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Quantitative investigations of amperometric spike feet suggest different controlling factors of the fusion pore in exocytosis at chromaffin cells

Christian Amatore *, Stéphane Arbault, Imelda Bonifas [1], Manon Guille

Laboratoire PASTEUR, Ecole Normale Supérieure, CNRS, UPMC Univ Paris 06, Département de Chimie, 24 Rue Lhomond, 75005, Paris, France

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ABSTRACT

Around 30% of exocytosis events recorded by amperometry at carbon fiber microelectrodes exhibit a pre-spike feature (PSF) termed a "foot". This wave is associated with the release of the neurotransmitters via a transitory fusion pore, whilst the large, main exocytotic spike is due to complete release. The amperometric data reported herein were obtained using bovine chromaffin cells stimulated with either potassium or barium ions, two commonly-employed elicitors of exocytosis. Identical trends are observed with both activators: (i) they induce the same ratio (close to 30%) of events with a foot in the population of amperometric spikes, and (ii) spikes with a foot can be divided into two primary categories, depending on the temporal variation of the current wave (viz. as a ramp, or a ramp followed by a plateau). Correlations between the characteristics of the whole current spike, and of its observed foot, have been sought; such analyses demonstrate that the maximum current of both foot and spike signals are highly correlated, but, in contrast, the integrated charges of both are poorly correlated. Moreover, the temporal duration of the PSF is fully uncorrelated with any parameter pertaining to the main current spike. On the basis of these reproducible observations, it is hypothesized that the characteristics (dimensions and topology, at least) of each secretory vesicle determine the probability of formation of the fusion pore and its maximum size, whilst molecular factors of the cell membrane control its duration, and, consequently, the amount delivered prior to the massive exocytosis of catecholamines observed as a spike in amperometry.

1. Introduction

Vesicular exocytosis is a key process used by living cells to deliver, with high specificity and precision, chemical messengers into their environment. This process allows the transmission of information at many levels, including within the chemical synapses between neurons [1,2], at the immunological synapse between immune cells [3,4] or into the blood by chromaffin cells from the adrenal gland [5,6]. The exocytosis of a secretory vesicle proceeds through a sequence of different steps initiated by the formation of a fusion pore between the membranes of the vesicle and of the cell [7,8]. Two major scenarios have been identified and addressed to characterize the release through the fusion pore kinetically and quantitatively. The first, concerned with pore size, was initially investigated by patch-clamp measurements of membrane conductance and capacitance. This work demonstrated that a nanometric channel is formed at the onset of exocytosis, and that this stage may be followed by a rapid expansion leading to a drastically increased rate of release of the vesicle content [9,10]. These conclusions were reinforced by near-field spectroscopies, including fluorescence microscopy under total internal reflection conditions (TIRFM), which effectively demonstrated the dynamics of the full fusion events [11–13]. The second case involved the amperometric measurement of the flux and temporal variation of messengers by oxidation at a carbon fiber microelectrode positioned at sub-micrometric distance from a secretory cell. This afforded the first direct analysis of the flux of species released during exocytosis [14,15]. By this dichotomic approach, each set of techniques documented different contributions of importance for vesicular exocytosis which are directly interrelated. Thus, great efforts have been made to propose combined methods for investigating simultaneously the conductance of the pore and the release of species, and the examination of their relationships [10,16]. In the present work, we wish to focus on the correlations between data provided by amperometric measurements of released fluxes during the pore stage (the foot) and the fusion event (the spike).

The amperometric detection of release (Fig. 1) through the transitory fusion pore can be observed as a foot preceding the amperometric spike [17]. However, the duration of this stage is usually extremely short since a clear foot feature is observed for only ca. one-third of the events detected. Consequently, most of the amperometric studies on exocytosis have remained at a descriptive level, merely reporting qualitative observations regarding the occurrence of the foot [18,19]. Very few studies have been designed so as to rationalize the occurrence probability or the kinetic or the quantitative characteristics of feet, primarily because of the analytical

* Corresponding author. Ecole Normale Supérieure, Département de Chimie, UMR 8640, 24 rue Lhomond, 75231 Paris cedex 05, France. Tel.: +33 1 4432 3388; fax: +33 1 4432 3863.
E-mail address: Christian.Amatore@ens.fr (C. Amatore).
[1] Present address: Institute for Scientific and Technological Research of San Luis Potosi, Department of Molecular Biology, 78216 San Luis Potosi, Mexico.

