

ATP: The crucial component of secretory vesicles

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The colligative properties of ATP and catecholamines demonstrated in vitro are thought to be responsible for the extraordinary accumulation of solutes inside chromaffin cell secretory vesicles, although this has yet to be demonstrated in living cells. Because functional cells cannot be deprived of ATP, we have knocked down the expression of the vesicular nucleotide carrier, the VNUT, to show that a reduction in vesicular ATP is accompanied by a drastic fall in the quantal release of catecholamines. This phenomenon is particularly evident in newly synthesized vesicles, which we show are the first to be released. Surprisingly, we find that inhibiting VNUT expression also reduces the frequency of exocytosis, whereas the overexpression of VNUT drastically increases the quantal size of exocytotic events. To our knowledge, our data provide the first demonstration that ATP, in addition to serving as an energy source and purinergic transmitter, is an essential element in the concentration of catecholamines in secretory vesicles. In this way, cells can use ATP to accumulate neurotransmitters and other secreted substances at high concentrations, supporting quantal transmission.

exocytosis | purines | quantum size | secretory vesicles | VNUT

Virtually most, and possibly all, types of secretory vesicles found in cells contain ATP, which often accumulates at high concentrations and, commonly, in conjunction with different types of neurotransmitters. However, the reason for this widespread distribution of ATP remains a mystery. Although ATP is present in all animal species, including primitive life forms like *Giardia lamblia* that lack Golgi complexes and mitochondria, the detection of ATP in the secretory vesicles of sympathetic neurons was considered to be the first example of cotransmission (1). However, given the ubiquitous accumulation of ATP in secretory vesicles, it might instead be considered that it is the other neurotransmitters that coincide with ATP, rather than the other way around (2). Indeed, perhaps ATP should be considered as the first molecule used as a transmitter in primitive forms of life.

Astonishingly high concentrations of releasable species are stored inside secretory vesicles, far exceeding those in the cytosol (3, 4). For example, the catecholamine content of adrenal secretory granules (SGs), a type of large dense core secretory vesicles also known as chromaffin granules, was 0.8–1 M when measured directly in adrenal chromaffin cells using patch amperometry (5, 6). In addition, SGs from chromaffin cells contain ATP at ~150 mM (7), calcium ~40 mM (8), about 2 mM of granins, ascorbate, peptides, and other nucleotides, all in an acidic pH ~5.5 environment.

ATP possesses intrinsic chemical characteristics that make it relevant to the accumulation of soluble substances in secretory vesicles. The formation of weak complexes between monoamines and ATP, the two main soluble compounds in chromaffin granules, has been demonstrated in vitro by NMR (9), ultracentrifugation (10), infrared spectroscopy, and calorimetry (11). This interaction was also demonstrated in isolated chromaffin granules (12), which are very similar structures to the large dense core vesicles present in many neurons and neuroendocrine cells. These vesicles are distinguishable from other vesicles through their size (~200 nm), and through their granin (chromogranins and secretogranins), enzymes, and peptide content, which constitute the

condensed vesicular core. However, in 1982 the interaction between these molecules and ATP was shown to behave as a non-ideal solution in terms of osmotic pressure (13). It was suggested that the ring stacking of catechol and adenosine groups forms a sandwich with a catecholamine/ATP stoichiometry between 1:1 and 1:4. Moreover, disulphide bonds and electrostatic forces between the β -carbon and hydroxyl groups of catecholamines and the phosphate or nitrogen groups of purines, respectively, also contribute to the formation of a complex at the inner vesicular pH (~5.5), providing osmotic stability to the complex (14).

The interaction of chromogranins (the most abundant vesicular proteins) with catecholamines was also proposed to be the mechanism responsible for reducing the osmotic forces that drive amine accumulation in the SGs of chromaffin cells (15). Indeed, we showed that complete ablation of chromogranins halved the amine content of SGs (16). However, even in the complete absence of chromogranins, the remaining amines are still theoretically hypertonic. Moreover, chromogranins are only present in dense core SGs and not in other organelles that are like synaptic vesicles, where the condensation of secretory products might rely on other mechanisms. For this reason we focused our attention on the colligative properties of ATP (13), a compound that accumulates strongly in SGs. The vesicular nucleotide carrier VNUT [or *SLC17A9* (17–21)] uses the electrochemical gradient of protons inside SGs to exchange H⁺ with nucleotides (17).

Because cells cannot be deprived of ATP, our strategy in this study was to manipulate VNUT to evaluate the functional role of vesicular ATP in the storage and exocytosis of catecholamines, using bovine chromaffin cells as a secretory model.

Significance

ATP is highly concentrated in secretory vesicles. In vitro experiments suggest that the association of ATP with catecholamines reduces their osmotic forces, permitting the extraordinary accumulation of amines within chromaffin granules. However, this has yet to be proved in living cells. Because functional cells cannot be deprived of ATP, we manipulated the vesicular nucleotide carrier, demonstrating that the extent of vesicular ATP is closely linked to the quantum catecholamine size. This is particularly evident in newly synthesized vesicles, the first to be released. This is the in vivo demonstration that vesicular ATP is an essential factor in the accumulation of neurotransmitters, which may well be a wider mechanism supporting quantal transmission.

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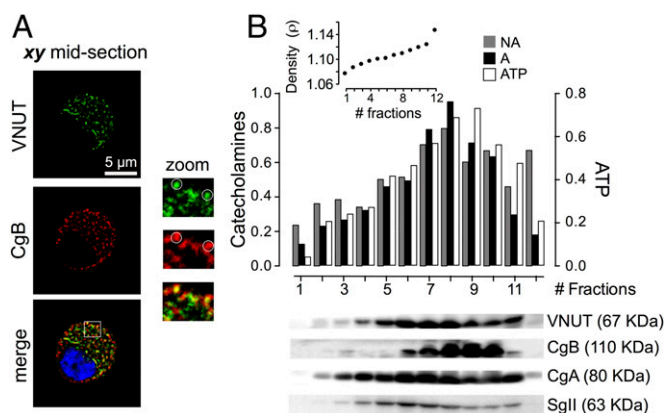


Fig. 1. (A) Endogenous VNUT codistributes with secretory chromaffin vesicles. Typical confocal microscopy images showing the cellular distribution of endogenous VNUT and CgB. The merged image also shows the distribution of endogenous VNUT and CgB. The zoomed images show the colocalization of VNUT and CgB in specific structures. Quantification was performed as described in *Materials and Methods*. (B) Normalized distribution of several components in chromaffin secretory vesicles. Twelve fractions of 450 μ L (#1 lighter, #12 heavier) were obtained by density gradient sedimentation. (Upper) Quantification of noradrenaline (NA), adrenaline (A), and ATP. The bars show pooled data from three independent gradients, and a typical chromaffin SG proteins distribution, data from one representative experiment (Lower). The *Inset* shows the iodixanol density profile of each fraction taken from a parallel ultracentrifuge tube. CgA, chromogranin A; CgB, chromogranin B; SgII, secretogranin II.

