ single channels before and after activation by 2 μM $InsP_3$ (added to the cis compartment). Openings are defined as downward deflections from the base line. *iii*, conditions are the same as for *ii*, except CGB (1 μg) was added to the trans compartment and mixed. The pH of the trans compartment was pH 5.5. *iv*, the pH in the trans compartment was changed by the addition of Tris (final concentration 55 mM) to pH 6.5. *v*, the trans pH was further changed by the addition of Tris (55 mM) to give a pH of 7.5. *vi*, the addition of heparin inhibited all channel activity. *B*, *top panel*, mean open times for $InsP_3R$ in the absence of CGB. One population of open times was observed with a value of 5.2 ± 1.5 ms. *Middle panel*, mean open times for $InsP_3R$ in the presence of CGB at trans pH 5.5. Two populations of open times were observed with values of 3.1 ± 0.3 and 217.4 ± 0.3 ms. *Bottom panel*, mean open times for $InsP_3R$ after partial dissociation of CGB by pH change (trans pH 7.5). Two populations of open times were observed with values of 4.8 ± 0.6 and 122.5 ± 0.7 ms. The open times were less than at pH 5.5, but had not returned to control levels. This experiment is typical of four similar but separate experiments.

minal cysteine, and anti-rabbit polyclonal antibody was raised. The polyclonal anti-rabbit antibody was affinity-purified on the immobilized peptide following the procedure described (23), and the specificity of the antibody was been confirmed (11).

$InsP_3R$ Interaction with Glutathione S-Transferase (GST)-CGA/CGB—To construct GST fusion proteins, bovine CGA cDNA (24) and CGB cDNA (25) were amplified by PCR and subcloned into vector pGEX-5T (Amersham Biosciences). GST-CGA and GST-CGB fusion proteins were expressed in *Escherichia coli* BL21 cells and purified on glutathione-agarose beads. The binding reactions were carried out either in a pH 5.5 buffer (20 mM sodium acetate, pH 5.5, 0.1 M KCl, 2 mM $CaCl_2$, and 0.1% Triton X-100) or in a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 2 mM $CaCl_2$, and 0.1% Triton X-100), and the interaction was determined by incubating an excess amount of purified GST-CGA or -CGB fusion protein (20 μg) with the purified $InsP_3R$ (0.5–0.7 μg) in 0.5 ml of buffer supplemented with 1× protease inhibitor mixture (Roche Applied Science) for 1 h at 4 °C. After incubation the reaction mixture was rinsed with 0.5 ml of buffer to remove unbound proteins. Elution of the bound $InsP_3R$ was carried out by using three bed volumes of the pH 7.5 buffer but with 1 M KCl. The eluted $InsP_3R$ was resolved on a 7.5% SDS-polyacrylamide gel and identified by chemiluminescence-based immunoblot analysis using the affinity-purified $InsP_3R$ antibody.

Purification of the $InsP_3R$—For flux studies the $InsP_3R$ type I was isolated from bovine cerebella as described previously (26). Briefly, 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 phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin), homogenized, and centrifuged at 2000 × *g* for 10 min at 4 °C. The supernatants were re-centrifuged at 105000 × *g* for 1 h to precipitate the membrane pellet, which was resuspended in buffer II (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin) containing 1% Triton X-100, stirred for 1 h, and then centrifuged at 32000 × *g* for 1 h at 4 °C. The resulting supernatant 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 phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin) and applied to an $InsP_3R$ antibody-coupled

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immunoaffinity column (0.35 × 1 cm) equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$. The protein-loaded column was washed with 20 bed volumes of this buffer, and the $InsP_3R$ 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 phenylmethylsulfonyl fluoride, 10 μM leupeptin, 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, 1 mM β-mercaptoethanol) and then applied to a benzamidine-Sepharose column equilibrated with buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 M KCl, and 3 M urea). $InsP_3R$-containing flow-through was collected and stored at −70 °C until use.

For bilayer experiments the $InsP_3R$ type I was solubilized in 1% CHAPS and purified from mouse cerebellum using heparin affinity and Con-A-Sepharose column chromatography, as described previously (27). The purified $InsP_3R$ was then incorporated into liposomes by the addition of 15 μg of purified protein to 1 ml of liposome solution (consisting of phosphatidylcholine in bilayer buffer), mixing, and then incubating on ice for 10 min.

