

Activation of the Inositol 1,4,5-Trisphosphate Receptor by the Calcium Storage Protein Chromogranin A*

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Secretory granules of neuroendocrine cells are inositol 1,4,5-trisphosphate (InsP₃)-sensitive Ca²⁺ stores in which the Ca²⁺ storage protein, chromogranin A (CGA), couples with InsP₃-gated Ca²⁺ channels (InsP₃R) located in the granule membrane. The functional aspect of this coupling has been investigated via release studies and planar lipid bilayer experiments in the presence and absence of CGA. CGA drastically increased the release activity of the InsP₃R by increasing the channel open probability by 9-fold and the mean open time by 12-fold. Our results show that CGA-coupled InsP₃Rs are more sensitive to activation than uncoupled receptors. This modulation of InsP₃R channel activity by CGA appears to be an essential component in the control of intracellular Ca²⁺ concentration by secretory granules and may regulate the rate of vesicle fusion and exocytosis.

CGA¹ is a member of the granin protein family and is stored in high concentrations in the large dense core secretory granules of most endocrine and neuroendocrine cells as well as in many nerve cells in the periphery and brain (1, 2). CGA, the first member of the granin family to be discovered (3–5), has a wide variety of functions, both extracellular and intracellular.

As one of its extracellular functions, CGA acts as a prohormone, a protein that contains numerous sites for proteolytic processing. Following secretion, extracellular proteases cleave CGA, generating several peptide fragments with biological activity, including pancreastatin (6, 7), vasostatins I and II (8–10), parastatin (11), catestatin (12), and chromacin (13). In healthy individuals, CGA and its peptide fragments are present in the circulatory system in low nanomolar quantities. However, in patients suffering from pheochromocytoma and other neuroendocrine tumors, concentrations are significantly higher (14). Elevated plasma levels of CGA are associated with a number of pathological conditions making the protein an ideal marker not only for neuroendocrine tumors but also for chronic heart failure and brain disorders such as Parkinson's and Alzheimer's diseases (15).

Among its intracellular roles, CGA has been shown to interact with ATP, catecholamines, and Ca²⁺ (16, 17), to acidify the intravesicular medium and to sort proteins for the regulated secretory pathway via a range of protein-protein interactions (15). These sorting functions include aggregation with chromogranin B, complexing with dopamine β -hydroxylase, *t*-plasma-activator, and binding secretory granule membrane constituents such as the InsP₃R (15).

In recent years, secretory granules of neuroendocrine cells have been identified as inositol (1,4,5)-trisphosphate (InsP₃)-sensitive Ca²⁺ stores (18–20). In the granules CGA forms a tetramer and appears to bind four molecules of the intraluminal loop of the InsP₃R at the intravesicular pH 5.5 (21–23). *In vitro* studies show that purified InsP₃R interact directly with CGA at this pH and dissociate from it at pH 7.5, a pH encountered when exocytosis occurs (24). Co-transfection of InsP₃R and CGA into COS-7 cells followed by co-immunoprecipitation demonstrates that these two proteins form a complex *in vivo* (24).

We have investigated the functional aspect of this coupling via InsP₃-mediated Ca²⁺ release studies using InsP₃R-reconstituted liposomes in the presence and absence of CGA. We have further characterized the molecular basis of this phenomenon at the single channel level using planar lipid bilayer studies. In the presence of CGA the open probability and mean open time of the InsP₃R channel increases significantly. Hence, modulation of InsP₃R channel activity by CGA appears to be an essential component in the control of intracellular Ca²⁺ concentration in secretory granules.

MATERIALS AND METHODS

Purification of the InsP₃ Receptor

For Flux Studies—The type I InsP₃ receptor was isolated from bovine cerebella as described previously (25). 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 PMSF, 10 μ M leupeptin, 10 μ M pepstatin), homogenized, and centrifuged at 2000 \times *g* for 10 min at 4 °C. The supernatants were re-centrifuged at 105,000 \times *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 PMSF, 10 μ M leupeptin, 10 μ M pepstatin) containing 1% Triton X-100, stirred for 1 h, and then centrifuged at 32,000 \times *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 PMSF, 10 μ M leupeptin, 10 μ M pepstatin), and applied to an InsP₃R antibody-coupled immunoaffinity column (0.35 \times 1 cm) equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM CaCl₂. The protein-loaded column was washed with 20 bed volumes of this buffer, and the InsP₃R was eluted by 10 ml of elution buffer (0.1 M glycine, pH 2.8, 0.2% Triton X-100, 0.5 M NaCl, 1 mM β -mercaptoethanol, 0.1 mM PMSF, 10 μ M leupeptin, 10 μ M 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); it was then applied to a benzamidine-Sepharose column equilibrated with Buffer V (20 mM

