Extracellular ATP Regulates the Vesicular Pore Opening in Chromaffin Cells and Increases the Fraction Released During Individual Exocytosis Events

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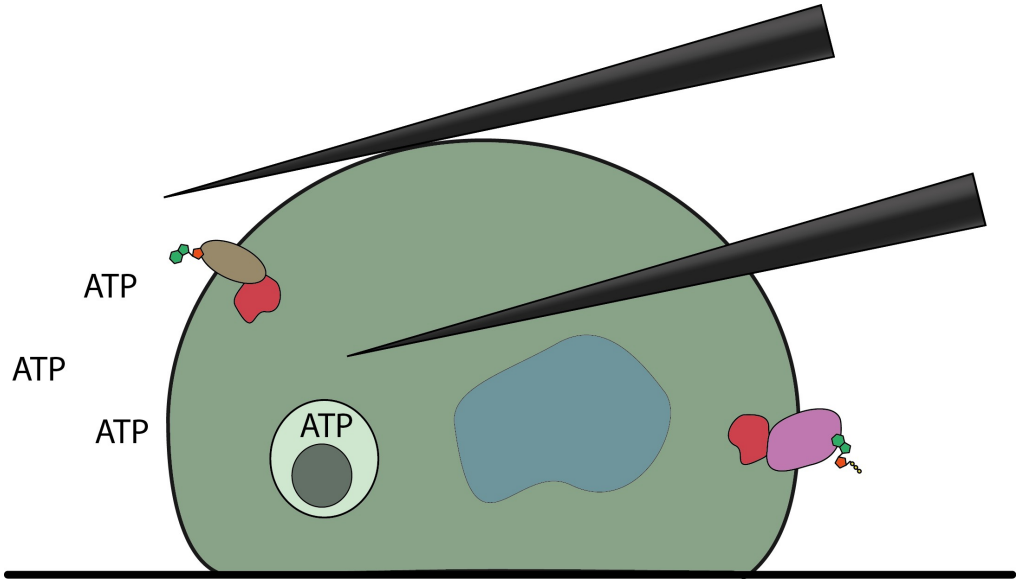
KEYWORDS: ATP • Amperometry • Vesicle impact cytometry • Exocytosis • Fusion pore • Partial release

TOC



ABSTRACT

Adenosine triphosphate (ATP) is the main energy source for cellular metabolism. Besides that, ATP is a neurotransmitter and a co-transmitter that act on purinergic receptors present either pre- or postsynaptically. Almost all types of secretory vesicles from any neuron or animal species contain high concentrations of ATP being an essential factor in the accumulation of neurotransmitters. In this work, we have studied the effects of ATP on quantum catecholamine release and vesicular storage in chromaffin cells. We have combined three electrochemical methods, conventional amperometry with *'intracellular vesicle impact electrochemical cytometry'* (IVIEC) and '*vesicle impact electrochemical cytometry'* (VIEC). We have found that extracellular ATP increased the released quantal fraction of catecholamine but not its vesicular content. Studying the dynamics of exocytosis events in ATP treated cells showed that ATP affects the release fusion pore. In order to elucidate the mechanisms of the observed ATP effects, cells and vesicles were pharmacologically treated with suramin (a purinergic blocker) and ARL-67156 (an inhibitor of ecto-ATPases). The data indicate that the catecholamine content of vesicles increased compared to control after these drugs. Our data suggest that ATP, acting on purinergic receptors increases the quantum releasable size through an increased fusion pore opening and that ARL and/or suramin protect the vesicle from neurotransmitter leakage by functioning as competitive inhibitors of ATP.

INTRODUCTION

Besides its role as the 'energetic coin', adenosine triphosphate (ATP) is a molecule involved in a plethora of biological cellular functions. ATP was probably the first neurotransmitter and it is accumulated in almost all secretory vesicles (SVs) in all animals including very primitive forms of life like *Giardia lamblia* or *Paramecium*.[1,2] With relation to SVs, ATP is known for its essential role in the acidification and loading of neurotransmitters, as well as in the critical steps of transport and priming of vesicles.[3] ATP also facilitates the accumulation of catecholamines inside vesicles by lowering the vesicular osmolarity.[4,5] In addition, most cells have purinergic receptors on their plasma membrane, meaning ATP itself acts as a neurotransmitter which is released either alone or accompanying other transmitters (co-transmission).[6] For instance, in the model system for transmission used here, bovine chromaffin cells, purinergic receptors such as P2Y and P1 (also known as adenosine receptors) have been identified.[7–9] Due to the widespread presence of ATP, this molecule might have further, yet unknown, roles regarding exocytosis.

