Localization of Three Types of the Inositol 1,4,5-Trisphosphate Receptor/Ca^{2+} Channel in the Secretory Granules and Coupling with the Ca^{2+} Storage Proteins Chromogranins A and B*

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Although the role of secretory granules as the inositol 1,4,5-trisphosphate (IP₃)-sensitive intracellular Ca^{2+} store and the presence of the IP₃ receptor (IP₃R)/Ca^{2+} channel on the secretory granule membrane have been established, the identity of the IP₃R types present in the secretory granules is not known. We have therefore investigated the presence of different types of IP₃R in the secretory granules of bovine adrenal medullary chromaffin cells using immunogold electron microscopy and found the existence of all three types of IP₃R in the secretory granules. To determine whether these IP₃Rs interact with CGA and CGB, each IP₃R isoform was co-transfected with CGA or CGB into NIH3T3 or COS-7 cells, and the expressed IP₃R isoform and CGA or CGB were co-immunoprecipitated. From these studies it was shown that all three types of IP₃R form complexes with CGA and CGB in the cells. To further confirm whether the IP₃R isoforms and CGA and CGB form a complex in the secretory granules the potential interaction between all three isoforms of IP₃R and CGA and CGB was tested by co-immunoprecipitation experiments of the mixture of secretory granule lysates and the granule membrane proteins. The three isoforms of IP₃R were shown to form complexes with CGA and CGB, indicating the complex formation between the three isoforms of IP₃R and CGA and CGB in the secretory granules. Moreover, the pH-dependent Ca^{2+} binding property of CGB was also studied using purified recombinant CGB, and it was shown that CGB bound 93 mol of Ca^{2+}/mol with a dissociation constant (K₅₀) of 1.5 mM at pH 5.5 but virtually no Ca^{2+} at pH 7.5. The high capacity, low affinity Ca^{2+}-binding property of CGB at pH 5.5 is comparable with that of CGA and is in line with its role as a Ca^{2+} storage protein in the secretory granules.

The secretory granules of endocrine cells, neurons, and neuroendocrine cells contain many hormones, ions, peptides and proteins, including 40 mM Ca^{2+} and 1–2 mM chromogranins A and B in addition to high concentrations of hormones (1–6). The secretory granule contents are secreted to the extracellular space and then into the bloodstream during exocytosis, which is initiated by a sudden increase of intracellular Ca^{2+} concentration. In bovine adrenal medullary chromaffin cells the secretory granules occupy ~10% of the total cell volume (8), thereby storing a majority of the intracellular Ca^{2+} of the cell in the secretory granules. Hence it appears inevitable for the secretory granules to participate in the control of intracellular Ca^{2+} concentrations.

Consistent with this notion, the secretory granules from adrenal medullary chromaffin cells (9), pancreatic acinar cells (10), and the goblet cells (11) were shown to release stored Ca^{2+} in response to IP₃. Using optical sectioning and fluorescent microscope techniques the participation of granular Ca^{2+} in the control of intracellular Ca^{2+} concentration has clearly been demonstrated by measuring the Ca^{2+} concentrations of both the intragranular milieu and of the cytoplasm that is immediately adjacent to the secretory granules simultaneously (11). Moreover, granular Ca^{2+} was shown to participate in the initiation of exocytosis (12–15), underscoring the importance of granular Ca^{2+} not only in the control of intracellular Ca^{2+} concentrations but also in exocytotic processes.

Despite the importance of the IP₃-sensitive intracellular Ca^{2+} store role of secretory granules, the study of IP₃R/Ca^{2+} channels in the secretory granules did not begin until the secretory granule Ca^{2+} storage protein chromogranin A was shown to interact with several integral secretory granule membrane proteins at pH 5.5, including the IP₃R/Ca^{2+} channel (16). Since then the existence of type 3 IP₃R (IP₃R-3) in the insulin-containing secretory granules of pancreatic β-cells had been reported (17) although this study was questioned later due to potential cross-interaction of the IP₃R-3-specific antibody used with insulin crystals found in the secretory granules (18). Nevertheless, the existence of IP₃R/Ca^{2+} channels in the secretory granules of insulin-containing pancreatic β-cells (19) and adrenal medullary chromaffin cells (20) has been confirmed. However, the identity of IP₃R/Ca^{2+} channel types present in the secretory granules is unknown. So far three types of IP₃R (IP₃R-1, -2, and -3) are known to exist, and they interact with each other to form homotetrameric and/or heterotetrameric structures in the native state (21–24), thus forming a Ca^{2+} channel (25). Each type of IP₃R/Ca^{2+} channel is known to have a different sensitivity to both IP₃ and surrounding Ca^{2+} concentrations (26–32). The IP₃ concentration dependence of Ca^{2+} release of these three types has been shown to be in the order of IP₃R-2>IP₃R-1>IP₃R-3, the type 2 being the most active and

