An amperometric study of the kinetics of exocytosis in mouse adrenal slice chromaffin cells: physiological and methodological insights

by

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Abstract

This study was designed to examine the kinetics of neurotransmitter release using the carbon fiber amperometric technique on cells in slices of mouse adrenal glands superfused with bicarbonate phosphate buffer-based solutions. The exocytic amperometric response evoked by electrical stimulation was significantly faster than that produced after exogenous application of ACh or K+. Splanchnic nerve-evoked neurotransmitter release was blocked by hexamethonium, indicating the involvement of ACh nicotinic receptors. We discuss the implications of our data for understanding the mechanisms underlying the vesicle fusion process.
1. Introduction

The kinetics of exocytotic events has been extensively explored mainly through capacitance recordings, as a measure of membrane fusion, or amperometric recordings, as a measure of neurotransmitter release (Neher 1998). The amperometric technique has been applied almost exclusively to cells in culture, with the exception of a few individual experiments in various reports (Barbara et al. 1998; Voets et al. 2001; Chan and Smith 2003). However, the behavior of chromaffin cells in the gland differs considerably from that of isolated cultured cells. For instance, Moser et al. (1997) discovered a fast exocytotic capacitance component in mouse adrenal slices, which was not seen in cultured cells (but see also Elhamdani et al. 1998, where action potentials induced fast secretion in cultured calf chromaffin cells). Moreover, it has been established that the expression of several Ca\(^{2+}\) channel subtypes varies considerably in the intact adrenal gland, compared to isolated cultured cells (Albillos et al. 2000; Benavides et al. 2004; Chan et al. 2005).

Tissue slices better approach physiological conditions and offer the unique opportunity of exploring the kinetics of single exocytotic events triggered by endogenously released ACh upon electrical field stimulation of splanchnic nerve terminals. Hence, we applied carbon fiber amperometry to mouse adrenal gland slices to explore the kinetics of exocytosis. We discovered that exocytotic events were significantly faster when evoked by electrical stimulation compared to exogenous stimuli such as ACh or K\(^+\). Moreover, the individual secretory spikes observed were much larger and faster than those previously reported for isolated mouse chromaffin cells (Herrero et al. 2002).
2. Materials and methods

2.1. Adrenal slice preparation and solutions

The experimental protocol was approved by the animal ethics committee of the Faculty of Medicine of our University (Universidad Autónoma de Madrid). We used two bicarbonate-buffered saline (BBS) solutions containing different CaCl₂ concentrations: the standard BBS solution, or solution 1, contained (in mM): 2 CaCl₂, 125 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, and 10 glucose; and solution 2, used for the slice preparations, was identical to solution 1, except that it contained 0.1 mM CaCl₂ and 3 mM MgCl₂. Both BBS solutions were adjusted to pH 7.4 by bubbling with 95% O₂ and 5% CO₂. The Krebs HEPES solutions used contained (in mM): 2 CaCl₂, 145 NaCl, 5.5 KCl, 1 MgCl₂, 10 HEPES and 10 glucose. When 100 mM K⁺ was used as a secretagogue, the 100 mM KCl replaced an equimolar amount of NaCl in solution 1. Adrenal glands were procured from 6-10 week-old decapitated mice and placed in cooled (0-2°C) BBS (solution 2). After removing overlying fatty tissue, the glands were embedded in 2% agarose (type VII-A, low melting point agarose) and cut into slices (100 µm thick) using a vibrating-blade microtome (Leica, Heidelberg, Germany). The cutting parameters were set at: speed 0.1 mm/s, frequency 80 Hz and amplitude 0.8 mm. The slices obtained were kept for 10 min at 37°C in a holding chamber containing solution 1 and then at room temperature. When used, ACh and K⁺ were prepared in solution 1 and delivered through a multibarrelled glass pipette (Carbone and Lux 1987) placed about 50 µm from the cell under study. All reagents were obtained from Sigma (Madrid, Spain).

2.2. Isolation and culture of mouse chromaffin cells

Mouse chromaffin cells were isolated according to the method of Hernández-Guijo and coworkers (Hernández-Guijo et al. 1998) and used in the experiments after 1 or 2 days of culture.

