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Invariance of Exocytotic Events Detected by Amperometry as a Function of the Carbon Fiber Microelectrode Diameter

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Etched carbon fiber microelectrodes of different radii have been used for amperometric measurements of single exocytotic events occurring at adrenal chromaffin cells. Frequency, kinetic, and quantitative information on exocytosis provided by amperometric spikes were analyzed as a function of the surface area of the microelectrodes. Interestingly, the percentage of spikes with foot (as well as their own characteristics), a category revealing the existence of sufficient long-lasting fusion pores, was found to be constant whatever the microelectrode diameter was, whereas the probability of overlapping spikes decreased with the electrode size. This confirmed that the prespike foot could not feature accidental superimposition of separated events occurring at different places. Moreover, the features of amperometric spikes investigated here (charge, intensity and kinetics) were found constant for all microelectrode diameters. This demonstrated that the electrochemical measurement does not introduce significant bias onto the kinetics and thermodynamics of release during individual exocytotic events. All in all, this work evidences that information on exocytosis amperometrically recorded with the usual 7 μm diameter carbon fiber electrodes is biologically relevant, although the frequent overlap between spikes requires a censorship of the data during the analytical treatment.

Exocytosis is the process by which a secretory cell releases biomolecules, like neurotransmitters or hormones, contained within intracellular cargo, namely, vesicles or granules. It plays a crucial role in communication between cells, such as between neurons in chemical synapses or during the hormonal liberation in the blood involved by the hypothalamo−hypophyseal−adrenocortical (HHA) axis. Vesicular exocytosis occurs through a sequence of different steps which are now well identified: trafficking, by which vesicles translocate in the cytoplasm over a significant distance to the vicinity of the plasma membrane; tethering,[1,2] by which vesicles create a link with the target membrane; docking and priming,[3,4] during which a tight core protein SNARE complex is formed to bring together the vesicle and plasma membranes;[5,6] and finally, the fusion and release steps. It was shown 2 decades ago that the fusion and release stages of vesicular exocytosis could be monitored by amperometry on carbon fiber microelectrodes,[7−12] for instance, on isolated adrenal chromaffin cells that release catecholamines (adrenaline, noradrenaline, dopamine) after appropriate stimulation. Thanks to the catecholamine's two-electron oxidation at the carbon electrode surface, each exocytotic event is displayed as a spike of current whose morphology (magnitude, area,...) reflects the dynamic of the event.[13] A cell secretion detected by amperometry then appears as a sequence of spikes, each amperometric spike detected by the microelectrode featuring one exocytotic event.[14,15]

In this context, "etched" carbon fiber microelectrodes of diameter smaller than 7 μm have been used by several groups to map amperometrically the cell surface during its secretion. Thus, Schroeder et al. exploited the small diameter of carbon ultramicroelectrodes (1 μm of radius) to map exocytotic release sites on the surface of bovine adrenal chromaffin cells. Fluorescent monitoring by confocal microscopy was used in addition to demonstrate the existence of specific zones of exocytotic activity (hot spots) on the surface of the cell.[16] Using nanoelectrodes (100−300 nm diameter) on PC12 cells, Wu et al. showed that dopamine release can be monitored in real time with high spatial resolution allowing one to differentiate hot spots even in a same

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active release zone.[17] In that case, the nanoelectrode sensing surface corresponded roughly to an average vesicle diameter and exocytosis was rarely detected when the nanoelectrode was randomly positioned at the top of the cell. Finally, Kawagoe et al. investigated the influence of diffusional filtering and accompanying dilution of the secretion when an etched microelectrode (electrode radius ~ 1 μm) is placed 1 μm from the cell surface.[18]

All these investigations have assumed that the electrode size does not alter the information recorded amperometrically (i.e., the features of the spikes analyzed), which is clearly not obvious since the detection is not passive and implies a near contact with the cell membrane. Furthermore, superimposition between subsequent spikes cannot be neglected.[13,19] Indeed, signal overlap could result when two events are temporally close but occur at different places on the cell or feature different modes of exocytosis, named composed or sequential exocytosis.[6,20,21] Since the overlap probability is expected to decrease with the electrode size, the overlap of spikes might follow up the same evolution. This suggests that the amperometric information would be more accurate and relevant (i.e., requires less censorship) for electrodes of smaller diameter. Conversely, decreasing the size too much leads to only the detection of rare events which may then not be statistically significant.[17]