Results

Endogenous VNUT Expression in Chromaffin Cells. The presence of VNUT in SGs of chromaffin cells was confirmed using an antibody against the cytoplasmic N-terminal domain of this nucleotide transporter, and it was compared with the distribution of chromogranin B (CgB), a major and constitutive component of SGs of chromaffin cells (22). In confocal microscopy images, endogenous VNUT presented a characteristic punctuate distribution that mostly colocalized with CgB ($\sim 75 \pm 3\%$, mean \pm SEM; $n = 278$ randomly taken spots from 7 different cells) (Fig. 1A). Furthermore, we confirmed that VNUT was specifically attached to SGs by transiently, yet simultaneously, expressing plasmids containing VNUT-EGFP and neuropeptide Y-coupled DsRed (NPY-DsRed). When analyzed 48 h after transfection, VNUT-EGFP and NPY-DsRed exhibited over 85% colocalization in confocal microscopy images (Fig. S1), showing that most of the newly synthesized VNUT was located in secretory vesicles.

To provide further evidence of the association of VNUT to SGs, we purified chromaffin granules (23) and fractionated the preparation by ultracentrifugation on a continuous iodixanol (OptiPrep) gradient. The presence of chromogranins detected in immunoblots cofractionated with catecholamines indicates their selective incorporation into SGs. We then examined the profile of VNUT and compared its distribution to that of ATP and to the catecholamine content, along with that of other granules well characterized as vesicular proteins (Fig. 1B). VNUT, ATP, and catecholamines exhibited a similar distribution (particularly in fractions 4–11), and VNUT coeluted with other chromaffin granule-specific and enriched proteins like CgB, CgA, and SgII. In contrast, the presence of the cytochrome *c* oxidase I (a mitochondrial protein) was negligible in all fractions recovered from the gradient (24). Hence, this fractionation and cellular codistribution data indicated that SGs contain VNUT, a protein that governs the transport of nucleotides.

Interfering with VNUT Expression Drastically Reduces the Secretion of ATP. Because cells cannot be deprived of ATP, our strategy to prevent its uptake into SGs was to selectively silence VNUT. We designed two species-specific short-interfering RNA (siRNA) oligonucleotides (siVNUT₁, siVNUT₂) that target different sequences in bovine VNUT (*SLC17A9*). Transient transfection of these oligonucleotides in chromaffin cells efficiently silenced VNUT compared with a nontargeted siRNA (scrambled). The efficiency of transfection of the Cy3-siRNA was $\sim 40\%$. To establish the most appropriate experimental conditions, the expression of *SLC17A9* mRNA was assayed 24 and 48 h after nucleofection by quantitative RT-PCR (qRT-PCR). The expression of these transcripts fell by $\sim 60\%$ (siVNUT₁) and $\sim 50\%$ (siVNUT₂) compared with the control cells (scrambled in Fig. 2A). A similar reduction in the total amount of VNUT was also detected in Western blots (Fig. 2B), where siVNUT reduced the amount of protein by ~ 65 (siVNUT₁) and $\sim 55\%$ (siVNUT₂). Conversely, the expression of CgB as shown in Fig. 2B was not modified by these siRNAs, indicating that our nucleofection procedure does not affect the expression of other components involved in the genesis and sorting of SGs. Because oligonucleotide siVNUT₁ produced a stronger effect and the maximal inhibition was obtained 24 h after transfection (Fig. 2A and C), we used these conditions for all further experiments.

ATP can be partially released by nonexocytotic mechanisms (25), so we quantified the exocytotic and nonexocytotic contributions to release. Cells seeded in 24-well plates (5×10^5 per well) were incubated for 10 min with the highly active plasmalemmal ectoATPase specific inhibitor, ARL 67156 (100 μ M)

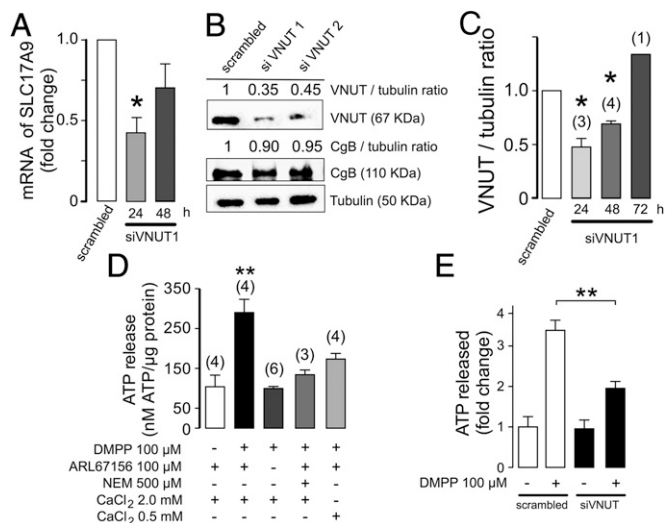


Fig. 2. Knockdown of the vesicular nucleotide transporter decreases ATP exocytosis. (A) VNUT mRNA assayed by qRT-PCR. The bars represent the mean expression \pm SD ($n = 3$) and the values were compared using a one-way ANOVA followed by Bonferroni's multiple-comparison test: $*P < 0.05$. (B) Representative Western blots of VNUT expression carried out 24 h after siRNA transfection. The effects of nucleofection with the siVNUT₁ and siVNUT₂ oligonucleotides were compared with those of a nontargeted siRNA (scrambled). Equal amounts of protein (20 μ g) were loaded per lane. (C) Quantitative analysis of the changes in VNUT expression at different times after transfection of siVNUT₁, expressed as the VNUT/ α -tubulin ratio in three different Western blots. Bonferroni's multiple-comparison test: $*P < 0.05$. (D) ATP release from chromaffin cells under resting conditions and after stimulation with the nicotinic agonist DMPP, in the presence or absence of the nucleotidase inhibitor ARL67156 or the exocytotic blocker NEM. (E) Decreased ATP release after VNUT knockdown. The experiments in D and E were performed at 37 $^{\circ}$ C and, unless specified, in the presence of 100 μ M of ARL67156. $***P < 0.01$, Mann-Whitney *U* test.