Flux Studies—$InsP_3R$ proteoliposomes were formed as described previously (26). Flux experiments were carried out using three types of proteoliposomes at both pH 5.5 and 7.5. Proteoliposomes either had 1) CGB encapsulated in them, 2) CGA and CGB encapsulated in them at the molar ratio of 1:1, or 3) no added chromogranin to be used for control experiments. Ca^{2+} efflux from the proteoliposomes was measured by observing changes in indo-1 fluorescence. Fluorometric measurements were carried out at 35 °C by using a Shimadzu RF-5301 PC spectrofluorometer equipped with a temperature-controlled cuvette holder. Fluorescence intensity was measured at the emission wavelength of 393 nm (excitation of 355 nm) with 10 nm of excitation band slit width and 10 nm of emission band slit width. For the kinetic analysis of $InsP_3$-induced Ca^{2+} release, the data were acquired every 20 ms after each addition of the indicated $InsP_3$ concentration to 0.5 ml of the proteoliposome solution. The fluorescent intensities of indo-1 were calibrated to free Ca^{2+} concentrations using a Ca^{2+}-EGTA buffering system (28). Fluorescence intensity after the addition of Triton X-100 was used to determine total intravesicular Ca^{2+}.

Potassium Iodide Quench Analysis—For the collisional fluorescence quenching of Trp residues in $InsP_3R$ 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.

Bilayer Experiments—Planar lipid bilayers were formed by painting a solution of phosphatidylethanolamine/phosphatidylserine (3:1; 30 mg/ml in decane) across a 100-μm aperture in a Teflon sheet bisecting a Lucite chamber. The hole was pre-painted with phosphatidylcholine/phosphatidylserine (3:1) before membrane formation. The two compartments are defined cis (corresponding to the cytosol) and trans (corresponding to the lumen of the ER). The cis (cytosolic) compartment consisted of 250 mM HEPES-Tris, pH 7.35, 0.5 mM EGTA, 300 nM $[Ca^{2+}]_{free}$, 0.5 mM ATP, 2 μM ruthenium red. The trans (luminal) compartment consisted of 250 mM HEPES adjusted to pH 5.5 (because purified $InsP_3R$ was used in these experiments the pH could be changed using 70 mM HCl) and 53 mM $BaOH_2$. Single channel currents were amplified using a bilayer clamp amplifier (Warner Instruments) and recorded on digital tape. Data were filtered with an eight-pole Bessel filter to 500 Hz, digitized to 2 KHz, transferred to a personal computer, and analyzed using pClamp 9.0 (Axon Instruments) software package.

$InsP_3R$ proteoliposomes were added to the cis compartment and mixed followed by the addition of 2 μM $InsP_3$ to the same compartment. Upon $InsP_3R$ activation, single channel activity was recorded. Either CGB (1 μg) or a mixture of CGA/CGB (1 μg) was added to the trans compartment and mixed. $InsP_3R$ single channel activity was recorded. The pH inside the trans compartment was changed by the addition of Tris (final concentration 110 mM) to pH 7.5, and $InsP_3R$ single channel activity was recorded.

These experiments were repeated in the presence of increasing concentrations of $InsP_3$ (over the range 0.2–2 μM) to the cis compartment and carried out in the presence and absence of 1 μg of CGB or the CGA/CGB mixture in the trans compartment at pH 5.5. $InsP_3R$ single channel activity was recorded. Because CGB does not completely dissociate from the $InsP_3R$ at pH 7.5, the $InsP_3$ concentration-response was repeated in the presence of luminal CGB or the CGA/CGB mixture at this pH. All data are presented as the mean ± S.E. of at least four similar experiments.

TABLE I
The effect of the chromogranins on the mean open time of the $InsP_3R$

Mean open times for the $InsP_3R$ single channel currents in the absence and presence of CGA, CGB, and CGA/CGB at pH 5.5 and 7.5. Values are generated from at least four experiments using each condition.

Control (−CGA pH 5.5)	+CGA pH 5.5	+CGA pH 7.5
ms	*ms*	*ms*
8.6 ± 0.5	3.3 ± 0.07 71.7 ± 0.08	10.0 ± 0.03
Control (−CGB pH 5.5)	**+CGB pH 5.5**	**+ CGB pH 7.5**
ms	*ms*	*ms*
5.2 ± 1.5	3.1 ± 0.3 217.4 ± 0.3	4.8 ± 0.6 122.5 ± 0.7
Control (−CGA/CGB pH 5.5)	**+CGA/CGB pH 5.5**	**+CGA/CGB pH 7.5**
ms	*ms*	*ms*
7.2 ± 0.2	11.1 ± 0.4 84.4 ± 0.3	7.4 ± 0.3 120.9 ± 0.3

TABLE II
The effect of the chromogranins on the open probability of the $InsP_3R$

Open probabilities for the $InsP_3R$ single channel currents in the absence and presence of CGA, CGB, and CGA/CGB at pH 5.5 and 7.5. Values are generated from at least 4 experiments using each condition.