Moreover, during exocytosis, the fusion step between a vesicle and the cell membrane is achieved through the formation of a nanometric pore. When this fusion pore is sufficiently long-lived (more than a millisecond), the low amount of electroactive species released before the pore expansion and oxidized at the microelectrode surface leads to a recognizable current event named a "prespike feature" (PSF) or amperometric foot.[22] The signature of this detected flux is a low amperometric current, in the picoampere range, which precedes the amperometric spike.[8,23−25] At chromaffin cells, under control experimental conditions, previous studies have shown that approximately 30% of spikes display such a foot.[26,27] Whereas the detection of a PSF is now commonly associated in the literature to the release through the fusion pore, there is no clear evidence that all PSFs represent foot releases. Indeed when the probability of overlap is significant, some may be artifact measurements due to the overlap between a small and a fast exocytotic event. Hence, reducing the electrode size is expected to offer a clear scrutiny concerning this issue.

In the present work, amperometric spikes were detected during exocytosis at chromaffin cells and analyzed as a function of the diameter of different carbon fiber microelectrodes in order to investigate if the size of the electroactive surface area of microelectrodes alters or not amperometric information (charge, kinetic parameters of amperometric spikes, and percentage of spikes with PSF as well as PSF characteristics). This accurate analysis showed that artifacts due to spikes superimposition which can occur with a microelectrode of largest surface area did not affect significantly the amperometric information as well as at the smallest electrodes. Additionally, these results definitively evidenced that the PSFs show real biological features in exocytosis and are not an experimental bias. Their probability of detection was found to be independent of the electrode size while that of accidental superimposition of independent events drops with the electrode dimension.

EXPERIMENTAL SECTION

Cell Culture and Preparation. Bovine chromaffin cells were prepared by collagenase digestion of the medulla of adrenal glands obtained from a local slaughterhouse (Meaux, France). Cells were purified and cultured using previously described methods.[28] They were then plated at a density of 4×10^4 cells/cm^2 on collagen−poly-L-lysine coated glass coverslips placed in 24 well plates and kept in a 5% CO_2 atmosphere at 37 °C in the incubator. Cells were used on day 3−10 after culture and 24 h maximum after plating.

Secretagogues. The two more often used secretagogues for chromaffin cells were employed in this work to elicit exocytosis: $BaCl_2$ at 2 mM and KCl at 55 mM in the presence of 2.5 mM $CaCl_2$. These two secretagogues do not induce the same secretion mechanisms in the cells and seem to select mainly some categories of vesicles.[29]

Electrode Preparation and Single-Cell Experiments. Carbon fiber microelectrodes (7 μm diameter, Thornel-Cytec Carbon Fibers, Greenville, SC) were constructed as described previously and back-filled with mercury for contact.[30] Etching of the tip was performed in a Bunsen flame in order to consume the carbon fiber before the fiber insulation. The carbon fiber was progressively and gently introduced into the flame for few seconds. The length of the fiber introduced in the flame did not exceed about half a centimeter.[14,18] We checked after this step the aspect of the tip, and only fibers with a cone-shaped tip were retained for experiments. Electrode tips were then polished at a 45° angle on a diamond dust-embedded micropipet beveling wheel (model EG-4, Narishige Co., London, U.K.) for 5−30 min depending on the diameter of the carbon fiber (see Figure 1). The duration of polishing was larger for lower carbon fiber diameters because of their lower pressure on the wheel and their higher flexibility. Only electrodes with a very stable amperometric baseline current were used for cell measurements.

