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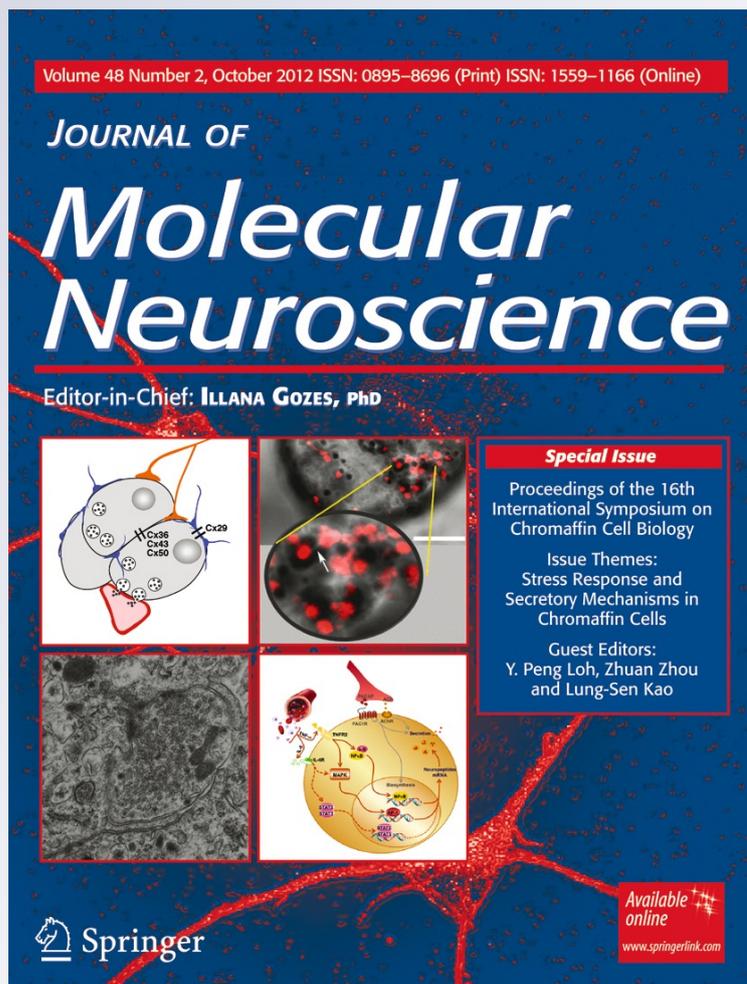
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The Functional Role of Chromogranins in Exocytosis

**Natalia Domínguez · Judith Estévez-Herrera ·
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Abstract Chromogranins A (CgA) and B (CgB) are the
main soluble proteins of large dense-core secretory vesicles
(LDCVs). Using CgA- and CgB-knockout (KO) mice, we
found that the absence of chromogranins A and B induces
significant changes in catecholamine (CA) accumulation
and the kinetics of exocytosis. By crossing these two knock-
out strains, we generated a viable and fertile double CgA/B-
KO mouse in which the catecholamine content in chromaf-
fin LDCVs was halved, and the secretory response signifi-
cantly reduced. Incubating cells with L-DOPA increased the
vesicular CA content in wild-type (WT) but not in Cg-KO
cells, which was not due to changes in amine transport, or in
the synthesis or degradation of cytosolic amines. Electron
microscopy revealed the presence of giant secretory vesicles
exhibiting significant alterations, with little or no electro-
dense inner matrix. Proteomic analysis confirmed the ab-
sence of CgA and B, and revealed small changes in SgII in
the LDCV-enriched fraction, as well as the overexpression
of fibrinogen and other proteins. In summary, our findings
indicate that the mechanisms responsible for vesicular accu-
mulation of CA are saturated in Cgs-KO cells, in contrast to
the ample capacity for further accumulation in WT cells. We
conclude that Cgs contribute to a highly efficient system that
directly mediates monoamine accumulation and exocytosis
in LDCVs.