(26), to avoid the rapid degradation of the ATP released that occurs in the absence of this inhibitor (Fig. 2D).

Because regulated exocytosis is a Ca^{2+} -dependent process, this contribution to the overall ATP release was also blocked in the presence of *N*-ethylmaleimide (NEM) (Fig. 2D). The nonexocytotic, constitutive release, or basal secretion in the absence of stimuli, was 75 nM of ATP per microgram of total protein, which increased significantly (threefold) in the presence of 2 mM Ca^{2+} and the nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium (DMPP; 100 μM , 5 min), an increase that was attenuated when the Ca^{2+} concentration was reduced to 0.5 mM (Fig. 2D). These results show that triggered release of ATP is a Ca^{2+} -dependent mechanism.

To validate the functional contribution of VNUT to the vesicular storage of ATP and its subsequent secretion, similar experiments were conducted to determine whether silencing VNUT expression affected ATP secretion. The evoked release of ATP measured between 24 and 48 h after transfection diminished about 40% in chromaffin cells nucleofected with siVNUT₁, whereas the control with scrambled siRNA showed no such effect. Moreover, nonexocytotic secretion remained unaffected (Fig. 2E). Note that only the newly synthesized fraction of SGs would be affected by the siRNA, so previously existing vesicles would presumably carry intact VNUT. These data may underestimate the real reduction in the ATP content of vesicles lacking active VNUT.

Adrenaline Secretion Is Impaired When Secretory Vesicles Lack VNUT.

To evaluate how VNUT knockdown (VNUT-KD) affects catecholamine exocytosis, we analyzed the basal and DMPP-evoked catecholamine release in the same experiments described above using HPLC coupled with electrochemical detection. The catecholamines secreted by control cells in response to DMPP were typically 30–40% noradrenaline and 70–60% adrenaline (see representative chromatogram in Fig. 3A, Right). However, the reduction in intravesicular ATP when VNUT was silenced lowered DMPP-evoked catecholamine secretion in a manner that appeared to be somewhat selective for adrenaline (Fig. 3A, C, and D). Conversely, neither the basal release (Fig. 3C and D) nor the total amount of catecholamines in the lysates of control or VNUT-KD cells were affected (Fig. 3B).

To better understand why the depletion of vesicular ATP preferentially modified adrenaline secretion, we separated purified SGs into 12 fractions using continuous gradients of iodixanol (Fig. 1B). The linear regression of both adrenaline and noradrenaline reflected a high linear dependency on ATP, yet the slope for adrenaline was more pronounced indicative of a tighter association between ATP and adrenaline (Fig. 3E). These results are consistent with those obtained when VNUT was silenced and ATP depletion was seen to mainly affect SGs containing adrenaline (Fig. 3C and D).

Newly Synthesized Granules Are the First to Be Released. Because RNA interference is most likely to selectively affect newly synthesized SGs, we explored whether new vesicles are the first to be released. To measure secretion as a function of granule age we labeled one component of SGs (NPY-EGFP) and compared its release with an endogenous releasable protein (CgB). We nucleofected cells with a plasmid that encodes NPY-EGFP, as described in *Materials and Methods*, and the released EGFP was assayed by dot blot. The set-up used for the on-line analysis of catecholamine release by electrochemistry and the subsequent fraction collection of the perfusate from chromaffin cells trapped on a column is shown in Fig. 4A (27, 28).

The cells were stimulated with repeated pulses of DMPP (10 μM , 20 s) applied at 3-min intervals, yet no autocrine effect was expected because the fast perfusion system washed out the released products (29). In this way, EGFP release will indicate the exocytosis of new vesicles, and quantification of the release of chromogranin B as an endogenous marker of exocytosis will

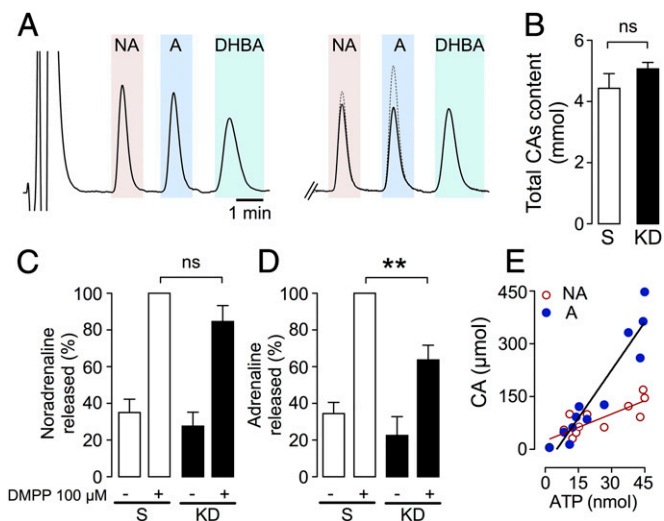


Fig. 3. Knockdown of VNUT preferentially impaired the vesicular exocytosis of adrenaline in chromaffin cells. (A) Typical HPLC chromatograms showing the detection of amines. (Left) External standard of noradrenaline (NA), adrenaline (A), and DHBA (internal standard). Cells were stimulated for 5 min with DMPP and the amines secreted into the media were analyzed. (Right) These traces show the catecholamines secreted from control cells (scrambled, dashed line) and VNUT-KD cells (solid line). (B) Average total catecholamine content in lysates from cells transfected with the scrambled (S) and siVNUT₁ (KD) siRNAs. ns, not significant. (C and D) Basal and DMPP-evoked noradrenaline and adrenaline secretion from cells stimulated by DMPP, showing greater release of noradrenaline. The data are the means \pm SEM of at least three independent cell cultures: * $P < 0.05$, ** $P < 0.01$ (Dunnett's paired *t* test). (E) Linear fit from the relation catecholamines/ATP obtained from purified SGs. The R^2 values were 0.9 and 0.7 for ATP/adrenaline and ATP/noradrenaline, respectively. The slopes of the linear equations were 2.5 vs. 9.1, with values from an experiment from two independent gradients of isolated vesicles shown.

reflect the release from new and old SGs. At the end of the experiment the cells were perfused with 0.1% Triton X-100 to quantify the total catecholamine content, which produced a big oxidation signal of about 4–4.5 μA , 75–80 μC , indistinguishable from that detected in scrambled cells.