Exocytosis is the main process of chemical communication between cells including neurons. It consists in the fusion of a SV with the plasma membrane thus allowing the release of its content to the external medium. This is a highly regulated process not only in the amount of SVs that undergo exocytosis but also in the amount of transmitter released in each quanta and their releasing kinetics. Growing experimental evidence has shown that exocytosis does not occur as a simple all-or-nothing process, but is adjustable and can occur through different modes such as kiss-and-run, open-and-closed, or full release.[10–12] These modes provide the cell with an additional step that can be fine-tuned and which can control and regulate the signaling strength in order to adapt to the current situation. However, currently not much is known of how this regulation occurs or which factors influence the mode of exocytosis, probably for the lack of technical approaches to determine the extent of the fraction of released upon each fusion event.

A widely used method for quantifying exocytosis with high temporal resolution is amperometry. This method is based on the electroactive properties of many biological amines such as dopamine, norepinephrine, epinephrine, histamine and serotonin and has been used to analyze single cell exocytosis for almost 30 years.[13] Briefly, a carbon-fiber microelectrode is placed onto to the cell surface with an applied potential sufficient to oxidize the transmitter of interest. When exocytosis occurs, the

released transmitters are detected at the electrode tip with a 'spike-like' feature of oxidative current. The analysis of the size and shape of the spike provides direct information of the dynamics of the releasing events.

As a complement to conventional single-cell amperometry (SCA), our group has recently developed two novel technical approaches to measure the total vesicular content. One of these is known as *'intracellular vesicle impact electrochemical cytometry'* (IVIEC), where a thin and sharp nanotip carbon-fiber electrode is placed inside the cell by carefully piercing the plasma membrane. Organelles such as SVs can then adsorb and stochastically break on the active microsurface of the electrode. IVIEC permits the amperometric detection of the electroactive content preserving the native environment of the organelle.[14,15] The other technique works by a similar mechanism, but instead of measuring vesicle content *in situ,* the vesicles are isolated and maintained in suspension in an intracellular physiological buffer. This technique is called *'vesicle impact electrochemical cytometry'* (VIEC) and has the advantage to allow the bathing media to be modified and thus to treat SVs without potential interferences from the rest of the cellular machinery.[16] While the amperometric techniques mentioned above are useful enough on their own, their true power becomes evident when used in combination. As it permits measuring the real quantum size and to compare with the apparent quantum size observed with conventional amperometry. Moreover, this allows the investigation of the different modes of exocytosis and their regulation to be studied in a detailed manner. For instance, their combination allows identifying with precision the locus of action of drugs, or environmental changes on exocytosis. This combination is also highly useful to define the role of proteins in exocytosis resulting from genetically modified animals, which can be quantified and, in part, localized as to where the site of action is: cellular or vesicular.

In this paper, we have used a combination of these three electrochemical tools, SCA, IVIEC and VIEC to investigate the effects of extracellular ATP on exocytosis in bovine chromaffin cells. We show that the fraction of released transmitters increases with added ATP and this effect can be partly attenuated through pharmacological manipulation. This suggests ATP might function as a neuromodulator by the presynaptic regulation of the strength of partial fusion.

RESULTS AND DISCUSSION

Extracellular ATP increases the fraction of released catecholamines in a concentration dependent manner

Exocytosis from chromaffin cells was measured by placing a nanotip electrode close to the surface of the cell. Secretion was elicited by pressure ejection of 2 mM Ba^{2+} solution for 5 s. The area under each current transient relates to oxidation of the catecholamines at the electrode after stimulation. Faraday's law, N = Q/nF, is used to quantify the number of molecules released (N), in which Q is the charge from the time integral of current transients or amperometric spikes, n is the number of electrons exchanged in the oxidation reaction (two electrons for catecholamines), and F is the Faraday constant (96,485 C mol^{-1} of electrons). The number of molecules released per exocytotic event after incubation of chromaffin cells for 30 min with different concentrations of ATP is plotted in pink in Figure 1.