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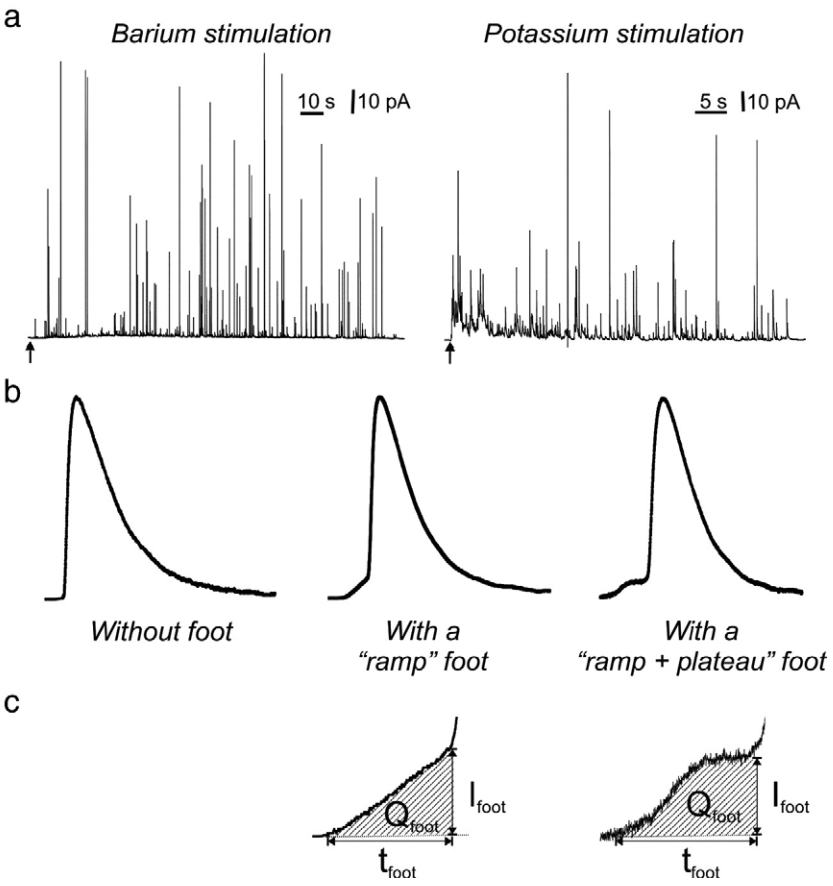


Fig. 1. a. Amperometric traces detected by a carbon fiber microelectrode on single bovine chromaffin cells stimulated with either a 2 mM Ba^{2+} or a 55 mM K^{+} solution. The arrow under each trace indicates the onset of the activator injection (duration of 10 s and 60 s for barium and potassium, respectively). b. The three main types of amperometric spikes detected on chromaffin cells: left, without a detectable foot; middle, with a "ramp" shaped foot; right, with a "ramp + plateau" shaped foot. c. Definition of the foot parameters on representative "ramp" and "ramp + plateau" feet.

challenge this represented, until recently. Owing to the small amplitudes and durations of foot currents, the quantitative interrogation of these requires fluxes as low as ca. 1000 molecules per millisecond to be monitored accurately. In this context, our laboratory, and others, have recently reported studies on adrenal chromaffin cells or PC 12 cells, in which precise measurements of the mean frequency of spikes with a foot in addition to the mean analytical parameters (overall duration, kinetics of release, total charge and maximum oxidative current) of the amperometric feet were reported and analyzed [20,21]. Moreover, statistical investigations revealed that the probability of detecting the amperometric foot is correlated with one specific parameter of the spike, viz. the integrated charge passed on electrolysis [22,23]. This latter parameter is proportional to the number of neurotransmitter molecules released during an exocytotic event, and thus represents, in all likelihood, an evaluation of the total vesicle content of the vesicle dimensions, since the internal concentration of catecholamines into the vesicle has been evaluated as relatively constant [24–27]. Consequently, we have shown that a detailed analysis of the pre-spike feature (PSF) parameters and their correlations with those of the whole subsequent spike may reveal important information about the factors that determine the observation of the foot, i.e. the occurrence of release through a transitory fusion pore of sufficiently long life-time to be distinctively observable during amperometric investigations.

A large set of exocytotic amperometric events detected on chromaffin cells, stimulated with either a barium (Ba^{2+}) solution or a potassium (K^{+}) solution, were analyzed to evidence novel characteristics of the PSF. First, these events can be separated into at least two distinct categories in terms of the temporal profile of their current, viz. of the corresponding kinetics of release. Thus, one of the two main shapes, i.e. a "ramp" or a "ramp + plateau" is essentially systematically observed. The exclusive occurrence of one of these two shapes reveals the existence of two different modes of release. Second, correlations between the foot and spike parameters clearly establish that the low flux of neurotransmitters released during the foot is in each case proportional to the large flux released during the spike; conversely, the fusion pore duration does not correlate with the other quantitative characteristics of the subsequent spike. These results enable the proposal of several hypotheses regarding the factors that govern the observation of spikes with and those without a foot, i.e. those involving a transitory fusion pore observable by amperometry, compared with those that appear to proceed directly to full fusion, respectively.

