# The Vesicular Transmitter Content in Chromaffin Cells can be Regulated via Extracellular ATP

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## ABSTRACT

The energy carrying molecule adenosine triphosphate (ATP) has been implicated for its role in modulation of chemical signaling for some time. Despite this, the precise effects and mechanisms of action of ATP on secretory cells are not well known. Here, bovine chromaffin cells have been used as a model system to study the effects of extracellular ATP in combination with the catecholamine transmitter norepinephrine (NE). Both transmitter storage and exocytotic release were quantified using complementary amperometric techniques. Although incubation with NE alone did not cause any changes to either transmitter storage or release, co-incubation with NE and ATP resulted in a significant increase that was concentration dependent. To probe the potential mechanisms of action, a slowly hydrolysable version of ATP, ATP-γ-s, was used either alone or together with NE. The result implicates two different behaviors of ATP acting on both the purinergic auto-receptors and as a source of the energy needed to load chromaffin cell vesicles.

## INTRODUCTION

The tightly regulated process of exocytotic release of catecholamines (dopamine, norepinephrine and epinephrine) has been extensively studied for some time. This is understandable given the widespread presence and variation of cellular

signaling affected by catecholamines including, regulation of fine movements, mood, memory and learning, and the flight-or-flight response. Exocytosis is initiated by a signal causing elevated cytosolic calcium.[1] In turn, this initiates fusion between the vesicular and plasma membranes, resulting in a pore exposing the lumen of the vesicle to the extracellular space.[2] The previously dominant hypothesis that the majority of vesicles completely collapsed into the plasma membrane and therefore released all of the content is facing increasing amounts of contradictory evidence.[3] In fact, in several cell types partial release of the transmitters and the subsequent recycling of the vesicle is now thought to be the main pathway for exocytotic signaling. This opens the possibility of exocytotic regulation at the single vesicle level, a feature seen by both our group and others and that appears to be malleable after pharmacological treatments.[4–9]

Another, yet related, aspect of exocytotic regulation occurs before transmitter release itself. Naturally, the total amount of transmitters loaded and stored inside vesicles can also provide a degree of regulation of the number of transmitters being released. Vesicles secreting catecholamines, in particular large dense core vesicles (LDCVs), are thought to contain high concentrations of transmitters perhaps as high as 0.5-1 M in chromaffin cells, whereas cytosolic levels are in the micromolar range.[10,11] To load the catecholamines across this substantial concentration gradient, the membrane potential and acidic interior of the vesicle is utilized by the vesicular monoamine transporter (VMAT).[12] The acidification is in turn driven by a V-ATPase, a crucial membrane protein present in all vesicles. V-ATPase uses adenosine triphosphate (ATP) as an energy source, to pump $H^+$ into the vesicular lumen.[12] Also, the vesicular catecholamine content can be manipulated using the VMAT inhibitor reserpine that significantly reduces the vesicular content and exocytotic response, or by cell incubation with the dopamine precursor, L-DOPA, which has the opposite effect.[13–15]

In addition, proteins of the granin family are expressed in LDCVs and are known to bind and condense catecholamines, a feature thought to be vital for the vesicle to maintain osmolarity despite the large amounts of solutes inside.[16–18] By genetically knocking out one or two of the main granins the amount of catecholamine released in exocytosis was halved.[19] Presumably this was due to the diminished loading capacity of the vesicles, although the content of the vesicles themselves was not quantified.[20,21]

Considering that even in the complete absence of granins, vesicles are still capable of concentrating catecholamines, we performed a detailed study of the colligative properties of intravesicular adenosine triphosphate (ATP) on the accumulation of catecholamines both *in vitro* and in living chromaffin cells.[22,23] Using this model, we have also shown that ATP enhances exocytotic release.[24] ATP, which is typically associated with the energetic metabolism of the cell, was added to the extracellular media. The resulting release was interpreted as prolonging the fusion pore opening time, leading to a larger amount of catecholamines released in each event.[24] Thus ATP appears to have multiple roles in the cellular machinery, one of which is to act as a neuromodulator during exocytotic release.

In this paper, we have studied the effects of ATP on vesicular loading and storage of transmitters as well as the interplay between ATP and norepinephrine (NE) using two electrochemical techniques, single cell amperometry (SCA) to measure exocytosis and intracellular vesicle impact electrochemical cytometry (IVIEC) to measure vesicle content in single living cells. We have found that NE itself does not increase vesicular content or the amount released, but combined extracellular exposure to NE and ATP significantly increased the vesicular content in a concentration dependent manner. This suggests that these two molecules work in concert to facilitate transmitter storage. In contrast, exposure to the slowly hydrolysable ATP-$\gamma$-S simultaneously to NE does not have an effect on vesicle content further suggesting a reliance of vesicle loading on the energy balance from ATP. Thus, our data suggest that ATP can both enhance vesicular transmitter storage and exocytotic release.

