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Relationship between amperometric pre-spike feet and secretion granule composition in Chromaffin cells: An overview

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Abstract

Amperometry is a simple and powerful technique to study exocytosis at the single cell level. By positioning and polarizing (at an appropriate potential at which the molecules released by the cell can be oxidized) a carbon fiber microelectrode at the top of the cell, each exocytotic event is detected as an amperometric spike. More particularly, a portion of these spikes has previously been shown to present a foot, i.e. a small pedestal of current that precedes the spike itself. Among the important number of works dealing with the monitoring of exocytosis by amperometry under different conditions, only a few studies focus on amperometric spikes with a foot. In this work, by coupling our previous and recent experiments on chromaffin cells (that release catecholamines after stimulation) with literature data, we bring more light on what an amperometric foot and particularly its features, represents.

Keywords: Catecholamines; Electrochemical analysis; Exocytosis; Pre-spike feature; Ultramicroelectrodes

1. Introduction

Vesicular exocytosis is a key mechanism in many processes of communication between living cells in complex organisms [1]. Usually studied as a relevant model of such a mechanism, chromaffin cells involve exocytosis to release catecholamine molecules towards the extracellular medium (Fig. 1A) [2–4]. By the mean of amperometry, oxidation of adrenaline and noradrenaline (the main catecholamines neurotransmitters stored into secretion granules) is usually detected at the surface of a carbon fiber microelectrode positioned near the cell membrane. It ensues that each exocytotic event, i.e., the release of the vesicular content, can be observed as an amperometric spike whose main features give information about the nature (kinetics, charge) of the whole process (Fig. 1B) [5,6].

In previous studies [7–10], we and others showed that under usual conditions of stimulation (for instance, exocytosis triggered by injection of a millimolar barium or potassium solution for chromaffin cells), an average 30% of the detected spikes present a "foot" which is a low (small plateau-like or ramping) current feature that precedes the spike itself. The existence of such a "foot" (called pre-spike feature or PSF) is usually ascribed to the oxidation of a small amount of material released through a nanometric fusion pore between the cell membrane and the vesicle interacting to fuse together [7,8,11–17]. Indeed, after the secretory vesicle has docked to the cell membrane supposedly by the mean of SNARE complexes [14], the fusion of vesicle and cell membranes leads to the formation of a nanometric fusion pore, through which the first ionic exchanges begin, thus inducing a swelling of the intravesicular matrix in which catecholamines are stored. Ionic exchanges with the extracellular medium continue so as to provoke the explosive expansion of the pore powered by swelling of the vesicular matrix and to the release of catecholamine molecules stored within the matrix [18]. Furthermore, the association of patch-clamp measurements and amperometry demonstrated the relationship between the fusion pore formation and the

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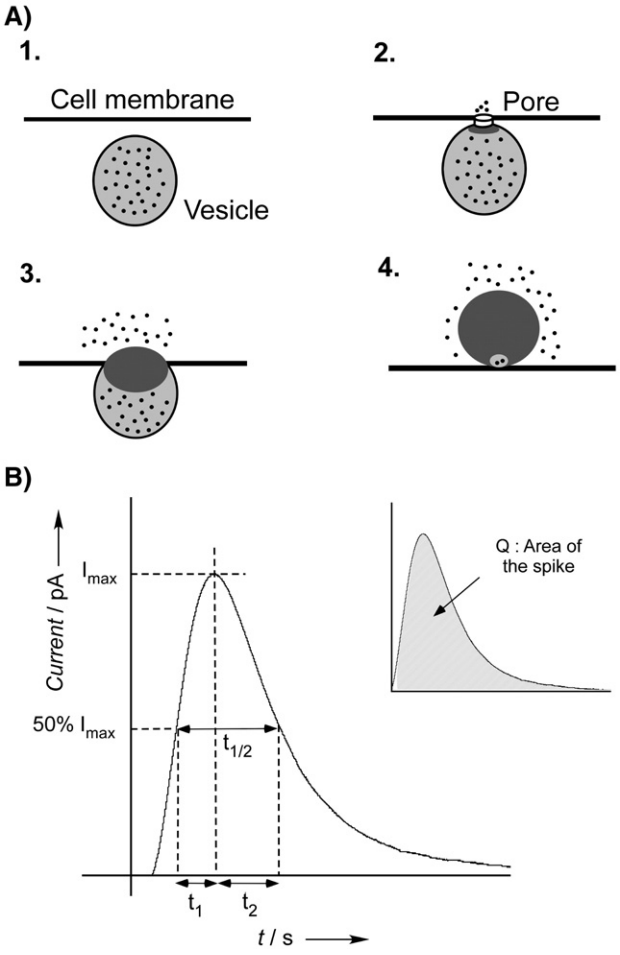