Keywords Amperometry · Intracellular electrochemistry ·
L-DOPA

N. Domínguez · J. Estévez-Herrera · M. R. Pardo · D. Pereda ·
J. D. Machado · R. Borges (✉)
Unidad de Farmacología, Facultad de Medicina,
Universidad de La Laguna,
38071 La Laguna, Tenerife, Spain
e-mail: rborges@ull.es

Introduction

Chromogranins are the main protein component of chromaffin
secretory vesicles, also known as chromaffin granules, which
are essentially the same organelles as the large dense-core
vesicles (LDCVs) found in many neuroendocrine cells and in
some neurons. Chromogranin A (CgA) was described in the
mid sixties (Blaschko et al. 1967) as the first of a series of
acidic proteins known as granins, of which nine members have
been identified to date (Borges et al. 2010; Taupenot et al.
2003). Chromogranins are characterized by highly hydrophilic
and acidic primary amino acid sequences (Huttner et al. 1991),
as well as the presence of multiple paired basic residues that
form cleavage sites in pro-hormones to generate bioactive
peptides (Helle et al. 2007; Lee and Hook 2009). They also
undergo a multitude of post-translational modifications
(Gasnier et al. 2004; Strub et al. 1997). CgA and CgB share
a tendency to self-aggregate at acidic pH values and high Ca^{2+}
concentrations, conditions typical of the lumen of the trans-
Golgi network and of secretory granules (Huttner et al. 1991;
Rosa and Gerdes 1994; Taupenot et al. 2003; Winkler and
Fischer-Colbrie 1992). Aggregated granins provide the physi-
cal driving force to induce budding of trans-Golgi network
membranes, resulting in the formation of dense core granules
(Koshimizu et al. 2010).

The most important chromogranins in chromaffin granules
are CgA and CgB, and to a lesser extent secretogranin II (SgII).
To date, four physiological roles have been attributed to Cgs:

(1) Cgs acts as pro-hormones, constituting a source of
biologically active peptides. These granins are secreted
during regulated exocytosis and they may fulfil hor-
monal, autocrine and paracrine activities through their
peptide derivatives (Helle 2004; Montero-Hadjadje et
al. 2009; Taupenot et al. 2003; Zhao et al. 2009).

(2) Down-regulation of CgA (Kim et al. 2001) and CgB (Huh et al. 2003) provokes a loss of secretory granules in PC12 cells, while overexpression induces the biogenesis of structures resembling secretory granules in non-endocrine cells, including CV-1, NIH3T3 or COS-7 cells. Indeed, these granule-like structures are able to release/secrete their contents (Beuret et al. 2004; Huh et al. 2003; Kim et al. 2001; Stettler et al. 2009). However, secretory granules can form independently of CgA expression in PC12 (Day and Gorr 2003) and mouse chromaffin cells (Hendy et al. 2006; Mahapatra et al. 2005; Montesinos et al. 2008), consistent with findings in CgB knockout mice (Diaz-Vera et al. 2010; Obermuller et al. 2010). Indeed, granule biogenesis and calcium-evoked secretory responses are both evident in chromaffin cells from Cgs-KO animals (Diaz-Vera et al. 2010, 2012).

(3) Cgs acts as chaperones for prohormone-mediated sorting and packaging of neuropeptides in granules within the trans-Golgi network (Iacangelo and Eiden 1995; Montero-Hadjadje et al. 2009; Natori and Huttner 1996; Rosa et al. 1985).

(4) Cgs are the main component of the dense core of granules, facilitating the storage of catecholamines and ATP (Helle et al. 1985; Machado et al. 2010; Nanavati and Fernandez 1993). Granins exhibit pH-buffering capacities and thus they help concentrate soluble products for secretion. This was the first function attributed to Cgs and is one of main interests of our research group.