## RESULTS AND DISCUSSION

### Incubation with norepinephrine alone does not affect catecholamine storage in chromaffin vesicles

After incubating cells for 30 min in a solution containing either 1.6 mM or 3.2 mM NE, we measured the exocytotic response using single cell amperometry with a nanotip electrode as described in the Methods. Exocytosis was elicited through a 5-s application of 2 mM $Ba^{2+}$. Interestingly, no significant increase in the amount of released catecholamine was detected at any of the concentrations used (Fig. 1A). To confirm that NE did not change the vesicular content, the study was complemented using IVIEC. As the electrode in IVIEC is inside the cytosol, *in situ* quantitative

measurement of the total catecholamine content is possible when vesicles break onto the electrode surface and can be compared to the measured released amounts assayed by conventional amperometry. To reduce experimental variance, both SCA and IVIEC measurements were performed on cells in the same dishes. As seen in Fig. 1B, no significant changes were found in vesicular content after incubation with NE alone. Extracellular NE does not appear to increase either the quantal release or the vesicular content.

The uptake of NE by chromaffin cells is far less efficient than in sympathetic nerve terminals probably as this mechanism of recycling catecholamines is not of physiological interest for adrenal cells, whereas it is crucial in synapses.[25] However, short time incubations with large NE concentrations also failed to promote a significant increase of catecholamine level in isolated chromaffin granules (Fig. S1). These results are in contrast with early literature looking at uptake of [$^3$H]-NE.[26] In fact, to regulate extracellular NE concentration, a NE transporter (NET) has been found in several cell types including chromaffin cells.[27] However, differences in culture conditions, and foremost methodology, could explain this discrepancy. The major difference is that early uptake studies were based on observing the [$^3$H]-NE turnover in whole cell populations, whereas we have carried out a direct quantal measurement in single vesicles. It is therefore possible that NE is taken up into chromaffin cells, but does not enhance the content inside LDCVs in the acute period examined.

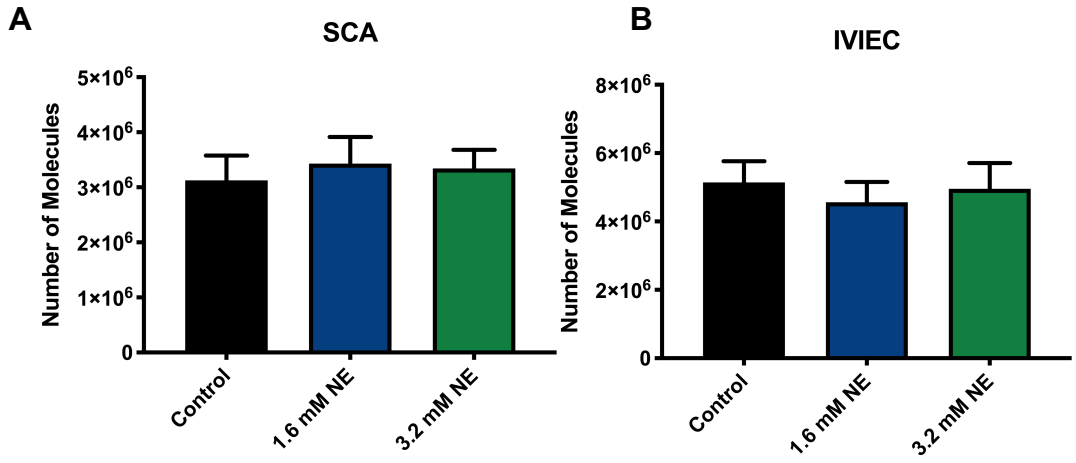


Fig 1. **A)** Released catecholamines as expressed by the number of molecules detected with SCA. **B)** Stored catecholamines in LDCVs as detected with IVIEC. Neither was significantly

different after 30 min of incubation with NE at the concentrations specified. Means of medians ± SEM, n >20 cells, Mann-Whitney two-tailed test.

