Title:

Stimulus Dependent Alterations in Quantal Neurotransmitter Release

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Running Head: Changes in Quantal Release Properties

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Abstract:

Neurotransmitter release is a steep function of the $[\text{Ca}^{2+}]_i$ at the release sites. Both the Ca$^{2+}$ amplitude and the time course appear to be important for specifying neurotransmitter release. Ca$^{2+}$-influx regulates the number of vesicles exocytosed as well as the amount of neurotransmitter each individual vesicle releases. In our study we stimulated mouse chromaffin cells in two different ways in order to alter Ca$^{2+}$ presentation at the release sites. One method, digitonin permeabilization followed by exposure to Ca$^{2+}$, allows for a large uniform global elevation of $[\text{Ca}^{2+}]_i$, while the second method, application of nicotine, depolarizes chromaffin cells and activates voltage-dependent Ca$^{2+}$-channels, thereby producing more phasic and localized changes in $[\text{Ca}^{2+}]_i$. Using amperometry to monitor catecholamine release, we show that both kinds of stimuli elicit the exocytosis of similar quantities of neurotransmitter per large dense core vesicle (LDCVs) released. Even so, the release process was quite different for each stimulus; nicotine-elicited events were small and slow while digitonin events were, in comparison, large and fast. In addition, the transient opening of the fusion pore, called the “foot”, was essentially absent in digitonin stimulated cells, but was quite common in nicotine-stimulated cells. Thus, even though both strong stimuli used in this study elicited the release of many vesicles it appears that the differences in the Ca$^{2+}$ levels at the release sites were key determinants for the fusion and release of individual vesicles.

Keywords: Nicotine, digitonin, amperometry, quantal content, neurotransmitter
Introduction

Neurotransmitter release from pre-synaptic nerve terminals or from secretory cells is mediated by the exocytosis of transmitter filled vesicles. Release is triggered by action potentials that activate voltage-dependent Ca\(^{2+}\) channels thereby allowing the influx of Ca\(^{2+}\) into the cells. This Ca\(^{2+}\)-influx strongly elevates the vesicle release probability for a brief period of time in a region localized to the vicinity of open Ca\(^{2+}\) channels (Sudhof 2004). Exocytotic efficacy depends critically on the intracellular calcium concentration near the release sites. Release is a steep function of Ca\(^{2+}\) entry; there is significant cooperativity among the Ca\(^{2+}\) ions that trigger neurotransmitter release (Dodge and Rahamimoff 1967), with the binding of 4-5 Ca\(^{2+}\) ions required for each release event (Bollmann and Sakmann 2005; Schneggenburger and Neher 2005). At synapses, [Ca\(^{2+}\)]\(_i\), is thought to rise and fall rapidly, with each action potential. This rapid rise and fall is consistent with tight co-localization of calcium channels and docked/primed vesicles (Harlow et al. 2001; Pumplin et al. 1981; Schneggenburger and Neher 2005; Simon 1985). In addition, to the [Ca\(^{2+}\)]\(_i\) levels the time course of the Ca\(^{2+}\) elevation at the release sites appears to be a another critical determinant of bulk neurotransmitter release (Bollmann and Sakmann 2005). In a similar manner, release of the neurotransmitter content of an individual vesicle may also depend on Ca\(^{2+}\) levels. For instance, in chromaffin cells quantal size appears to depend on Ca\(^{2+}\) influx (Elhamdani et al. 2001). In hippocampal neurons low frequency stimulation appears to initiate release via a kiss and run mechanism while high frequency stimuli induce full vesicle fusion with the plasma membrane (Aravanis et al. 2003). These different stimulation frequencies undoubtedly produced markedly different elevations in both the amplitude and time course of [Ca\(^{2+}\)]\(_i\) at the release sites. On the other hand experiments from a
different lab have suggested that elevated extracellular Ca\textsuperscript{2+} causes release to shift to kiss and run from full fusion (Ales et al. 1999).