2.3. Electrical stimulation of adrenal slices

Electrical field stimulation was achieved by means of two tungsten electrodes glued together (about 150 µm apart) connected to an electrical stimulator (Cibertec model CS 220, Madrid, Spain) (Iijima et al. 1992; Barbara and Takeda 1996). The pulse intensity was selected by applying single pulses over the range of currents 0.7 to 7 mA, using the values described by Iijima et al. (1992) for rat adrenal gland slices and the same stimulation electrodes as reference. The minimum current at which an amperometric response was observed was 6 mA, such that we selected this current for our experiments. A rectangular single pulse of 1 ms duration and 6 mA intensity was delivered through an isolation unit (ISU 165, Cibertec, Madrid, Spain) to the slices. Some cells were stimulated by applying 10 pulses of 1 ms at 10 Hz. This frequency was chosen because it covers the normal firing rate measured for activated chromaffin cells (Brandt et al. 1976; Kidokoro and Ritchie 1980; Wakade 1981). Since results obtained at this frequency did not exhibit any difference with those obtained with a single stimulation pulse, we included those cells in the analysis. Solution 1 was continuously added to the cell under study using the multibarrelled glass pipette.

2.4. Amperometric recordings

Carbon fiber electrodes were prepared by cannulating a 10 µm-diameter carbon fiber in polyethylene tubing (diameter: outer, 1 mm; inner, 0.5 mm). The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp headstage, and back filled with 3 M KCl to connect
to the Ag/AgCl wire, which was kept at +700 mV. Amperometric currents were recorded using an EPC-9 amplifier and PULSE software running on an Apple Macintosh computer. Sampling was performed at 14.5 kHz and samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments using 50 µM adrenaline as standard solution. Only fibers that rendered 200-300 pA of current increment after a 50 µM adrenaline pulse were used for the experiments. The tip of the fiber was recut for each experiment and calibrated again. Slices were fixed in the recording chamber using a grid of nylon threads. The slices were mounted onto the stage of an upright microscope (BX50WI, Olympus) and individually observed using a 40X lens. Recordings were obtained from the cleanest cells, most exposed to the surface and positioned between the two edges of the stimulation electrode. When needed, we used a borosilicate glass pipette of tip diameter around 10 µm to clean the surface of the cell under study (Moser and Neher 1997; Albillos et al. 2000).

2.5. Electrophysiological Ca$^{2+}$ current measurements

Ca$^{2+}$ currents were measured in the whole-cell configuration of the patch-clamp technique. Solution 1 was used as the external solution. The composition of the solution in the pipette was (in mM): 145 Cs glutamate, 10 HEPES, 9.5 NaCl, 0.3 BAPTA, 2 MgATP. Initially, a ramp was applied to obtain the peak current voltage, and then step depolarizations of 500 ms duration were conducted from a holding potential of –80 mV to the peak current voltage.

2.6. Analysis of data

Spike analysis was performed using IGOR Pro software, which includes a macro package that allows the analysis of single events and the rejection of overlapping spikes (see for details Segura et al. 2000). A threshold of 4.5 times the first derivative of the noise standard deviation was calculated to clearly detect amperometric events. Next, among the events whose first derivative was above this threshold, only those showing one peak and one rising and one falling phase, were considered as single spikes. To minimize variability among cells, we used the overall mean of average spike values recorded in several single cells. After establishing a normal distribution of data with the Kolmogorov-Smirnov test, we used the Student’s t-test to compare our data.
3. Results

3.1. ACh versus K⁺ stimulation in the chromaffin cells of mouse adrenal gland slices

To characterize the amperometric exocytotic signals produced by chromaffin cells in the mouse adrenal slices, we first applied exogenous 1 mM acetylcholine (ACh) or 100 mM K⁺ in the BBS-based solutions (Figs. 1A and B, respectively) to the same cells. Each secretagogue was perfused three or four times, and then a new set of pulses was delivered using the other secretagogue several times. Pulses of 5 s duration were applied every 1 min. In both cases, a burst of amperometric spikes was recorded. Repetitive stimuli evoked similar responses. Figures 1C and D show typical single fast amperometric events evoked by ACh or K⁺, respectively, and details of their kinetic variables. The following factors were determined: \( I_{\text{max}} \) (peak amplitude); \( Q \) (charge); \( m \) (ascending slope, calculated from the linear portion of the trace between 25% and 75% of the \( I_{\text{max}} \)); \( t_{1/2} \) (half-width or duration of the amperometric signal at 50% of its peak amplitude); and \( t_p \) (time-to-peak, time from the start of the spike until the peak in seconds).