2. Results

2.1. Quantitative and kinetic parameters of the amperometric spikes with a foot detected on chromaffin cells

The results presented herein could only be obtained via a substantial improvement of amperometric detection limits (time and current resolution) at carbon fiber microelectrodes during the analysis of secretory processes at single cells. In these experiments, the rms electrical noise was sufficiently reduced so as to fall in the range of 0.3 to 0.5 pA in conjunction with a response time of 50 μs. This allowed the measurement, with extreme precision, of amperometric transients of amplitude as small as 1 pA, and which would have been otherwise ignored. These conditions enabled a precise investigation of the PSF, or foot, that often precedes the amperometric spikes observed

during the exocytosis of catecholamines at chromaffin cells. The kinetic and quantitative characteristics of the spikes with a foot were analyzed (Fig. 1a) under stimulation of cell secretion by either the injection of a potassium solution (KCl 55 mM), in the presence of calcium ($CaCl_2$ 2 mM) in the bath, or by the injection of a barium solution ($BaCl_2$ 2 mM). Experiments with each type of activator were conducted on the same batch of cells allowing a direct comparison of their results.

In agreement with results for chromaffin cells reported previously by several groups [18,28,29], we determined in the present set of experiments that about 30% (27% and 31% for the barium and for the potassium activation, respectively) of the amperometric spikes displayed a significant foot current (S/N ratio = 3, at least). In spite of our increased accuracy for low currents and fast kinetics measurement, the frequency of spikes displaying a PSF was similar to that reported in earlier works, indicating that if the previous observations of PSF suffered owing to kinetic limitations of the accuracy with which they were recorded, the measurements were sufficiently precise so as to identify qualitatively the presence of a PSF whenever this occurred. This suggests that the observation of an amperometric foot manifests a characteristic threshold intrinsic to exocytotic events, rather than merely representing a function of measurement accuracy.

In each amperometric trace corresponding to the response of a single cell, two main types of current transients with PSF could be continually identified (see Fig. 1b). They differ according to their waveshape, i.e. the temporal profile of the foot current prior to the onset of the spike, and which displayed either (1) a unique, monotonically increasing current in the form of a ramp, or (2) a short ramp followed by a current plateau lasting up to the onset of the spike. The repartition between these two categories of spikes with a foot feature, named "ramp" and "ramp + plateau", was equivalent for both type of exocytosis elicitor (see Table 1) and seemingly constant for each amperometric trace, viz. approximately 70% of "ramp" and 20% of "ramp + plateau" feet. The remaining 10% of PSF could not be classified using the above taxonomy since their shape was more complex, comprising several rising or stationary phases. Furthermore, the amplitude (current or charge) of these exceptional PSFs was often an important proportion of the whole spike. Such events may originate from the accidental superimposition of spikes of different amplitudes. Indeed, these PSFs were not observed in experiments with etched carbon fiber microelectrodes, wherein a decreased frequency of detected spikes and a reduced extent of their superimposition was evident [30]. For these different reasons, such meaningless events were excluded from our present investigations. Taken together, these results establish that true differences exist between amperometric spikes with and without a foot, as well as between the different categories of events with a foot based on their characterized kinetic profile.

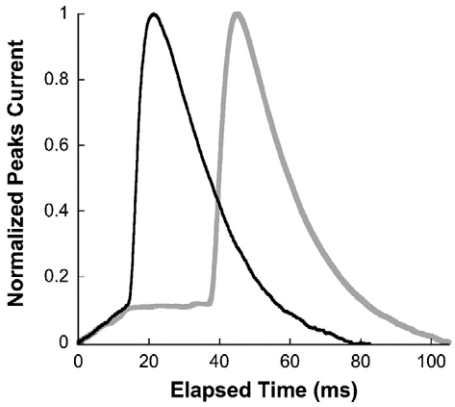


Fig. 2. Superposition of two representative spikes, normalized vs. their maximum current, preceded by either a "ramp" foot or a "ramp + plateau" foot.

The kinetic and quantitative parameters of all spikes with a foot were analyzed for each activator (barium or potassium) and classified within each category ("ramp" or "ramp + plateau"). Although the two activators lead to slight differences in the global characteristics of the exocytotic events, e.g. a smaller amplitude (Q and I_{max}) and faster kinetics ($t_{1/2}$) when induced by the potassium stimulation, the same trends were observed for spikes of each category. The type of PSF, i.e. "ramp" or "ramp + plateau", did not correlate with any sensible modification of kinetics (mainly the half-width) or charge of the respective exocytotic events. The main quantitative difference between the two categories appeared at the level of the foot parameters (the foot duration t_{foot}, and electrical charge Q_{foot}). Indeed, although the maximum current I_{foot} values were very similar for the "ramp" and "ramp + plateau" feet, the second category corresponded to a much longer (t_{foot}) and hence larger (Q_{foot}) release of catecholamines (see Table 1). Furthermore, this depended on the elicitor. For "ramp + plateau" feet t_{foot} was observed to increase by a factor of 1.9 (K^+ stimulation) or 3.0 (Ba^{2+} stimulation), on average, and Q_{foot} increased by a factor of 2.8 (K^+ stimulation) or 4.2 (Ba^{2+} stimulation), on average, compared with those detected for ramp feet.