Chromogranins are currently considered to be high capacity and low affinity buffers. For example, CgA can bind 32 mol adrenaline per mol with a K_d of 2.1 mM (Videen et al. 1992), and depending on the granin type, chromogranins can bind ≈ 50 mol Ca^{2+} per mol with a K_d of 1.5–4 mM (Yoo 2010). The ability of CgA and CgB to form dimers or hetero-tetramers with one another has been studied to further elucidate the interactions of Cgs with Ca^{2+} (Yoo 1996; Yoo and Albanesi 1991). Similar interactions with soluble species such as catecholamines and ATP are also likely to occur, as the presence of multiple dibasic groups in the chromogranin structure increases their ability to concentrate solutes (Park et al. 2002; Yoo 1996; Yoo and Albanesi 1990). CgA and CgB are the most abundant soluble proteins in LDCVs and thus, they are the main candidates for facilitating the condensation of soluble species to generate the functional matrix (Helle et al. 1985). This matrix probably corresponds to the electron-dense core observed in electron microscopy images (Ehrhart et al. 1986).

The ability of secretory vesicles to actively accumulate enormous concentrations of solutes has intrigued scientists for decades. This process is crucial in cells whose primary function is to efficiently secrete substances such as neurotransmitters and hormones, as few exocytotic events can provoke sufficiently large secretory responses. The strong accumulation of vesicular solutes is probably the main mechanism used to reduce the pressure produced by the concentration delimited in the vesicular membrane. Thus, this vesicular cocktail is reminiscent of Groucho Marx's infamous "Stateroom" in the film *A Night at the Opera* (Fig. 1). In this limited space, amines, Ca^{2+} and ATP concentrate, representing the mobile components whose concentration gradients relative to the cytosol are maintained by special carriers. By contrast, Cgs constitute the immobile components that form the dense core of LDCV that aggregates the majority of solutes.

H^+ is an important component of vesicles and it is concentrated by a specific V-ATPase to maintain an inner pH of 5.5, approximately coinciding with the isoelectric point of Cgs. As the association of Cgs with other solutes is pH-dependent (Helle et al. 1985), vesicular pH may also regulate the ability of CgA to form aggregates (Taupenot et al. 2005), thereby playing a functional role in the dynamics of vesicular Ca^{2+}, ATP and catecholamines.

Two CgA-KO mice have been developed using distinct strategies (Mahapatra et al. 2005), and a CgB-KO mouse was developed later (Obermuller et al. 2010). By crossbreeding these two strains, we recently developed the first double CgA/B-KO mouse, which was viable and fertile in homozygosis (Diaz-Vera et al. 2012). These three strains constitute valuable tools to analyze the role of Cgs in cargo concentration and exocytosis in chromaffin vesicles.

Catecholamine Exocytosis in the Absence of Chromogranin A

The absence of CgA appears to trigger compensatory mechanisms that include the overexpression of CgB (Mahapatra et al. 2005; Montesinos et al. 2008). However, the redistribution of

Fig. 1 Estimated composition of the content of chromaffin granules. In the vesicular components, mobile solutes (catecholamines, calcium, ATP, ascorbate) can be distinguished from immobile species such as Cgs and enzymes. The calcium concentrations refer to free versus bound cation. While catecholamines are efficiently packaged in normal vesicles, some room for the uptake of newly synthesized catecholamines remains. This ability is lost in the absence of either CgA or CgB

Cgs has drastic effects on the storage and release of catecholamines from the LDCVs of adrenal chromaffin cells. Using amperometry, we showed that despite the similar frequency of exocytotic events (Fig. 2a), CgA-KO cells released≈40% less catecholamines than wild-type (WT) cells upon stimulation (Fig. 2b), which is probably due to a reduction in the net catecholamine quantum content (Fig. 2c) and an increase in exocytosis. These kinetic changes mainly affected the latter (descending) portion of the spikes (Fig. 5). Taken together, it appears that in the absence of CgA, the LDCV matrix is less capable of concentrating and retaining catecholamines, resulting in more rapid exocytosis (Montesinos et al. 2008).