Control (−CGA pH 5.5)	+CGA pH 5.5	+CGA pH 7.5
%	%	%
4.0 ± 1.0	33.0 ± 8.5	3.0 ± 1.6
Control (−CGB pH 5.5)	**+CGB pH 5.5**	**+CGB pH 7.5**
%	%	%
5.0 ± 1.0	80.0 ± 9.0	40.0 ± 2.0
Control (−CGA/CGB pH 5.5)	**+CGA/CGB pH 5.5**	**+CGA/CGB pH 7.5**
%	%	%
3.0 ± 1.0	77.0 ± 3.0	48.0 ± 2.0

RESULTS

pH-dependent Interaction of $InsP_3R$ with CGA, CGB, and CGA/CGB—To determine whether there was a direct interaction between the purified $InsP_3R$ and CGA or CGB, GST fusion forms of CGA and CGB were expressed in *E. coli* BL21 and purified. The interaction between the $InsP_3R$ and GST-CGA and -CGB fusion proteins was examined at pH 5.5 and 7.5 (Fig. 1). As shown in Fig. 1 (*left side* of the *figure*), CGA, CGB, and a mixture of CGA and CGB (CGA/CGB) all interacted with the $InsP_3R$ at pH 5.5. When tested at pH 7.5 CGA failed to interact with the $InsP_3R$ (Fig. 1, *right side*), but CGB still interacted with the $InsP_3R$, albeit at a reduced level (Fig. 1, *right side*), reflecting a stronger affinity of CGB for the $InsP_3R$.

Effects of CGB and CGA/CGB on $InsP_3$-mediated Ca^{2+} Release—Ca^{2+} release studies were employed to investigate the effects of CGB on the $InsP_3$ concentration-response for $InsP_3R$ type I. As previously described for CGA (19), $InsP_3$-induced Ca^{2+} release from $InsP_3R$-reconstituted liposomes was monitored at two different pH values both in the presence and absence of CGB (Fig. 2*A*). $InsP_3$-induced Ca^{2+} efflux was determined using proteoliposomes containing 300 μM Ca^{2+}. The total amount of $InsP_3$-releasable Ca^{2+} was estimated to be 62–70%. The $InsP_3$-induced Ca^{2+} release obtained in the absence of CGB gave a K_{app} value for $InsP_3$ of 0.68 μM. When CGB was present inside the vesicle at pH 5.5, the pH value at which CGA and CGB coupled with the $InsP_3R$ (18), $InsP_3$-induced Ca^{2+} release was markedly enhanced (see Fig. 2*A*), yielding a K_{app} value for $InsP_3$ of 0.16 μM. Interestingly, when the pH was maintained at 7.5, the fluxes measured at each $InsP_3$ concen-

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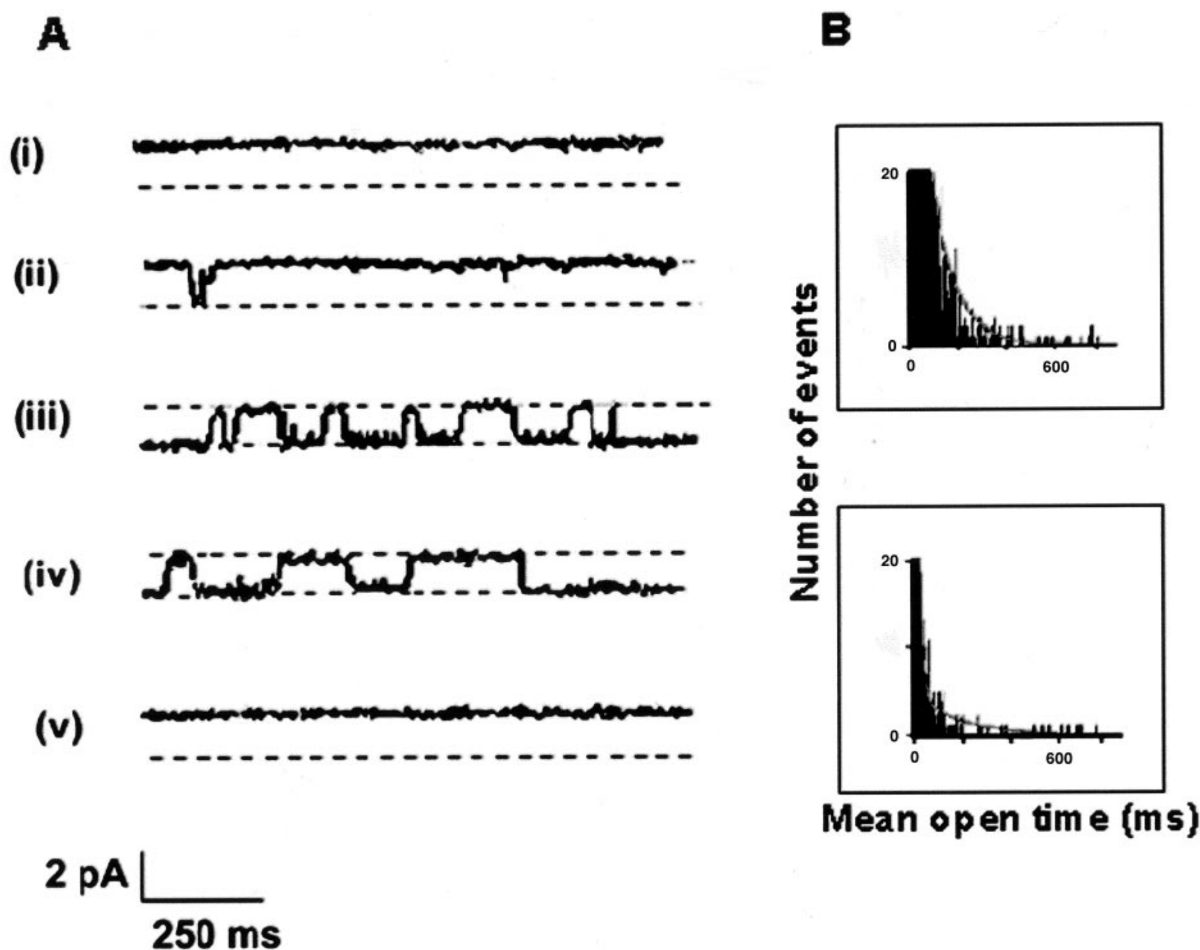