**Presence of ATP allows additional loading of NE into vesicles**

As previously mentioned, catecholamines are loaded into LDCVs though the VMAT. This active transport is dependent on the pH gradient across the vesicular membrane driven by V-ATPase.[28] Although the requirement of cytosolic ATP for loading catecholamines into chromaffin granules has been well reported in the literature, the role of extracellular ATP is still unknown. It should be highlighted here that chromaffin cells store and release large concentrations of ATP.[23,29] We incubated cells with ATP and NE for 30 min. The ratio of ATP to NE (1:4) was decided based on previous literature showing the ratio of both species in LDCVs.[22,30] Measurements of quantal release and vesicular content were again assessed using SCA and IVIEC.

When exposed to both ATP and NE, cells released significantly larger amounts of catecholamines (Fig. 2A). This effect was concentration-dependent and also increased the total vesicular content (Fig. 2B). This effect was also observed for isolated vesicles (Fig. S2). This indicates that the larger release of catecholamines is driven by a larger vesicular cargo. An enhancement of catecholamine uptake in the presence of ATP has been shown previously in PC12 cells where a concentration dependent increase of both NE and dopamine uptake was observed, supporting the results here.[31]

Our group has successfully used the combination of SCA and IVIEC to estimate the fraction of transmitter molecules released during exocytosis by comparing the amount that is released to the total vesicular content of transmitters, in other words estimate the degree of partially released vesicle cargo. In fact, several pharmacological and chemical treatments such as cocaine, methylphenidate, lidocaine, zinc, and DMSO have been shown to affect the release fraction making this parameter of possible interest to study for regulation and plasticity in cellular communication.[6–8,32] However, in the case of co-incubation with ATP and NE, the change in fraction released is relatively small. For control cells, 61% of the vesicular content is released (comparable to previous studies), compared to 64% and 53% released following incubation with the lower and higher concentrations ATP and NE used, respectively.[5,33] This result shows that the increase in released transmitter is due to a pre-existing increase in vesicle content rather than a change in the fraction released.

Several amperometric peak parameters are known to correlate to steps in the exocytotic release, but for ATP and NE incubation these are not significantly different from control experiments (Fig. S3).[34] This confirms that co-treatment using ATP and NE does not alter the exocytotic mode nor its dynamics, but rather affects the signal strength by enhancing the transmitter storage efficiency of vesicles. The similarity in calculated percentages of released transmitters also supports this hypothesis.

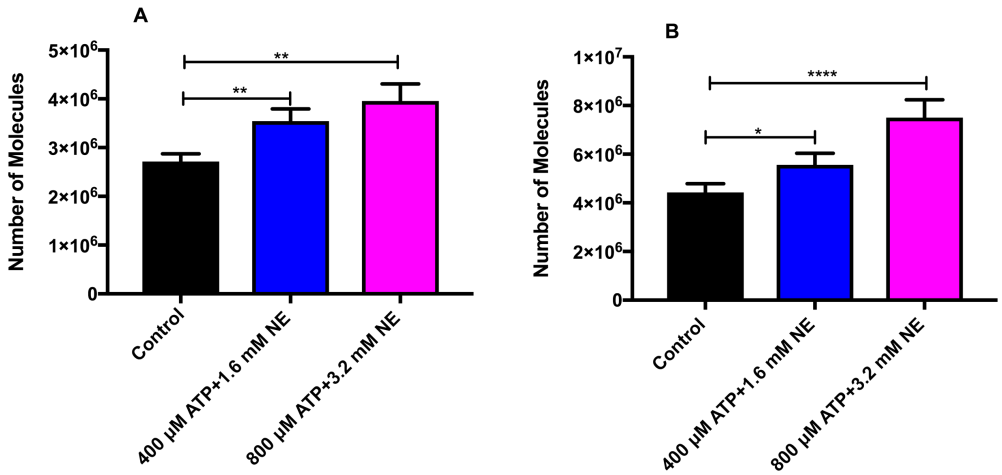


Fig 2. Co-incubation of ATP and norepinephrine for 30 min increase the transmitter release (A) and content (B) in a concentration dependent manner. Means of medians ± SEM, n >20 cells, Mann-Whitney two-tailed test with p-values indicated by * >0.0332, ** >0.0021, **** <0.0001.