Cells were prepared by placing each coverslip into a 35 mm plastic dish filled with 5 mL of isotonic physiological saline. The composition of this buffer was different for the two secretagogues used throughout this work. For $BaCl_2$ we used 154 mM NaCl, 4.2 mM KCl, 0.7 mM $MgCl_2$, 11.2 mM glucose, 10 mM HEPES,

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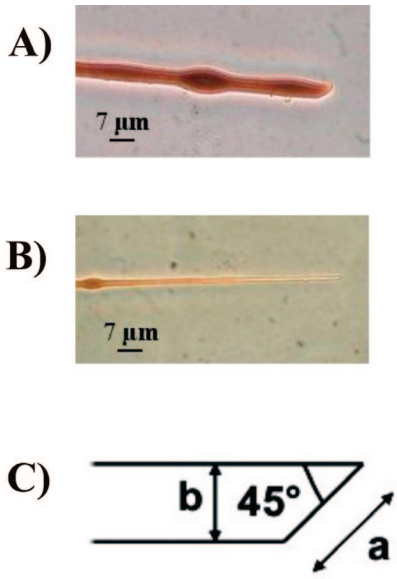

Figure 1. (A) Microphotography of a usual microelectrode, surface area of 90 μm^2. (B) Microphotography of an etched microelectrode surface area of 15 μm^2. (C) Measurement of the geometrical surface area of a microelectrode through microscope examination.

pH 7.4, whereas for KCl, Locke buffer supplemented with calcium was used, e.g., 154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5.6 mM glucose, 3.6 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 15 mM HEPES, pH 7.4. After positioning the dish onto the stage of an inverted microscope (Axiovert-135, Carl Zeiss, Germany), the carbon fiber microelectrode surface was positioned with a micromanipulator (model MHW-103, Narishige Co., London, U.K.) in contact with the membrane of an isolated chromaffin cell. The close proximity of the electrode surface to the cell surface was confirmed by a slight dumpling in the outline of the cell. We always chose to position the center of the microelectrode surface at the top of the cell, i.e., at the center of the cell's apical pole. We proceeded like that in order to get better collection efficiency when using an etched carbon fiber microelectrode like the ones of 15 μm^2 surface area. Then, a glass microcapillary (10−20 μm diameter) was positioned with a second micromanipulator at a distance of 20−30 μm from the cell and used to inject (Femtojet injector, Eppendorf Inc., Hamburg, Germany) the stimulating solution toward the cell surface. Cell responses usually, after the end of the 10 s injection of $BaCl_2$, last for a few minutes (depending on cellular variability) while the one to KCl lasted during the whole injection (that is, 1 min). The microelectrode was kept in place during the stimulation and all along the secretion process. Each cell was only stimulated once. All experiments were performed at room temperature.

Measurement of Microelectrode Surface Area. The measurement of the electrode surface area was based on observations in normal and contrast-phase modes on an inverted microscope (Axiovert-135, Carl Zeiss, Germany). Each microelectrode was placed in order that their 45° angle beveled tip was perfectly observable (Figure 1, parts A and B). Two measurements (diametral axes, a and b, as shown on Figure 1C) were necessary to estimate the elliptic surface area of microelectrode. a was the major axis and b was the minor axis of the ellipse. The apparent polished area of microelectrode was then calculated as equal to $\pi ab/4$.

Data Acquisition and Data Analysis. Electrodes were held at +0.65 V versus a silver/silver chloride reference electrode (Ag/AgCl) using a modified picoamperometer (model AMU-130, Radiometer Analytical Instruments, Copenhagen, Denmark), for which the adjustable time response was set at 0.05 ms. The output was digitized at 40 kHz, displayed in real time, and stored on a computer (Powerlab-4SP A/D converter and software Chart, ADinstruments, Colorado Springs, CO) with no subsequent digital filtering. Each amperometric trace acquired during cell secretion was visually inspected, and signals were designated as exocytotic spikes if their maximum current values were 3 times higher than the rms noise (0.4−0.7 pA) of the baseline current recorded prior to each signal. Spikes were designated as having a foot by the existence of a current increase and an inflection point or a slope discontinuity distinguishing the end of the foot portion of the trace from the onset of the main event. Main spike and foot characteristics, i.e., the maximum oxidation current I_{max} (pA), the total electrical charge Q (fC), and the rise time t_{20-90} (ms) of the spike, as well as foot intensity I_{foot} (pA), charge Q_{foot} (fC), and duration t_{foot} (ms), were determined using home made software. All values are reported as the mean ± SEM.