In the example shown in Fig. 4B, the first stimulus caused a secretory response of about 25 nA, which was followed by a characteristic pattern of amperometric peaks that reflected the gradual growth in the size of the releasable pool during the first five pulses. This pattern was also observed in cells nucleofected with the scrambled oligonucleotide. In contrast, the EGFP secretory profile indicated that maximal release was achieved during the first two stimuli (note the clear differences in the time course of both secretory components in Fig. 4C). These data suggest that the youngest SGs are the first to undergo exocytosis.

Silencing VNUT Selectively Affects Catecholamine Release from Newly Synthesized Granules.

To test whether silencing VNUT synthesis affects the early releasable pool of vesicles distinctly, we analyzed the time course of catecholamine secretion in response to repetitive stimuli. Compared with the cells nucleofected with the scrambled siRNA, the silencing of VNUT had a selective impact on the catecholamine release from this newly created SG pool, whereas the older granules seem to remain unaffected (as summarized in Fig. 4D). Note that silencing VNUT halved the initial secretory responses compared with the control nucleofected cells.

VNUT-KD Affects the Rate of Granule Recruitment Inherent to DMPP-Induced- $\Delta[\text{Ca}^{2+}]$.

Because the reduction in catecholamine secretion observed after knockdown of VNUT could be caused by a

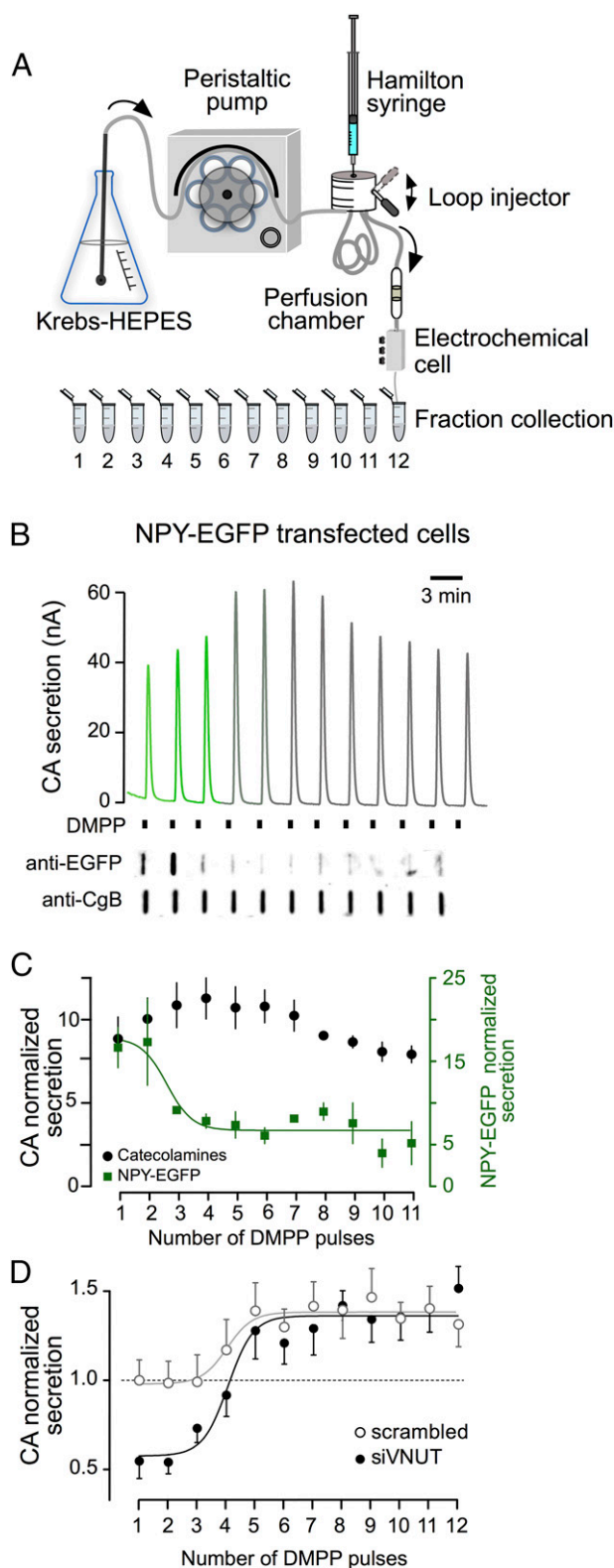


Fig. 4. VNUT knockdown predominantly affects catecholamine secretion from newly synthesized vesicles. The capacity of cells to recruit vesicles from the early- and late-releasable pools was studied using 12 successive DMPP stimulations. (A) The scheme shows the perfusion system used for the on-line electrochemical analysis of catecholamine release and the fraction collection for protein quantification. (B) Representative traces of the electrochemical recording from perfused chromaffin cells expressing NPY-EGFP. Each current peak indicates catecholamine secretion. A representative dot blot probed for NPY-EGFP and CgB obtained from the same experiment. (C) Time course

reduction in the number of exocytotic events or the amount of amines released per quantum, we examined the effect of siVNUT on the frequency of exocytotic events. Transfected cells were identified for amperometry by using Cy3-tagged oligonucleotides (nucleofected cells were greater than three times more fluorescent than the SD of the background) (Fig. 5A, *Inset*) and these cells were stimulated for 10 s with DMPP (10 μ M) using a repetitive protocol similar to that used for the on-line studies (Fig. 5A). The recordings were made on the same day using the same calibrated carbon-fiber electrode and alternating recordings from control and siRNA VNUT nucleofected cells to minimize the bias. The application of DMPP produced exocytotic bursts of \sim 8 s that were significantly shorter in VNUT-KD cells (Fig. 5D and E). Furthermore, and as expected given this effect, the integrated secretory response was weaker in silenced cells (defined as the catecholamine release per pulse) (Fig. 5B).

It is well known that regulated exocytosis is a Ca^{2+} -dependent phenomenon and that the frequency of exocytosis is therefore mainly controlled by intracellular calcium. The release of less ATP could be lowering potential regulatory effects of purinergic autoreceptors. This possibility can be ruled out in the on-line perfusion experiments because the flow (1 mL/min) assures the fast washout of secreted products preventing autocrine effects on Ca^{2+} entry (29). When the time course of intracellular Ca^{2+} was analyzed by calcium-imaging, in perfused cells—in response to four successive stimulations with DMPP—no differences were evident in either the initial peaks or in the time course of Ca^{2+} signals (Fig. S2). Hence, the effects on the frequency of exocytosis did not appear to be a result of intracellular calcium dynamics. In addition, when we studied whether siVNUT affected the biogenesis of SGs by quantifying NPY-EGFP expression and their cellular distribution, this did not appear to be the case (Fig. S3). Thus, the reduction in the frequency of secretory spikes does not appear to be caused by changes in cytosolic calcium or vesicle biogenesis.