**NE needs hydrolysable, energy providing ATP to load into vesicles**

To further test how ATP could facilitate the loading of NE into vesicles, a pharmacological approach was used. The ATP analog, ATP 5'-(γ-thiotriphosphate) (ATP-γ-S), is known

to be slowly hydrolysable, effectively decreasing the potential energy provided to the cell.[35]

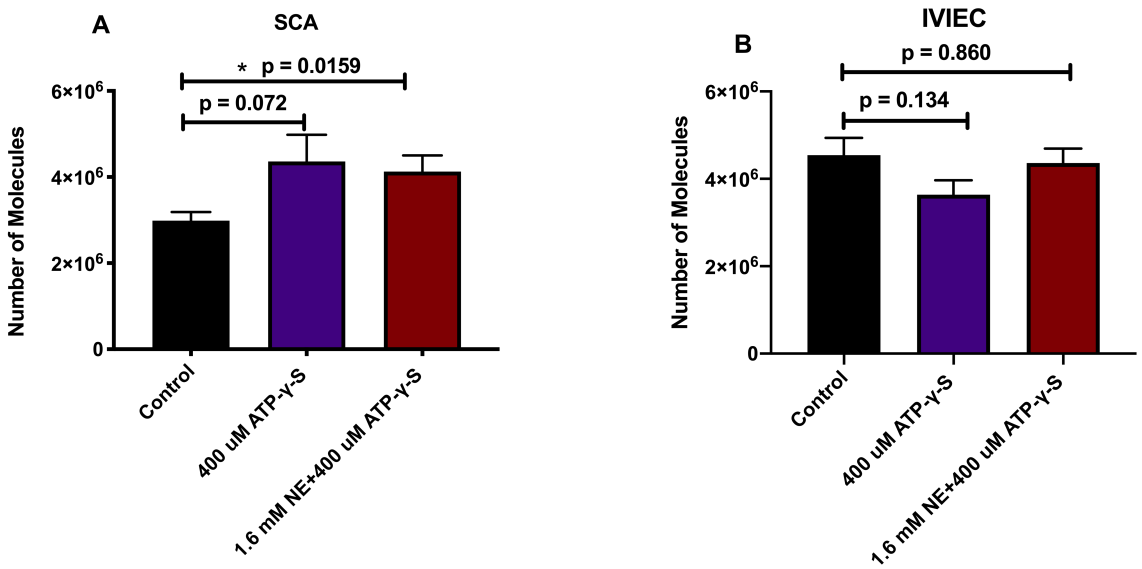


Fig 3. Pharmacological investigation using ATP-γ-s reveals that the effect of ATP on exocytosis (measured by SCA) is not dependent on the energetic abilities of ATP (A). On the contrary, enhanced loading (as measured by IVIEC) into the vesicles is dependent on hydrolysable ATP and is thereby limited when using ATP-γ-s (B). Means of medians ± SEM, n> 11 cells, Mann-Whitney two-tailed test with p-values indicated in the graphs.

ATP-γ-S can stimulate $P2X_2$ receptors with similar affinity to ATP.[36] This analog was added to the extracellular medium either alone or together with NE for 30 min before amperometric measurements. When measuring the released number of transmitters using SCA, both incubation with ATP-γ-S alone and ATP-γ-S combined with NE increased the number of molecules released during exocytosis, although only ATP-γ-S and NE together makes a significant difference at the 95 % confidence limit (Fig. 3A). In contrast, the vesicular content as measured by IVIEC is not significantly different for either treatment (Fig. 3B).

These different effects of ATP-γ-S can be used to dissect the two main mechanisms involved in NE accumulation and secretion, the purinergic auto-receptor activation which is common to ATP and ATP-γ-S and the energy-driven (i.e. V-ATPase) process that is solely activated by the natural ATP form. ATP and analogues such as ATP-γ-S appear to exert effects on the plasma membrane receptors that would influence the fusion pore and thus mainly cause changes in the exocytotic release

process. In the end, however, additional NE is needed for ATP to increase the content of messengers stored in each vesicle.

## CONCLUSIONS

We have described here novel mechanisms of quantum size regulation caused by ATP. We have shown that NE transport into secretory vesicles can be directly increased by a double mechanism compatible with the activation of purinergic receptors and by V-ATPase hyperactivation. When combined, extracellular exposure of NE and ATP results in an increase in the vesicular content of catecholamine, indicating that these two molecules work in concert to facilitate transmitter storage. This increase is dependent on the energy providing capabilities of ATP added with NE, and thus the slowly hydrolysable ATP-γ-S does not have a significant effect on vesicle content. Interestingly, ATP-γ-S has a similar enhancing effect on exocytotic release to ATP on intact cells. This could result from the function of ATP-γ-S as a purinergic receptor agonist. Given the long evolutionary history of ATP across all forms of life, it is not surprising that this basic molecule has different effects and acts in several roles for cellular communication making ATP a highly interesting molecule to study in relation to regulation of the exocytotic process.