Table 1 provides average values for the spike kinetic variables obtained (808 spikes when the stimulus was ACh, and 756 spikes when the stimulus was K⁺), applying both types of stimulus to the same cell (n=7). No significant differences were detected between the two stimuli. Figure 1E also shows frequency histograms of the kinetic variables. Note that similar distributions were obtained for both types of stimulus, indicating exocytosis was identical.

The baseline of the recordings increased considerably when an exogenous stimulus, ACh or K⁺, was applied. A possible explanation for this is that there could be contamination by vesicles from stimulated cells adjacent to the cell being recorded, in response to ACh or high K⁺ delivered by the multibarrelled glass pipette. In contrast, this interference due to catecholamines, appearing as a wavy baseline, was not observed following electrical stimulation, since splanchnic nerve terminals innervate less chromaffin cells (Coupland 1965). To test this hypothesis, simultaneous recordings of whole-cell Ca²⁺ currents and amperometry were performed on a single cell, such that only one cell was stimulated. To do this, we applied depolarizing pulses of 500 ms duration from a holding potential of –80 mV to the peak current voltage (Fig. 2). In 8 cells tested, superimposed spikes were obtained, but the wavy baseline was not observed. This hypothesis is also supported by the fact that the baseline did not increase when cultured cells, plated at a very low density, were superfused with high K⁺ (see below, Fig. 4A). Furthermore, the amperometric recordings without wavy baseline obtained by Voets and coworkers (Voets et al. 2001) confirmed this hypothesis, since only one single mouse slice chromaffin cell was stimulated by flash photolysis. Also, we perfused ACh or K⁺ in vitro, on the carbon fiber, and no wavy baseline was observed (n=16 carbon fibers).

3.2. Electrical field stimulation of the splanchnic nerve innervating the chromaffin cells of mouse adrenal gland slices

Splanchnic nerve terminals in mouse adrenal slices were also subjected to electrical field stimulation. As shown by Barbara and Takeda (Barbara and Takeda 1996), an all-or-none response was elicited as the stimulus intensity was increased. The threshold current at which an exocytotic response started to be observed was 6 mA. Figures 3A and B show typical responses. To ensure the integrity of the cell membrane after applying the electrical stimulus, two or three pulses of 100 mM K⁺ (5 s duration applied every 1 min) were delivered before and after the
electrical stimulus, giving healthy responses. Amperometric exocytotic events evoked by electrical stimulation of the presynaptic nerve were blocked by the nicotinic receptor antagonist hexamethonium at a concentration of 5 µM (n=4). This blockade, shown in Figure 3C, indicates that, under our experimental conditions, the amperometric responses obtained could be attributed to electrical stimulation of the splanchnic nerve and the subsequent release of endogenous ACh, stimulating the chromaffin cell being recorded.

The kinetic variables m, t1/2 and tp of the spikes obtained after electrical stimulation of the splanchnic nerve differed significantly from those estimated for the response to ACh or K^+ (Fig. 3D and Table 1). The apparent difference in the kinetic variable Q when comparing the histograms of Fig. 1E and Fig. 3D is due to a large proportion of events in Fig. 3D coming from a small subset of cells.

Out of the whole pool of data, those obtained for K^+ (applied at the beginning of the experiment) and electrical stimulation applied to the same cells are provided in Table 2 to compare results derived from the same cells. Thus, exocytosis was faster when ACh was released endogenously.