The pre-fusion release of small amounts of neurotransmitter was originally observed in chromaffin cells and mast cells (Alvarez de Toledo 1993; Chow 1992). “Feet” are thought to reflect a slow release of neurotransmitter through a narrow fusion pore. Many amperometric studies have observed “feet. Interestingly, Amatore et al. (Amatore et al. 2005) found that small amperometric events had few “feet” but for large events “feet” were quite common. They suggested that amperometric events with feet correspond to vesicles with higher catecholamine content (Amatore et al. 2005). Han et al. (Han et al. 2004) reported that mutations in syntaxin could alter the “feet” observed in PC12 cells, suggesting that syntaxin was a constituent of the fusion pore and that like other protein channels constituent groups were important for its operation. Our own results, while not directly bearing on those of Han et al., show that alterations in Ca\textsuperscript{2+} at release sites can produce changes in amperometric “feet’, raises the possibility, however unlikely, that mutations in syntaxin somehow results in the alteration of the local Ca\textsuperscript{2+} concentration.

In our study we stimulated mouse chromaffin cells either by permeabilizing them with digitonin and then exposing them to Ca\textsuperscript{2+} or by the direct application of nicotine to chromaffin cells. Amperometric recordings allowed for the monitoring of catecholamine release. Although the quantal content for each vesicle released was similar for both stimuli, there were nonetheless significant differences observed in the amperometric recordings. Cells stimulated with digitonin showed few, if any, “feet”, while they were quite common in nicotine-stimulated cells. Nicotine stimuli produced amperometric events that were small and slow, while digitonin produced events
that were large and fast in comparison. Our results suggest that the kinetics of release of the neurotransmitter content of individual LDCVs is under significant regulatory control.
**Materials and Methods**

**Cell Culture**

Animals were housed and handled as required by the Animal Resource Council (University of Chicago, IL). Cells were prepared similar to previously published methods (Obukhov and Nowycky 2002). Cells were plated on glass cover slips coated with Matrigel (Discovery Labware Inc, Bedford, MA, USA) and maintained in a 37°C, 5% CO2 incubator. EM, confocal and amperometry experiments were carried out 2 and 3 days post tissue harvest.

**Amperometry**

Carbon fiber electrodes were fabricated with 7 µm diameter carbon fibers (Fortafil Fibers Inc., Knoxville, TN), as previously described (Grabner et al. 2005). The electrode was pressed gently against the cell during the recording. A newly cut surface or a new electrode was used for each cell. Recordings were made with an EPC-7 amplifier (HEKA Electronics, Lambrecht, Germany). The amperometric signal was low-pass filtered at 2 kHz (8-pole Bessel; Warner Instruments, Hamden, CT, USA) and sampled into a computer at 10 KHz using a 16-bit analog-to-digital converter (National Instruments Corp., Austin, TX). Records with rms noise greater than 2 pA were not analyzed. Amperometric spike features, quantal size and kinetic parameters, were analyzed using Minianalysis (Synaptosoft, Decatur, Georgia, USA) or via a series of macros written in Igor Pro (Wavemetrics Inc.) kindly supplied by Dr. Eugene Mosharov (Columbia University). The detection threshold for an event was set 4 – 5 times the baseline rms noise, and the spikes were automatically detected. Overlapping events were rejected. Overlapping events were relatively rare. The area under individual amperometric spikes is equal to the charge (pC) per release event, referred to as Q. The number of oxidized neurotransmitter
molecules (N) was calculated using the Faraday equation, \( N = \frac{Q}{ne} \), with \( n = 2 \) electrons per oxidized molecule of transmitter; \( e \) is the elemental charge (1.603 \( \times \) 10\(^{-19} \) Coulomb).

**Recording Solutions and Stimulation Protocols**

Amperometric recordings were made from adherent cells that were under constant perfusion (flow rate of ~1.0 mL/min; chamber volume ~150 µL). All recording solutions had the following composition: 145 mM NaCl, 2.0 mM KCl, 10 mM HEPES and 1.0 mM MgCl\(_2\). Ca\(^{2+}\)-free solutions contained 100 µM EGTA. All solutions used during and after cell permeabilization contained 1.0 mM Na\(_2\)ATP. All experiments were performed at ambient temperature (23\(^\circ\) ± 2\(^\circ\)C). Cells were stimulated repeatedly using the following protocol: (1) 2 min in a Ca\(^{2+}\)-free solution, (2) permeabilized with 20 µM digitonin (Ca\(^{2+}\)-free) for 10 sec, (3) then stimulated 2 min with a solution containing 100 µM free Ca\(^{2+}\), (4) washed for 1 min in Ca\(^{2+}\)-free media before the cycle was repeated starting at step (2). Cells were typically stimulated 3 – 4 cycles (at most six cycles) or until the cell membrane changed from its initial, bright-field dark appearance to a granular texture (for details see ref (Jankowski et al. 1993)).