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¹ The abbreviations used are: CGA, chromogranin A; InsP₃, inositol 1,4,5-trisphosphate; InsP₃R, InsP₃ receptor; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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 type I InsP_3 receptor was solubilized in 1% CHAPS and purified from mouse cerebellum using heparin affinity and concanavalin A-Sepharose column chromatography as described previously (26). The purified InsP_3R was then incorporated into liposomes by adding 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_3 Dose Response for InsP_3R — InsP_3R proteoliposomes were formed as described previously (25). Some of these proteoliposomes had CGA encapsulated in them, and the remainder was 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 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 (27).

InsP_3 dose-dependent Ca^{2+} release was also measured by the intensity of indo-1 fluorescence after each InsP_3 addition and was compared with the fluorescence intensity after the addition of Triton X-100 instead of InsP_3 . In these experiments, 10 μM indo-1 was used, which is a high enough concentration to buffer released Ca^{2+} , thus precluding the possibility of Ca^{2+} regulation of the InsP_3 -induced release.

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) prior to membrane formation. The two compartments are defined as *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 ($[\text{Ca}^{2+}]_{\text{free}} = 200$ nM), ATP 0.5 mM, and ruthenium red 2 μM .

The *trans* (luminal) compartment consisted of 250 mM HEPES, adjusted to pH 5.5 (as purified InsP_3R was used in these experiments, the pH could be changed using 70 mM HCl, and 53 mM $\text{Ba}(\text{OH})_2$). Single channel currents were amplified using a bilayer clamp amplifier (Warner Instruments) and recorded on digital tape. Data was filtered with an eight-pole Bessel filter to 500 Hz, digitized to 2 kHz, transferred to a personal computer, and analyzed using the pClamp 6.0 (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. CGA (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 adding Tris (final concentration 110 mM) to pH 7.5 (to dissociate CGA from InsP_3R), and InsP_3R single channel activity was recorded.

These experiments were repeated (i) in the presence of increasing doses of InsP_3 (over the range of 0.2–2 μM) to the *cis* compartment and (ii) at a fixed InsP_3 concentration of 2 μM in the presence of increasing free Ca^{2+} concentrations (over the range of 0.01–1 μM) to the *cis* compartment. Both steps i and ii were carried out in the presence and absence of 1 μg of CGA in the *trans* compartment, and InsP_3R single channel activity was recorded.

RESULTS

Effect of CGA on InsP_3 -mediated Ca^{2+} Release—The effect of CGA on InsP_3 dose response for type I InsP_3R from bovine cerebella was investigated initially using Ca^{2+} release studies. InsP_3 -induced Ca^{2+} release from InsP_3R -reconstituted liposomes was monitored both in the presence and absence of CGA (Fig. 1). InsP_3 -induced Ca^{2+} efflux through the proteoliposomes (300 μM Ca^{2+} inside) was determined by the change of indo-1 fluorescence at 393 nm. The total amount of Ca^{2+} in the liposomes was determined by adding 1% Triton X-100, and this was the value set at 100%. Given this information, the total