3.3. Comparing the kinetics of exocytosis in isolated cells versus adrenal slice cells

Herrero et al. (2002) reported the following kinetic variables for cultured mouse chromaffin cells stimulated with 1 mM ACh in KH solutions: I_{max}=22.8±1.8 pA, Q=0.48±0.02 pC, t1/2 =19.9±6.1 ms, m=5.2±0.6 nA/s. In addition, Fulop et al. working with cultured mouse chromaffin cells reported a mean amplitude of 50 pA for the amperometric spikes rendered by cells stimulated at 14 Hz, and less than 20 pA when cells were stimulated at 0.5 Hz. In a report by Voets et al. (2001), amperometric spike amplitudes elicited by flash photolysis were around 17 pA (the exact value was not given by these authors). Given the vast differences of these results with respect to our present data, we speculated they could be attributed to the distinct preparations used. We thus went on to perform a further set of experiments, in which mouse adrenal gland chromaffin cells in culture were stimulated with 1 mM ACh, but superfused with the same solution as for the slices, i.e., a BBS-based solution (Fig. 4A). The results obtained are summarized in Table 3. Surprisingly, we observed no significant differences, except in the case of t1/2 and tp, indicating an even faster response in the cultured cells.

3.4. Comparing the kinetics of exocytosis in the presence of KH versus BBS in adrenal slice cells

Given the discrepancies between the data published by Herrero et al. (2002) and our results did not seem to be due to the distinct preparations used, we then explored the possible effects of the different buffer solution used. To this end, we alternately superfused chromaffin cells in tissue slices with KH-based and BBS-based solutions for 10 min before applying a 5 s pulse of 100 mM K^+ (Fig. 4B). Once again we detected no significant differences. These data are provided in Table 4.

4. Discussion

This work is the first detailed and extensive study on the kinetics of catecholamine release in
Our findings indicate that catecholamine release in response to electrical stimulation of the splanchnic nerve is faster than that elicited by exogenous superfusion with ACh or high K\(^+\). This is shown by the higher release rate given by \(m\), and the lower \(t_{1/2}\) and \(t_p\) values of the spikes obtained by electrical field stimulation, compared to those of the spikes provoked by ACh or high K\(^+\) (Table 1 and 2). We demonstrate that the wavy baseline reflects capture by the carbon fiber electrode of catecholamines from neighboring cells. The recent work by Zhan Zhou’s group (Chen et al., 2005) reported that three compounds coreleased with catecholamines during the exocytotic bulk (ATP, opioids or somatostatin), accelerated the amperometric spikes shown by a reduction in their half-widths, by a mechanism that involves G\(_{i/o}\)-protein activation through G\(\beta\gamma\) and PKC. Endogenous neurotransmitters also inhibited catecholamine secretion (Fig. 6), but unfortunately half-width values were not studied in this case. It could be that the huge amount of compounds stored in the chromaffin granules (see for a review Winkler and Carmichael, 1977) and coreleased with the catecholamines had an opposite effect that the only three neurotransmitters studied in that work (ATP, opioids, somatostatin). In fact, Chen and coworkers demonstrated (Fig. 4) that ATP (via P\(_{2\gamma}\) receptors) and ACh (via mACh receptors) had an opposite effect. Also, low concentrations of ATP had been demonstrated to act synergistically with cholinergic secretagogues by potentiating catecholamine release, while high concentrations of the neurotransmitter inhibits secretion (Diverse-Pierlussi et al., 1991). Another peptide derived from chromogranin A, which is also released during the exocytotic bulk, catestatin, had been shown to inhibit ACh evoked secretion without affecting the kinetic parameters of the exocytotic amperometric events elicited by the ACh (Herrero et al., 2002). Other compounds corealased with the catecholamines had been reported to modulate positively or negatively secretion in chromaffin cells: GABA (Kataoka et al., 1984, 1988; Oset-Gasque and Aunis, 1989; Castro et al., 1989), and NPY (Higuchi et al., 1988).

Thus, the possibility exists that the compounds accompanying the catecholamines, detected as the wavy baseline recorded in the slices, interfere with the bulk release of vesicular contents, slowing down the release process. In effect, the puzzling results obtained in cultured cells compared to the slices may also be interpreted on this basis. Hence, the faster \(t_{1/2}\) and \(t_p\) shown by amperometric events recorded in cultured cells could be attributed to a lack of modulation of the release event by the neurotransmitters coreleased with catecholamines.