## METHODS

### Chemicals and solutions

All reagents were obtained from Sigma-Aldrich, unless otherwise stated. Locke's stock buffer contained NaCl (1540 mM), KCl (56 mM), $NaHCO_3$ (36 mM), glucose (56 mM), and HEPES (50 mM) and 1% (v/v) penicillin, pH 7.4. This stock solution was diluted 10× with distilled water the day before the experiment and used for gland storage and rinsing the adrenal gland vein. $Ca^{2+}$ free isotonic solution: NaCl (154 mM), KCl (4.2 mM), $MgCl_2 \cdot 6H_2O$ (0.7 mM) , glucose (11.2 mM), HEPES (10 mM), pH 7.4. Barium stimulation solution: $BaCl_2$ (2mM), $MgCl_2$ (0.7 mM) in diluted Locke's buffer, pH 7.4. Collagenase P (from Clostridium histolyticum) was obtained from Roche, Sweden. Cell culture medium: DMEM:Ham's F12 1:1 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin, 1% (v/v) cytosine β-D-arabinofuranoside, 0.1% (v/v) 5-fuoro-2′-deoxyuridine. Homogenizing buffer: sucrose (230 mM), EDTA (1mM), $MgSO_4$

(1mM), HEPES (10 mM), KCl (10 mM), cOmplete enzyme inhibitor (Roche, Sweden), DNase I (10 µg/ mL) (Roche), 0.001 oligomycin, pH 7.4, ~310 mOsm.

**Cell Isolation**

Bovine adrenal glands were obtained from a local slaughterhouse, and transported in cold Locke's buffer. The chromaffin cells were isolated as previously described.[37] Briefly, after sterilizing the glands with a 70% solution of ethanol, fat and connective tissue were trimmed away. Blood cells were cleared by rinsing the veins with Locke's buffer. The medulla was isolated after digesting the gland with collagenase P (0.2%, Roche, Sweden) treatment. Digested medulla was filtered over steel sieves and diluted with Locke's buffer to reduce the activity of collagenase P. The cells were pelleted at 300 g for 10 min at room temperature and the pellet obtained was resuspended in Locke's buffer and filtered over a sterile 100 µm nylon mesh. The chromaffin cell suspension was mixed with sterile Percoll/Locke's buffer (10:1) and centrifuged at 18 600 g (Avanti J- 20XP) for 20 min at room temperature. The top layer of the density gradient was collected and filtered over 100 µm nylon mesh. Again, the cell suspension was diluted with Locke's buffer and centrifuged at 300g for 10 min at room temperature to exclude Percoll. About 500 000 cells were seeded on 60 mm collagen (IV) coated plastic dishes (Corning Biocoat, VWR, Sweden) and maintained in a humidified incubator at 37 °C, 5% $CO_2$ for a maximum of 3 days prior to experiments.

**Single-cell experiments**

In order to detect and quantify both the number of transmitters released and stored inside vesicles, the electrochemical technique amperometry was used. Electrochemical recordings from single cells were performed on an inverted microscope (Olympus), in a Faraday cage. The working electrode was held at + 700 mV versus an Ag/AgCl reference electrode by using an Axon 200B potentiostat (Molecular Devices, Sunnyvale, CA). Nanotip carbon fiber electrodes were prepared as previously described.[5] Briefly, a 5 µm carbon fiber was aspirated into a borosilicate capillary (1.2 mm o.d., 0.69 mm i.d., Sutter Instrument Co., Novato, CA) and pulled in half. The exposed carbon fiber at the end of the capillary was cut to a length of 100-150 µm and then flame etched in a butane burner to create a sharp, conical tip with a tip diameter of ~100 nm. Nanotip electrodes were sealed using epoxy (Epo-Tek,

Billerica, MA) followed by a 10 s acetone wash to clean the fiber from epoxy. Before experiments, electrodes were tested in a 100 μM solution of dopamine using cyclic voltammetry, and only electrodes with diffusion limited steady-state current and low charging current were used. To measure the amount of transmitters released from a cell, the electrode was placed in close vicinity of the plasma membrane in a technique known as single cell amperometry. Upon stimulation, vesicles release transmitters through the fusion pore and these are detected as a current spike. By integrating the area under the spike, the measured charge can be related to the number of transmitters detected through Faraday's law.[38] A similar principle was applied quantify the catecholamine content of vesicles inside the cell. In the technique known as intracellular vesicular electrochemical cytometry, the nanotip electrode is carefully placed inside a cell by piercing the plasma membrane. However, instead of using stimulation to initiate exocytosis, the electrode is simply left in contact with the cytosol to allow vesicles to diffuse and eventually attach to the electrode surface. Due to the applied potential, some vesicles will electroporate and the transmitter content will be oxidized and measured through a similar spike as for SCA.[5,33]