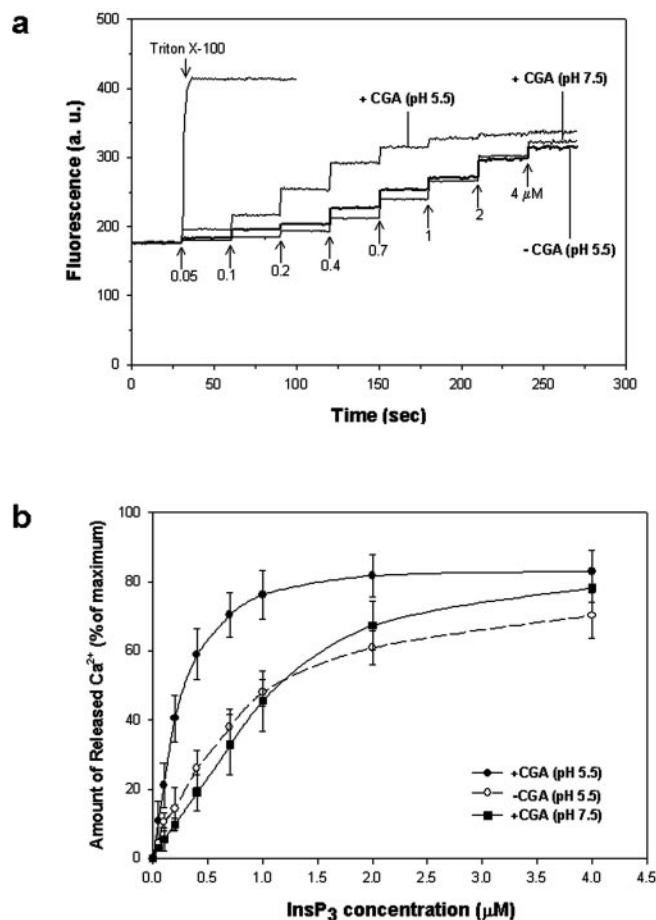


FIG. 1. Effects of CGA on InsP_3 -induced Ca^{2+} release from InsP_3R -reconstituted liposomes. *a*, InsP_3 -induced Ca^{2+} efflux through the proteoliposomes (300 μM Ca^{2+} inside) was determined by the fluorescence change of indo-1 at 393 nm after a series of incremental additions of InsP_3 (4.0 μM final) to the proteoliposome solution. The intraliposomal pH was pH 5.5 unless stated otherwise. InsP_3 -induced Ca^{2+} release was also measured in the presence of encapsulated CGA at intraliposomal pH of 5.5 and 7.5. *b*, the amount of released Ca^{2+} measured after a series of incremental additions of InsP_3 (4.0 μM final) to the proteoliposome solution containing 1 μM indo-1 at 35°C . InsP_3 -induced fluorescent changes were compared with that obtained by the addition of Triton X-100 (this value was set at 100%).

amount of InsP_3 -releasable Ca^{2+} was estimated to be 60%. When CGA was present inside the vesicle at pH 5.5, the pH value at which CGA associates with the InsP_3R , InsP_3 -induced Ca^{2+} release was significantly enhanced (see Fig. 1*a*). A K_{app} value for InsP_3 of 0.2 μM was obtained. When the pH was maintained at 7.5, however, the fluorescent changes seen at each InsP_3 dose more closely resembled those seen in the absence of CGA at pH 5.5 (K_{app} values for InsP_3 of 0.8 and 0.9 μM , respectively), further supporting the pH dependence of the $\text{InsP}_3\text{R}/\text{CGA}$ interaction. The presence of CGA at pH 5.5 markedly increased the apparent affinity of the receptor for InsP_3 when compared with the Ca^{2+} release obtained in the absence of CGA. This result complements the effect of CGA on InsP_3 binding to its receptor (25). Even at InsP_3 concentrations lower than those published previously (starting at 0.05 μM) (25), an increase in apparent affinity for InsP_3 was seen (Fig. 1*b*).

Effect of CGA upon InsP_3R Channel Activity—The enhancement of Ca^{2+} release from InsP_3R -reconstituted liposomes as a result of the pH-dependent interaction of CGA with InsP_3R , illustrates a functional phenomenon associated with this coupling. To further define the actual mechanism of action, we investigated these effects at the single channel level using