The capacity of LDCVs to concentrate their cargo can be explored using the catecholamine precursor L-DOPA. L-DOPA penetrates the chromaffin cell membrane and it is rapidly converted to dopamine, which is then taken up by LDCVs and converted to noradrenaline by dopamine-β-hydroxylase. Thus, the usual effect of L-DOPA incubation is a notable increase in vesicular catecholamine content (Colliver et al. 2000; Gong et al. 2003; Sombers et al. 2007), as observed in WT cells. By contrast, no increase in amine uptake was detected in the LDCVs of CgA-KO chromaffin cells.

To determine whether this deficit in catecholamine uptake was due to a reduction in the availability of cytosolic catecholamines, we analyzed the intracellular electrochemistry in the presence of the monoamine oxidase inhibitor, pargyline. The technique used was a modified version of patch-amperometry using the whole-cell configuration, thereby allowing a carbon fibre electrode to contact the cytosol (Mosharov et al. 2003). Chromaffin cells from KO animals contained less free cytosolic catecholamines than their WT counterparts. However, a dramatic increase in free cytosolic amines was observed in CgA-KO mice after incubation with L-DOPA (100 μM for 90 min) when compared with the WT controls. This finding suggests that saturation

of the LDCV matrix prevents the uptake of newly synthesized catecholamines (Montesinos et al. 2008).

The storage and release properties of LDCVs lacking CgA were studied in more detail using patch-amperometry in the cell-attached configuration, simultaneously monitoring vesicle size (capacitance) and catecholamine release (amperometry) in the same vesicle (Albillos et al. 1997; Montesinos et al. 2008). The results revealed a decrease in vesicular catecholamine concentration from 870 mM in WT to 530 mM in CgA-KO mice. Taken together, these findings indicate a dramatic reduction in the capacity of chromaffin cell LDCVs to concentrate catecholamines in the absence of CgA, despite the apparent compensatory overexpression of CgB.

Catecholamine Exocytosis in the Absence of Chromogranin B

The absence of CgB in CgB-KO mice was confirmed by immunohistochemistry and western blotting, also revealing the overexpression of CgA (Diaz-Vera et al. 2010). Hence, the secretory characteristics of chromaffin cells in these mice were then analyzed as described for the CgA-KO strain. Despite of critical role of CgB in the genesis and sorting of LDCVs, sustained exocytotic catecholamine release was described in chromaffin cells from CgB-KO mice (Glombik et al. 1999; Kromer et al. 1998; Natori and Huttner 1996). We observed similar secretory patterns in chromaffin cells from WT and CgB-KO mice, with no differences in spike number (Fig. 3a). However, the total catecholamine release from CgB-KO cells was 33% lower than from control cells (Fig. 3b), roughly coinciding with the reduction observed in the amount released per quanta (Fig. 3c). Careful analysis of the kinetic properties of secretory spikes showed that the reduction in exocytosis primarily affected the initial (ascending) portion of the spikes (Fig. 5), in contrast to the pattern observed in
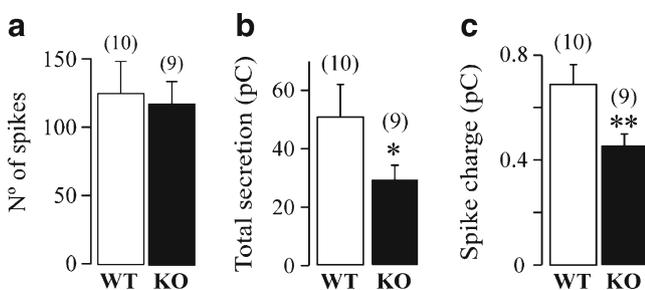