FIG. 5. **Single channel activity and mean open time in the presence and absence of CGA/CGB.** *A*, *i* and *ii*, $InsP_3R$ single channels before and after activation by 2 μM $InsP_3$ (added to the cis compartment). Openings are defined as downward deflections from the base line. *iii*, conditions are the same as for the control, except CGA/CGB (1 μg) was added to the trans compartment and mixed. The pH of the trans compartment was pH 5.5. *iv*, the pH in the trans compartment was changed by the addition of Tris (final concentration 110 mM) to give a pH of 7.5. *v*, the addition of heparin inhibits all channel activity. *B*, *top panel*, mean open times for $InsP_3R$ in the presence of CGA/CGB at trans pH 5.5. Two populations of open times were observed with a values of 11.1 ± 0.4 and 84.4 ± 0.3 ms. *Bottom panel*, mean open times for $InsP_3R$ in the presence of CGA/CGB after pH change (trans pH 7.5). Two populations of open times are observed with values of 7.4 ± 0.3 and 120.9 ± 0.3 ms, and the open times were less than at pH 5.5, but they had not returned to control levels. This experiment is typical of four similar but separate experiments.

tration were similar to those seen at pH 5.5 in the presence of CGB (K_{app} value for $InsP_3$ of 0.17 μM). This result is distinct from that obtained with CGA where pH had a profound effect, and the response at pH 7.5 was similar to that measured in the absence of CGA (19).

Ca^{2+} release from the $InsP_3R$-reconstituted liposomes was also monitored in the presence of an equimolar mixture of CGA/CGB (Fig. 2*B*). The K_{app} value for $InsP_3$ was approximately the same in the presence of the mixtures as that measured in the presence of CGB alone (K_{app} value for $InsP_3$ was 0.15 μM at pH 5.5 and 0.18 μM at pH 7.5).

Changes in $InsP_3R$ Conformation—To investigate possible conformational changes of the $InsP_3R$ by its interaction with monomeric CGB or a heteromeric CGA/CGB, we utilized collisional quenching of the intrinsic tryptophan (Trp) fluorescence of the $InsP_3R$ by iodide (Fig. 3). By determining the extent of quenching it was possible to determine whether the Trp environment of the $InsP_3R$ is changed as a result of a change in the $InsP_3R$ conformation. As shown in Fig. 3, the $InsP_3R$ Trp fluorescence was quenched by iodide in the presence of both CGB or the CGA/CGB mixture. The emission fluorescence at 340 nm was measured, and the results were plotted according to the Stern-Volmer equation (29), $F_o - F = K_{sv}[I^-] + 1$, where 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 2.94 M^{-1} for the reconstituted $InsP_3R$ in the absence of CGB. When CGB was present, this value decreased to 2.51 and 1.55 M^{-1} at the intraliposomal pH 7.5 and 5.5, respectively (Fig. 3*A*). From this experiment it is clear that at least some Trp residues of the $InsP_3R$ are less exposed to the solvent when CGB is present, demonstrating the CGB-induced conformational changes of the $InsP_3R$. Furthermore, the K_{sv} values suggest that the Trp residues are less exposed when the intraliposomal pH was maintained at 5.5 than at 7.5.

There also were conformational changes of the $InsP_3R$ in the presence of a CGA/CGB mixture in the liposome (Fig. 3*B*). The K_{sv} value of 3.11 in the absence of CGA/CGB was decreased to 2.25 and 2.06 M^{-1} at the intraliposomal pH 7.5 and 5.5, respectively, in the presence of the CGA/CGB mixture, indicating the conformational changes of the $InsP_3R$ in the presence of CGA/CGB.