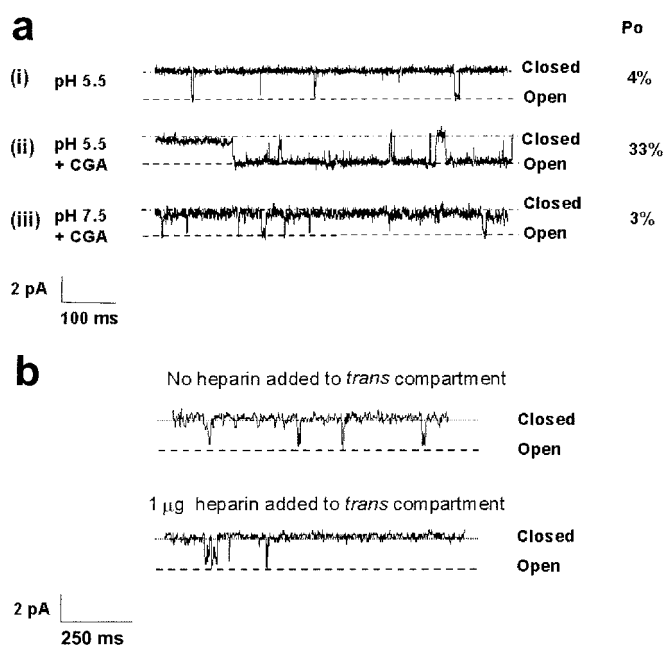


FIG. 2. *a*, single channel activity in the presence of InsP_3R . Trace *i*, InsP_3R single channels activated by $2 \mu\text{M}$ InsP_3 (added to the *cis* compartment). Openings are defined as downward deflections from the base line. Trace *ii*, conditions are same as for trace *i*, except CGA ($1 \mu\text{g}$) was added to the *trans* compartment and mixed. The pH of the *trans* compartment was pH 5.5. Trace *iii*, The pH inside the *trans* compartment was changed by the addition of Tris (final concentration 110 mM) to pH 7.5 (to dissociate CGA from IP_3R). *b*, effect of heparin, a charged macromolecule, on InsP_3R function. Top trace, InsP_3R single channels activated by $2 \mu\text{M}$ InsP_3 (added to the *cis* compartment). Openings are defined as downward deflections from the base line. Lower trace, heparin ($1 \mu\text{g}$) was added to the *trans* compartment and mixed. The pH of the *trans* compartment was pH 5.5. As CGA is a highly charged protein, these control experiments exclude the possibility that any charged macromolecule could be responsible for the effects observed in this study.

InsP_3R incorporated into planar lipid bilayers.

Under control conditions, in the absence of luminal CGA and in the presence of cytosolic free Ca^{2+} (300 nM) and InsP_3 ($2 \mu\text{M}$), mouse InsP_3R type I, single channel activity was observed (see Fig. 2*a*, trace *i*). Single channel currents of $\sim 2 \text{ pA}$ were seen, and the presence of a pH gradient between the *trans* and *cis* compartments (pH 5.5:pH 7.35) did not affect channel activity. Two populations of mean open times were seen with values of 0.864 ± 0.039 and $8.84 \pm 0.014 \text{ ms}$ (Fig. 3*a*). The data set is further expanded (Fig. 3*b*) to emphasize the complete population of longer open times. The open probability (P_o) was $4.0 \pm 1.0\%$ (S.E.) ($n = 4$).

Less than 1 min after the addition of $1 \mu\text{g}$ of CGA to the *trans* compartment, a dramatic change in channel activity was observed (Fig. 2*a*, trace *ii*). The magnitude of the single channel current remained unaltered; however, significant differences were apparent in mean open times and P_o . Two populations of mean open time were evident, as before, but were greatly increased over control values (Fig. 3*c*). Values of 2.61 ± 0.024 and $103.5 \pm 0.003 \text{ ms}$ were obtained, illustrating an approximate 3-fold and 12-fold increase of open time in each respective population. This is displayed even more clearly when the scale from Fig. 3*c* is expanded to focus on the population of longer open times (Fig. 3*d*). Furthermore, a large increase in P_o over control levels was seen with a value of $33.0 \pm 8.5\%$ (S.E.) ($n = 4$).

By changing the pH of the *trans* compartment to 7.5, a condition known to cause dissociation of CGA from InsP_3R , channel activity essentially reverted to control levels (Fig. 2*a*,

trace *iii*). The two populations of mean open time were reduced to 1.05 ± 0.014 and $7.19 \pm 0.013 \text{ ms}$ ($n = 4$, Fig. 3*e*, and compare with Fig. 3*a*), and the P_o was reduced to $3.0 \pm 1.6\%$ (S.E.), a value close to that seen for the control. The addition of heparin, an InsP_3R -specific antagonist, to the *cis* compartment inhibited channel activity completely.

A similar study was carried out using microsomes from mouse cerebellum to see whether the effects of CGA would still be seen in native tissue the same as in purified protein. These experiments were complicated by the fact that HCl was present in the *trans* compartment, a condition necessary for maintaining the pH at 5.5. Thus chloride channels present in the microsomes were activated, making the analysis difficult. Nonetheless, under comparable control conditions to those described for the purified receptor, the open probability was 8%; and upon addition of CGA to the *trans* compartment this increased to 52%. Altering the pH of the *trans* compartment lowered the open probability to $\sim 1\%$.

The addition of CGA to the *trans* compartment in the absence of InsP_3R had no effect upon the bilayer itself, and CGA did not potentiate any InsP_3R channel activity in the absence of cytosolic InsP_3 . Furthermore, the addition of $1 \mu\text{g}$ of CGA to the *cis* compartment in the presence of InsP_3R and InsP_3 did not affect channel activity.

As CGA is a highly charged protein, control experiments were carried out to exclude the possibility that any charged macromolecule could be responsible for the effects observed in this study. Heparin is one such charged macromolecule and is known for its inhibitory effects on the InsP_3R when exposed to the cytosolic face of the receptor, although it is not known to bind to its luminal face. The addition of $1 \mu\text{g}$ of heparin to the *trans* compartment (see Fig. 2*b*) did not alter channel open probability (5% for the control compared with 4.4% in the presence of luminal heparin), indicating that CGA does have a specific modulatory effect on InsP_3Rs .