The Quantum Size of Exocytosis Is Drastically Reduced in VNUT-KD Cells. The kinetic features of single exocytotic events were studied by single-cell amperometry. Secretion was elicited by a 5-s pressure ejection of 5 mM BaCl_2 , an especially useful secretagogue that does not require receptor activation and that produces a low frequency of exocytosis, enabling the quantification of individual secretory events. Indeed, the probability of two independent and undistinguished exocytotic overlapping events in our experimental conditions was very low ($P = 0.018$; $n = 1,201$ events).

The distribution of spikes in control (Fig. 6A, scrambled, gray trace) and VNUT silenced chromaffin cells (Fig. 6A, siVNUT, black trace) was obtained from amperometric recordings. In our conditions, the typical profile of barium-evoked secretion from naïve cells is identical to that observed in control cells, whereas cells nucleofected with siVNUT do not show the initial spike burst that usually lasts for 1 min but that maintains a slow pace of about 0.8 Hz. The quantum size was drastically reduced during the 70 s after stimulation in cells nucleofected with siVNUT (Fig. 6A, *ii*). The prespike feature “foot” duration, which has been assigned to the fusion pore stability, was significantly reduced in siVNUT cells. These differences in the kinetics of release and quantum size are more evident when considered in these two cell types after 70 s of recording (Table 1).

of catecholamine and newly synthesized NPY (pooled results from four experiments). (D) Time course of catecholamine release from control (scrambled) and knockdown-VNUT (siVNUT) nucleofected chromaffin cells. To correct the bias of different cell batches, the data were normalized to the mean of the first secretory peak from the control cells (scrambled), considering the initial response as 1.

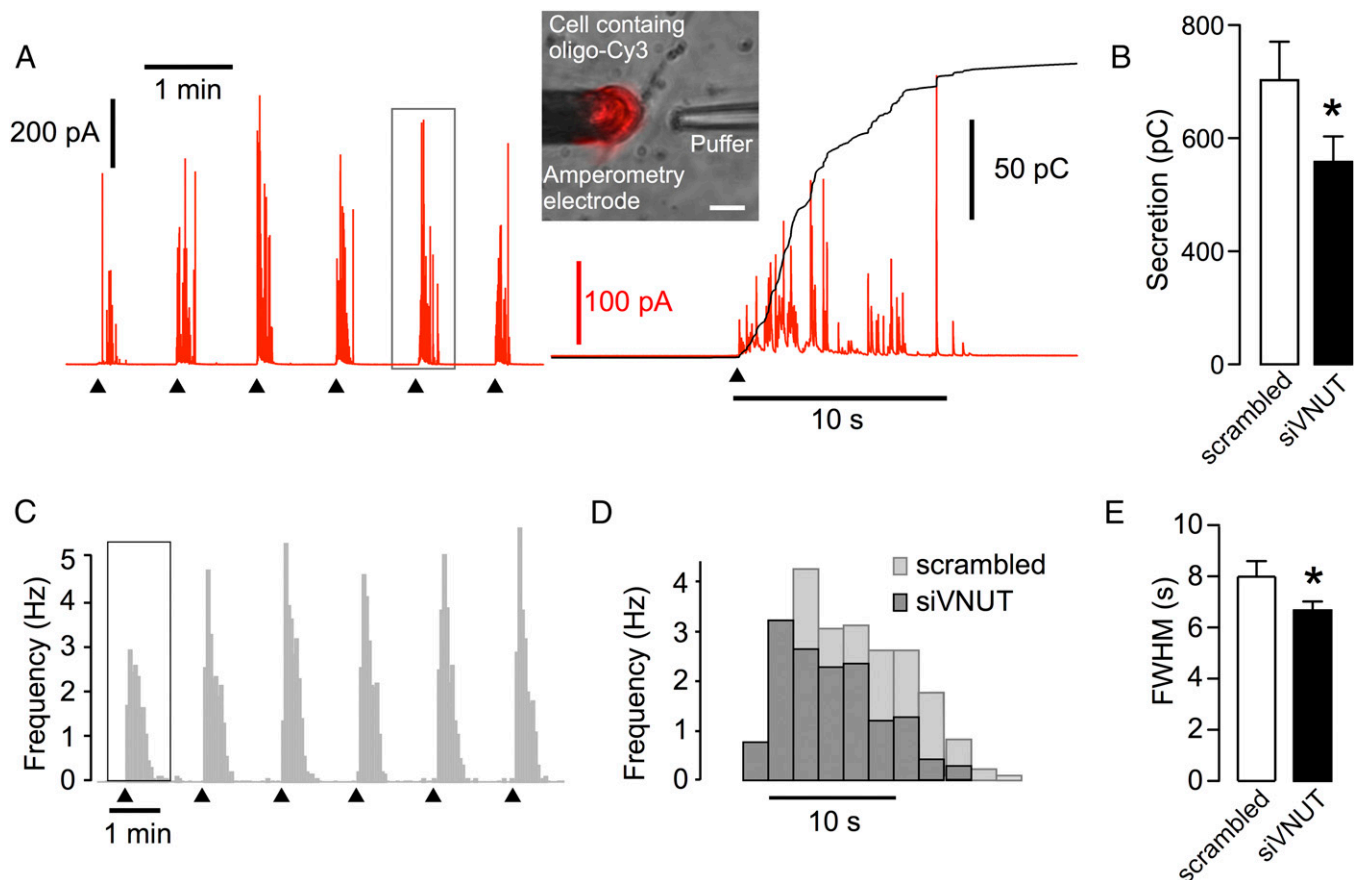


Fig. 5. The number of exocytotic events is reduced by VNUT interference. (A) Representative amperometric recording from a control cell. Secretion was stimulated every minute by a 10-s pressure ejection of 10 μ M DMPP from a micropipette placed 30 μ m from the cell (triangles). After each stimulus the micropipette was moved 500- μ m up using a motor-driven micromanipulator to avoid receptor desensitization caused by DMPP leakage. An amplified view of the fifth pulse is shown revealing the typical firing pattern of exocytosis (red trace) and the cumulative secretion obtained by integrating the amperometric signals (superimposed black trace). (Inset) A cell labeled with the fluorophore (Cy3-oligonucleotide) indicates successful nucleofection. The image shows the position of the carbon fiber electrode and the glass pipette used for DMPP injection (puffer, on the right). (Scale bar: 10 μ m.) (B) Total secretion responses from control (scrambled) and siVNUT cells. The data represent the mean \pm SE secretion pooled from six secretory responses elicited by 10- μ M DMPP pulses. (C) Spike-frequency analysis (1-s bins). The image shows the frequency of exocytosis. Each bar represents the average spike frequencies from control cells ($n = 10$). The maximum number of spikes was reached 5 s after stimulation. (D) The first histogram is amplified and the spike frequency (recorded in 2-s bins) from the control (scrambled) and VNUT silenced cells superimposed. Note that spikes from VNUT-KD cells stopped firing before the control cells. (E) Pooled data from 10 cells showing the full width at half maximum (FWHM) from the sigmoidal fits of frequency histograms. * $P < 0.05$, Mann-Whitney U test.