Fig. 2 The exocytotic process in cells from CgA-KO versus wild-type control mice. Data were obtained from mouse chromaffin cells by carbon fibre amperometry. **a** Average number of secretory spikes counted over 2 min following a 5-s pulse of 5 mM BaCl₂. **b** The same recordings were integrated to measure total catecholamine release. **c** Quantum size of exocytotic events for individual spikes, *p<0.05, Mann–Whitney test. Cell number is indicated in brackets. Modified from Montesinos et al. (2008)
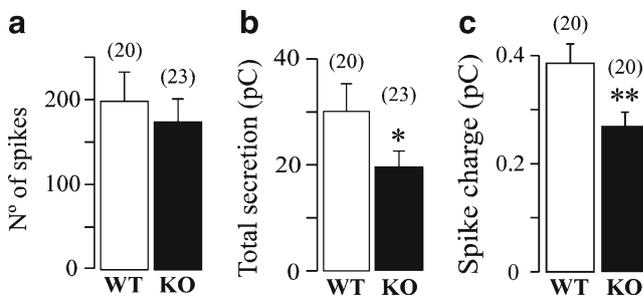

Fig. 3 The exocytotic process in cells from CgB-KO versus wild-type control mice. Data were obtained from mouse chromaffin cells by carbon fibre amperometry. **a** Average number of secretory spikes counted over 2 min following a 5-s pulse of 5 mM BaCl₂. **b** The same recordings were integrated to measure total catecholamine release. **c** Quantum size of exocytotic events for individual spikes, *p<0.05, **p<0.01, Mann–Whitney test. Cell number is indicated in brackets. Modified from Diaz-Vera et al. (2010)
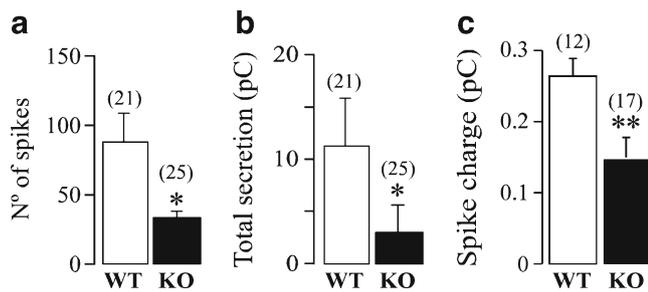
Fig. 4 The exocytotic process in cells from CgA/B-KO versus wild-type control mice. Data were obtained from mouse chromaffin cells by carbon fibre amperometry. **a** Average number of secretory spikes counted over 2 min following a 5-s pulse of 5 mM $BaCl_2$. **b** The same recordings were integrated to measure total catecholamine release. **c** Quantum size of exocytotic events for individual spikes, *$p < 0.05$, **$p < 0.01$, Mann–Whitney test. Cell number is indicated in brackets. Modified from Diaz-Vera et al. (2012)

CgA-KO mice (Diaz-Vera et al. 2010). Moreover, L-DOPA overloading revealed that LDCVs in CgB-KO cells were unable to take up more catecholamines, with excess amines remaining in the cytosol.

Catecholamine Exocytosis in the Absence of Chromogranins A and B

The recent generation of double CgA/B KO mice allowed us to analyze catecholamine secretion in the absence of both chromogranins. Electron microscopy images of the adrenal medulla revealed the presence of giant granules with little or no vesicular matrix (Diaz-Vera et al. 2012). The large vesicular size is likely the result of osmotic decompensation, and it may explain the dramatic reduction in the frequency of exocytotic firing in CgA/B KO mice, which was not observed in the absence of CgA or CgB alone. Granule membranes were usually broken, indicating a high susceptibility to the osmotic changes associated with the fixation procedure.
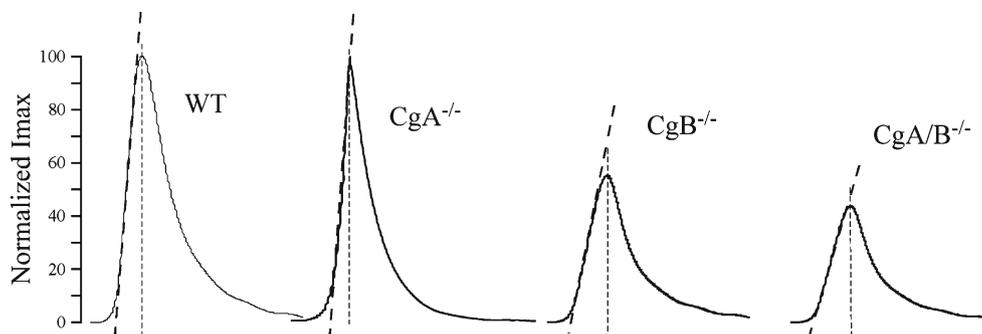
Total amine secretion was strongly reduced (Fig. 4b) in CgA/B-KO mice due to a combination of low spike firing (Fig. 4a) and the small quantum size (Fig. 4c). When determined by amperometric spikes, the kinetics of exocytosis differed clearly from those of control mice and they bore a greater resemblance to CgB-KO rather than CgA-KO cells. Indeed, the Imax value was halved and the slope of the ascending region of the spikes was not as steep as in the WT controls. This apparent general slowdown of exocytosis may have been influenced by the very low catecholamine concentration. However, the kinetic changes observed appear to have been produced more by a combination of the limited amounts of amines and the very large size of the secretory vesicles (Fig. 5).