Effects of CGB and CGA/CGB on $InsP_3R$ Channel Activity—The effects of CGB and CGA/CGB on $InsP_3R$ function were investigated at the single channel level using $InsP_3R$ incorporated into planar lipid bilayers. In the absence of luminal CGB and in the presence of cytosolic-free Ca^{2+} (300 nM) and $InsP_3$ (2 μM) $InsP_3R$ single channel activity was observed (see Fig. 4*A*, *trace iii*). Single channel currents of ~2 pA were seen, and a

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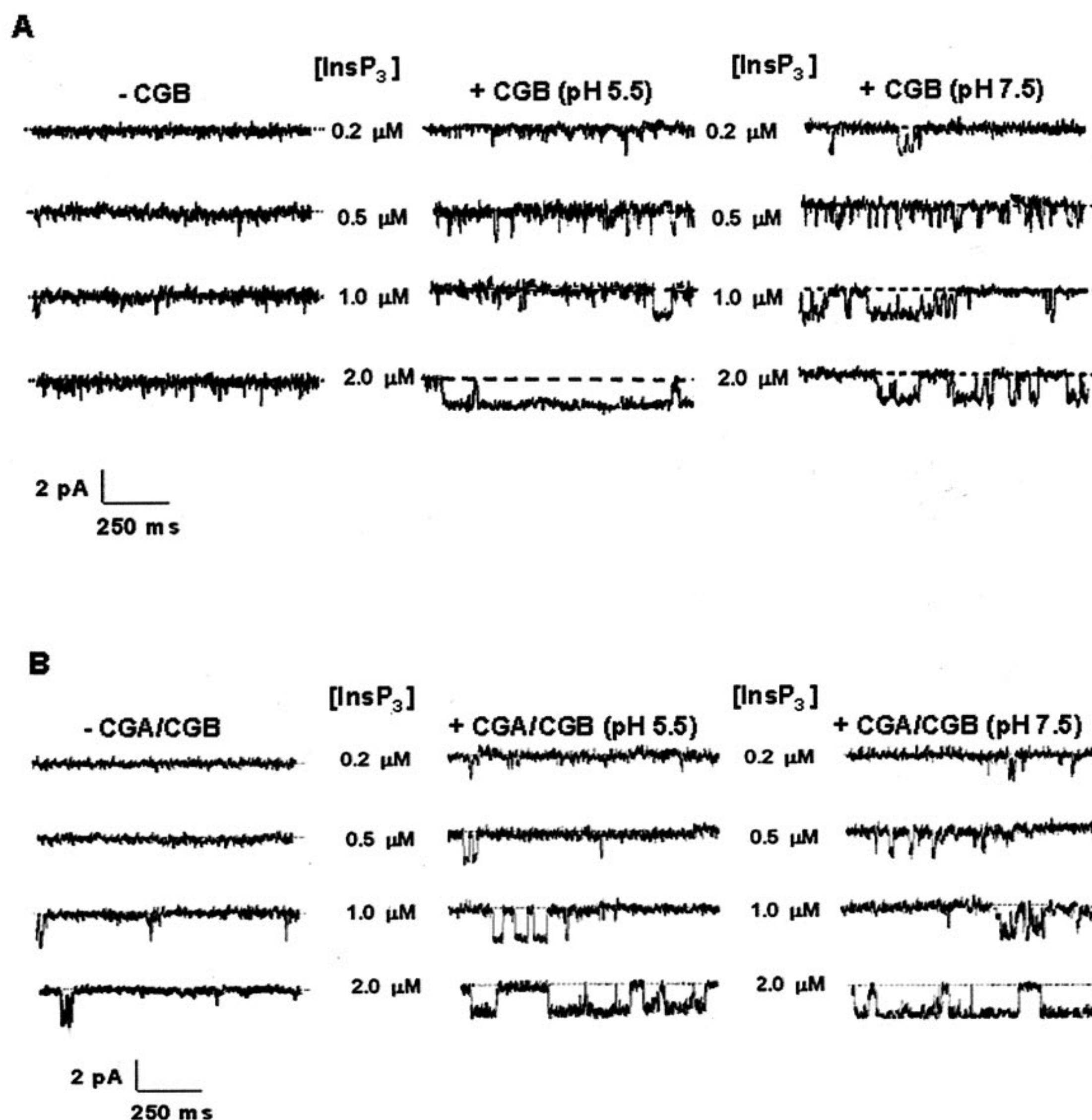