Effect of CGA on InsP_3 Dose Response for InsP_3R —Single channel activity as a function of InsP_3 concentration, both in the presence and absence of CGA, was characterized next (Fig. 4, *a* and *b*). Over a range of InsP_3 concentrations starting at $0.2 \mu\text{M}$, the open probability was greater in the presence of CGA, with a 14-fold increase observed at $2 \mu\text{M}$ InsP_3 . When the pH of the *trans* compartment was changed to 7.5, the P_o was reduced to that seen in the control experiments. The results obtained from the single channel experiments concur with those seen in the Ca^{2+} release studies (see Fig. 1) in that, at each InsP_3 concentration and in the presence of CGA, a significant increase in P_o is concomitant with an increased amount of released Ca^{2+} .

Effect of CGA on Ca^{2+} Dependence for InsP_3R —The Ca^{2+} -dependence of the InsP_3R was investigated in the absence and presence of CGA (Fig. 5, *a* and *b*) at a fixed InsP_3 concentration of $2 \mu\text{M}$ and over a Ca^{2+} concentration range of 0.01 – $1.0 \mu\text{M}$. As the Ca^{2+} concentration in the *cis* compartment increased successively from 0.01 to $0.3 \mu\text{M}$, the P_o increased, reaching a maximum value of 4% at $1 \mu\text{M}$ Ca^{2+} ($p\text{Ca} 6$) in the absence of CGA (Fig. 5*b*). At Ca^{2+} concentrations higher than $0.3 \mu\text{M}$ ($p\text{Ca} 6.5$), no inhibition was seen (see Fig. 5*b*, expanded section). This lack of inhibition by free Ca^{2+} has been observed previously for purified receptor (28, 29) and contrasts to the inhibition seen with microsomes (30). Repetition of this experiment, with the addition of CGA to the *trans* compartment, produced dramatic increases in channel activity (Fig. 5*a*). At a Ca^{2+} concentration of $0.01 \mu\text{M}$ ($p\text{Ca} 8$) the P_o is effectively zero in the absence of CGA, in contrast to the P_o observed when CGA is present, which expressed as a percentage of total open probability is 34%. Furthermore, the P_o effectively remains at this level