Representative events from each category of spikes with foot were superimposed in normalized coordinates (vs. the spike current maxima) to allow their comparison (Fig. 2). This showed that the normalized slopes of the current rise were similar for both types of PSF (mean slopes of 0.35 and 0.38 pA/ms for "ramp" and "ramp + plateau" feet, respectively), and it suggests that the ramp stage is a common feature of all PSF, although it may be followed either by the immediate onset of the spike ("ramp" category) or by a stationary current ("ramp + plateau" category), i.e. a constant flux of release, before the onset of the spike. This stationary phase is manifested via an increase in the number of molecules (Q_{foot}) released during the PSF, and in a delay (t_{foot}) of the massive exocytosis stage. Yet this occurs without significantly modifying

Table 1
Mean values of the parameters of the amperometric spikes with a foot detected during elicited exocytosis at bovine chromaffin cells.

	Stimulation with $BaCl_2$			Stimulation with KCl		
	All spikes with foot	Shape of a ramp	Shape of a ramp + plateau	All spikes with foot	Shape of a ramp	Shape of a ramp + plateau
	$n = 367$	$n = 262$	$n = 105$	$n = 388$	$n = 294$	$n = 94$
I_{max} (pA)	31.8 ± 1.8	33.3 ± 2.2	28.0 ± 2.7	25.4 ± 1.1	25.3 ± 1.2	25.6 ± 2.5
Q (fC)	1076 ± 62	1062 ± 73	1111 ± 119	816 ± 34	826 ± 39	785 ± 65
$t_{1/2}$ (ms)	32.1 ± 1.2	31.3 ± 1.4	34.1 ± 2.2	27.7 ± 0.7	28.0 ± 0.8	26.8 ± 1.2
I_{foot} (pA)	3.7 ± 0.2	3.6 ± 0.2	3.9 ± 0.3	3.3 ± 0.1	3.2 ± 0.2	3.9 ± 0.3
Q_{foot} (fC)	25.8 ± 2.5	13.5 ± 1.7	56.3 ± 6.8	28.0 ± 2.0	19.4 ± 1.7	53.9 ± 5.6
t_{foot} (ms)	16.5 ± 1.1	10.5 ± 0.7	31.5 ± 3.2	17.7 ± 0.8	14.6 ± 0.8	27.3 ± 1.9
Foot type (%)	–	69	22	–	65	22

Values are given as mean ± standard error. The percentages of spikes with a foot having a different unclassified shape were 9% for the stimulation with $BaCl_2$ and 13% for the stimulation with KCl.

the total charge of the whole event (i.e. the foot and its associated spike), since the overall Q values were found to be comparable for both categories of spikes with foot.

2.2. Correlations between parameters of amperometric spikes with a foot

The above results question the nature of biological or physicochemical factors which participate in the control of the fusion pore stability and determine the dynamics of release through the fusion pore. This was investigated further by searching for correlations which may exist between the foot parameters and the general parameters of amperometric spikes.

The dependence of the foot maximum current I_{foot} on the spike maximum current I_{max} was first investigated. The ratio I_{foot}/I_{max} was found to be rather constant: $11.6 \pm 0.5\%$ and $13.0 \pm 0.3\%$ on average, respectively for barium- and potassium-evoked responses of chromaffin cells. This correlation between these two currents reflects the interdependence of the physicochemical mechanisms sustaining two main fluxes of release during the exocytosis process, although PSFs reflect the fusion pore, whilst the spike is associated with the membrane fusion and exposure of the vesicular matrix to the extracellular medium. This issue was further reinforced upon plotting the foot and spike currents for each event (Fig. 3a and b). For both types of activator and each sub-population of foot features ("ramp"; "ramp + plateau"), good correlations with unity slope (mean R^2 of 0.7, see legend of Fig. 3) between I_{foot} and I_{max} were observed, evidencing that the maximum flux released through the initial fusion pore is a constant fraction of the maximum flux of catecholamines secreted overall by the vesicle matrix.

We reported previously, based on a large set of data obtained with chromaffin cells, that the probability of occurrence of a pre-spike feature increased with the total charge of the amperometric event, i.e. somehow with the dimensions of the secretory vesicles since the intravesicular of catecholamines is a priori constant [22]. The probability of detection of a spike with a foot increased with the spike charge value and reached 50% at most for spikes providing charges larger than 1000 fC. The same qualitative conclusion could also be drawn from the present set of experiments (data not shown) for either mode of exocytosis activation. However, from a more quantitative approach allowed by our present accuracy, it was observed (Fig. 3c–d and e–f) that either the foot maximum current or the foot charge only weakly correlated (R^2 values below 0.58) with the spike charge. Clouds of data displayed roughly linear trends however with very broad distributions, especially for the foot charge-spike charge correlations. Linear correlations were even more doubtful in the case of spikes with foot displaying only a ramp of current (the main category) since at many instances the same charge Q_{foot} could be measured for different spikes whose total charges Q ranged over almost two orders of magnitude.