FIG. 6. **Effect of CGB and CGA/CGB on the $InsP_3$ concentration-response for $InsP_3R$.** Single channel experiments were done using the conditions as described for Figs. 4 and 5, except that a range of $InsP_3$ concentrations was monitored. $InsP_3R$ single channels activated by 0.2–2 μM $InsP_3$ (added to the cis compartment) are shown. The $InsP_3$ concentration used is shown in the figure next to the relevant traces. Openings are defined as downward deflections from the base line. *A*, the channel traces shown on the *left* of the figure are in the absence of CGB. Channel traces shown in the *middle* of the figure are in the presence of CGB (1 μg) in the trans compartment, maintained at pH 5.5. Channel traces on the *far right* are in the presence of CGB at pH 7.5. *B*, channel traces shown in the *middle* of the figure are in the presence of CGA/CGB (1 μg) in the trans compartment, maintained at pH 5.5. Channel traces on the *far right* are in the presence of CGA/CGB at pH 7.5. The data sets shown are typical of four similar experiments.

single population of mean open times was obtained with a value that ranged between 5.2 and 8.6 ms (Fig. 4*B*, *top panel*; Table I). The open probability (*P*o) was 5 ± 1% (n = 4; Table II).

After the addition of 1 μg of CGB to the luminal compartment a dramatic increase in channel activity was observed (Fig. 4*A*, *trace ii*). Although the size of the single channel current remained unaltered, significant differences in mean open times and *P*o were seen. Two populations of mean open time were apparent, but the values were noticeably increased over control values (Fig. 4*B*, *middle panel*). Although the short open time remained in the same range as control values, a new, longer open time was evident that was at least 10-times larger than the open time observed under control conditions (3.1 ± 0.3 and 217.4 ± 0.3 ms are the open times in the presence of CGB; Table I). Furthermore, the *P*o increased from 5% in control conditions to 80 ± 9% (n = 4; Table II) in the presence of CGB.

After changing the pH of the luminal compartment to pH 6.5, a condition causing partial dissociation of CGB from the $InsP_3R$, the *P*o was reduced to 63 ± 2%. Although this is a decrease in channel activity, the *P*o was still elevated when compared with control levels (Fig. 4*A*, *trace iv*). A further change in luminal pH to 7.5 (Fig. 4*A*, *trace v*) caused the *P*o to fall even further, but the value of 40 ± 2% still exceeded control levels, indicating that CGB remained coupled to the $InsP_3R$. The mean open times were reduced to 4.8 ± 0.6 and 122 ± 0.7 ms (n = 4; Fig. 4*B*, *bottom panel* and Table I). Addition of heparin, an $InsP_3R$-specific antagonist, to the cytoplasmic compartment inhibited channel activity completely (Fig. 4*A*, *trace vi*).

In the next series of experiments a mixture of CGA/CGB was tested. In the absence of CGA/CGB the amplitude of the single channel currents was 2 pA (see Fig. 5*A*, *trace ii*), the *P*o was 3.0 ± 1.0% (n = 4), and the mean open time was similar to that obtained in the previous control experiments (7.2 ± 0.2 ms). After the addition of 1 μg of CGA/CGB to the luminal compartment (pH 5.5) a large increase in channel activity was observed

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(Fig. 5*A*, *trace iii*). The *Po* was 77% ± 3% (*n* = 4), and two populations of mean open time were evident (11.1 ± 0.4 and 84.4 ± 0.3 ms; Table I). Although the values for the open times were increased over control values (Fig. 5*B*, *top panel*) the longer population of mean open times was less than that seen with CGB alone and more closely resembled that seen with CGA alone.

A subsequent change in the luminal pH to 7.5 (Fig. 5*A*, *trace iv*) caused *Po* to fall, but the value of 48 ± 2% again exceeded control levels. The elevated *Po* indicates a continued coupling of CGA/CGB with the $InsP_3R$, potentially via the CGB component of the heteromer. The mean open times were 7.3 ± 0.3 and 120.9 ± 0.3 ms, values closer to CGB alone at pH 7.5 (*n* = 4; Fig. 5*B*, *bottom panel*; Table I). The addition of heparin to the cytoplasmic compartment inhibited channel activity completely (Fig. 5*A*, *trace v*).

As with previous studies demonstrating the effect of CGA on the activity of the $InsP_3R$ (19), several control experiments were done. 1) The addition of CGB or CGA/CGB in the absence of cytosolic $InsP_3$ did not potentiate any $InsP_3R$ channel activity; 2) the addition of CGB or CGA/CGB to the luminal compartment in the absence of $InsP_3R$ had no effect upon the bilayer currents; 3) the addition of CGB or CGA/CGB to the cytoplasmic compartment in the presence of $InsP_3R$ and $InsP_3$ did not affect channel activity.