**Data Acquisition and Analysis**

Current transients were recorded and digitized using a Digidata1440A (Molecular Devices) and digitized at 10 kHz and filtered at 2 kHz using a 4-pole Bessel filter. The data were converted in Matlab (The MathWorks, Inc.) and processed in an IgorPro 6.22 routine originating from David Sulzer's group (Columbia University). The detection limit was set to 5 × RMS of the noise measured from the initial baseline in each measurement. In addition, traces were manually checked for potential false detections done by the software. Spike characteristics were determined as number of molecules based on the charge measured in each spike, the rise time, $t_{rise}$, which was defined as the time separating 25% of the maximum from 75% of the maximum on the ascending part of the spike; the half peak width $t_{1/2}$, which was defined as the width of the exocytotic peak at half of its magnitude; the fall time, $t_{fall}$, which was defined as the time separating 75% of the maximum from 25% of the maximum on the descending part of the spike. The medians from all cells were pooled, and groups were statistically analyzed using Prism 7 (GraphPad, La Jolla, CA) with an unpaired two-tailed Mann−Whitney rank sum test due to non-normal distributions.

## ASSOCIATED CONTENT

*Supporting Information

Methodology for vesicular impact electrochemical cytometry (VIEC).

Figure S1. Analysis of transmitter content in isolated vesicles after NE treatment.

Figure S2. Transmitter content in isolated vesicles increases after NE + ATP treatment.

Figure S3. Analysis of SCA peak parameters after ATP and NE treatment.

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## AUTHOR CONTRIBUTIONS

All authors have given approval to the final version of the manuscript. Anna Larsson and Soodabeh Majdi conceived the idea in discussion with Ricardo Borges and Andrew Ewing. Larsson and Majdi carried out the experiments, analysed and interpreted the data, and wrote the manuscript. Ewing and Borges were involved in intellectual discussions and editing the manuscript. Andrew Ewing was involved in supervising, discussions of data interpretation, designing additional experiments, outlining the manuscript, revisions and editing.

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## ABBREVIATIONS

ATP adenosine triphosphate; LDCV large dense core vesicle; IVIEC intracellular vesicle impact electrochemical cytometry; NE norepinephrine; NET norepinephrine transporter; SCA single cell amperometry; VIEC vesicle impact electrochemical cytometry; VMAT vesicle monoamine transporter.