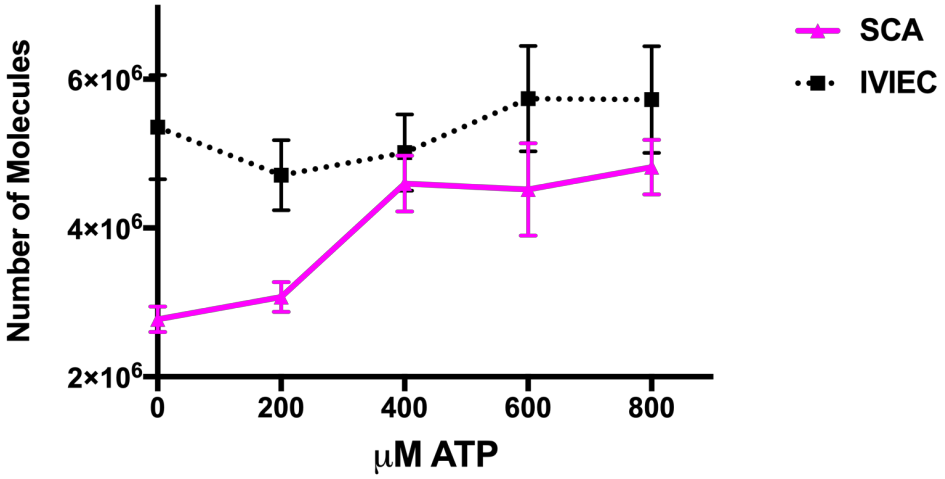


Figure 1. Vesicle content (measured by IVIEC) and released (measured by SCA) in different concentration of ATP. Extracellular ATP does not significantly change, the vesicle content, but largely increased the released fraction. Data are means of median ± standard errors. Details for p-values are shown in table S1. n= 25 cells from 3 different chromaffin cultures.

To investigate whether ATP caused any effect on vesicle content, IVIEC experiments were carried out. For IVIEC, an electrode of the same construction was used to pierce the plasma membrane to detect vesicle content *in situ*. The results presented in black in Figure 1 show no changes in catecholamine content regardless the presence of ATP. However, the comparison with SCA shows that only 52% of the vesicle content was released during exocytosis when external ATP was absent. However, after incubation of the cells in a high concentration ATP solution (800 μM) for 30 min, this apparent quantal release of catecholamines increased to 85%. These

comparisons show that although ATP does not significantly change vesicle content, it enhances the released fraction by a membrane-dependent mechanism. Our results are compatible with a switching mode of exocytosis from short towards longer fusion pore expansion. Due to the high temporal resolution of amperometric measurements, this method is not only quantitative but can provide information about the dynamics of the exocytotic events. Subsequently, to investigate the effect of ATP on the fusion pore opening during exocytosis we used this advantage of the amperometric method to study the dynamics of the exocytotic process affected by ATP.

ATP decreases exocytotic dynamics seemingly through fusion pore action

Spike parameters obtained from the shape of exocytosis events at different concentrations of ATP show that treatment leads to a significant increase in the rise time (t_{rise}) and width ($t_{1/2}$) of the amperometric event as measured by amperometry (Figure 2), whereas the fall time (t_{fall}) does not change significantly (Figure S1). The

