Amperometric Study of the Kinetics of Exocytosis in Mouse Adrenal Slice Chromaffin Cells: Physiological and Methodological Insights

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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; Chan and Smith 2003; Voets et al. 2001). 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 ccf chromaffin cells). Moreover, it has been established that the expression of several Ca²⁺ channel subtypes varies considerably in the intact adrenal gland, compared with 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 acetylcholine (ACh) on electrical field stimulation of splanchnic nerve terminals. Thus 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 with 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).

METHODS

Adrenal slice preparation and solutions

The experimental protocol was approved by the animal ethics committee of the Faculty of Medicine of the 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% O₂-5% CO₂. The Krebs–HEPES (KH) 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- to 10-wk-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 multibarreled glass pipette (Carbone and Lux 1987) placed about 50 μm from the cell under study. All reagents were obtained from Sigma (Madrid, Spain).

Isolation and culture of mouse chromaffin cells

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

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 (Model CS 220, Cibertec, Madrid, Spain) (Barbara and Takeda 1996; Iijima et al. 1992). 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) 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). Because results obtained at this frequency did not exhibit any difference from 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 multibarreled glass pipette.

Amperometric recordings

Carbon fiber electrodes were prepared by cannulating a 10-μm-diameter carbon fiber in polyethylene tubing (diameters: 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 backfilled 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 ×40 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

![FIG. 1. Amperometric spikes recorded in mouse adrenal slice chromaffin cells elicited by acetylcholine (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\(^+\).](http://jn.physiology.org/10.1152/jn.00756.2006)
diameter around 10 μm to clean the surface of the cell under study (Albillos et al. 2000; Moser and Neher 1997).

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, after which step depolarizations of 500-ms duration were conducted from a holding potential of −80 mV to the peak current voltage.

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 (for details see Segura et al. 2000). A threshold of 4.5-fold the first derivative of the noise SD 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 phase 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.

RESULTS

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 ACh or 100 mM K$^+$ in the BBS-based solutions (Fig. 1, A 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. Figure 1, C and D shows 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

**TABLE 1.** Mean kinetic variables of amperometric spikes recorded in response to ACh, K$^+$, and electrical field stimulation

<table>
<thead>
<tr>
<th></th>
<th>$I_{\text{max}}$, pA</th>
<th>$Q$, pC</th>
<th>$m$, nA/s</th>
<th>$t_{1/2}$, ms</th>
<th>$t_p$, ms</th>
<th>Number of Spikes</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>81.2 ± 11.7</td>
<td>0.77 ± 0.11</td>
<td>69.9 ± 12.9</td>
<td>7.9 ± 0.74</td>
<td>4.1 ± 0.44</td>
<td>808</td>
<td>7</td>
</tr>
<tr>
<td>K$^+$</td>
<td>91.8 ± 10.0</td>
<td>0.83 ± 0.12</td>
<td>67.6 ± 11.2</td>
<td>6.7 ± 0.34</td>
<td>4.0 ± 0.58</td>
<td>756</td>
<td>7</td>
</tr>
<tr>
<td>Electric</td>
<td>76.1 ± 10.2</td>
<td>0.66 ± 0.10</td>
<td>167.3 ± 28.7</td>
<td>5.0 ± 0.57***</td>
<td>2.9 ± 0.36**</td>
<td>6,850</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are means ± SD. The variables $I_{\text{max}}$, $Q$, $m$, $t_{1/2}$, and $t_p$ indicate the peak amplitude, charge, ascending slope (calculated from the linear portion of the trace between 25 and 75% of the $I_{\text{max}}$), half-width (duration of the signal at 50% of its peak amplitude), and the time-to-peak (time from the start of the spike until the peak in seconds), respectively. *$P$ ≤ 0.05, ACh versus Electric stimuli; **$P$ ≤ 0.01, ACh versus Electric stimuli; ***$P$ ≤ 0.01, K$^+$ versus Electric stimuli.
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 multibarreled glass pipette. In contrast, this interference arising from catecholamines, appearing as a wavy baseline, was not observed after electrical stimulation because splanchnic nerve terminals innervate fewer 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 eight cells tested, superimposed spikes were obtained, although 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 following text, Fig. 4A). Furthermore, the amperometric recordings without wavy baseline obtained by Voets and coworkers (2001) confirmed this hypothesis because 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).

**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 (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. Figure 3, A and B shows 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.

**FIG. 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: initial spike labeled with # arises from the electrical stimulus artifact. Amperometric spike marked with an asterisk in A is represented in B along with its kinetic variables, using an expanded time-scale. C: nicotinic receptor antagonist hexamethonium (5 μM) blocked the responses evoked by electrical stimulation. 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.
ric 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 Fig. 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 \( m, t_{1/2}, \) and \( t_p \) 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). In a comparison of the histograms of Figs. 1E and 3D, the apparent difference in the kinetic variable \( Q \) is explained by 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.

### 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: \( I_{\text{max}} = 22.8 \pm 1.8 \text{ pA}, Q = 0.48 \pm 0.02 \text{ pC}, t_{1/2} = 19.9 \pm 6.1 \text{ ms}, \) and \( m = 5.2 \pm 0.6 \text{ nA/s}. \) In addition, working with cultured mouse chromaffin cells, Fulop et al. (2005) reported a mean amplitude of 50 pA for the amperometric spikes rendered by cells stimulated at 14 Hz and <20 pA when cells were stimulated at 0.5 Hz. As reported 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 attributable 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 that 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 \( t_{1/2} \) and \( t_p, \) indicating an even faster response in the cultured cells.