Incubation of cells with L-DOPA showed that the uptake of newly synthesized catecholamines granules was impaired in CgA/B-KO cells. No increase in the net charge of granules was detected after incubation with L-DOPA, although the free cytosolic catechols increased as granules cannot easily remove the catecholamines from cytosol (Diaz-Vera et al. 2012).

While the concentration of catecholamines accumulated in CgA/B-KO chromaffin vesicles was significantly reduced, it remained above that required to reach isotonicity with the cytosol. As such, we cannot rule out the possibility that other components of the vesicular cocktail, such as ATP (Kopell and Westhead 1982) and/or H^+ (Camacho et al. 2006, 2008), contribute to the maintenance of amine accumulation.

To determine whether other granins could fulfil the role of Cgs in forming the dense matrix, we performed a proteomic analysis of the enriched LDCV fraction from the adrenal medulla of the CgBKO and CgAB-KO mice (Diaz-Vera et al. 2012). While no significant changes in the amount of SgII or other granins were observed, surprisingly significant amounts of fibrinogen were detected, for which the three chains (α, β and γ) were only present in the LDCVs of KO mice. In addition to its crucial role in clot formation, fibrinogen has been associated with the sorting of constitutive vesicles (Glombik et al. 1999). However, no other protein appears to be

Fig. 5 Kinetic profiles of amperometric spikes from CgA-KO, CgB-KO and CgA/B-KO chromaffin cells. Traces illustrate the kinetic changes in exocytosis observed in cells lacking CgA, CgB or both Cgs. The profiles were constructed by averaging the spikes from WT, CgA-KO, CgB-KO and CgA/B-KO cells, normalized to the Imax (100%) of their corresponding controls. *Discontinuous lines* indicate the ascending slopes obtained by linear fitting of the 25–75% segment of the ascending portion of the spikes

capable of fulfilling the role of Cgs as a matrix-condenser for soluble intravesicular components (Diaz-Vera et al. 2010).

Concluding remarks

Since their discovery, Cgs have captivated the attention of scientists and they have been implicated in several processes, including granule biogenesis and sorting, the production of bioactive peptides, tumour marking, and the pathophysiology of neurodegenerative diseases. New data from Cg-KO mice provides direct evidence implicating Cgs in vesicular storage and in the exocytotic release of catecholamines. While the frequency of secretory events is maintained, even in the complete absence of Cgs, the absence of Cgs impairs vesicular catecholamine accumulation. Hence, although LDCV biogenesis does not appear to be affected, the saturation of vesicular storage capacity might well be. Protein analysis of the secretory vesicle fraction revealed the compensatory overexpression of CgA in the absence of CgB, and vice versa. Unexpectedly, other proteins that are apparently unrelated with secretion were only present in the adrenomedullary tissue of CgB-KO animals. In conclusion, Cgs are highly efficient direct mediators of monoamine accumulation, influencing the kinetics of exocytosis in LDCVs.

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