## REFERENCES


(1) García, A. G., García-De-Diego, A. M., Gandía, L., Borges, R., and García-Sancho, J. (2006) Calcium signaling and exocytosis in adrenal chromaffin cells. *Physiol. Rev. 86*, 1093–1131.
(2) Burgoyne, R. D., and Morgan, A. (2003) Secretory granule exocytosis. *Physiol. Rev. 83*, 581–632.
(3) Ren, L., Mellander, L. J., Keighron, J., Cans, A.-S., Kurczy, M. E., Svir, I., Oleinick, A., Amatore, C., and Ewing, A. G. (2016) The evidence for open and closed exocytosis as the primary release mechanism. *Q. Rev. Biophys. 49*, e12.
(4) Hu, R., Ren, B., Lin, C.-J., Oleinick, A., Svir, I., Tian, Z.-Q., and Amatore, C. (2016) How "Full" is "Full Fusion" during Exocytosis from Dense Core Vesicles? Effect of SDS on "Quantal" Release and Final Fusion Pore Size. *J. Electrochem. Soc. 163*, H853–H865.
(5) Li, X., Majdi, S., Dunevall, J., Fathali, H., and Ewing, A. G. (2015) Quantitative Measurement of Transmitters in Individual Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes. *Angew. Chemie - Int. Ed. 54*, 11978–11982.
(6) Ren, L., Pour, M. D., Majdi, S., Li, X., Malmberg, P., and Ewing, A. G. (2017) Zinc Regulates Chemical-Transmitter Storage in Nanometer Vesicles and Exocytosis Dynamics as Measured by Amperometry. *Angew. Chemie - Int. Ed. 56*, 4970–4975.
(7) Majdi, S., Najafinobar, N., Dunevall, J., Lovric, J., and Ewing, A. G. (2017) DMSO Chemically Alters Cell Membranes to Slow Exocytosis and Increase the Fraction of Partial Transmitter Released. *ChemBioChem 18*, 1898–1902.
(8) Zhu, W., Gu, C., Dunevall, J., Ren, L., Zhou, X., and Ewing, A. G. (2019) Combined Amperometry and Electrochemical Cytometry Reveal Differential Effects of Cocaine and Methylphenidate on Exocytosis and the Fraction of Chemical Release. *Angew. Chemie - Int. Ed. 58*, 4238–4242.
(9) Wu, Q., Zhang, Q., Liu, B., Li, Y., Wu, X., Kuo, S., Zheng, L., Wang, C., Zhu, F., and Zhou, Z. (2019) Dynamin 1 Restrains Vesicular Release to a Subquantal Mode in Mammalian Adrenal Cheomaffin Cells. *J. Neurosci. 39*, 199–211.
(10) Albillos, A., Dernick, G., Horstmann, H., Almers, W., Alvarez de Toledo, G., and Lindau, M. (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature 389*, 509–512.
(11) Mosharov, E. V., Gong, L. W., Khanna, B., Sulzer, D., and Lindau, M. (2003) Intracellular patch electrochemistry: Regulation of cytosolic catecholamines in chromaffin cells. *J. Neurosci. 23*, 5835–5845.
(12) Wimalasena, K. (2011) Vesicular monoamine transporters: Structure-function, pharmacology, and medicinal chemistry. *Med. Res. Rev. 31*, 483–519.
(13) Pothos, E., Desmond, M., and Sulzer, D. (2002) l-3,4-Dihydroxyphenylalanine Increases the Quantal Size of Exocytotic Dopamine Release In Vitro. *J. Neurochem. 66*, 629–636.
(14) Colliver, T. L., Pyott, S. J., Achalabun, M., and Ewing, A. G. (2000) VMAT-Mediated Changes in Quantal Size and Vesicular Volume. *J. Neurosci. 20*, 5276–5282.
(15) Lovrić, J., Dunevall, J., Larsson, A., Ren, L., Andersson, S., Meibom, A., Malmberg, P., Kurczy, M. E., and Ewing, A. G. (2017) Nano Secondary Ion Mass Spectrometry Imaging of Dopamine Distribution Across Nanometer Vesicles. *ACS Nano 11*, 3446–3455.