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1 The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate; IP₃R, inositol 1,4,5-trisphosphate receptor; CGA, chromogranin A; CGB, chromogranin B; ER, endoplasmic reticulum; HA, hemagglutinin; PBS, phosphate-buffered saline.
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Type 3 being the last active (27). Moreover, the Ca2+ release property of these channels is shown to be dependent on the surrounding Ca2+ concentrations; the Ca2+ release via the IP3R-1 exhibited a biphasic Ca2+ dependence whereas that of IP3R-3 did not exhibit a biphasic Ca2+ dependence (27–32).

Although the secretory granules of bovine adrenal chromaffin cells contain 40 mM Ca2+, more than 99.9% of the intragranular Ca2+ stays bound to intragranular proteins chromogranins, thus leaving only 24 µM free Ca2+ inside the granules (33). Such low concentrations of intragranular free Ca2+ have also been found in mouse pancreatic acinar cell (10), gooblet cell (11), and mast cell (34). Of the chromogranins, chromogranin A and chromogranin B are the two major proteins in virtually all secretory granules of endocrine cells, neurons, and neuroendocrine cells (1–6). For instance, CGA is the most abundant protein in the bovine adrenal medullary chromaffin cells (1–6), whereas CGB is the most abundant protein in human adrenal medullary chromaffin cells (35, 36). Further, CGA is known to bind 55 mol of Ca2+/mol with a Kd of 4 mM at the intragranular pH 5.5 and 32 mol of Ca2+/mol with a dissociation constant (Kd) of 2.7 mM at a near physiological pH 7.5 (37). The high capacity, low affinity Ca2+ binding property of CGA has been proposed to be responsible for the IP3-sensitive Ca2+ store role of secretory granules of bovine adrenal medullary chromaffin cells (9, 37).

We have previously demonstrated direct interaction between the IP3R-1 and CGA and CGB using purified IP3R-1 (20). Furthermore, coupling of CGA and CGB with the IP3R-1 was shown to enhance the IP3-induced Ca2+ release through the IP3R-1/Ca2+ channel reconstituted in the liposomes (38). In addition, the open probability and mean open time of the IP3R-1/Ca2+ channel were also shown to increase in the lipid bilayer as a result of coupled CGA (39). We have therefore investigated in the present study the identity of IP3R types that are present in the secretory granules and the pH-dependent Ca2+ binding property of CGB. Moreover, the potential interaction between each IP3R type and the secretory granule Ca2+ storage proteins chromogranins A and B was also studied.

EXPERIMENTAL PROCEDURES

Antibodies—Production of polyclonal anti-rabbit CGA and CGB antibodies were raised against intact bovine CGA and recombinant CGB. Monoclonal hemagglutinin (HA) antibody was from Roche Molecular Biochemicals. IP3R antibodies specific to the terminal 10–13 amino acids of type 1 (HPHPHMVNPQPOQPA), type 2 (SNTPHENHHMPPA), and type 3 (FVDVQNCMSR) were synthesized with a carboxyl-terminal capping peptide following the procedure described for the IP3R antibody production (16). Monoclonal anti-bovine CGB antibody was produced following the manufacturer’s recommended protocol (British Biocell International). After etching and washing, the grids were placed on 50–µl droplets of solution A (phosphate saline solution, pH 8.2, containing 4% normal goat serum, 1% bovine serum albumin, 0.1% Tween 20, 0.1% sodium azide) for 30 min. Grids were then incubated for 2 h at room temperature in a humidified chamber on 50–µl droplets of each type-specific anti-rabbit IP3R antibody appropriately diluted in solution B (solution A but with 1% normal goat serum), followed by rinses in solution B. The grids were then incubated with 10-nM gold-conjugated goat anti-rabbit IgG diluted in solution A. Controls for the specificity of IP3R immunogold labeling included 1) omitting the primary antibody, and 2) replacing the primary antibody with preimmune serum. After washes in PBS and deionized water, the grids were stained with uranyl acetate (7 min) and lead citrate (2 min), and were viewed with a Zeiss EM912 electron microscope.