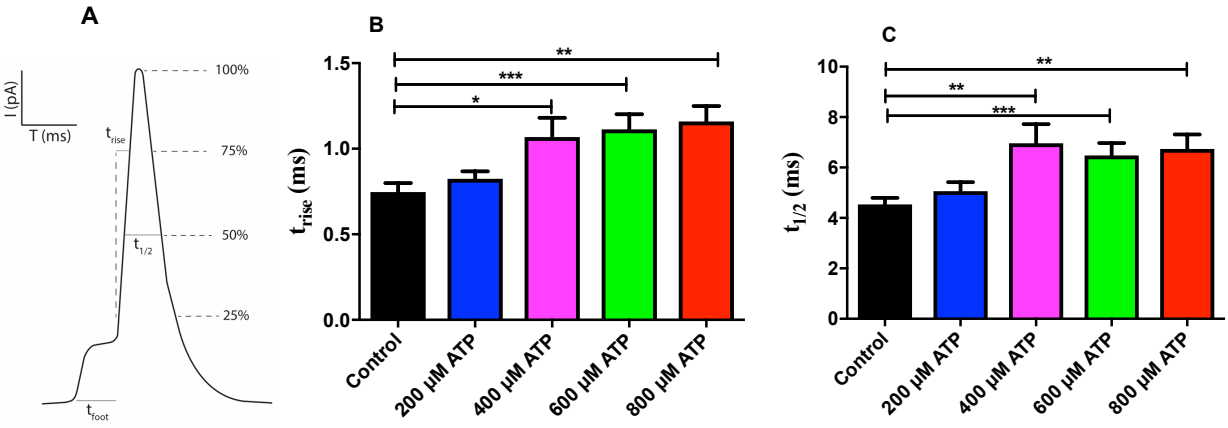


Figure 2. Spike parameters from exocytosis obtained by SCA after treatment of cells with different concentrations of ATP. Secretion was elicited by 5 s pulse of Ba^{2+} (2 mM) **A)** Typical amperometrical spike with different parameters used for peak analysis, t_{rise}: rise time, $t_{1/2}$:half peak width, t_{foot} :foot duration. **B)** Rise time of amperometrical spikes obtained from cells incubated with different concentrations of ATP. **C)** The effect of ATP on the half peak width duration. Data show means ± standard error. Unpaired, nonparametric, Mann-Whitney test, 95% confidence level, P-value: (*) 0.0332, (**) 0.0021, (***) 0.0002. n= 27 cells from 3 different chromaffin cell cultures.

occurrence of changes in the first part of the spike, with negligible alteration in the falling part suggest that ATP influences the fraction of catecholamine released during exocytosis by favoring the pore expansion. If this is correct, then the pre-spike opening might be affected as well.

The data from the pre-spike feature, the so-called 'foot', obtained with SCA show that increasing the concentration of ATP does increase both the duration of the foot (t_{foot}) and subsequently increases the amount of catecholamines released via the initial

fusion pore (Figure 3). This is compatible with the exposure to extracellular ATP stabilizing the initial fusion pore making it last longer prior to continued expansion. As the fall time of the spikes is not altered (Figure S1) it seems likely that ATP does not affect the fusion pore closure and it does not act on the vesicle matrix. One of the several proteins suggested to regulate the fusion pore is actin, the filamentous component of the cytoskeleton. Effects of actin on the fusion pore have been seen by both electrochemical detection of exocytosis as well as using STED imaging.[17,18]

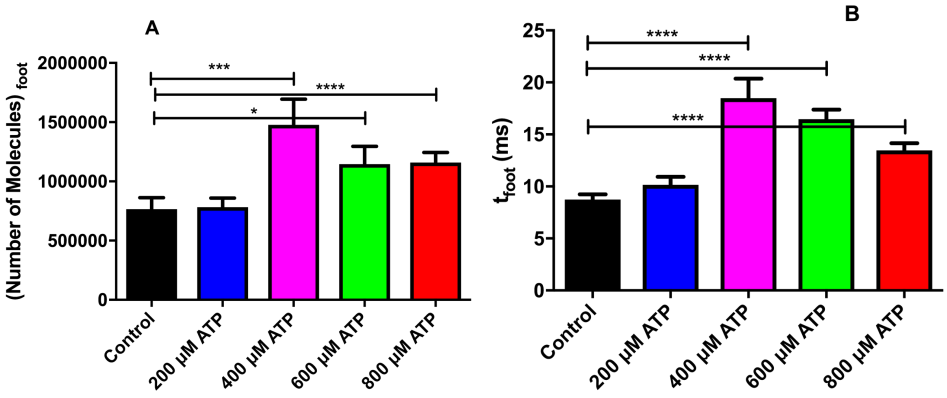


Figure 3. Effects of ATP on pre-spike foot duration. Bar plots from exocytosis experiments measured by SCA under different concentrations of ATP. **A)** The average number of catecholamine molecules released and **B)** foot duration. Data show mean of median ± standard error. Unpaired, nonparametric, Mann-Whitney test, 95% confidence level, P-value: (*) 0.0332, (**) 0.0021, (***) 0.0002, (****) <0.0001. n= 27 cells from 3 different chromaffin cell cultures.

Interestingly, besides being the substrate for the ATPase activity of actin, a higher level of ATP has been suggested to cause more stiffness in actin filaments.[19] Such changes in the physical properties of the cytoskeleton could be a potential cause for the slower exocytotic dynamics seen here as opposed to changing the kinetics of actin polymerization.