RESULTS AND DISCUSSION

Features of the Amperometric Spikes as a Function of the Microelectrode Diameter. Exocytotic events were measured under usual control conditions without any modification of the cells. Their secretory responses were elicited by two different secretagogues (barium or potassium) and detected at carbon microelectrodes of several disk geometric areas: 15, 25, 65, and 90 μm^2. Examples of cell responses (amperometric traces) acquired for both secretagogues with the larger and the smaller diameter of microelectrodes are reported on Figure 2. As shown in Figure 2A (top), on the trace recorded with a 90 μm^2 surface area microelectrode after potassium stimulation, the high frequency of exocytosis spikes, particularly during the first 20 s, caused a significant spike overlap, leading to a difficult extraction of isolated events. The frequency slowly decreased afterward, allowing the selection and the analysis of most spikes detected at the end of the amperometric recording. In the present studies, spikes were selected and analyzed only when they were perfectly defined, i.e., with a flat baseline before and after the spike and no hint of merging with other spikes. This selection was required to characterize accurately the kinetic and charge parameters of each amperometric spike. An example of two superimposed spikes (detected on an amperometric recording obtained under potassium stimulation and with a 90 μm^2 surface area microelectrode), and therefore not analyzable, is given on Figure 2C. Conversely, when the cell response was acquired with a 15 μm^2 surface area microelectrode (Figure 2A, bottom) and under the same stimulation, the overall average frequency of the amperometric trace was lower, each spike being well separated from its neighbor. As a consequence, most of the spikes could be selected for analysis. This change was more severe when exocytosis was triggered by a barium solution. The particularly high spikes frequency detected at 90 μm^2 surface area microelectrodes and the resulting high rate