A consequence of the lower number of spikes is a drastic fall in the total amount of amines secreted by the siVNUT cells (Fig. 6B). Indeed, the reduction in the quantum size was accompanied with a parallel decrease in the I_{max} [maximum oxidation current, expressed in picoampere (pA)] and $t_{1/2}$ (spike width at half height,

expressed in milliseconds) (Fig. 6D), suggesting that the accumulation of ATP and catecholamines is linked. It is important to emphasize here that the reduction in both frequency of secretory events and quantum size were observed exclusively in the first spikes, which roughly coincided with the newly synthesized

Table 1. Characteristics of the secretory spikes obtained from chromaffin cells nucleofected with scrambled or siVNUT₁ oligonucleotides

Time	I_{max} (pA)	Q (pC)	$t_{1/2}$ (ms)	Mean (pA/ms)	Foot duration (ms)	n (cells)	n (spikes)
0–70 s							
Scrambled	117.7 \pm 15.1	1.68 \pm 0.28	13.1 \pm 1.2	36.9 \pm 4.4	6.9 \pm 0.3	9	990
siVNUT ₁	55.2 \pm 21.1*	0.72 \pm 0.28*	8.4 \pm 1.1*	30.7 \pm 5.9	4.9 \pm 0.4**	9	468
Change	-53.1%	-57.1%	-64.1%	-19.8%	-29%		
70–180 s							
Scrambled	99.0 \pm 17.8	1.49 \pm 0.31	13.0 \pm 1.5	20.5 \pm 8.3	7.7 \pm 0.3	9	866
siVNUT ₁	68.7 \pm 24.2	1.05 \pm 0.47	10.3 \pm 1.9	21.8 \pm 5.9	6.9 \pm 0.3**	9	820
Change	-30.6%	-29.5%	-20.7%	+ 6.3%	-11%		

For explanation, see *Materials and Methods* and ref. 30. This is a representative experiment from four replicas carried out in different cell cultures, performed alternating scrambled and siVNUT cells. The data are expressed in the units in parentheses. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney U test).

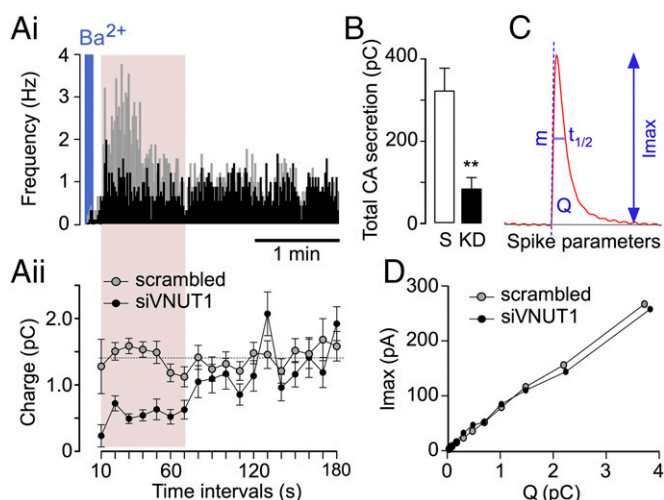


Fig. 6. Quantum catecholamine size from VNUT-KD SGs. (A, *i*) Spike-frequency analysis of isolated cells nucleofected with the scrambled (gray traces) or siVNUT oligonucleotides (black traces) and stimulated for 5 s with 5 mM BaCl₂. Each spike represents the catecholamine released from a single vesicle. Frequency bins are the average number of spikes in 1 s. (A, *ii*) Quantum size of the exocytotic events recorded in 10-s bins over 3-min intervals. Each open circle (scrambled) and solid circle (siVNUT₁) represents the average charge (in picocoulombs, nine cells each). (B) Total secretion (mean ± SEM) comparing the control (scrambled, S) and siVNUT₁ (KD). The cumulative charges were measured for 3 min after stimulation and they are expressed as picocoulombs. ***P* < 0.01, Student's *t* test. (C) Parameters obtained from each secretory spike: *I*_{max}, maximum oxidation current; *t*_{1/2}, spike width at the half-height; *Q*, net catecholamine charge; *m*, ascending slope of the spike. (D) Spike amplitude (*I*_{max}) versus quantum size (*Q*) of secretory spikes from siVNUT and control nucleofected cells: Mann–Whitney *U* test with the Bonferroni correction (the data are averaged from the experiment shown in Table 1).

SGs that are the most strongly affected by VNUT-KD. These data are also consistent with the on-line secretion from the perfused cells.

The Overexpression of VNUT Increases Quantum Size of SGs. To validate the above results indicating a specific role of intravesicular ATP as crucial factor in the accumulation of catecholamine, we conducted single-cell amperometric recordings on chromaffin cells overexpressing VNUT-EGFP. This approach also overrides the problems derived from double transfection in primary cultures of a cell like chromaffin cells when rescue strategies are required.

Chromaffin cells were nucleofected with VNUT-EGFP or EGFP (control cells) as described in *Materials and Methods* and the amperometry recordings were performed 48-h later. Secretory response was elicited by a 5-s pulse of BaCl₂. Nevertheless, the quantum size increased ~66% in cells that overexpressed VNUT. Table 2 summarizes these results. Note the quantitative differences between the control values from this and Table 1, as electrodes were different. In addition, control conditions are not

identical (scrambled vs. EGFP overexpression) and cells came from different cultures and experimental conditions (30).

Discussion

Phylogenetically speaking, ATP was probably the first neurotransmitter in the early forms of eukaryotic life and is now present in almost all of the SGs found in living organisms. Given this widespread distribution, the concept that ATP is costored with neurotransmitters should perhaps be reversed, such that almost all “secretable” substances are costored with ATP. The physiological significance of the high concentrations of ATP in SGs could reflect its intrinsic chemical properties. ATP has a high capacity for self-aggregation, behaving as a nonideal solute, which results in solutions with a far lower osmolarity than theoretically predicted. This physico-chemical interaction becomes even more evident when the osmotic behavior of ATP/catecholamine mixtures is studied (2, 13).