Since the correlations between the currents (Fig. 3a and b) are evident, it was of interest to examine if the dispersions in Fig. 3c–f resulted from poor correlations of the foot duration (t_{foot}) and the spike charge ($Q_{foot} \propto t_{foot}\, I_{foot}$). Fig. 3f and g confirmed this view since no correlation could be observed for any stimulus employed here. The large and diffuse clouds of data in Fig. 3g–h establish that the duration of release through the fusion pore does not depend on the content of the vesicle. The duration of the fusion pore may thus depend more significantly on local molecular factors of the cell membrane, which prevail at the site of fusion and may control the dynamics of membranes fusion [31–34].

3. Discussion

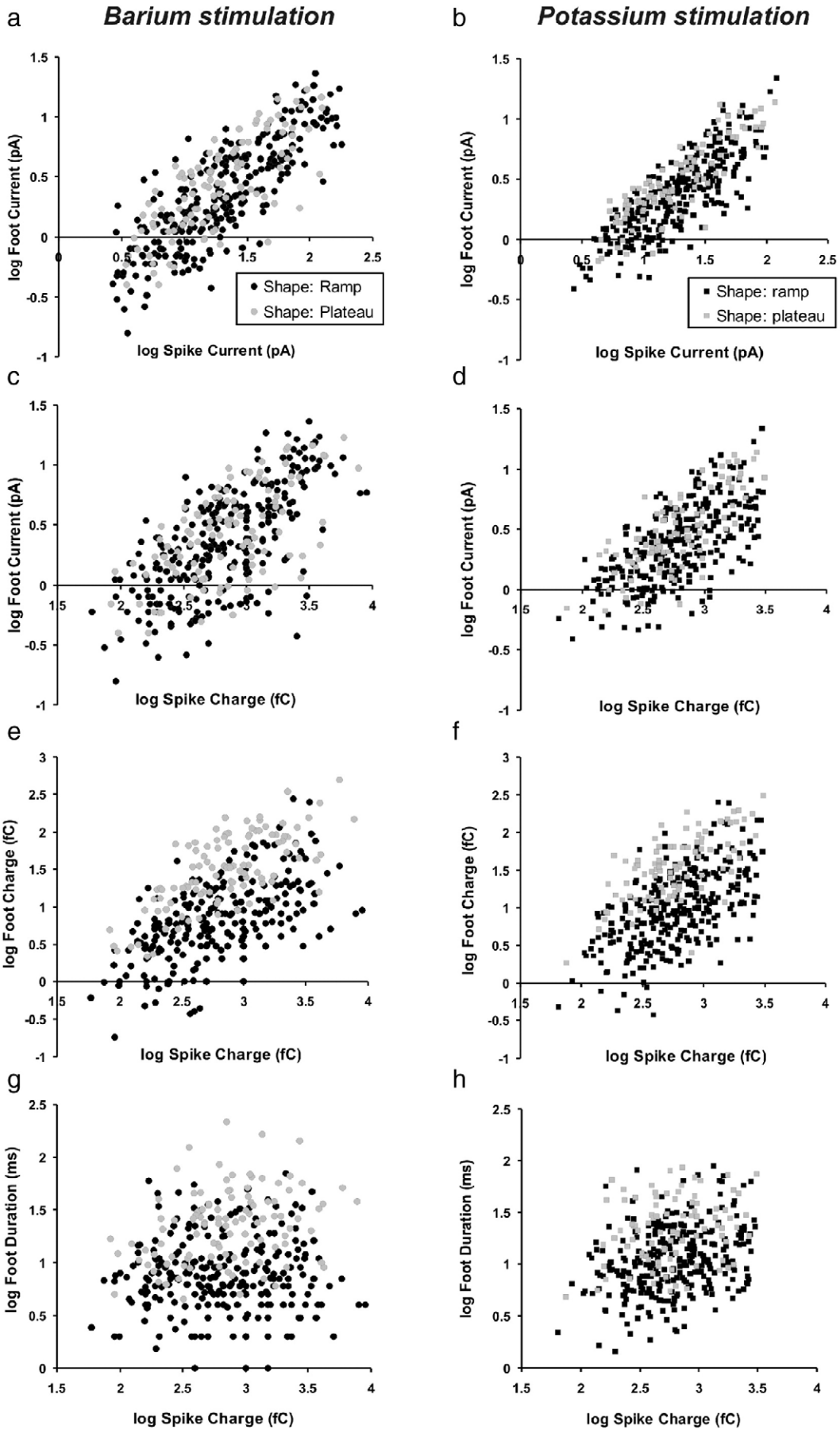
3.1. Evidence of different modes of release by exocytosis

Pre-spike features (PSF) in the amperometric detection of exocytotic events have been described since the early 1990s [18,35]. They have been ascribed to the observation of release through the fusion pore by comparison with the electrophysiological data obtained on the same cells, and more particularly, owing to studies made by combined patch-amperometry [10,36]. These latter studies showed that the amperometric detection of a small current preceding the amperometric spike is temporally correlated with the opening and conductance of a nanometric-sized initial fusion pore. Conversely, the subsequent expansion of the fusion pore leads to the massive release of catecholamines (in chromaffin cells) detected as a current spike. This was shown to be consistent with the refrained swelling of the granular matrix within the vesicle, inducing an internal pressure and the ensuing development of a Laplace tension on the vesicle membrane [24]. In our view, such mechanical constraints favor the pore opening and initiation of the fusion of membranes [37,38].