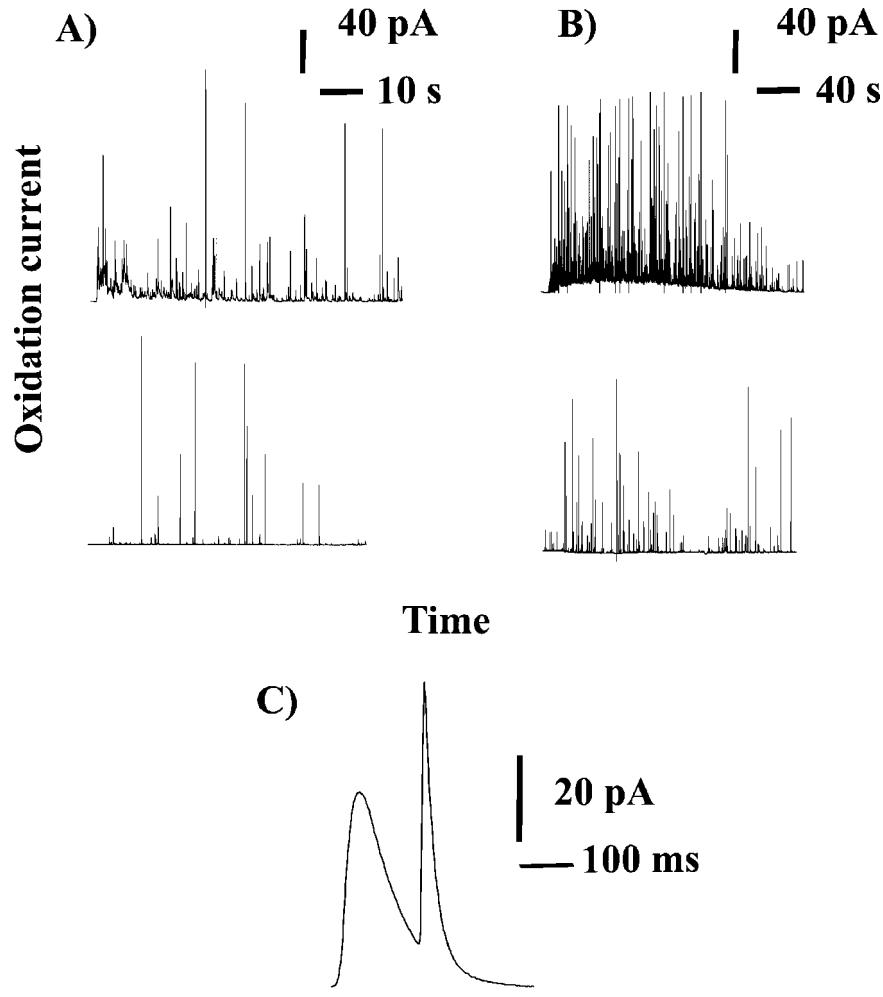

Figure 2. Representative exocytotic responses of a bovine chromaffin cell detected by amperometry at a carbon fiber microelectrode. The secretagogue was K^+ (A) or Ba^{2+} (B). Responses were acquired with a classical microelectrode, 90 μm^2 surface area (top), and an etched carbon fiber microelectrode, 15 μm^2 surface area (bottom). (C) Example of two superimposed spikes detected on the amperometric trace obtained with K^+ as a secretagogue and a 90 μm^2 surface area microelectrode.

of superimposition of spikes induced a deformation of the baseline of current for the whole trace producing a global envelope by convolution of the end decays of each spike (Figure 2B, top). Conversely, with a microelectrode surface area of 15 μm^2 and still after barium stimulation, the baseline was stable and flat, almost all spikes being then separated and analyzable (Figure 2B, bottom). The probability of spikes superimposition as a function of the microelectrode surface areas was calculated and is presented in the Supporting Information.

The variations of the cumulative number of amperometric events were analyzed as function of microelectrode calculated surface areas. This parameter increased with the microelectrode surface area for both potassium and barium stimulations (Figure 3, parts A and B, respectively), which reflected that a large probed surface area allowed the collection of a high number of events per unit of time. The exposed surface of a chromaffin cell, with an average diameter of about 10 μm, can be evaluated to $\sim$80 μm^2 so that with a 90 μm^2 surface area microelectrode the whole surface of the apical pole of the cell is covered. Upon decreasing the microelectrode surface area, a lower surface of the cell is covered and, consequently, fewer events are detected. Interestingly, during certain experiments it was observed that secretion could not be detected with a 15 μm^2 surface area electrode, positioned at the center of the cell apex (apical pole). On the same cell, a slight translation of the electrode by a few micrometers, without injuring the cell membrane, was sufficient to monitor exocytotic events. This experimental observation also confirmed that there are zones of inactivity and "hot spots" on chromaffin cell membranes.[16,17,31−34] Since our goal in this work was not to take advantage of this effect to map the releasing sites over the cell surface but to compare spikes features for different microelectrode diameters, we always searched for releasing areas exclusively.

As the frequency of detected events drastically decreased with small carbon fiber microelectrodes and less superimposed spikes were observed, we were interested in correlating the two observations and, consequently, estimating the probability of superimposition for two amperometric spikes as a function of microelectrodes diameter. For that, a simple model was built to evaluate the probability of spikes superimposition as a function of the mean

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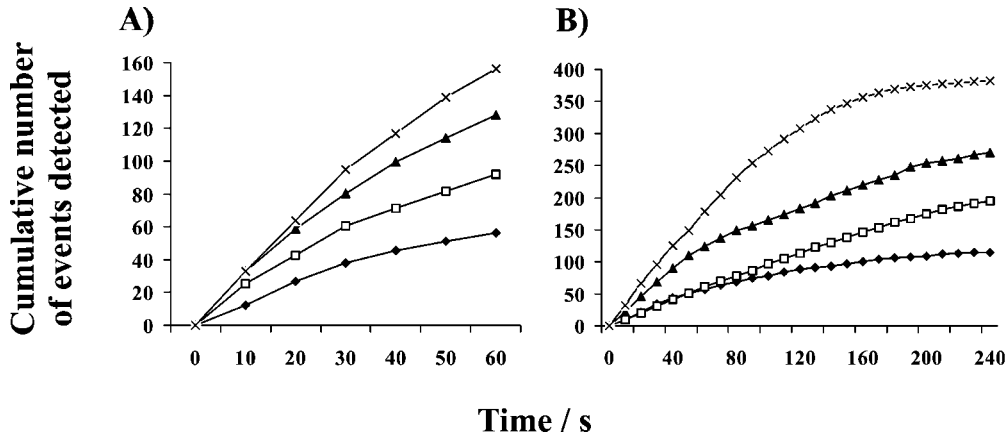

Figure 3. Cumulative number of secretion events for, respectively, the secretagogues K^+ (A) and Ba^{2+} (B) for four microelectrode surface areas: 90 μm^2 ($n = 7$ and 5 cells; symbol, ×), 65 μm^2 ($n = 7$ and 3 cells; symbol, ▲), 25 μm^2 ($n = 12$ and 11 cells; symbol, □), and 15 μm^2 ($n = 30$ and 10 cells; symbol, ◆).