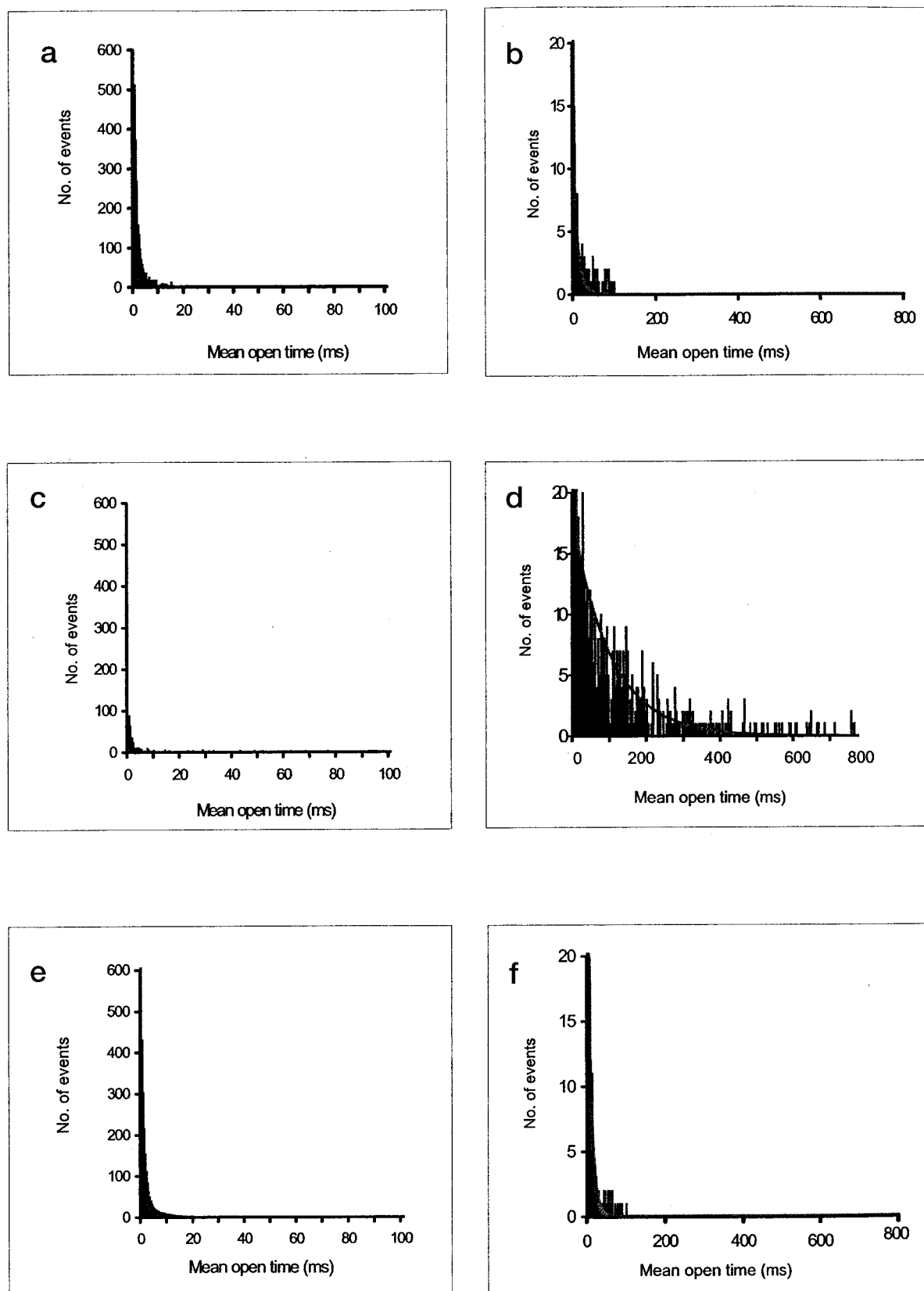


FIG. 3. Mean open times for InsP_3R in the presence and absence of CGA. *a*, mean open times for InsP_3R in the absence of CGA. Two populations of open times are observed with values of 0.864 ± 0.039 and 8.84 ± 0.014 ms. *b*, an expanded section with a fit to the data. This experiment is typical of four similar but separate experiments. *c*, mean open times for InsP_3R in the presence of CGA. Again, two populations of open times are observed with values of 2.61 ± 0.024 and 103.5 ± 0.003 ms, but now the number of longer openings has increased, and the mean open time is greater. *d*, an expanded section with a fit to the data. The increase in the number of longer open times is illustrated clearly. This experiment is typical of four similar but separate experiments. *e*, mean open times for InsP_3R following dissociation of CGA by pH change (pH 5.5 to pH 7.5). Two populations of open times are observed with values of 1.05 ± 0.014 and 7.19 ± 0.013 ms, and the open times have returned to control levels. *f*, an expanded section with a fit to the data ($n = 4$).

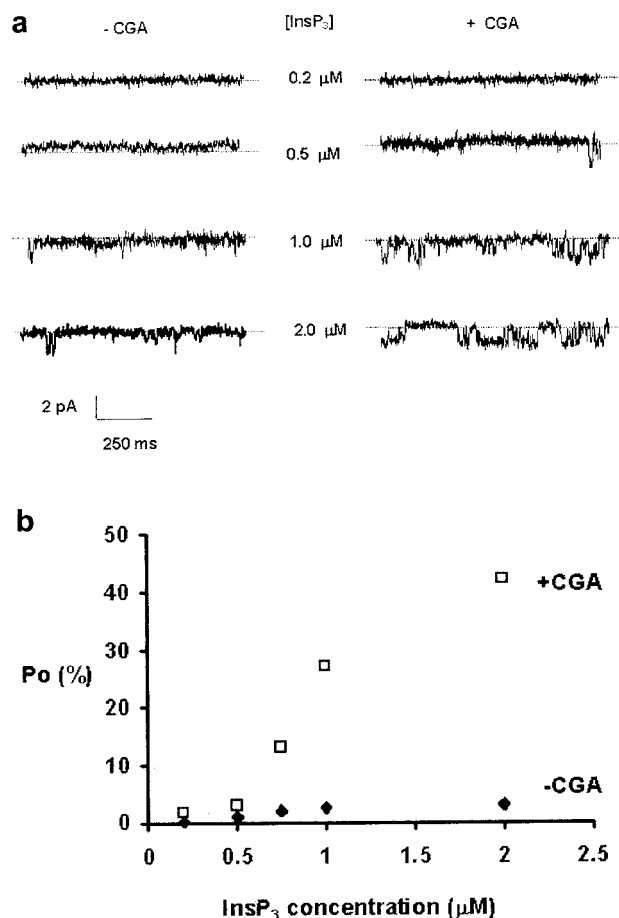


FIG. 4. Effect of CGA on InsP_3 dose response for InsP_3R . Bilayer experiments were repeated, same conditions as before, except over a range of InsP_3 concentrations. *a*, InsP_3R single channels activated by 0.2–2 μM InsP_3 (added to the *cis* compartment) are shown. The InsP_3 concentration used is shown in the figure next to the relevant traces. Openings are defined as downward deflections from the base line. The channel traces shown on the *left* are in the absence of CGA. Channel traces shown on the *right* are in the presence of CGA (1 μg) in the *trans* compartment maintained at pH 5.5. *b*, at each InsP_3 concentration the open probability was measured in the presence (\square) and absence (\blacklozenge) of CGA. CGA significantly enhances P_o by at least 10-fold. The data set shown is typical of four similar experiments.