Co-transfection and Co-immunoprecipitation of IP3R-2 or -3 and CGA or CGB—The full-length cDNAs for bovine IP3R type 2 (Y. S. Oh, M. K. Kang and S. H. Yoo, GenBank accession no. AF402600) and type 3 (M. K. Kang, Y. S. Oh, and S. H. Yoo, GenBank accession no. AF402601) were subcloned into the vector pcDNA 3.1(+) and tagged with HA to create recombinant plasmid pc-b2 and -b3. NIH3T3 and COS-7 cells, along with CGB expressing pCI-CGB (42) and CGB expressing pCI-CGB (43) cells, were cultured in DMEM medium supplemented with 10% fetal bovine serum. The pc-b2 or -b3 was co-transfected either into NIH3T3 cells along with CGB expressing pCI-CGA, or into COS-7 cells along with CGB expressing pCI-CGB. Expression of IP3R-2 or IP3R-3 as well as CGB or CGB in NIH3T3 and COS-7 cells was confirmed by Western blot analysis (not shown). The rest of the co-immunoprecipitation experiments followed the procedure described for the co-transfection and co-immunoprecipitation of IP3R-1 and CGB on CGB (20).

Co-immunoprecipitation of Three Isoforms of IP3R and CGB and CGA from Secretory Granules of Bovine Adrenal Chromaffin Cells—To perform co-immunoprecipitation experiments, 20 µg of secretory granule lysate proteins and 200 µg of secretory granule membrane proteins (16) were mixed first in 220 µl of the pH 5.5 buffer (20 mM sodium acetate, pH 5.5, 0.1 M KCl, and 0.1% Triton X-100). The mixture was precleared by incubating with protein A-Sepharose for 1 h at 4 °C, followed by centrifugation at 22,000 × g for 5 min. Immunoprecipitation was carried out using the supernatant (∼200 µl) by incubating with 10 µl (1 µg/µl) of the polyclonal anti-rabbit CGB antibody for 1 h at 4 °C. Then 15 µl of a 1:1 slurry of protein A-Sepharose in the pH 5.5 buffer was added to the mixture and incubated for 1 h. The complexes were pelleted by centrifugation at 3,500 × g for 1 min, and the immunoprecipitate was washed five times with the pH 5.5 buffer. The bound proteins were separated on 7.5% SDS-polyacrylamide gels and subjected to immunoblot analysis using each isoform-specific IP3R antibodies and monoclonal CGB and polyclonal CGB antibodies.

Purification of Recombinant CGB and Ca2+ Binding Study—Recombinant bovine CGB was expressed in Escherichia coli and purified as described (42). For the CGB Ca2+ binding study, purified recombinant CGB was coupled to 1 ml of cyanogen bromide-activated Sepha- rose 4B according to the method suggested by the manufacturer (Amersham Pharmacia Biotech). The amount of CGB coupled to Sepharose 4B was determined by extracting with a strong alkali treatment as described previously (20). Upon completion of the coupling, it was estimated that 1 mg of CGB was coupled to 1 ml (wet volume) of Sepharose 4B, and this was used for “Ca2+” binding studies. The Ca2+ binding studies were carried out according to the procedure described for the Ca2+ binding to CGB (20).

RESULTS

Specificity of Isoform-specific IP3R Antibodies—To determine whether each IP3R antibody interacts with each isoform-specific IP3R, the proteins that interact with each isoform-specific IP3R antibody were purified from bovine cerebellum using each isoform-specific antibody-coupled immunoaffinity column (Fig. 1A). Visualization of the purified proteins on an SDS-polyacrylamide gel showed proteins with molecular sizes of 260–280 kDa (Fig. 1A); the proteins purified by type 1 and type 3 specific antibodies were shown in a ~280-kDa region while the protein purified by the type 2-specific antibody was shown in a ~260-kDa region, indicating that each IP3R isoform-specific antibody interacted with different proteins in the 260–280 kDa range. These proteins were shown to bind 300–320 pmol of IP3/mg as determined by a method described previously (21), thus confirming them as IP3Rs.
To further determine whether each of the purified proteins interact with only one isoform-specific IP₃R antibody or not, the purified IP₃Rs were separated on 7.5% SDS-polyacrylamide gels and subjected to immunoblot analysis using each isoform-specific IP₃R antibody. Results in Fig. 1B show that the different IP₃R isoform purified reacts only with the IP₃R antibody with which it is purified and not with other isoform-specific IP₃R antibodies, thus demonstrating the isoform-specific nature of the IP₃R antibodies used.