Digitonin was purchased from Calbiochem (La Jolla, CA, USA). Cells stimulated with nicotine were exposed to 10 µM nicotine for 3 min in 2 mM Ca\(^{2+}\), followed by a 2 min wash without nicotine. Two additional stimulations repetitions were carried out.

Statistical analyses between experimental groups are presented as mean ± standard error of the mean, and two-tailed P-values were made using the Mann-Whitney test for unpaired, nonparametric data (GraphPad; San Diego, CA). All plots were performed in Origin (Origin Corp., Northampton, MA, USA).
Results

Amperometry was used to assay catecholamine release from mouse adrenal chromaffin cells treated with different secretagogues. Release was stimulated by the application of 10 µM nicotine perfused into the bath for 3 minutes, resulting in a large number of amperometric events over the period of the exposure (Fig. 1A). Digitonin permeabilization followed by application of Ca\(^{2+}\) was the second form of stimulation employed in this study. Fig. 1B shows the response of a digitonin permeabilized chromaffin cell after exposure to 100 µM Ca\(^{2+}\) (Jankowski et al. 1992). Amperometric events were rare in the absence of Ca\(^{2+}\), even after digitonin permeabilization, which suggests that the secretory machinery functions properly in the presence of digitonin (Graham et al. 2002; Holz et al. 1989; Jankowski et al. 1993; Jankowski et al. 1992). The two traces, elicited by different secretagogues, show that the individual amperometric events were on average larger in digitonin-permeabilized cells than in cells stimulated with nicotine. The amperometric event in Fig. 1A and 1B were aligned to their rising phase and averaged; Figs. 1C and 1D plot the average amperometric traces. The average spike amplitude and shape were clearly different with the nicotine-stimulated cell exhibiting a smaller spike with slower release kinetics in comparison to the digitonin-stimulated cell.

Some features of the amperometric events observed for either nicotine or digitonin stimulation were further analyzed and are plotted in Fig. 2. Fig. 2A shows that the average area under each amperometric event, corresponding to the amount of neurotransmitter released under each condition, was approximately the same. Nicotine elicited events were on average 0.295 ± .042 pC, while digitonin events were 0.286 ± .095 pC. Figure 2B plots amperometric event amplitude and shows that digitonin-elicited events were about twice as large as those elicited by
nicotine. The average nicotine elicited event was 23.3 ± 6.1 pA while the average digitonin event was 42.1 ± 4.9 pA. This difference was significant (p < 0.05).

The similarity in quantal size but difference in spike amplitude, measured in digitonin permeabilized and nicotine-stimulated cells, predicts that the release kinetics for each group must be distinct. Fig. 2C shows that the rise time of nicotine-elicited spikes were significantly slower than those elicited by digitonin. The 10%-90% rise time for nicotine stimulated events was 3.8 ± 0.42 msec while the digitonin stimulated events significantly faster at 2.4 ± 0.28 msec (p<0.01). Thus, even though the nicotine-elicited amplitudes were smaller, the fact that they were slower meant that about the same amount of neurotransmitter was released per event. A more detailed analysis of rise times can be found in Fig. 2D, which plots rise time distributions for each stimulation condition.

Plotting amperometric event amplitude as a function of charge shows the difference between events recorded in nicotine-stimulated cells versus those from digitonin permeabilized cells (Fig. 3A). With the exception of small quantal events, the amplitudes observed for amperometric events from nicotine-stimulated cells were always smaller than those from digitonin-permeabilized cells, for a given quantal charge. Fig. 3B, plots rise time as a function of amperometric spike charge for the two stimulation protocols. This data suggests that kinetic differences are most pronounced for large quantal events.

Another important difference between nicotine and digitonin stimulation is shown in Fig. 4A. The majority of events appear as a single spike that rises rapidly and falls at a slower rate; however, in some cells a fraction of the events exhibit the small elevation from baseline prior to the spike known as a “foot”, which is thought to arise from neurotransmitter release from a partially open fusion pore (Chow 1992). Feet were almost never observed in digitonin-
stimulated cells but were quite common in the nicotine-stimulated cells. To explore the differences in “feet” in more detail, data from three nicotine- and from three digitonin-stimulated cells were averaged. Every amperometric event from these cells was included in the average. The averages were scaled to the same height and are plotted in Fig. 4B. Only the bottom one third of each ensemble average is plotted, in order to view the “feet” at a higher gain. Fig. 4 clearly shows that nicotine elicited currents with “feet”, while digitonin did not. Furthermore, Fig. 4B reinforces the observation that nicotine stimulated events were slower than were their digitonin counterparts.
Discussion