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

Because the discrepancies between the data published by Herrero et al. (2002) and our results did not seem to be a result of 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.

### Discussion

This work is the first detailed and extensive study on the kinetics of catecholamine release in adrenal slice chromaffin cells, using the carbon fiber amperometric technique.

Our findings indicate that catecholamine release in response to electrical stimulation of the splanchnic nerve is faster than

![Fig. 4. Amperometry recordings obtained in K⁹⁺ 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 K⁹⁺ in BBS-based solutions in 2 different cells in culture. B: original recordings obtained for a 5-s stimulus of 100 mM K⁹⁺ applied every 10 min to a cell in situ. Cell in the slice under study was alternately perfused with BBS- and KH-based solutions for 10 min before stimulation. Order of perfusion with the solutions was varied in different experiments.](http://jn.physiology.org/_DOWNLOAD/2017/06/20/220/246)
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 with those of the spikes provoked by ACh or high K+ (Tables 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 Zhuang Zhou’s group (Chen et al. 2005) reported that three compounds co-released 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\( \beta \gamma \) protein and protein kinase C. Endogenous neurotransmitters also inhibited catecholamine secretion (see Fig. 6 in Chen et al. 2005), 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 (for a review see Winkler and Carmichael 1982) and co-released with the catecholamines had an opposite effect than that of the only three neurotransmitters studied in that work (ATP, opioids, somatostatin). In fact, Chen and coworkers demonstrated (Fig. 4) that ATP (by P\( _{2Y} \) receptors) and ACh (by mACh receptors) had an opposite effect. Also, low concentrations of ATP had been demonstrated to act synergistically with cholinergic secretagogues by potentiating catecholamine release, whereas high concentrations of the neurotransmitter inhibit secretion (Diverse-Pierlussi et al. 1991). Another peptide derived from chromogranin A, catestatin (which is also released during the exocytotic event), 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 co-released with the catecholamines had been reported to modulate positively or negatively secretion in chromaffin cells: \( \gamma \)-aminobutyric acid (Castro et al. 1989; Kataoka et al. 1984, 1988; Osei-Gasque and Aunis 1989) and neuropeptide Y (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 with the slices may also be interpreted on this basis. Thus 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 co-released with catecholamines.

The intense, rapid amperometric events recorded here in mouse chromaffin cells in situ (\( I_{\text{max}} = 81.2 \pm 12 \) pA, \( Q = 0.77 \pm 0.1 \) pC, \( t_{1/2} = 7.9 \pm 0.2 \) ms, \( m = 73.5 \pm 3.5 \) nA/s, stimulus ACh) contrast with the less-intense, slower events previously reported using ACh as stimulus in cultured mouse chromaffin cells in the work of Herrero et al. (2002) (\( I_{\text{max}} = 22.8 \pm 1.8 \) pA, \( Q = 0.48 \pm 0.02 \) pC, \( t_{1/2} = 19.9 \pm 6.1 \) ms, \( m = 5.2 \pm 0.6 \) nA/s). This difference, however, could not be reproduced in our present study by varying the type of preparation (slices vs. 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. Because 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, and 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 to study 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 co-released 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, because modulation of the exocytotic event by compounds co-released with catecholamines could slow down the release process.

**Acknowledgments**

The authors thank Dr. Y. Kidokoro for the kind gift of tungsten electrodes and the Drs. Sánchez for help with data analysis.

**Table 3.** Mean kinetic variables obtained from individual events recorded in response to K+ in cultured versus slice cells

<table>
<thead>
<tr>
<th>Type of Preparation</th>
<th>( I_{\text{max}}, ) pA</th>
<th>( Q, ) pC</th>
<th>( m, ) nA/s</th>
<th>( t_{1/2}, ) ms</th>
<th>( t_p, ) ms</th>
<th>Spikes</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>96.0 ± 15.1</td>
<td>0.52 ± 0.08</td>
<td>84.3 ± 14.5</td>
<td>4.7 ± 0.5**</td>
<td>2.6 ± 0.2**</td>
<td>1,142</td>
<td>20</td>
</tr>
<tr>
<td>Slices</td>
<td>85.2 ± 10.9</td>
<td>0.61 ± 0.07</td>
<td>83.7 ± 20.1</td>
<td>7.7 ± 1.1</td>
<td>4.1 ± 0.6</td>
<td>1,002</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are means ± SD. The variables are as described in Table 1. **p ≤ 0.01.

**Table 4.** Mean kinetic variables obtained from individual events recorded in response to K+ in KH- and BBS-based solutions

<table>
<thead>
<tr>
<th>Type of Solution</th>
<th>( I_{\text{max}}, ) pA</th>
<th>( Q, ) pC</th>
<th>( m, ) nA/s</th>
<th>( t_{1/2}, ) ms</th>
<th>( t_p, ) ms</th>
<th>Spikes</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH</td>
<td>101.1 ± 10.7</td>
<td>0.76 ± 0.07</td>
<td>76.9 ± 13.7</td>
<td>6.6 ± 0.67</td>
<td>3.9 ± 0.43</td>
<td>844</td>
<td>8</td>
</tr>
<tr>
<td>BBS</td>
<td>97.9 ± 10.5</td>
<td>0.75 ± 0.07</td>
<td>76.3 ± 16.5</td>
<td>6.4 ± 0.68</td>
<td>3.9 ± 0.50</td>
<td>716</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are means ± SD. The variables are as described in Table 1.
GRANTS

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