**Subcellular Localization of IP₃R-1, -2, and -3**—To determine the subcellular location of each type of IP₃R in the adrenal medullary chromaffin cells, immunogold cytochemistry was carried out with bovine adrenal medulla using each IP₃R type-specific antibody (Fig. 2, A-C). As shown in Fig. 2A, the IP₃R type 1 (IP₃R-1)-reacting gold particles were localized in the secretory granules whereas none of the gold particles was localized in the mitochondria. More than half of the gold particles found in the secretory granules are localized in the periphery of the secretory granules along the secretory granule membrane, consistent with the known location of the IP₃R on the secretory granule membrane (20).

To further determine the presence of IP₃R type 2 (IP₃R-2) in the secretory granules, the IP₃R-2-specific antibody was used in the immunogold cytochemistry (Fig. 2B). The IP₃R-2-reacting gold particles were primarily localized on the secretory granule membranes whereas no gold particles were found in the mitochondria (Fig. 2B). In a similar study using IP₃R type 3 (IP₃R-3) specific antibody (Fig. 2C), the IP₃R-3-reacting gold particles were also shown to localize in the secretory granules of bovine adrenal medullary chromaffin cells, more than half of the gold particles localizing on the periphery of the secretory granules (Fig. 2C).

In parallel control experiments that had been carried out either without the primary antibody or with preimmune serum, virtually no gold particles were found in the identical chromaffin cell tissues (not shown), further demonstrating the specificity of the immunogold labeling.

To ensure that the IP₃R antibodies react specifically with the IP₃R on the secretory granule membrane and that each type-specific IP₃R antibody react specifically with the corresponding IP₃R type, the secretory granule membrane proteins from bovine adrenal medulla were separated on a 7% SDS-polyacrylamide gel and subjected to immunoblot analysis using each type-specific IP₃R antibody (Fig. 3). The IP₃R-1-specific antibody reacted exclusively with a ~280-kDa secretory granule membrane protein, suggesting that the IP₃R-1 antibody used in the present study specifically reacted with different IP₃Rs on the secretory granule membrane protein. Some of the type 2 IP₃R appeared to be degraded as an ~83-kDa protein also reacted with the antibody.

**Complex Formation between the IP₃R-1, -2, and -3 and Chromogranins A and B**—We have previously demonstrated the
of the NIH3T3 cell extracts, which had been co-transfected with the same each type-specific IP₃R (IP₃R-1, -2, and -3) antibodies used in the immunogold cytochemical studies. Ten µg of integral membrane proteins were visualized with Coomassie Blue staining (memb. prot.), and the immunoblot results obtained with the IP₃R-1 (type 1), IP₃R-2 (type 2), and IP₃R-3 (type 3) antibodies are shown. A 260–280 kDa membrane protein specifically reacted with each IP₃R antibody.

physical interaction between the IP₃R-1 and CGA and CGB (24). However, the potential physical interactions between the chromogranins and either the IP₃R-2 or the IP₃R-3 have not been studied. Hence, to determine whether the IP₃R-2 and chromogranins A and B are physically linked in the cells, each type of IP₃R and CGA or CGB were co-transfected into NIH3T3 or COS-7 cells and co-immunoprecipitation was carried out (Figs. 4 and 5). As shown in Fig. 4, A and B, immunoprecipitation of the NIH3T3 cell extracts, which had been co-transfected with IP₃R-2 and CGA, by monoclonal HA antibody and immunoblot analysis by CGA and HA antibodies (Fig. 4A) indicated the co-immunoprecipitation of the IP₃R-2 and CGA. Moreover, immunoprecipitation of the same cell extracts with CGA antibody and immunoblot analysis by CGA and HA antibodies (Fig. 4B) also indicated the co-precipitation of CGA and IP₃R-2, demonstrating the complex formation between the IP₃R-2 and CGA in the cell. Furthermore, in similar experiments NIH3T3 cells were co-transfected with the IP₃R-3 and CGA to determine the complex formation between the IP₃R-3 and CGA. As shown in Fig. 4, C and D, immunoprecipitation of the NIH3T3 cell extracts that had been co-transfected with the IP₃R-3 and CGA by monoclonal HA antibody and immunoblot analysis by CGA and HA antibodies (Fig. 4C) indicated the co-immunoprecipitation of the IP₃R-3 and CGA. Further, immunoprecipitation of the same cell extracts with CGA antibody and immunoblot analysis by CGA and HA antibodies (Fig. 4D) also indicated the co-precipitation of CGA and IP₃R-3, indicating the complex formation between the IP₃R-3 and CGA in the cell. Identical results were also obtained with COS-7 cells.