Effect of CGB or CGA/CGB on the $InsP_3$ Concentration-Response for $InsP_3R$—Single channel activity was observed over a range of $InsP_3$ concentrations both in the presence of CGB at pH 5.5 and 7.5 and in its absence (Fig. 6*A*). Starting at an $InsP_3$ concentration of 0.2 μM, the *Po* was greater in the presence of CGB (luminal pH 5.5) when compared with control levels, with an ~18-fold increase in *Po* at 2 μM $InsP_3$ (Fig. 6*A*, *middle panel*). At pH 7.5 (Fig. 6*A*, *right panel*) the channel again has a higher *Po* at the lower $InsP_3$ concentration range, although at 2 μM $InsP_3$ the *Po* is less than that observed at pH 5.5.

Similar experiments to examine the $InsP_3$ concentration dependence were done in the presence of the CGA/CGB heteromer (Fig. 6*B*). Again, in the presence of CGA/CGB, over a range of $InsP_3$ concentrations starting at 0.2 μM, the open probability was greater in the presence of CGA/CGB (luminal pH 5.5) when compared with control levels (Fig. 6*B*, *middle panel*). Repetition of the experiment at pH 7.5 (Fig. 6*B*, *right panel*) indicated that the *Po* at the lower $InsP_3$ concentrations were similar to those seen for CGA/CGB at pH 5.5, but at 2 μM $InsP_3$ the *Po* was less at pH 7.5 than that seen at pH 5.5.

The $InsP_3$ concentration dependence of channel activity after the addition of either CGB or the mixture of CGA/CGB was compared at pH 5.5 and 7.5 (Fig. 7). Regardless of the pH used the effect of CGB alone or CGA/CGB was similar (Fig. 7), suggesting that the properties of CGB, especially the ability to bind to the $InsP_3R$ at pH 7.5, predominates.

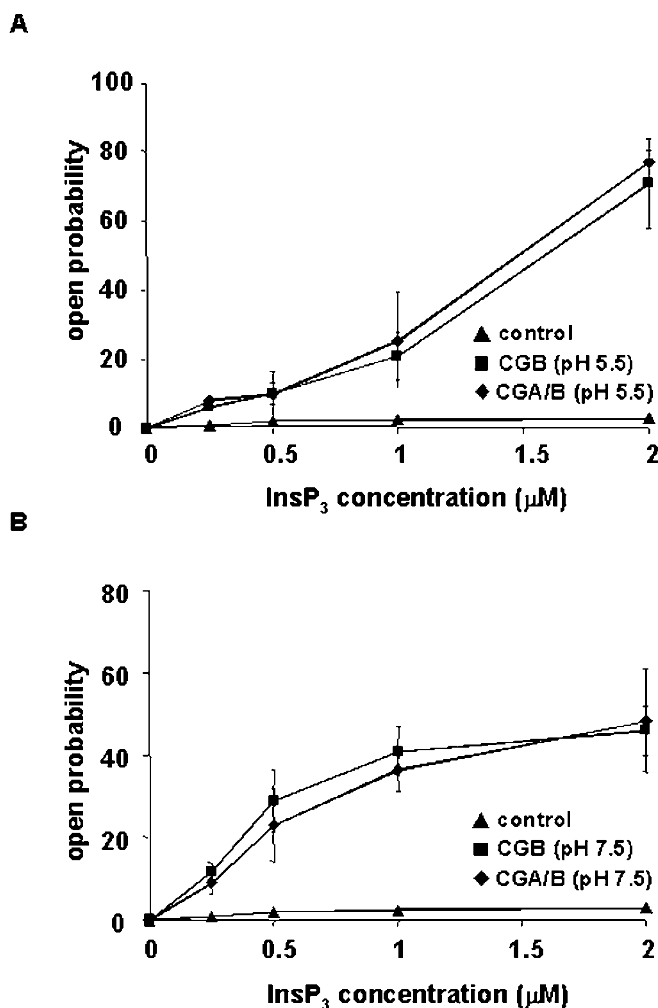

FIG. 7. **Effect of CGB and CGA/CGB on the $InsP_3$ concentration-response for $InsP_3R$.** At each $InsP_3$ concentration shown in Fig. 6, the open probability was measured. *A*, the $InsP_3$ dependence of the addition of CGB (■), CGA/CGB (♦), or in its absence (△) at pH 5.5. *B*, the $InsP_3$ dependence of the addition of CGB (■), CGA/CGB (♦), or in its absence (△) at pH 7.5. Note that the $InsP_3$ dependence is similar over the entire range of $InsP_3$ concentrations tested. The data sets shown are typical of four similar experiments.