Because a cell cannot survive without cytosolic ATP, we diminished the expression of the vesicular nucleotide carrier, VNUT (20), to specifically reduce the ATP content of SGs without affecting its cytosolic concentration. Although the role of VNUT in ATP secretion from platelets has already been evaluated (31), to the best of our knowledge this is the first time that the influence of vesicular ATP on stored neurotransmitters has been studied in living cells. The lack of suitable antibodies or specific pharmacological blockers has impeded the characterization of VNUT. Moreover, many of the drugs widely used to study VNUT also have other effects, like the potent blocker DIDS (4,4'-diisothiocyano-2,2'-disulphonic stilbene acid) that is also a potent blocker of Cl⁻ channels (32, 33), or Evans' blue (another widely used VNUT blocker) that is also an antagonist of P2X purinergic receptors (34). Moreover, the strong color of the latter prevents the use of the luciferase method to measure ATP. In addition, glyoxylate is another compound thought to act as a selective VNUT inhibitor (31), although it is well known to strongly interfere with the cellular production of ATP.

Although the estimated concentration of vesicular ATP in chromaffin cells varies among studies, it is generally found to be hundreds of millimolars. Furthermore, the gross averaged catecholamine/ATP ratios are also found to vary from 4:1–9:1 (7, 35, 36) depending on the study and the amine (adrenaline or noradrenaline) measured (see below). These astonishingly high concentrations of ATP make it a unique candidate to be an intravesicular factor that stoichiometrically associates with catecholamines and, probably, many other neurotransmitters. Chromogranins A and B are the main protein components of chromaffin granules and these large, dense core vesicles still retain half of their catecholamine content even after the complete ablation of chromogranins (16). The remaining amine content (~400 mM) is still above theoretical isotonicity and, thus, the only remaining candidate to adsorb catecholamines must be ATP.

Here we silenced the expression of VNUT in bovine chromaffin cells and, as expected, the exocytotic component of ATP release was dramatically reduced. These data are consistent with those of earlier studies on PC12 cells (20) and hippocampal neurons (37). The reduction of vesicular ATP was accompanied

Table 2. The overexpression of VNUT-EGFP increases the quantum size of exocytosis

Experiment	<i>I</i> _{max} (pA)	<i>Q</i> (pC)	<i>t</i> _{1/2} (ms)	<i>m</i> (pA/ms)	<i>n</i> (cells)	<i>n</i> (spikes)
EGFP	47.0 ± 8.4	1.18 ± 0.18	22.0 ± 1.6	5.2 ± 0.9	12	1,135
VNUT-EGFP	68.3 ± 14.9	1.96 ± 0.36*	27.2 ± 3.4	7.5 ± 1.6	8	1,153
Change	+45.3%	+66.1%	+23.6%	+44.2%		

This is a representative experiment from two experiments carried out in different cell cultures, performed alternating cells that either overexpressed EGFP (control cells) or VNUT-EGFP cells. The data are expressed in the units in parentheses. **P* < 0.05 (Mann–Whitney *U* test).

by a decrease in catecholamine release without affecting noradrenaline, and with no measurable change in the total cell amine content. The explanation for this is that, even in the control situation only a fraction of the total SGs (roughly 30%) is releasable even by strong stimuli. From this population, only a small number of SGs are newly synthesized and consequently affected by siRNA. Because newly synthesized vesicles are released first (Fig. 4B), the siRNA affects only the first two to three stimuli and is not expected to measurably modify the total cellular amine content. When measuring the total cellular amine content we are underestimating the real extent of the role of VNUT (Fig. 5B). The fraction of SGs affected by VNUT silencing is smaller than that reported in the VNUT-KO mouse (21); these authors also reported a reduction in catecholamine secretion.

On density gradients we found ATP to be more tightly associated with adrenaline than noradrenaline (Fig. 3E). The most feasible explanation for this might be related to the different rates of the steps in adrenaline synthesis. Dopamine is taken up by SGs in a few minutes and there converted into noradrenaline. Some of the noradrenaline then slowly leaves the vesicle to become converted into adrenaline in the cytosol. The whole process ends in the uptake of adrenaline into SGs, requiring about 24 h (38). Because this last process is likely to be impaired, in the siVNUT-treated cells the expected concentration of released adrenaline must be smaller. This preferential effect on adrenaline is similar to that observed in the absence of chromogranins (16).

In the experiments of Fig. 3, cells were stimulated with a high concentration of the nicotinic agonist DMPP for a long period (5 min), whereby most nicotinic receptors should be desensitized and the secreted ATP is most likely to act on purinergic autoreceptors (39). To avoid these effects, rapid perfusion was used and VNUT-KD was seen to mainly affect only the first two to three secretory responses, the later secretory peaks remaining unaffected. Moreover, the selective presence of EGFP (from NPY-EGFP) in these two peaks demonstrated that the newly synthesized SGs are preferentially the first to be released and that these vesicles contain far fewer catecholamines and ATP than their controls (scrambled siRNA + NPY-EGFP-treated cells). These results support the “last synthesized/first released” theory postulated from evanescent wave microscopy (40, 41). However, our strategy using NPY-EGFP offers more direct evidence, given that the quantification in dot-blots can be compared with that of catecholamines and chromogranin B.

Total catecholamine secretion is reduced in VNUT-KD cells, in conjunction with a similar decrease in the frequency of secretory events, with VNUT-KD cells failing to maintain the number of secretory events as long as control cells. We do not have an explanation for this reduction in the number of spikes. One possible explanation is the presence of SGs with either no catecholamines or almost none, which is not detected by amperometry (42). In addition, the prefilled state of SGs has been recently proposed as a factor that modified the probability of exocytosis either in glutamate- (43) or GABA-neurons (44). It might be also argued that reduced activation of presynaptic purinergic receptors might occur because of the removal of feedback, although the intracellular calcium measurements suggest that purinergic regulation does not contribute to the reduction in spike frequency.

The overall effect of the reduction in vesicular ATP is a fall in the quantum release of catecholamines, particularly evident in the exocytotic events occurring within the first 70 s. As the quantum size of the spike feet decreases in parallel with the quantum size of spikes (45), it is expected that the number of spikes with foot and the foot duration was reduced (Table 1). Because I_{\max} and Q [net spike charge, expressed in picocoulomb (pC)] are highly correlated parameters (Fig. 6D), we conclude that the reduction in quantum size resulted in scalable smaller spikes without any kinetic qualitative differences indicating a

single phenomenon, contrary to that observed with other manipulations, like the suppression of chromogranins (16).