The detection of amperometric PSFs has now been reported for many secretory cell types in culture, including the chromaffin and PC 12 cells (derived from a sarcoma of the adrenal glands), mast cells, beta-cells and neurons [9,20,39–41]. All these observations have demonstrated that the specific experimental data representing the amperometric foot provide significant information that are certainly biologically relevant, despite the fact that the exact physiological or physicochemical parameters which govern the intensity and duration of PSFs remain unclear.

However, several quantitative studies aimed at rationalizing the characteristics or even the frequency of PSFs, particularly on the model of PC12 or chromaffin cells have been reported [20,22]. Until recently, most of the published studies had to deal with the problem at a descriptive level since the accurate characterization of an amperometric foot remained an analytical challenge. The required criteria for the accurate and precise detection of such infrequent and small events are indeed stringent: first, they are not very frequent, approximately one-third of all analyzable events; second, their amplitudes are limited to a few picoamperes as shown in the experiments reported herein; last, it is easy to mistake PSFs with the seldomly-observed accidental superimposition of two different spikes of different amplitude being monitored within the same time-window by the microelectrode, but occurring at different locations at the cell membrane. However, studies on PC12 and chromaffin cells reported over the last decade have shown that a significant frequency (ca. 30%) of events with a PSF [20,22] is always detected (at least in control experiments), provided that the amperometric sensitivity is sufficient.

As reported here and before [21,22], an amperometric foot may essentially be characterized by three main parameters: its maximum current (I_{foot}), its time-length (t_{foot}) and the charge (Q_{foot}) released during this phase before the spike onset. The present set of data, based on statistically-meaningful populations of spikes, and obtained with two types of exocytosis activators, afforded mean values for the foot parameters, detected on the model of bovine chromaffin cells. Thus, a mean amperometric foot is a specific event of 3.5 pA maximum current, 17 ms duration and 27 fC coulometric charge, corresponding to the release of ca. 100,000 molecules of adrenaline, each being detected by the transfer of two electrons per molecule [42]. Nevertheless, the present studies additionally reveal that the duration and charge parameter of the foot must be considered with care, since two main types of PSF were reproducibly observed in all our experiments. The majority of foot events were observed as a linear current rise ("ramp", 2/3 of cases) whilst a second, less frequent category was composed of feet displaying a stable current plateau that occurred after the ramp stage ended and before the spike onset ("ramp + plateau"). This second category was shown definitively to represent a different release regime and not any artifact, e.g. resulting from the superimposition of spikes (at least two) of different amplitude, such like a small one (possibly distorted by diffusion) and a larger one giving rise to the main spike. For example, the present comparison of the rates of release during the ramp stages (see Fig. 2) of each category demonstrated unambiguously that events consisting of a ramp followed by a plateau were true PSFs


a
Barium stimulation
b
Potassium stimulation
log Foot Current (pA)
log Spike Current (pA)
Shape: Ramp
Shape: Plateau
Shape: ramp
Shape: plateau
c
d
log Spike Charge (fC)
e
f
log Foot Charge (fC)
g
h
log Foot Duration (ms)

associated with the following current spike. This second category may derive from the first one ("ramp" only), i.e. when the dynamic structure giving rise to the ramp "freezes" after reaching a maximum release rate instead of yielding to the onset of full fusion. In this view, both categories would be differentiable through the stability of the pore structure (viz. with or without a lag time before full fusion occurs) after the release rate reached same maximum values.

3.2. Evidence for controlling factors of the fusion pore in exocytosis

Given the reproducible observation of two different main types of PSF by amperometric measurements at chromaffin cells, it is insightful to enquire as to the identity of which factors govern the fusion pore stability. Several types of proteins (syntaxin, synaptobrevin, SNAP25, sytI…), located either on the vesicle surface or on the internal membrane surface, have been designated as participants in the initial formation of the fusion pore but also into the control of its expansion [6,7,32,43,44]. Some of these proteins and enzymes are suspected of controlling the switching between different modes of exocytosis, i.e. full fusion, "kiss and run" or multi-granular fusion [45–48]. We, and others, have revealed that other factors of physicochemical nature may also participate to the control of the fusion pore dynamic [24,29,33,49,50]. It has been demonstrated that amperometric feet are observed preferentially (probability >50%) for spikes that correspond to large charges, suggesting the possibility that a specific population of large vesicles is prone to deliver their content with different kinetics than those involving a population of smaller ones [22]. Taking advantage of the increased accuracy in the measurement of such PSF events, we decided in the present study to pursue a quantitative investigation into the existence of possible correlations between the characteristics of feet with the global characteristics of their corresponding spikes within each class of PSF, viz. consisting of a ramp only or a ramp followed by a plateau.