Data available about the role of ATP in chromaffin cells are still controversial, probably due to the different experimental approaches used. One possible explanation for the observed effects of externally applied ATP could be an increase in the amount of free cytosolic Ca^{2+}. However, our results show that ATP in the presence of physiological external Ca^{2+} does not change intracellular divalent cation levels upon Ba^{2+} stimuli (Figure S2). It is also widely accepted that ATP acts on purinergic auto-receptors on the chromaffin cell membrane. The reported effects caused by this stimulation on secretion are controversial, hence Harkins & Fox reported an inhibition that is in agreement with Gandia et al., who described the modulation of Ca^{2+} currents by purinergic autoceptors.[20,21] This seems to be mediated by P2Y.[22] Conversely, it has

also been reported that purinergic receptor P2Y stimulation can potentiate secretion probably by acting on IP3 production coupled with an increase in cytosolic free calcium.[23–25] Nevertheless, to our knowledge the effect of ATP on the apparent quantum size of SVs has not been reported. This action seems to involve the latest stages of exocytosis, probably by prolonging the opening time of the fusion pore as the kinetic characteristics of amperometric spikes (increasing the ascending part of spikes accompanied with no effects on the decaying part) indicate that other phenomenon like the affinity for the vesicular matrix are not affected.

The blockade of purinergic receptors antagonizes the effect of ATP

The potential interactions of ATP on the purinergic receptors were tested by using the un-specific purinergic blocker suramin, which antagonizes P2X and P2Y receptors. We also used ARL-67156, an inhibitor of ecto-ATPases (the enzyme responsible for degrading ATP to ADP and AMP).[26–28] ARL-67156 is also reported to bind with low affinity to P2Y receptors in bovine chromaffin cells.[29,30] Inhibition of ecto-ATPases should therefore decrease any effects seen due to activation of receptors selective to the ATP metabolites, e.g. P1 receptors which are selective for adenosine.[9]

Results obtained with SCA suggest that the effects of ATP are caused by purinergic receptors and that ARL-67156, at the concentration used in this study (100 μM) is acting basically as a purinergic competitor of ATP and not as an ecto-ATPase blocker (Figure 4A). On the right panel and this time measuring catecholamines by IVIEC it is shown that ATP itself does not cause any change in granule content. This would support the idea that purinergic receptor activation is responsible for the ATP effects. Surprisingly, the IVIEC data shown in Figure 4B indicate that incubation with suramin and ARL-67156 reduce the quantum content of vesicles below control values, unveiling the presence of cytosolic ATP. As an interesting note, simultaneous treatment with ATP and all the inhibitors significantly increases the fraction of catecholamine released in each event (Table S2).

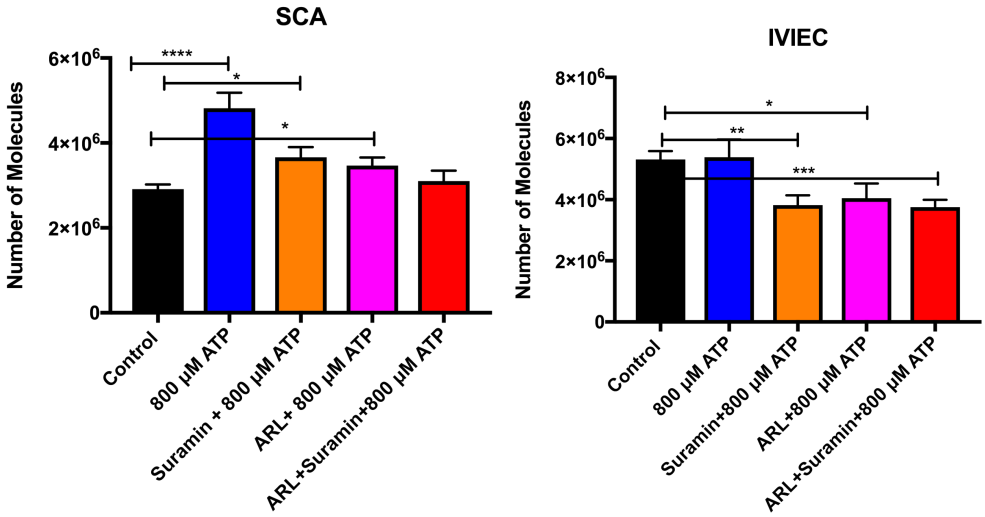


Figure 4. Effects of purinergic blockade and ecto-ATPase inhibition on quantum secretion and content. A) Catecholamine release as measured by SCA and **B)** vesicular catecholamine content as measured by IVIEC from chromaffin cells incubated for 30 min with ATP in the presence of 30 µM of suramin or 100 µM ARL-67156. Data are mean of medians ± standard error of the number of catecholamine molecules detected. Unpaired, nonparametric, Mann-Whitney test, 95% confidence level, P-values: (*) 0.0332, (**)0.0021, (***) 0.0002, (****) <0.0001. n= 28 cells from 3 different adrenal cultures.