The main objective of this paper has been manipulate the intravesicular concentrations of ATP and study how these changes affected the accumulation of catecholamines inside SGs. Chromaffin cells are probably the best model to study neurosecretion using amperometry; however, they have some limitations when rescue protocols for siRNA of vesicular proteins are required. One such limitation is the restricted temporal window for obtaining a good number of amperometrical recordings. In addition, the efficiency of double transfection is very low. For these reasons we decided to promote the opposite effect: increase the vesicular ATP by overexpressing VNUT (46) in single-cell experiments. Table 2 clearly shows a substantial increase in the quantal amine release. Note that the overall parameters that described the kinetics of exocytosis also indicate the acceleration of the phenomenon, which is similar to that observed after catecholamine overload caused by L-DOPA (47, 48).

It should be noted that our data are probably underestimating the magnitude of the effect of ATP reduction in amine content as only a fraction of cells (35–40%) and only a fraction of SGs are affected by RNA interference. To the best of our knowledge, this is the first demonstration of the crucial role of vesicular ATP in the storage of a cotransmitter, probably based on its colligative properties (7).

The widespread distribution of vesicular ATP in most SGs from all known animal species has not received much attention from the scientific community. However, the participation of vesicular ATP in the storage and exocytosis of adrenal catecholamines raises new exciting questions regarding its possible participation in the homeostasis of other neurotransmitters.

Materials and Methods

A detailed description of the materials, antibodies, plasmid constructs, RNA isolation, cDNA synthesis, qRT-PCR, and SL17A9 mRNA knockdown generation, as well as Western blotting procedure can be found in *SI Materials and Methods*.

Cell Culture and Transfection. Bovine chromaffin cells were isolated by adrenal medulla digestion and cultured under standard conditions (*SI Materials and Methods*). To maximize the interference effect, we reduced the number of mature SGs by exposing isolated bovine chromaffin cells to a high K^+ solution (35 mM) 5 min before transfection. A detailed description of the procedure is given in *SI Materials and Methods*.

Immunofluorescence and Confocal Microscopy. Chromaffin cells (5×10^5 cultured cells) grown on 12-mm diameter poly-D-lysine-coated glass coverslips were fixed following a standard procedure. The analysis of codistribution was performed as described elsewhere (49); a detailed description can be found in *SI Materials and Methods*.

Isolation of Bovine Chromaffin Secretory Vesicles. Adrenal medullas were triturated and vesicles isolated by ultracentrifugation in continuous gradient of iodixanol, as described in *SI Materials and Methods*.

ATP Secretion Assay. Unless indicated, the selective inhibitor of ectoATPases ARL 67156 (100 μ M) was present during the whole procedure. The ATP was measured in 50 μ L of the supernatant using a luciferin-luciferase assay kit (Molecular Probes, Invitrogen) and quantified on sextuplicates in a standard luminometer (see a detailed description in *SI Materials and Methods*).

On-Line Analysis of Catecholamine Released from Perfused Bovine Chromaffin Cells. Chromaffin cells (3×10^6 cells) plated in plastic Petri dishes (92-mm diameter) were nucleofected with siRNA oligos and cultured for 24 h in standard conditions. The cells were then gently removed from the bottom of the dish with a cell scraper, centrifuged, and packaged in a 100- μ L microchamber sealed with a cigarette filter (cut to a 3-mm section). The cells were then perfused with Krebs-Hepes solution at the rate of 1 mL/min and the liquid emanated was conducted to an electrochemical detector (LC-4B, BioAnalytical Systems), connected to a PowerLab 8/30 (ADInstruments). The cells were stimulated every 3 min to secrete their catecholamines with 10-s

pulses of a Krebs-Hepes solution containing DMPP (10 μ M). Secretion was quantified by integration of the curves above basal levels.

Amperometric Detection of Exocytosis. Chromaffin cells transfected with Cy3 tagged siRNA oligonucleotides were viewed by epi-fluorescence under an inverted microscope (Olympus IX51, using a 40 \times /0.60 NA objective). Excited light (mercury lamp, X-cite EXFO series, 120W) was band-pass-filtered (BP545/25) and the emitted light was passed through a dichroic 565-lpxr and band-pass filter (BP650/70; all filters were from Chroma Technology). Carbon-fiber microelectrodes (6- μ m radius; Thorne P-55, Amoco) were prepared as described previously (30, 50), and electrochemical recordings were obtained using a VA-10X potentiostat (NPI Electronics) connected to a PowerLab 8/30 (ADInstruments) (see refs. 6 and 51, and *SI Materials and Methods* for details). All amperometric measurements were performed with a calibrated electrode gently touching the cell membrane. Cell release was stimulated by a 5-s pressure ejection of a BaCl₂ solution (5 mM) or DMPP (10 μ M) from a micropipette positioned 40 μ m from the cell.

Amperometric signals were low-pass-filtered at 1 kHz and collected at 4 kHz. The data were analyzed using locally written macros for IGOR that extract the following parameters from each spike (Wavemetrics) (52): I_{\max} , maximum oxidation current, expressed in picoampere; $t_{1/2}$, spike width at half height, expressed in milliseconds; Q , net spike charge, expressed in picocoulombs; m , ascending slope of spike, expressed in nanoamperes per second (nA/s). The discrimination threshold was fixed at 2.5 SD of the basal noise of the first derivative of each recording. It usually includes spikes with

an I_{\max} over 2.5 pA. The kinetic parameters were calculated as mean values from at least 20 spikes per cell. To avoid the bias caused by the different number of spikes produced by each cell, the average values of the spike's parameters recorded from each cell were considered as $n = 1$ (53).

HPLC Analysis of Catecholamines. Chromaffin cells were triturated in ice-cold lysis buffer containing perchloric acid (0.05 N) and 3,4-dihydroxybenzylamine (DHBA, 200 nM) as internal standards. The homogenates were centrifuged and the cleared supernatants were analyzed by HPLC coupled to electrochemical detection (54).

Statistics. The datasets are expressed as the means \pm SE. The statistical significance of the data from groups of experiments was assessed with the nonparametric Mann-Whitney U test or Student's t test as appropriate. Differences were considered significant at the level of $P < 0.05$. The data were analyzed with the Prism program (Graphpad Software).

The methods related to the measurement of cytosolic-free calcium are described in *SI Materials and Methods*.

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