We observed that the maximum foot current and spike current are well correlated. The ratio between the two currents is impressively constant irrespective of the stimulus employed. This may appear quite surprising, since it may be envisioned that the release and diffusion of a small quantity of catecholamines through the fusion pore (PSF) and those that occur after the fusion of the membranes, the exposure of the vesicular matrix and its swelling (spike phase), obey different laws. These fluxes are exclusively linked to the surface area of matrix exposed through the membrane and to the diffusivities D of catecholamines in the swelling matrix [29,51]. The diffusivity of catecholamines during the foot phase is most certainly similar to the one prevailing at the moment of the maximum delivery (the spike) since both characterize diffusion in the swollen area of the matrix. Hence the ratio between the two currents I_{max} and I_{foot} should reflect a ratio between the aperture areas A_{max} and A_{foot}:

$$I_{max} / I_{foot} = (D_{max} / D_{foot}) \cdot (A_{max} / A_{foot}) \approx \gamma (A_{max} / A_{foot}) \quad (1)$$

where γ is a constant featuring the two different regimes of diffusion (spherical convergent during the foot phase; spherical divergent during the spike phase). In addition, since at A_{max} the area of the matrix exposed to the extracellular fluid is proportional to the square of the vesicle radius r_0, it follows from Eq. (1) that a correlation between I_{foot} and $Q^{2/3}$ should be observed. Results from Fig. 3c–d support this hypothesis since the gradients of the regression line of between both parameters fall between 0.6 and 0.7. Hence these correlations and their slopes suggest that $I_{foot} \propto r_0^2$, i.e. that the size reached by the fusion pore prior to its full expansion, and which characterizes the foot current, is related to the size of the vesicle undergoing exocytosis.

Accordingly, it follows that the charge released during the foot phase may be proportional to the total amount released by the vesicle during its exocytosis. However, Fig. 3e–f demonstrates that the two types of charge are only weakly correlated in both conditions of exocytosis stimulation (barium or potassium). Furthermore, there is no correlation between the duration of the foot and the spike charge (Fig. 3g–h). In other words, the stability or duration of the fusion pore is not related to the integrated charge or size of the vesicle. In spite of our previous work indicating that the probability of observing the fusion pore release is high for large vesicles (in content or volume, which are both equivalent for a stationary intravesicular concentration of catecholamines), the stability of the pore (measured by t_{foot}) is likely to be also controlled by other factors. As mentioned above, local molecular factors including proteins (SNAREs), enzymes (phospholipases) or specific lipids, prone to control the cell membrane features (viscosity, mechanical tension, curvature, et hoc genus omne) at the site of fusion, might surely offer a plethora of ways for the control of the dynamics of release [34].

4. Conclusion

The present amperometric studies of exocytosis at chromaffin cells enabled the characterization of novel features of the pre-spike feature termed the foot, observed for about 30% of the exocytotic events. Mean quantitative and kinetic characteristics of the amperometric feet were determined for two common elicitors of exocytosis, viz. barium and potassium (under a calcium gradient). Interestingly, we observed that the kinetic profiles of the foot current were remarkably reproducible and fall into two categories, depending on their shape: "ramp" or "ramp + plateau" amperometric feet. Quantitative analyses have demonstrated that the parameters characterizing the foot current are not necessarily correlated with parameters of the following spike. The present analyses demonstrated also that the foot duration, i.e. the fusion pore life-time is not related to the charge of the vesicle undergoing exocytosis. Conversely, the flux released during the foot, i.e. at the maximum pore size, is strongly correlated to the flux of catecholamines that may be released during the full exocytosis. These results suggest that if the maximal size achieved by the fusion pore before the fusion stage is related to the vesicle size, its dynamics are controlled by local structural factors featuring the involvement of specific biological components or of factors of physicochemical nature. Indeed, local perturbations in the medium osmolarity, of membrane content in lipids, or of ionic composition, directly modify the characteristics of the pre-spike events, as observed in previous works. [38,52] Owing to the precise quantitative analyses allowed by the present high analytical temporal resolution, it appears that investigations focusing on the pre-spike event variations should provide novel and new information on the fusion pore, which is the first event leading to release in the whole exocytosis process.