Data obtained with SCA and IVIEC were obtained from intact (or near intact cells) meaning that receptor-transduction signaling should be preserved. In order to know whether the effects of ATP on the quantum content of secretory vesicles is in fact mediated by plasma membrane receptors, we performed the experiments summarized in Figure 5, this time using VIEC on isolated chromaffin granules. Also, the effect of the aforementioned inhibitors on isolated vesicles was studied with VIEC to determine if the IVIEC effects could be seen even without the presence of the majority of the cellular machinery. The data show the catecholamine content in isolated vesicles increases after incubating the vesicles with different concentrations of ATP for 30 min and the maximum effect is found at 400 µM ATP (Figure 5A). Incubation of vesicles with ATP and the inhibitory drugs (suramin and ARL) indicates a slight, but not significant, increase in the number of molecules (Figure 5B), which is contrary to the same treatment for cells where the presence of inhibitors significantly decreased the vesicular content (Figure 4B). This suggests that the changes shown in Figure 4B are dependent on plasma membrane receptors and/or other pathways absent from isolated vesicles. For instance, an inhibitory effect caused by suramin on the vesicular H^+-ATPase seems highly unlikely during the SCA or IVIEC measurements. Interestingly, after incubating the isolated vesicles with the drugs (ARL+suramin) alone we observed higher amounts of catecholamines in the vesicles compared to incubation

with drugs and ATP. The suggested mechanism for this might be that one or both of the drugs act as a competitive inhibitor with ATP and protect the vesicles from neurotransmitter leakage to a larger extent than 400 µM ATP alone. ATP is required to avoid leakage of catecholamines from isolated vesicles, at least in our hands, as it preserves the activity of V-ATPase, which maintains the granule acidity. ATP, ATP and suramin and ARL, or suramin and ARL all lead to more ATP to maintain the pH gradient across the vesicle membrane and to avoid leakage.

As both suramin and ARL-67156 disturb the effect of ATP on the exocytotic process, it is difficult to pinpoint which receptor or signaling pathway is responsible for the effects observed. ATP and its derivatives are widely used in biological systems and