The intense, rapid amperometric events recorded here in mouse chromaffin cells in situ (\(I_{max}=81.2\pm12\) pA, \(Q=0.77\pm0.1\) pC, \(t_{1/2}=7.9\pm0.2\) ms, \(m=73.5\pm3.5\) nA/s, stimulus ACh) contrast with the less intense, slower values previously reported using ACh as stimulus in cultured mouse chromaffin cells in the work of Herrero et al. (2002) (\(I_{max}=22.8\pm1.8\) pA, \(Q=0.48\pm0.02\) pC, \(t_{1/2}=19.9\pm6.1\) ms, \(m=5.2\pm0.6\) nA/s). This difference, however, could not be reproduced in our present study by varying the type of preparation (slices versus cultures) under the same experimental conditions. The measurements obtained for the cells in slices or culture were similar, with the exception of \(t_{1/2}\) and \(t_p\), which were faster for the cultured cells. The type of buffer solution used, Krebs-HEPES versus BBS also failed to affect the results. Since kinetic parameters are very sensitive to experimental conditions, other methodological differences could explain the distortion of the data mentioned above: sampling rate of 1-4 kHz versus the rate of 14.5 kHz used.
here, carbon fibers not calibrated versus calibrated electrodes in the present study, analysis of mean spike values versus mean cell values used in this work.

In conclusion, our findings provide valuable information for understanding the exocytotic event. The use of chromaffin cells in slices of tissue offers a unique opportunity for studying the kinetics of catecholamine release under conditions closer to the in vivo situation. Under these experimental conditions, we obtained exocytotic amperometric responses evoked by field electrical stimulation that are significantly faster than those produced after exogenous application of ACh or K⁺, which would seem to indicate that compounds coreleased with ACh during the exocytotic bulk might be regulating the release process. Our data also suggest that results obtained by perfusing slice chromaffin cells with secretagogues should be interpreted with caution, since modulation of the exocytotic event by compounds coreleased with catecholamines could slow down the release process.
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Figure legends

Figure 1. Amperometric spikes recorded in mouse adrenal slice chromaffin cells elicited by ACh or K+. A and B, Original recordings obtained in response to a 5 s stimulus of 1 mM ACh or 100 mM K+, respectively, applied every 1 min. C and D, Typical amperometric spikes (marked with asterisks in A and B) and their corresponding kinetic variables obtained by superfusion with ACh or K+, respectively. E, Frequency histograms for the different kinetic variables calculated for the amperometric spikes recorded in response to ACh or K+.

Figure 2. Simultaneous measurement of Ca²⁺ currents and catecholamine release recorded with carbon fiber amperometry in a voltage-clamped mouse adrenal slice chromaffin cell, using the whole-cell configuration of the patch-clamp technique. 500 ms depolarizations were applied from a holding potential of −80 mV to the peak current voltage, which was -5 mV in this cell.

Figure 3. Amperometric spikes triggered by field electrical stimulation of mouse adrenal slice chromaffin cells. A pulse of 1 ms duration and 6 mA intensity was applied. A, The initial spike labeled with # is due to the electrical stimulus artefact. The amperometric spike marked with an asterisk in A is represented in B along with its kinetic variables, using an expanded time scale. C, The nicotinic receptor antagonist hexamethonium (5 µM) blocked the responses evoked by electrical stimulation. The stimulus was a single rectangular pulse of 1 ms duration and 6 mA intensity. D, Frequency histograms showing the kinetic variables obtained for the amperometric spikes rendered by electrical stimulation.

Figure 4. Amperometry recordings obtained in K⁺ stimulated mouse adrenal chromaffin cells in culture (A) or in slices, using Krebs HEPES (KH) and bicarbonate buffered saline solutions (BBS) (B). A) Original recordings obtained for a 5 s stimulus of 100 mM K⁺ in BBS-based solutions in 2 different cells in culture. B) Original recordings obtained for a 5 s stimulus of 100 mM K⁺ applied every 10 min to a cell in situ. The cell in the slice under study was alternately perfused with BBS- and KH-based solutions for 10 min prior to stimulation. The order of perfusion with the solutions was varied in different experiments.
Fig. 3

A

B

C

D

$I_{\text{max}} = 473.31 \text{ pA}$

$Q = 1.16068 \text{ pC}$

$m = 221.4 \text{ nA/s}$

$t_{1/2} = 1.242 \text{ ms}$

$t_p = 0.977 \text{ ms}$

Hexamethonium