irrespective despite increasing Ca^{2+} concentrations, and again no inhibition by Ca^{2+} is seen. The activating phase of the Ca^{2+} dependence seen in the absence of CGA is not apparent in its presence. The channel has reached maximal open probability at $p\text{Ca}$ 8. Thus, the lack of dependence on Ca^{2+} for activation in the presence of CGA and 2 μM InsP_3 is clearly illustrated.

DISCUSSION

Secretory granules of endocrine and neuroendocrine cells have been shown to serve as InsP_3 -sensitive intracellular Ca^{2+} stores (18, 31), and additional evidence from goblet cells has demonstrated their direct participation in the control of cytoplasmic Ca^{2+} (20). Chromogranins are Ca^{2+} storage proteins that are found in secretory granules in millimolar concentrations (1–2 mM) (1, 32), and CGA binds 32 mol of Ca^{2+} /mol with a dissociation constant of 2.7 mM at pH 7.5 and 55 mol of Ca^{2+} /mol with a dissociation constant of 4 mM at pH 5.5 (33). Given the high capacity Ca^{2+} binding of CGA, most of the 40 mM intravesicular Ca^{2+} remains bound, thus yielding a total free Ca^{2+} concentration of $\sim 24 \mu\text{M}$ inside the granules (34). As secretory granules occupy about 10% of the total cell volume (at least in bovine chromaffin cells) (32) and have a high storage

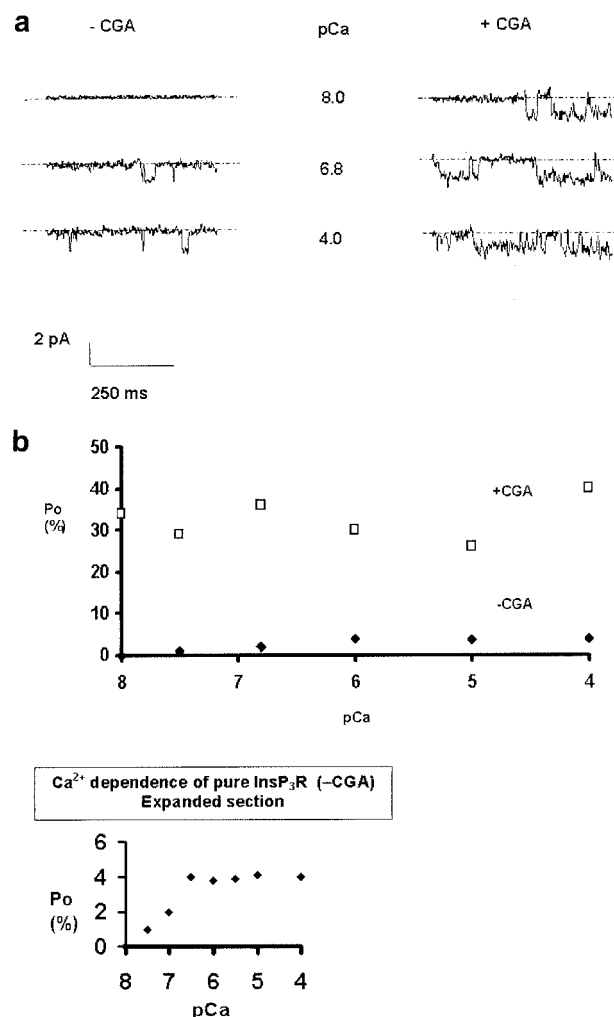


FIG. 5. Effect of CGA on Ca^{2+} dependence for InsP_3R . Bilayer experiments were repeated under the same conditions as described under “Materials and Methods” except over a range of cytosolic Ca^{2+} concentrations (0.01–10 μM). *a*, InsP_3R single channels activated by 2 μM InsP_3 and in the presence of different Ca^{2+} concentrations (added to the *cis* compartment) are shown. The free Ca^{2+} concentration used is shown next to the relevant traces. Openings are defined as downward deflections from the base line. The channel traces shown on the *left* are in the absence of CGA. Channel traces shown on the *right* are in the presence of CGA (1 μg) in the *trans* compartment. *b*, at each Ca^{2+} concentration the open probability was measured in the presence (\square) and absence (\blacklozenge) of CGA. The Ca^{2+} dependence in the absence of CGA is shown (expanded section is shown to emphasize that purified InsP_3Rs are not inhibited at higher cytosolic Ca^{2+} concentrations). Again, CGA significantly enhances P_o . At the lowest Ca^{2+} concentration the P_o of the channel is already at 34%, with no indication of an activation phase. The data set shown is typical of four similar experiments.

capacity for Ca^{2+} , they may play an important role in governing intracellular Ca^{2+} dynamics.