A recent study by Bollman and Sakmann (Bollmann and Sakmann 2005) showed that the Ca$^{2+}$ sensor that responded to the increases in presynaptic [Ca$^{2+}$], to trigger neurotransmitter release also responded to the time course of the Ca$^{2+}$ waveform. Using Ca$^{2+}$ uncaging and low affinity Ca$^{2+}$ buffers the authors were able to apply rapid Ca$^{2+}$ transients or multiple transients at the vesicle release sites. The rise time and the amplitude of the excitatory postsynaptic currents recorded depended on the time course of the [Ca$^{2+}$]$_i$ transients. The release of individual vesicles can also be modified by alterations in [Ca$^{2+}$]. Elhamdani et al (Elhamdani et al. 2001) showed that increasing the frequency of action potentials or elevating Ca$^{2+}$-influx into chromaffin cells produced an elevation in the amount neurotransmitter released per event i.e the quantal size. In this study the authors showed that with increased stimulation, quantal size rose continuously, until it peaked. Other studies suggest that increases in stimulation frequency, which are expected to produce alterations in Ca$^{2+}$ at the vesicle release sites, can alter the mode of exocytosis between kiss and run and full-fusion (Aravanis et al. 2003). Working on the fly neuromuscular junction, Pawlu et al., showed that the decay phase of excitatory postsynaptic currents recorded were variable suggesting postfusional plasticity (Pawlu et al. 2004). Our own studies suggest that strong stimuli, which we have previously shown completely empty the vesicle of its’ neurotransmitter content (Grabner et al. 2005), can still produce dramatic differences in quantal release. The amperometric events elicited by nicotine were significantly smaller and slower than those elicited by digitonin and subsequent exposure to Ca$^{2+}$. The differences in amplitude and kinetics were accomplished without any alteration in quantal content and both stimulation methods produced amperometric event charge distributions that were bimodally distributed, suggesting the existence of two distinct populations of vesicles in chromaffin cells (Grabner et
Thus, even strong stimuli can exhibit altered release properties as long as they present the release sites with different intracellular Ca$^{2+}$ profiles.

A recent study suggested that amperometric “feet” were altered by the neurotransmitter content of LDCVs in chromaffin cells; Amatore et al. showed that small quantal events had few “feet” while “feet” were quite common for large events (Amatore et al. 2005). Surprisingly, the same group reported that small events had a higher frequency of exhibiting “feet” in PC12 cells (Sombers et al. 2004). We found that few feet in digitonin stimulated cells but large numbers of feet in nicotine stimulated cells (or in high-K$^+$ stimulated cells, data not shown). Although neither of the stimuli that we used was physiological, nicotine does activate endogenous Ca$^{2+}$-permeable nicotinic ACh receptors that depolarize the cells and thereby activating voltage-gated Ca$^{2+}$ channels. It is expected that digitonin will produce a larger and more uniform elevation in [Ca$^{2+}$].

Under what condition would the presentation of Ca$^{2+}$ at the release sites be significantly altered? It has been known for decades that Ca$^{2+}$ channels are plastic. Single channel studies have shown that they can enter states where openings are very long lasting, which would allow the influx of considerable Ca$^{2+}$ into cells (Delcour et al. 1993b; Hivert et al. 1999; Luvisetto et al. 2004; Pietrobon 1990). Ca$^{2+}$ channels can sojourn in these ‘modes’ for seconds. They can also stay in modes where channels open for brief periods (Delcour and Tsien 1993a). They can open during depolarization or following repolarization, when the driving force is extremely large, resulting in large Ca$^{2+}$ influx in a brief period of time (Hivert et al. 1999). In addition, channels can be modulated by neurotransmitters (both positive and negative regulation) (Bean 1989; Elmslie 1990; Penington 1991), which can result in dramatic changes in activation rates.
Recent studies have suggested that Ca\textsuperscript{2+} channels can either be inhibited or facilitated by calmodulin or other Ca\textsuperscript{2+} binding proteins (Lee et al. 2002; Liang et al. 2003). In addition, changes in action potential waveform (activation of K\textsuperscript{+} channels, synaptic channels etc), will alter Ca\textsuperscript{2+} at the release sites. Thus, the presentation of Ca\textsuperscript{2+} at release sites may vary dramatically, even more than the differences between digitonin and nicotine outlined in this manuscript, simply allowing for the plastic nature of Ca\textsuperscript{2+} channels and the large repertoire of different activation states available to them.