Similarly, immunoprecipitation of the COS-7 cell extracts, which had been co-transfected with the IP₃R-2 and CGB, by monoclonal HA antibody and immunoblot analysis by CGB and HA antibodies (Fig. 5A) indicated the co-precipitation of CGB and IP₃R-2. Immunoprecipitation of the same cell extracts with CGB antibody and immunoblot analysis by IP₃R-2 and CGB antibodies (Fig. 5B) also indicated the co-precipitation of CGB and IP₃R-2, further demonstrating the complex formation between the IP₃R-2 and CGB in the cell. Likewise, immunoprecipitation of the COS-7 cell extracts that had been co-transfected by the IP₃R-3 and CGB by monoclonal HA antibody and immunoblot analysis by CGB and HA antibodies (Fig. 5C) indicated the co-precipitation of CGB and IP₃R-3. Moreover, immunoprecipitation of the same cell extracts with CGB antibody and immunoblot analysis by IP₃R-3 and CGB antibodies...
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(Fig. 5D) also indicated the co-precipitation of CGB and IP₃R-3, again demonstrating the complex formation between the IP₃R-3 and CGB in native state. Again, identical results were also obtained with NIH3T3 cells, further suggesting the universal nature of complex formation between the IP₃Rs and chromogranins A and B.

To further determine whether all three isoforms of IP₃R form complexes with CGB and CAG in the secretory granules of adrenal chromaffin cells, the mixture of secretory granule lysate proteins and the granule membrane proteins of bovine adrenal chromaffin cells was immunoprecipitated with polyclonal anti-rabbit CGB antibody. The presence of each isoform-specific IP₃R, along with CAG and CGB, in the immunoprecipitate was tested using each isoform-specific IP₃R antibodies and monocolonal CGB and polyclonal CAG antibodies (Fig. 6). As shown in Fig. 6, immunoprecipitation of the secretory granule proteins by polyclonal anti-rabbit CGB antibody precipitated all three types of IP₃R (A, type 1; B, type 2; C, type 3) in addition to CAG and CGB, clearly demonstrating the complex formation between all three types of IP₃R and CAG and CGB in the secretory granules of adrenal chromaffin cells.

Ca²⁺ Binding to CGB—Although the pH-dependent low affinity, high capacity Ca²⁺ binding property of CGB has been determined before (37), the low affinity, high capacity Ca²⁺ binding property of CGB remained to be determined. To determine the Ca²⁺ binding property of CGB, we have first expressed bovine CGB in E. coli and obtained large amounts of purified recombinant CGB as described (43). One of the difficulties of determining the Ca²⁺ binding property of CGB was its tendency to aggregate in the presence of Ca²⁺ (44). To avoid the aggregation problem, CGB was first coupled to Sepharose 4B before measuring its Ca²⁺ binding property. As shown in Fig. 7A, Ca²⁺ binding to the immobilized CGB showed drastically different pH-dependent profiles. Large amounts of Ca²⁺ bound to CGB at the intragranular pH 5.5 and the Ca²⁺ binding profile indicated saturation as the free Ca²⁺ concentration increased. In contrast, there was virtually no Ca²⁺ binding to CGB at a near physiological pH 7.5. Scatchard analysis (45) of the binding result (Fig. 7B) showed that CGB bound 93 mol of Ca²⁺/mg (93 mol of Ca²⁺/mol) at pH 5.5 with a dissociation constant (Kᵣ) of 1.5 mM. Although another high capacity, low affinity Ca²⁺-binding protein CAG is also known to bind more Ca²⁺ at pH 5.5 than at pH 7.5 (37), the drastic difference in the Ca²⁺-binding property of CGB at the two pH levels is surprising. Given that the acidic pH is expected to reduce ionic interaction between negatively charged acidic protein CGB and positively charged calcium ions, the high capacity Ca²⁺ binding to CGB at pH 5.5 appears to be due to the CGB conformation at the acidic pH.