(16) Videen, J. S., Mezger, M. S., Chang, Y., Daniel, T., and Connor, O. (1992) Calcium and Catecholamine Interactions with Adrenal Chromogranins. *J. Biol. Chem. 267*, 3066–3073.
(17) Yoo, S. H., Albanesi, J. P., and Jameson, D. M. (1990) Fluorescence studies of nucleotide interactions with bovine adrenal chromogranin A. *Biochim. Biophys. Acta (BBA)/Protein Struct. Mol. 1040*, 66–70.
(18) Helle, K. B., Reed, R. K., Pihl, K. E., and Serck-Hanssen, G. (1985) Osmotic properties of the chromogranins and relation to osmotic pressure in catecholamine storage granules. *Acta Physiol. Scand. 123*, 21–33.
(19) Díaz-Vera, J., Camacho, M., Machado, J. D., Domínguez, N., Montesinos, M. S., Hernández-Fernaud, J. R., Luján, R., and Borges, R. (2012) Chromogranins A and B are key proteins in amine accumulation, but the catecholamine secretory pathway is conserved without them. *FASEB J. 26*, 430–438.
(20) Diaz-Vera, J., Morales, Y. G., Hernandez-Fernaud, J. R., Camacho, M., Montesinos, M. S., Calegari, F., Huttner, W. B., Borges, R., and Machado, J. D. (2010) Chromogranin B Gene Ablation Reduces the Catecholamine Cargo and Decelerates Exocytosis in Chromaffin Secretory Vesicles. *J. Neurosci. 30*, 950–957.
(21) Montesinos, M. S., Machado, J. D., Camacho, M., Diaz, J., Morales, Y. G., Alvarez de la Rosa, D., Carmona, E., Castaneyra, A., Viveros, O. H., O'Connor, D. T., Mahata, S. K., and Borges, R. (2008) The Crucial Role of Chromogranins in Storage and Exocytosis Revealed Using Chromaffin Cells from Chromogranin A Null Mouse. *J. Neurosci. 28*, 3350–3358.
(22) Taleat, Z., Estévez-Herrera, J., Machado, J. D., Dunevall, J., Ewing, A. G., and Borges, R. (2018) Electrochemical Investigation of the Interaction between Catecholamines and ATP. *Anal. Chem. 90*, 1601–1607.
(23) Estévez-Herrera, J., Domínguez, N., Pardo, M. R., González-Santana, A., Westhead, E. W., Borges, R., and Machado, J. D. (2016) ATP: The crucial component of secretory vesicles. *Proc. Natl. Acad. Sci. 113*, E4098–E4106.
(24) Majdi, S., Larsson, A., Najafinobar, N., Borges, R., and Ewing, A. G. (2019) Extracellular ATP Regulates the Vesicular Pore Opening in Chromaffin Cells and Increases the Fraction Released During Individual Exocytosis Events. *ACS Chem. Neurosci. 10*, 2459–2466.
(25) Wakade, A. R., and Wakade, T. D. (1984) Absence of Catecholamine Uptake Mechanism in the Isolated Perfused Adrenal Gland of the Rat. *Neurosci. Lett. 50*, 139–143.
(26) Banerjee, D. K., Lutz, R. a, Levine, M. a, Rodbard, D., and Pollard, H. B. (1987) Uptake of norepinephrine and related catecholamines by cultured chromaffin cells: characterization of cocaine-sensitive and -insensitive plasma membrane transport sites. *Proc. Natl. Acad. Sci. U. S. A. 84*, 1749–1753.
(27) Mandela, P., and Ordway, G. A. (2006) The norepinephrine transporter and its regulation. *J. Neurochem. 97*, 310–333.
(28) Johnson, R. G. (2017) Accumulation of biological amines into chromaffin granules: a model for hormone and neurotransmitter transport. *Physiol. Rev. 68*, 232–307.
(29) Burnstock, G. (2006) Historical review: ATP as a neurotransmitter. *Trends Pharmacol. Sci. 27*, 166–176.
(30) Kopell, W. N., and Westhead, E. W. (1982) Osmotic pressures of solutions of ATP and catecholamines relating to storage in chromaffin granules. *J. Biol. Chem. 275*, 5707–5710.
(31) Echleman, A., Dunigan, C., Shamoo, A., and Eldefrawi, M. (1995) ATP enhances catecholamine uptake into PC12 cells. *Life Sci 56*, 1613–1621.
(32) Ye, D., Gu, C., and Ewing, A. G. (2018) Using Single-Cell Amperometry and Intracellular Vesicle Impact Electrochemical Cytometry to Shed Light on the Biphasic Effects of Lidocaine on Exocytosis. *ACS Chem. Neurosci.* 6–12.
(33) Lovric, J., Najafinobar, N., Dunevall, J., Majdi, S., Svir, I., Oleinick, A., Amatore, C., and Ewing, A. (2016) On the mechanism of electrochemical vesicle cytometry: chromaffin cell vesicles and liposomes. *Faraday Discuss.* DOI: 10.1039/C6FD00102E.
(34) Oleinick, A., Lemaître, F., Collignon, M. G., Svir, I., and Amatore, C. (2013) Vesicular release of neurotransmitters: Converting amperometric measurements into size, dynamics and energetics of initial fusion pores. *Faraday Discuss. 164*, 33–55.
(35) Welford, L. A., Cusack, N. J., and Hourani, S. M. O. (1987) The structure-activity

relationships of ectonucleotidases and of excitatory P2-purinoceptors: evidence that dephosphorylation of ATP analogues reduces pharmacological potency. *Eur. J. Pharmacol. 141*, 123–130.
(36) Burnstock, G. (2007) Purine and pyrimidine receptors. *Cell. Mol. Life Sci. 64*, 1471–1483.
(37) Connor, D. T. O., Mahata, S. K., Mahata, M., Jiang, Q., Hook, V. Y., and Taupenot, L. (2007) Primary culture of bovine chromaffin cells. *Nat. Protoc. 2*, 1248–1253.
(38) Leszczyszyn, D. J., Jankowski, J. a, Viveross, H., Diliberto, E. J., Nears, J. a, and Wightman, R. M. (1990) Nicotinic Receptor-mediated Catecholamine Secretion from Individual Chromaffin Cells. *J. Biol. Chem. 265*, 14736–14737.
(39) Pardo, M. R., Estévez-Herrera, J., Castañeyra, L., Borges, R., and Machado, J. D. (2017) Isolation of mouse chromaffin secretory vesicles and their division into 12 fractions. *Anal. Biochem. 536*, 1–7.