What are the consequences of the differences in release described in this paper? Slower release will lead to reduced neurotransmitter concentrations in the synaptic cleft. For instance, at glutamate synapses it has been suggested that slow release of neurotransmitter may activate high-affinity NMDA receptors preferentially over lower-affinity AMPA receptors (Krupa and Liu 2004). In a similar manner, alterations in release kinetics in chromaffin cells may have effects on the activation level of nearby receptors, including autoreceptors. Slow release kinetics may prevent the activation of classes of receptors that would otherwise be activated.
Acknowledgements

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References


Figure Legends

Figure 1 - Differences in digitonin- or nicotine-elicited amperometric events. (A), Shown here is a representative two-minute amperometric trace with multiple events. The cell was exposed to nicotine (10 µM), producing 175 events. (B), The cell was permeabilized with digitonin (20 µM) and then exposed to Ca^{2+} (100 µM); 49 events were detected. (C), All 175 amperometric events from the experiment shown in panel A were aligned in time and then averaged to produce the current trace. (D), All 49 amperometric events from the experiment shown in panel B were aligned in time and then averaged to produce the current trace. From this data it is clear that the events elicited by nicotine differ considerably in amplitude and overall shape from those elicited by digitonin.

Figure 2 – Nicotine stimulated amperometric events were smaller and slower than those elicited by digitonin. (A), The mean charge (Q) per release event recorded from cells stimulated with either nicotine or digitonin. Error bars represent SEM. Average charge was calculated for each individual cell and then averaged. The quantal size recorded in response to digitonin was similar to that obtained in cells stimulated with nicotine. (B), In contrast, the mean event peak amplitude was significantly smaller when nicotine was used to stimulate the cells, in comparison to digitonin. (C), The mean event rise time was significantly slower when nicotine was used to stimulate the cells, in comparison to digitonin. (D), Plots rise time distributions for both nicotine- (black bars) and digitonin-stimulated (gray bars) cells. The data was measured from 9 digitonin and 6 nicotine treated cells.
Figure 3 – Digitonin permeabilized cells have larger amperometric spike amplitudes and faster rise times for a given spike charge than do nicotine-stimulated cells. (A), Plot of amperometric event amplitude (pA) as a function of amperometric event charge (pC). Data from nicotine-stimulated cells are plotted as black squares and digitonin permeabilized cells as gray circles. (B), Plot of rise-time (10%-90%, in ms) as a function of amperometric event charge (pC). Data from nicotine-stimulated cells (n=6) are plotted as black squares, while data from digitonin-permeabilized cells (n=9) are plotted as gray circles. 746 amperometric events from digitonin-permeabilized cells were analyzed for the graphs shown and 814 amperometric events from nicotine-stimulated cells were analyzed.

Figure 4 – Amperometric events stimulated with nicotine commonly showed “feet”, while cells stimulated with digitonin did not. A, Individual amperometric events are plotted from cells stimulated with either nicotine or digitonin, on an expanded time scale. Note that the nicotine-stimulated event was smaller, slower and exhibited a prominent “foot”. In contrast, the digitonin-stimulated event was large, fast and showed no “foot”. These events are representative of all the data obtained. B, Ensemble averages of amperometric events recorded from cells stimulated with nicotine showed “feet”, while ensemble averages of amperometric events from cells stimulated with digitonin, did not. Three cells were included in each group. All amperometric events recorded were used to construct the averaged event. Amperometric events were aligned by their rising phases. The average trace made from each cell was normalized to unity and then data from the three cells was then averaged. Only the bottom 35% of each ensemble average is plotted. The solid line at the bottom represents zero current. 458 amperometric events were
included in the ensemble average for digitonin and 346 amperometric events were included in the nicotine average. Three cells from each group were used to construct the ensemble plots.
Nicotine Digitonin

Average Nicotine Event (n=175)
Average Digitonin Event (n=49)

100 pA
20 sec
10 pA
100 psec
A

Mean Peak Height (pA) Mean Charge Per Event (pC)

Nicotine Digitonin

B

Mean Peak Height (pA)

Nicotine Digitonin

C

Mean Rise Time (ms)

Nicotine Digitonin

D

Number of Events

Rise Time (ms)