![Co-immunoprecipitation of all three isoforms of IP₃R and CGB with CGB in the secretory granule proteins of bovine adrenal chromaffin cells](image)

**Fig. 6.** Co-immunoprecipitation of all three isoforms of IP₃R and CGB with CGB in the secretory granule proteins of bovine adrenal chromaffin cells. The mixture of 20 μg of secretory granule lysate proteins and 200 μg of secretory granule membrane proteins were immunoprecipitated with 10 μg of polyclonal anti-rabbit CGB antibody, and the immunoprecipitates were analyzed by immunoblotting for the presence of IP₃R-1 (A), -2 (B), and -3 (C) using each isoform-specific antibodies, and polyclonal CAG and monoclonal CGB antibodies.

![Binding of Ca²⁺ to chromogranin B immobilized on Sepharose 4B and Scatchard analysis](image)

**Fig. 7.** Binding of Ca²⁺ to chromogranin B immobilized on Sepharose 4B and Scatchard analysis. Ca²⁺ binding to CGB coupled to Sepharose 4B was measured at pH 5.5 and pH 7.5 and was analyzed according to Scatchard (45). Buffers used were either 20 mM sodium acetate, pH 5.5, 0.1 mM KCl, or 20 mM Tris-HCl, pH 7.5, 0.1 mM KCl. A, binding to Ca²⁺ to CGB was measured as described previously (37). Data are means ± S.E. of three representative experiments at pH 5.5 (●) and pH 7.5 (○). B, Scatchard analysis of the Ca²⁺ binding data at pH 5.5 in panel A. Chromogranin B bound 1290 nmol of Ca²⁺/mg (93 mol of Ca²⁺/mol) with a Kᵣ of 1.5 mM at pH 5.5. B/F, bound/free.

**DISCUSSION**

Since the demonstration that the secretory granules of adrenal medullary chromaffin cells release Ca²⁺ in response to IP₃ (9), the IP₃-sensitive Ca²⁺ store role of secretory granules has also been shown in other secretory cells (10, 11). The presence of IP₃R on the secretory granule membrane has been shown in adrenal chromaffin cells (16, 20) and pancreatic β-cells (19). Further, the IP₃R was demonstrated to co-localize with chromogranin A in the secretory granules of bovine adrenal medullary chromaffin cells (20). Nevertheless, it was not known what isoforms of IP₃R exist on the secretory granule membrane. Present results demonstrate the presence of all three types of IP₃R in the secretory granules of bovine adrenal medullary chromaffin cells (20). By co-transfection of IP₃R-1 and CAG or CGB into COS-7 cells, followed by co-immunoprecipitation of the expressed proteins, we have shown previously that the IP₃R-1 and CAG or CGB form complexes in the cell (20).

Likewise, co-transfection and co-immunoprecipitation studies using the IP₃R-2, -3 and chromogranins A and B further demonstrated the complex formation between CAG and the IP₃R-2 or -3 (Fig. 4) and between CGB and the IP₃R-2 or -3 (Fig. 5). The complex formation between the IP₃Rs and chromogranins A and B appeared to be an intrinsic property of these Ca²⁺ channel and Ca²⁺ storage proteins, reflecting the functional coupling in the secretory granules of secretory cells. It is hence of great importance that the results in Fig. 6 clearly demon-
strate that the secretory granules of bovine adrenal chromaffin cells not only contain all three isoforms of IP\(_3\)R but also they form complexes with CGA and CGB. This is the first time that chromogranins A and B were shown to form complexes with all three isoforms of IP\(_3\)R in native state. The widespread presence of CGA and CGB in the secretory granules of secretory cells appears to strongly imply the physiological importance of the coupling between the IP\(_3\)Rs and the Ca\(^{2+}\) storage proteins in neurons, endo/exocrine cells, and neuroendocrine cells.