DISCUSSION

In the present study we examined the functional interaction between the $InsP_3R$ type I and CGB monomers or CGA/CGB heteromers and found that both CGB and CGA/CGB heteromers enhanced activation of the $InsP_3R/Ca^{2+}$ channel in the presence of $InsP_3$. A direct physical interaction between the $InsP_3R$ and CGA or CGB was demonstrated (17), and the molecules co-localized in intact cells (5). Furthermore, a mixture of CGA/CGB interacted with the purified $InsP_3R$ at both pH 5.5 and 7.5, although the interaction at pH 7.5 was markedly reduced compared with that at pH 5.5 (Fig. 1). The stronger interaction between the $InsP_3R$ and the chromogranins at acidic pH is consistent with the physiological roles of secretory granules because the intragranular pH of secretory granules decreases and the Ca^{2+} content increases as the granules move from the trans-Golgi network to the plasma membrane.

The interaction of the $InsP_3R$ with CGB monomers and with CGA/CGB heterotetramers at pH 5.5 elicited an open probability for the $InsP_3$-gated Ca^{2+} channel of 80 and 77%, respectively, which is the highest activity of the $InsP_3R$ observed to date in a bilayer. This functional interaction at pH 5.5 is likely related to the association of these proteins in secretory granules (18). An elevation of intracellular Ca^{2+} is necessary for the process of fusion and exocytosis of secretory vesicles, and it is likely that the $InsP_3R$/CGB interaction assists in this process. As the vesicles mature, the intraluminal pH becomes more acidic, and the Ca^{2+} is elevated (30). The mature vesicles accumulate near the plasma membrane, and exocytosis is more likely to occur for more acidic secretory granules (30). The increased association between CGB and the $InsP_3R$ as the vesicles mature will enhance the probability of exocytosis because the protein complex shows an increased sensitivity of the $InsP_3R$ to activation by $InsP_3$. This enhanced channel activity will quickly release Ca^{2+} into the microregion between the vesicle and the plasma membrane where the fusion apparatus awaits the Ca^{2+} required for exocytosis.

The functional interaction between the $InsP_3R$ and CGB

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remains even when the pH is at a near physiological level, suggesting that this protein association has roles in numerous locations in a cell. The addition of CGB or CGA/CGB heteromers to the $InsP_3R$ at pH 7.5 elicited an open probability of 40 and 48%, respectively. Again, the level of activation at this pH was elevated from those reported previously when using isolated $InsP_3R$ (31, 32). The association between the $InsP_3R$ and CGB at pH 7.5 can have important regulatory roles in the ER, where CGB is found intraluminally and the $InsP_3R$ is associated with the ER membrane. Although the distribution of the $InsP_3R$ is assumed to be relatively homogeneous, CGB has been shown to be distributed in a regionally specific manner, as seen in neuronally differentiated PC12 cells (5). In these cells CGB is preferentially localized to the neurites, which is the region where intracellular Ca^{2+} signals initiate (5). It is likely that this scenario will be found in other cell types as additional signaling microdomains are identified, and the association of CGB with the $InsP_3R$ may become an important modulator and amplifier of intracellular Ca^{2+} signaling.

Interestingly, the effect of adding a mixture of CGA/CGB is the same as adding CGB alone. When mixed *in vitro*, CGA and CGB form a CGA-CGB heterodimer at pH 7.5 and a CGA_2CGB_2 heterotetramer at pH 5.5 (22). In the experiments presented here the response of the $InsP_3$-gated channel was similar after the addition of CGB alone or a mixture of CGA/CGB at a ratio of 1:1 or 10:1. Unlike CGA, which dissociated completely from the $InsP_3R$ at pH 7.5 (18) and was without any activating effect on the $InsP_3R/Ca^{2+}$ channel (19), the $InsP_3R/Ca^{2+}$ channel-activating effect of CGB at pH 7.5 implies an important physiological role. Because the ratios of CGA to CGB vary among cells (10) and the intraluminal pH levels of the organelles in which CGA and CGB are found also differ, the $InsP_3R/Ca^{2+}$ channel-activating roles of CGA and CGB will also differ. We suggest, therefore, that the different ratios of CGA to CGB found in cells could be very important in modulating the time needed to achieve maturity in different Ca^{2+} storage organelles because the pH dependence of the enhanced Ca^{2+} release will be diminished in CGB-containing vesicles. Similarly, this difference in the ability of CGA and CGB to regulate the $InsP_3R$ could influence the pattern of $InsP_3$-mediated Ca^{2+} release as CGA/CGB ratios vary in different regions of the cell. In particular, the ability of CGB to activate the $InsP_3R/Ca^{2+}$ channel at pH 7.5 is likely to have a big impact on $InsP_3$-mediated Ca^{2+} release from the ER.

In conclusion, we have identified a functional interaction between the intracellular Ca^{2+} release channel, the $InsP_3R$, with the Ca^{2+} storage protein, chromogranin. Interactions occur with CGB, CGA, and with CGA/CGB heteromers. In all of these cases the interactions elicit the highest increase in the purified $InsP_3R$ activity observed. Because these interactions can be modulated, the chromogranins have the potential to serve as robust amplifiers of Ca^{2+} release in a variety of cell types both from secretory granules and from the ER.

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