Table 1. Mean Spike Frequency, Predicted Overlapping Probability, and Proportion of Spikes with Foot for the Four Microelectrode Surface Areas and the Two Secretagogues (K^+ 55 mM and Ba^{2+} 2 mM)[a]

secretagogue	microelectrode surface area (μm^2)	spike frequency $1/\Delta t$ (Hz)	overlapping probability (%)	% spikes with foot
K^+ ($n = 30$)	15	1.40	17	29
K^+ ($n = 12$)	25	2.49	26	32
K^+ ($n = 7$)	65	3.42	38	26
K^+ ($n = 7$)	90	5.01	51	33
Ba^{2+} ($n = 10$)	15	1.18	16	29
Ba^{2+} ($n = 11$)	25	1.15	16	35
Ba^{2+} ($n = 3$)	65	1.30	19	35
Ba^{2+} ($n = 5$)	90	2.24	32	27

[a] n represents the number of cells.

frequency and features of spikes under each experimental condition (see Table 1 and the Supporting Information). On the one hand, this showed that the overlapping probability of two successive amperometric spikes increased, as expected, with the diameter of microelectrodes. On the other hand, this allowed quantifying this probability which resulted far from being negligible (see Table 1). Indeed, superimposition probability of two spikes reached 51% for the potassium secretagogue and 32% for the barium secretagogue for a 90 μm^2 sensing surface area (7 μm diameter). This percentage decreased to, respectively, 17% and 16% for an etched microelectrode of 15 μm^2 surface area. This overlapping probability was therefore found to be highly dependent on the microelectrode diameter, due to the change in the number of hot spots analyzed simultaneously. The high decrease of the superimposition probability when moving from a 90 μm^2 surface area to a 15 μm^2 one allowed a lower censorship of the amperometric traces with small carbon microelectrodes.

In order to examine if the electrode size could alter the features of individual spikes, three main parameters were investigated for all the analyzed spikes: Q (area of the spike in fC), I_{max} (maximum current in pA), and t_{20-90} (ms) which corresponds to the time between 20% and 90% of maximum oxidation current during the rising phase of a spike. It was first observed that the two secretagogues investigated here led to differences in the