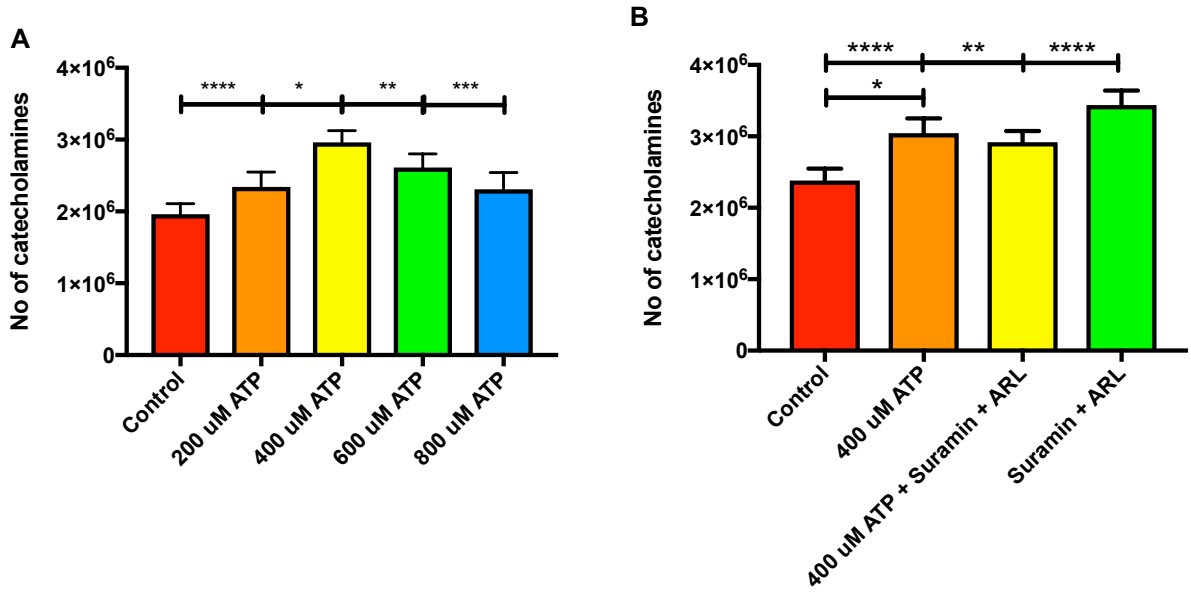


Figure 5. Number of molecules in vesicles as measured by VIEC A) incubated 30 min in different concentration of ATP and B) after the isolated vesicles were incubated for 10 min with ARL and suramin followed by 30 min incubation with ATP. Error bar: standard error of the mean. Unpaired, nonparametric, Mann-Whitney test, 95% confidence level, P-values: (***) 0.0002, (****) <0.0001. n=3 separate vesicle isolations.

any effects might be due to a combination of interactions and subsequent results. In addition, depending on which receptor is activated the secondary response in the cell might differ. Extracellular ATP has previously been reported to cause increases in both inositol phosphates and cyclic AMP, both of which are known to affect the exocytotic process and its regulation.[31–33]

CONCLUSIONS

Extracellular application of ATP was observed to increase the fraction of catecholamine molecules released during exocytosis from chromaffin cells. This

increase was due to enhanced exocytotic release and not changes in vesicular content as the content measured by IVIEC did not change, and this points towards a role for purinergic receptors moving the mode of exocytosis toward full fusion. The secondary messenger through which the receptors act is not known at this point, although cAMP and inositol phosphate are two likely messengers. The enhanced exocytotic effect can be compared with previous work from our group where for example DMSO had a similar effect of increasing the fraction of released transmitters through enhanced exocytosis.[34] The fraction released was also changed in the related cell type PC12 cells when treated with zinc, a compound that has been suggested to increase cognitive abilities. However, in contrast to DMSO zinc affects the catecholamine content inside vesicles, but not the number of released molecules.[35] In addition, the effect of ATP seems to be similar to that found by increasing intracellular Ca^{2+} or cAMP.[32,36] ATP also significantly changed the dynamics of exocytotic release observed as changes in the shape of the amperometric spike in SCA. The rise time increased in a concentration dependent manner, and the pre-spike feet were maintained for a longer time with ATP. The results presented here indicate that ATP causes a larger, more stable fusion pore, thus allowing more of the vesicular content to be released to the extracellular space. Several proteins have been suggested to regulate the fusion pore and one of them, actin, has also been known to interact with ATP, suggesting one mechanism of how ATP causes this stabilizing effect on the pore. To narrow down the molecular mechanism through which ATP act on exocytosis, a pharmacological approach was used to inhibit ATP related enzymes and receptors. The results from SCA and IVIEC indicate that purinergic auto-receptors are in fact involved as inhibition decreases the effects caused by ATP. Drugs that affect the ATPase might also act as competitive inhibitors and protect the vesicle from leakage. This is the first report demonstrating the effect of ATP on exocytosis is mediated by an auto-receptor.

METHODS

Chemicals and solutions

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich. Locke's buffer (mM): 1.54 M NaCl, 56 KCl, 36 $NaHCO_3$, 56 glucose, 50 HEPES, 1% (v/v) penicillin, pH 7.4. This stock solution was diluted 10x with distilled water the day before the experiment. Ca^{2+} free isotonic solution (mM): 154 NaCl, 4.2 KCl, 0.7 $MgCl_2$*$6H_20$,

11.2 Glucose, 10 HEPES, pH 7.4, ~310 mOsm. Barium stimulation solution (mM): 2 $BaCl_2$, 0.7 $MgCl_2$ in diluted Locke's buffer, pH 7.4. 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 (mM): 230 sucrose, 1 EDTA, 1 $MgSO_4$, 10 HEPES, 10 KCl, cOmplete enzyme inhibitor (Roche, Sweden), pH 7.4, ~310 mOsm.