This hypothesis is further supported by the discovery that chromogranins A and B interact directly with type I InsP_3Rs located on the secretory granule membrane (24) and that this coupling is functional, at least in terms of CGA (25). The present results obtained from our bilayer studies reemphasize the functional importance of the interaction and provide the first mechanistic insight at the level of a single InsP_3R . The effects of CGA on InsP_3R channel activity, at the intravesicular pH of 5.5 (the pH at which the coupling occurs), are very profound. The increase in both mean open time and open probability (Fig. 3) demonstrates clearly that CGA causes the channel to open more frequently and, once open, to stay open for longer times, which when translated to the cellular level, im-

plies a greater release of Ca^{2+} . When the intravesicular pH is altered to 7.5, the effects of CGA are seen to dissipate almost instantly (Fig. 2, trace iii, and Fig. 3, e and f) and resemble more closely those seen at pH 5.5 in the absence of CGA. The InsP_3 concentration dependence both in bilayer studies and flux studies carried out at comparable concentrations complement one another. At the single channel level the InsP_3R is seen to have a greater chance of opening when CGA is present, and in the flux studies, the apparent affinity for InsP_3 is greater. Again, a change in pH to 7.5 causes the effect to revert to control levels. As for Ca^{2+} dependence, the InsP_3R is already active at maximal levels when CGA and InsP_3 are present even when the level of cytosolic free Ca^{2+} is only 10 nM (Fig. 5). The data shown in Figs. 4 and 5 indicate that in the presence of coupled CGA and a sufficient concentration of InsP_3 (2 μM), the characteristic effects of cytosolic Ca^{2+} on the InsP_3R channel disappear. It appears that the conformation of the InsP_3R is in such a state in the CGA-coupled condition, that high InsP_3 dictates the channel activity regardless of the presence of Ca^{2+} .

How do these results relate to the physiological situation? In the presence of CGA at intravesicular pH 5.5, the InsP_3R is primed to respond to low levels of InsP_3 . Hence, when a secretory granule reaches the surface of the cell and docks with the inner surface of the plasma membrane, it is fully loaded with Ca^{2+} and sensitized for release. Generation of InsP_3 , even in small quantities, will cause a large elevation in local Ca^{2+} concentration (values as high as 100 μM have been seen immediately prior to exocytosis) (23), initiating secretory processes. During exocytosis, the vesicle contents are exposed to the extracellular pH of 7.4, thus causing dissociation of CGA from the InsP_3R resulting in altered channel properties and hence Ca^{2+} release. Following these events, vesicular contents dissociate from the vesicle membrane, with secretory cargo moving to the extracellular space and then into the bloodstream.

The chromogranins, particularly CGA, appear to play a role in intracellular Ca^{2+} dynamics and secretion, which also has significant implications to human disease. For example, patients suffering from pheochromocytoma and other neuroendocrine tumors have significantly higher concentrations of CGA measured in the plasma (14, 15). This high plasma CGA indicates that high levels must have been stored in order to be released. Individual cells contain increased CGA, and there are more CGA-containing cells. In various cholestatic liver diseases, a striking increase is seen in the number of bile ductules (35). These reactive bile ductules differ from their normal counterparts in that they display neuroendocrine features, in particular the expression of CGA. Hence, the presence of elevated CGA can lead to abnormal Ca^{2+} release within these cells, thus activating specific metabolic mechanisms in the cell, which leads to unnecessary growth. The increased release of neuroendocrine substances, including CGA, may also play an autocrine or paracrine regulatory role in ductular metaplasia of hepatocytes or bile ductule growth. Thus, although the role of CGA in

disease is largely attributed to extracellular CGA and its derivatives, a contribution may arise also from intragranular CGA with respect to its effects on intracellular Ca^{2+} release and exocytosis.

In conclusion, our present work is the first electrophysiological study detailing the very important physiological phenomenon arising from the interaction between CGA, a Ca^{2+} storage protein, and an intracellular Ca^{2+} release channel, InsP_3R . We have shown that there is an order of magnitude increase in the open probability and open time of the InsP_3 -gated Ca^{2+} channel. These effects of the interaction of CGA and InsP_3R have implications for exocytosis and neuroendocrine cell function in disease.

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