In line with this importance, the coupling of CGA with the IP\(_3\)R was indeed shown to modulate the IP\(_3\)/Ca\(^{2+}\) channel function (38). Using liposomes incorporated with the purified IP\(_3\)R-1 in the presence and absence of encapsulated CGA, it was found that the coupled CGA changed the conformation of IP\(_3\)R-1 such that more IP\(_3\) bound to the incorporated IP\(_3\)R-1 and more Ca\(^{2+}\) were released from the proteoliposomes in the presence of coupled CGA (38). The IP\(_3\)/Ca\(^{2+}\) channel modulatory role of CGA was observed only when the intraliposomal pH was maintained at 5.5 (38), the intragranular pH at which the IP\(_3\)R and CGA remained coupled (16, 20). But when the intraliposomal pH was changed to 7.5, CGA failed to exhibit any effect (38) due to its dissociation from the IP\(_3\)/Ca\(^{2+}\) channel (16, 20). It was further shown that the open probability and mean open time of the IP\(_3\)/Ca\(^{2+}\) channel were markedly increased in the presence of coupled CGA as determined by single channel recordings using lipid bilayers (39). But when CGA was decoupled from the IP\(_3\)/Ca\(^{2+}\) channel by changing the pH from 5.5 to 7.5, the mean open time and open probability of the IP\(_3\)/Ca\(^{2+}\) channel returned to the levels obtained in the absence of CGA (39).

In addition to the increased binding of IP\(_3\) and the conformational change of the IP\(_3\)/Ca\(^{2+}\) channel as a result of CGA coupling, our preliminary results also suggested that the IP\(_3\) sensitivity of the IP\(_3\)/Ca\(^{2+}\) channel increases at least 5-fold in the presence of coupled CGA, i.e. 5-fold less IP\(_3\) is needed to induce release of same amount of Ca\(^{2+}\) in the presence of coupled CGA than in the absence. Moreover, the IP\(_3\)/Ca\(^{2+}\) channel modulatory effect of CGA was also similarly exhibited by CGB. From these results it appears that the coupled Ca\(^{2+}\) storage proteins CGA and CGB directly modulate the IP\(_3\)/Ca\(^{2+}\) channel such that not only the IP\(_3\)-mediated Ca\(^{2+}\) release property but also the IP\(_3\) sensitivity of the IP\(_3\)/Ca\(^{2+}\) channels are altered. In light of the fact that chromogranins are most abundant in the secretory granules, it is likely that the secretory granules will be more sensitive to IP\(_3\) than other organelles such as the ER and nucleus. Interestingly, it has continuously been shown that despite the presence of IP\(_3\)R/Ca\(^{2+}\) channels in the nucleus (46, 47), endoplasmic reticulum (21–32), and secretory granules, a given dose of IP\(_3\) does not necessarily evoke a uniform release of Ca\(^{2+}\) from these organelles (29). This difference in the amount of Ca\(^{2+}\) released from each IP\(_3\) sensitive store in response to a same concentration of IP\(_3\) is likely to be influenced by the presence or absence of chromogranins inside the IP\(_3\)/Ca\(^{2+}\)-containing organelles.

In this regard, the example of different sensitivity of IP\(_3\) sensitive intracellular Ca\(^{2+}\) store to IP\(_3\) has clearly been shown in the case of pancreatic acinar cell (10, 48, 49). The pancreatic acinar cell is highly polarized that all the zymogen granules are localized in the apical secretory granule region whereas the endoplasmic reticulum and nucleus are localized in the basal region of the cell (50). The ER-rich, nucleus-containing cytoplasmic area encircles the apical granule region where the cluster of zymogen granules is found. Due to this kind of highly polarized morphology of pancreatic acinar cells, any IP\(_3\) that is produced at a localized plasma membrane region away from the granule area should travel through the ER-rich, nucleus-containing cytoplasm before it can reach the secretory granule area. Indeed, exposure of pancreatic acinar cells to the IP\(_3\)-producing agonist such as acetylcholine and cholecystokinin has been shown to release Ca\(^{2+}\) from the secretory granule area first although the IP\(_3\)-producing agonist was applied to the cell surface far removed from the secretory granule-rich area of the cell (10, 48, 49). Therefore, the release of Ca\(^{2+}\) from the secretory granules of pancreatic acinar cell, ahead of the ER and nucleus (10, 48, 49), clearly demonstrates the fact that the secretory granules are more sensitive to IP\(_3\) than the ER or the nucleus.