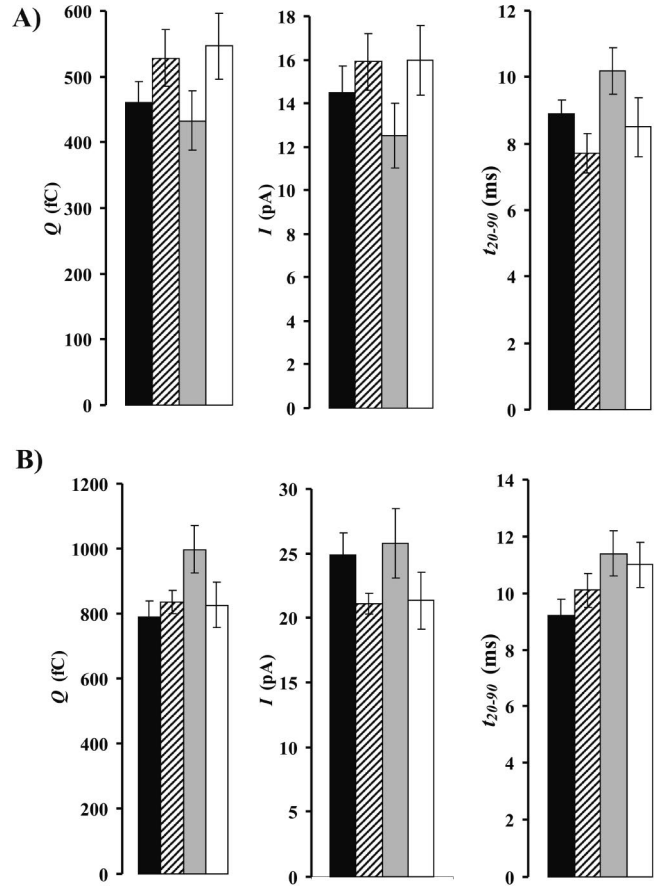

Figure 4. Charge Q, current I_{max}, and half-width t_{20-90} of all amperometric spikes for different microelectrode surface areas for the secretagogues K^+ (A) and Ba^{2+} (B) (black 15, striped 25, gray 65, and white 90 μm^2 surface area electrodes).

general features of the amperometric spikes. Indeed, the potassium stimulation induced amperometric events of smaller amplitude (charge and maximum current of spike) and of faster kinetics (t_{20-90}) than with barium stimulation. This observation is in full agreement with previous reports.[9] Conversely, as shown in Figure 4, for each elicitor, the parameters (charge and flux released, kinetic parameters) collected on all the amperometric spikes did not evidence any significant variation with the diameter of the microelectrode when overlapping spikes were

censored. These data are given as means for all the spikes, i.e., with and without foot. Thus, no striking differences appeared in view of the SEM error bars between the four different microelectrodes. Therefore, provided that care is taken to avoid spikes accidental superimposition, the main features of the spikes are not dependent on the microelectrodes diameter, and hence, reflect biological or physiological parameters involved in the mechanisms of exocytosis. Overall, the study reported here clearly evidences that 7 μm diameter carbon microelectrodes do not distort the information recorded amperometrically on exocytosis which gave reassurance on all amperometric information collected presently and in the past with these usual carbon microelectrodes.

Could the Amperometric Foot Be an Experimental Artifact? Release through fusion pores is frequently ascribed to the observation of PSFs in amperometry.[35] However, there is still no absolute proof that the foot preceding an amperometric spike is always representative of a fusion pore release. One can reasonably wonder if a substantial fraction of the amperometric PSFs may arise from the accidental superimposition of two distinct exocytotic spikes which are temporally close together or even possibly correlated. Furthermore, with our present experimental precision (rms noise comprised between 0.4 and 0.7 pA), the amperometric spikes with foot could also be composed of a small step of current ("kiss-and-run" cases or events involving small released quantities or spikes distorted by diffusion) superimposed to a large straight spike, both events being detected simultaneously though occurring at different points of the sensing area of the microelectrode.

However, these hypotheses can be ruled out based on present results which showed that the percentage of amperometric feet remained globally invariant with the electrode size. Present data established that this proportion did not vary significantly with the surface area of the microelectrode and the secretion frequency (see Table 1) but ranged between 26% and 35%, which is the habitual span of values for control conditions reflecting solely the cellular variability. On average, about 30% of the amperometric events display an analyzable foot (see Table 1), i.e., with a maximum current I_{foot} larger than 1.5 pA (signal/noise = 3) and a time duration t_{foot} greater than 1 ms.[27]

If a significant proportion of the spikes with foot would arise from superimposition of two independent events, this percentage should obviously increase with the probability of superimposition, i.e., with the microelectrode surface area. Hence, a constant foot percentage excludes that the spikes with foot could result from a detection artifact related to accidental superimposition of separate events. To investigate if the monitored foot features depended on the microelectrodes electroactive areas, we also examined if any variation occurred in the main parameters describing a foot. Q_{foot}, t_{foot}, and I_{foot}, respectively, the mean charge, duration, and intensity of the feet detected for all conditions are given in Figure 5. The constancy of these parameters with the electrode size showed that PSFs are intrinsic characteristics of release and do not depend on the conditions of their measurement. This conclusion is in full agreement with a recent work by Hafez et al.[36] Through investigations of exocytosis at single chromaffin cells