Cell and vesicle isolation

Bovine adrenal glands were obtained from a local slaughterhouse and the chromaffin cells were isolated as previously described.[37] Briefly, the vein was perfused with Locke's buffer to clear away blood cells. The medulla was isolated after collagenase (0.2%, Roche, Sweden) treatment, and cells were isolated using a series of homogenization and centrifugation steps. For single cell experiments, ~500 000 cells were seeded on 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. For vesicle isolation, a protocol developed by the R. Borges group was used. Briefly, the medulla was mechanically homogenized in homogenizing buffer, and the vesicles were purified using a series of centrifugation steps: 1 000*g for 10 min to remove whole cells followed by 10 000*g to pellet vesicles. All centrifugation was performed at 4°C. The final pellet of vesicles was resuspended and diluted in homogenizing buffer and subsequently used for electrochemical measurements the same day.

Drug treatment

Cultured cells were rinsed three times with isotonic solution before incubation. Stock solutions of ATP, suramin, and ARL-67156 were prepared in Ca^{2+} free isotonic solution and added to the final concentration as specified. For incubation with norepinephrine, the isotonic solution was first purged with argon (AGA, Sweden) before the norepinephrine was added. Aliquots of this stock solution were procured immediately before the start of the cell incubation. Incubation of ATP occurred for 30 min at incubator conditions. When using suramin or ARL, these were added 10 min prior to ATP in order to block targets before ATP addition. For treatments of vesicles, a portion of the vesicles were pelleted down and resuspended in homogenizing solution with

ATP, suramin, ARL and/or norepinephrine. The suspension was then left in room temperature for 30 min before pelleting down the vesicles again and resuspension in pure homogenizing solution for electrochemical analysis.

Electrochemistry

Nanotip electrodes were prepared as previously described.[14] Briefly, a 5 μm carbon-fiber was aspirated into a borosilicate capillary and pulled in half. The protruding carbon fiber was cut to a length of 100-150 μm and then flame etched in a butane burner. Electrodes with narrow and sharp fibers 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, symmetric voltammograms and low charging current were used. In all amperometric experiments, a potential of +700 mV *vs* a Ag|AgCl reference electrode was applied using an Axon 200B potentiostat (Molecular Devices, San Jose, CA). Single cell amperometry and IVIEC were performed successively in the same dishes in a randomized manner. Release was stimulated through a 5 s ejection of Ba^{2+} stimulation solution through a glass micropipette and the release events were detected using a nanotip electrodes placed on the cell surface. For IVIEC, the nanotip electrode was carefully placed inside the cell by piercing the plasma membrane. Detection of vesicular content was performed for 3 min after placing electrode inside the cell. Only data from cells that appeared morphologically similar before and after the IVIEC measurement was used. VIEC measurement of vesicle content in samples of isolated vesicles was done as previously described.[16] A nanotip electrode was used for detection to ensure similar signal-to-noise ratio between methods. The nanotip electrode was placed in a vesicle suspension solution and left for 20 min during which current transients were recorded.

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 was converted in Matlab (The MathWorks, Inc.) and processed in IgorPro (Wavemetrics, Lake Oswego, OR).[38] A binomial filter was set to 1 kHz and 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, t_{rise}= time from the 25% of the maximum to 75% of maximum during the increase of the spike, t_{fall}= time from 75% of maximum to 25% of maximum during the decrease of the spike, $t_{1/2}$= full spike width at half maximum, t_{foot}=duration of pre-spike foot from initial increase from base line to the start of the spike, Number of $molecules_{foot}$=calculated from the charge measured in the foot. The median from all cells were pooled and groups were statistically analysed using Prism 7 (GraphPad, La Jolla, CA) with an unpaired two-tailed Mann-Whitney rank sum test.

ASSOCIATED CONTENT

* Supporting Information

Table S1. Statistical analysis of single cell amperometry and IVIEC

Figure S1. Analysis of t_{fall} for exocytotic spikes

Methodology for calcium imaging

Figure S2. Calcium transient responses with and without ATP treatment

Table S2. Amperometrical measurements of catecholamine content using IVIEC and SCA

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Author Contributions

All authors have given approval to the final version of the manuscript. Soodabeh Majdi conceived the idea and, she and Anna Larsson carried out the experiments, analyzed and interpreted the data, and wrote the manuscript. Neda Najafinobar carried out part of the exocytosis experiments. Ricardo Borges was 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; IVIEC intracellular vesicle impact electrochemical cytometry; VIEC vesicle impact electrochemical cytometry; SCA single-cell Amperometry; SVs secretory vesicles;

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