In line with the high capacity, low affinity Ca\(^{2+}\) binding property of CGA at two different conformations (37), CGB also bound 93 mol of Ca\(^{2+}\)/mol with a \(K_d\) of 1.5 mM at pH 5.5, but bound virtually no Ca\(^{2+}\) at pH 7.5 (Fig. 7). These results clearly point out a close relationship between the Ca\(^{2+}\) binding property and the conformation of chromogranins A and B. Considering that binding of IP\(_3\) to the IP\(_3\)/Ca\(^{2+}\) channel is known to change the conformation of the IP\(_3\)/Ca\(^{2+}\) channel (51), it is likely to assume that the conformational change of the IP\(_3\)/Ca\(^{2+}\) channel that results from IP\(_3\) binding will automatically be transmitted to the coupled chromogranins, which in turn could lead not only to the change in conformation of the chromogranins but also to the change in affinity for Ca\(^{2+}\). This change in affinity for Ca\(^{2+}\) is expected to change the free Ca\(^{2+}\) concentrations inside the secretory granules instantaneously, probably changing from low to high free Ca\(^{2+}\) concentrations. Therefore, given the large amount of calcium (40 mM) in the secretory granule the sudden dissociation of even a small fraction of the bound Ca\(^{2+}\) from the chromogranins is likely to produce a relatively high free Ca\(^{2+}\) concentration that could be released into the cytoplasm through the IP\(_3\)/Ca\(^{2+}\) channels. Given the direct physical coupling between the IP\(_3\)/Ca\(^{2+}\) channels and the Ca\(^{2+}\) storage proteins CGA and CGB in the secretory granules, the whole train of events from the binding of IP\(_3\) to the release of Ca\(^{2+}\) is expected to take place very fast, in accordance with the fast stimulus secretion responses of secretory cells.

In light of the lack of other examples of direct coupling between an ion channel and its cognate ion storage protein, the coupling between the IP\(_3\)/Ca\(^{2+}\) channel and chromogranins A and B stands out as the only known example of direct coupling between an ion channel and its cognate ion storage protein. In particular, in view of the fact that the IP\(_3\)/Ca\(^{2+}\) channel exists as homo- and/or heterotetrameric structure (21–24) and that chromogranins A and B can also exist either in homotetrameric CGA (52, 53) or in heterotetrameric CGA\(_A\)CGB\(_B\) structure (54), the coupling between these two classes of proteins presents the possibility of coupling between tetrameric IP\(_3\)/Ca\(^{2+}\) channel and tetrameric chromogranin (54). In this regard, the present results demonstrating the complex formation between all three types of IP\(_3\)/Ca\(^{2+}\) channels and chromogranins A and B in the secretory granules further underscore this possibility. Given that the IP\(_3\)/Ca\(^{2+}\) channels in the cell respond differently even to same amount of IP\(_3\) (26–32) our present results shed further light on the molecular structural basis of why the IP\(_3\)/Ca\(^{2+}\) channels respond differently to a given concentration of IP\(_3\) in the cell.

Although the ER also contains calreticulin, which is a high capacity, low affinity Ca\(^{2+}\) storage protein (55), binding 25–50 mol of Ca\(^{2+}\)/mol with a \(K_d\) of 1–2 mM (56–58), it is not known whether calreticulin is directly coupled to the IP\(_3\)/Ca\(^{2+}\) channel in the ER. Calreticulin is estimated to represent 1–2% of total microsomal proteins (57), whereas chromogranins A and

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(59) is far lower than 40 mM Ca\(^{2+}\) in the secretory granules (1, 2, 33). Hence, these facts further highlight the need for cells to better control the intracellular Ca\(^{2+}\) concentrations through fine-tuning of the Ca\(^{2+}\) storage/release mechanisms of the secretory granules. In this respect, the existence of all three types of IP\(_3\)/Ca\(^{2+}\) channel and the abundant presence of Ca\(^{2+}\) storage proteins chromogranins A and B in the secretory granules will be pivotal for the secretory granules in fine-tuning the intracellular Ca\(^{2+}\) concentrations in the secretory cells.

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Localization of Three Types of the Inositol 1,4,5-Trisphosphate Receptor/Ca\(^{2+}\) Channel in the Secretory Granules and Coupling with the Ca\(^{2+}\) Storage Proteins Chromogranins A and B
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