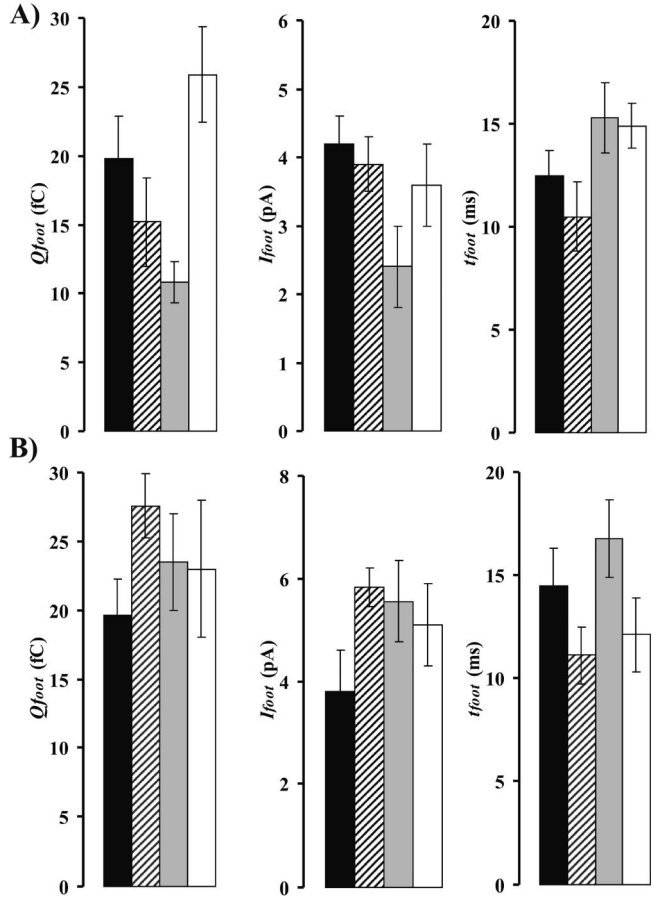

Figure 5. Charge Q_{foot}, current I_{foot}, and duration t_{foot} as measured with microelectrodes of different surface areas for the secretagogues K^+ (A) and Ba^{2+} (B) (black 15, striped 25, gray 65, and white 90 μm^2 surface area electrodes).

positioned on an electrochemical array of four Pt electrodes these authors showed that the amperometric PSF features (frequency, intensity, charge, duration) were similar to those acquired with larger carbon fiber microelectrodes.

Thus, the present observations reinforce the conclusion that the percentage of spikes with foot corresponds to the exocytosis of vesicles with special physiological features relative to nearly 30% of events under all the experimental conditions tested here. These last results unequivocally demonstrate that the observation of amperometric feet and their frequency are directly linked to biological, physiological, and/or physical properties of the fusing vesicles and membranes and not to any measurement artifact, as we questioned initially.[27]

CONCLUSION

This work examined quantitatively the influence of the microelectrode diameters on the amperometric traces reflecting the exocytosis of bovine chromaffin cells. The frequency of detected events was highly reduced with microelectrodes of small surface area due to a lower coverage of cell secretion surface. Henceforth, microelectrodes of smaller area allowed reducing significantly the problem of spikes superimposition which is helpful for systematic kinetic analyses of spikes along a recorded amperometric trace. The global parameters of all amperometric spikes (with and without foot) were not significantly modified at smaller diameter

(35) De Toledo, G. A.; Fernandez-Chacon, R.; Fernandez, J. M. *Nature* **1993**, *363*, 554–558.

(36) Hafez, I.; Kisler, K.; Berberian, K.; Dernick, G.; Valero, V.; Yong, M. G.; Craighead, H. G.; Lindau, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13879–13884.

microelectrodes, i.e., less than 7 μm, reinforcing the confidence in results obtained in the past by many groups with such usual carbon microelectrodes, provided the amperometric traces were censored to eliminate accidentally superimposed spikes. The percentage of spikes with foot was found to be constant with the microelectrode diameter, i.e., around 30%. As the proportion of spikes with foot was independent of electrode area, whereas the proportion of overlapping spikes was not, this observation supported the important contention that the feet are not an artifact resulting from overlapping events. The mean percentage of spikes with foot must thus then be considered as a real biological property, and not to measurement artifact, probably corresponding to the exocytosis of particular vesicles present in the chromaffin cells, such as halo-equipped.[26,27]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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