5. Experimental section

5.1. Cell culture and preparation

Bovine chromaffin cells were prepared by the collagenase digestion of the medulla of adrenal glands obtained from a local slaughterhouse (Meaux, France). Cells were purified and cultured using methods previously described [53]. They were then plated (4×10^4 cells/cm^2) on

Fig. 3. Correlations between the specific parameters of feet and the whole parameters of their respective spikes. The two categories of spikes with foot defined in the present study were analyzed separately (black symbols for "ramp" feet; gray symbols for the "ramp + plateau" feet) in both cases of Ba^{2+} and K^+-evoked release from single bovine chromaffin cells. a, b. Correlations between the foot maximum current and the spike maximum current parameters (a. $R^2 = 0.68$ for ramp and 0.58 for plateau, b. $R^2 = 0.65$ for ramp and 0.74 for plateau). c, d. Correlations between the foot maximum current and the spike charge parameters (c. $R^2 = 0.36$ for ramp and 0.45 for plateau, d. $R^2 = 0.43$ for ramp and 0.57 for plateau). e, f. Correlations between the foot charge and the spike charge parameters (e. $R^2 = 0.31$ for ramp and 0.49 for plateau, f. $R^2 = 0.35$ for ramp and 0.46 for plateau). g, h. Correlations between the foot duration and the spike charge parameters (g. $R^2 = 0.00$ for ramp and 0.09 for plateau, h. $R^2 = 0.09$ for ramp and 0.06 for plateau).

collagen–poly-L-lysine coated glass coverslips (24 wells plates) and incubated in a CO_2-atmosphere (5%) at 37 °C. Cells were used on days 3–10 after culture and 24 h maximum after plating.

5.2. Electrode preparation and single cell experiments

Carbon fiber microelectrodes (7-μm diameter, Thornel Carbon Fibers, Cytec Engineered Materials, Greenville, SC, USA) were constructed as described previously [40]. Electrode tips were polished (45° angle) on a diamond dust-embedded micropipette beveling wheel (Model EG-4, Narishige Co., Tokyo, Japan) for 5–10 min before experiments. Only electrodes with a very stable amperometric baseline current were used for cell measurements.

Cells were prepared by placing each coverslip into a plastic dish (35 mm) filled with isotonic physiological saline (154 mM NaCl, 4.2 mM KCl, 0.7 mM $MgCl_2$, 11.2 mM glucose, 10 mM HEPES, pH 7.4, 5 mL). 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., Tokyo, Japan) at sub-micrometric distance from the membrane of an isolated chromaffin cell. The close proximity of the electrode surface to the cell surface was often seen by a slight deformation in the outline of the cell. Then, a glass microcapillary (20–30 μm diameter) was positioned with a second micromanipulator near the cell (20–30 μm) and used (Femtojet injector, Eppendorf Inc., Hamburg, Germany) to inject towards the cell surface a stimulating solution composed of either: $BaCl_2$ 2 mM in Locke buffer supplemented with 0.7 mM $MgCl_2$, without carbonates; or KCl 55 mM in Locke buffer supplemented with 2.5 mM $CaCl_2$. The injection duration was 10 s and 60 s for Ba^{2+} and K^+, respectively. The microelectrode was kept in place during the stimulation and all along the secretion process (mean time-length: about 5 min). Each cell was only stimulated once. All experiments were performed at room temperature.

5.3. Data acquisition and data analysis

Electrodes were held at +0.65 V vs. a silver/silver chloride reference electrode using a modified picoamperometer (model AMU-130, Radiometer Analytical Instruments, Copenhagen, DK), for which the adjustable time–response was set at 50 μs. The output was digitized at 40 kHz, displayed in real time and stored on a computer (Powerlab-4SP A/D converter and software Chart 5.0, ADinstruments, Colorado Springs, CO, USA) with no subsequent digital filtering.

Each amperometric trace obtained during cell secretion was visually inspected and signals were designated as exocytotic spikes whenever their maximum current values were 3 times higher than the RMS noise (0.2 to 0.5 pA) of the baseline current (30 ms minimum time-length) recorded prior to each individual spike. Special attention was applied to check the baseline stability before and after each spike in order to avoid spike superimposition. Generally, 50 to 200 spikes could be isolated from each trace following these criteria. Spikes were designated as having a pre-spike feature 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. Furthermore, the minimum accuracy of the PSF determination was set at a current equal to 3× the above noise level [21]. As a consequence, two types of kinetic were mainly detected for amperometric feet: ramp or ramp + plateau. In combination with their individual visual inspection and comparison, classification within each of the two categories was additionally achieved by calculating the variations of slope in current rise. A decrease of the slope was considered as an indicator of current stabilization, i.e. a plateau, before the onset of the main amperometric spike. In addition, amperometric feet whose current slightly decreased (a negative slope of current) were excluded from the analyses and ascribed to a superimposition of two independent spikes. Moreover, contrary to the procedure that seems to be applied in other works, each spike that displays a foot whose maximum current amplitude is larger than one third of the maximum amplitude of the spike ($I_{foot}/I_{max} > 1/3$) was also considered as a superimposition of two independent exocytotic events and therefore excluded from the analysis. Indeed, assuming that the pore radius is reflected by the foot current, [28,51] a spike whose foot current would be comparable to the spike current itself would not make physical sense.

The amperometric foot characteristics (Q_{foot}, I_{foot}, t_{foot}) were determined using home-made software. All values are reported as the mean ± SE ($\sigma / \sqrt{n}$, where s is the standard deviation and n the number of values considered for the mean).

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