Fig. 1. A) Schematic and simplified representation of the main phases of exocytosis during fusion of dense-core vesicles (catecholamines are stored into a matrix that is a polyelectrolytic gel) with cell membrane. 1. Docking: after appropriate stimulation, vesicles which are primed to undergo exocytosis dock to the cell membrane by the mean of SNARE complexes (not shown). 2. It ensues the formation of a fusion pore, through which catecholamines begin to diffuse out the matrix. 3. The ionic exchanges (catecholamines cations versus Na^+ or H^+) between the intravesicular and extracellular media provokes the matrix swelling and thus the rupture and exponential expansion of the initial fusion pore. 4. If it is not stopped or reverted by biological controls, this process may pursue until the vesicular matrix is fully exposed to the extracellular medium. B) Representative amperometric spike without pre-spike feature (70% of the amperometric spikes), indicating the spike characteristics generally analyzed: the maximum oxidation current I_{max} (pA) (i.e. the maximum flux of molecules released), the total electrical charge Q (fC) (i.e. the total amount of molecules released), the t_1 and t_2 times (the time interval between $I_{max}/2$ on the current rise and I_{max}; the time interval between I_{max} and $I_{max}/2$ on the current fall; in ms), the half-spike time width ($t_{1/2}=t_1+t_2$).

conductance through this pore as well as the variation of capacitance once the pore formed [13,19–21]. All in all, the amperometric foot appears as electrochemical evidence of the existence and of the short life of the fusion pore. This view implies that only 30% of the exocytotic events have a sufficiently long-lived fusion pore to be electrochemically detectable. In that way, the features of the fusion pore (life time, amount of catecholamines released during this phase, frequency) would thus be expected to directly depend on physical parameters (viscosity of the membrane, tension surface energy…).

In previous studies, we investigated amperometrically how changes in the cell membrane properties (membrane tension and viscosity by modifying external osmolarity, membrane curvature by inserting exogenous compounds) affect the mean characteristics of the exocytotic events without mainly focusing the work on the amperometric feet features [22,23]. Nevertheless, we noticed that the relative number of spikes with a foot remained constant. Since disrupting the fusion pore stability should have a direct effect on its life time and in fact, on the number of detected spikes with a PSF, we wish to present and discuss in this report additional measurements dealing with the other features of the amperometric foot (intensity, duration, charge). Furthermore, as described below, other complementary experiments have been performed to validate this issue, i.e., to understand how the effects on the stability of the fusion pore affects its intrinsic amperometric properties (frequency, life span…).

First of all, results dealing with the monitoring of exocytosis at chromaffin cells under different conditions (incorporation of geometrical lipids in the cell membrane, modification of osmolarity of the extracellular medium) which are expected to induce physical constraints on the cell membrane (and thus affect the stability of the fusion pore) and modify the frequency of spikes with an amperometric foot, will be presented. In a second way, we will compare these results with those obtained when chromaffin cells were treated with L-DOPA or reserpine, whose expected effects are the increase or the decrease of the amount of catecholamine molecules present in chromaffin granules respectively. Finally, an additional series of experiments that deals with the influence of the temperature will be also commented and discussed.

Our results seem to indicate that the detection of an amperometric foot is not only related on the stability of the fusion pore and on the external physico-chemical parameters. Indeed, the percentage of spikes with a foot is clearly not modified when different constraints (curvature, tension, viscosity…) were applied on chromaffin cells membranes. Conversely, modifying the granule composition (effect of L-DOPA or reserpine) induces changes in the frequency of an amperometric foot detection. PSF existence could thus be linked to the structure and the nature of the secretory vesicles, i.e. to the presence of an intravesicular medium in which the diffusion coefficient of catecholamines would be larger than into the packed dense-core matrix.

2. Results and discussion

2.1. Features of an amperometric pre-spike foot

The nature of the fusion pore during the exocytotic process remains under debate despite its major role in the regulatory mechanisms of exocytosis [24–26]. Amperometrically, the main difficulty stems from the ability to monitor its presence and life span with a high precision (500–1000 molecules per milliseconds and a RMS noise of 0.2 to 0.5 pA). Indeed, as

we introduced above, in chromaffin cells, ca. 30% of the amperometric spikes are preceded by a small "pedestal", called the foot of the spike. The small current during the phase of this pre-spike feature (PSF) represents a small release of the catecholamines stored into the vesicle through the pore [17].

Typically, two different types of amperometric spikes may be isolated from recordings of exocytosis: [27] (1) amperometric spikes without PSF (see Fig. 1B), ca. 70% of cases. (2) spikes that are preceded by a PSF, i.e. a ramping (Fig. 2A) or ramp-plus-plateau-like (Fig. 2B).

Whatever the morphology of the PSF is (ramp-plus-plateau or ramp shape), four usual parameters can be analyzed (Fig. 2): the life-time (t_{foot}), the charge (Q_{foot}), which is proportional to the number of molecules released through the pore, and the maximum current (I_{foot}) that reflects the flux of catecholamines released. The fourth parameter that is classically retained is the percentage of the spikes with a foot (or PSF frequency) among the ensemble of all the spikes analyzed. As already said above, this amounts approximately to 30% under control conditions (i.e. when exocytosis is triggered by a barium or potassium solution stimulation) [27].

In the following sections, we will consider and discuss the influence of different modifications (cell membrane curvature, external osmolarity, temperature…) on the four above parameters which characterize amperometric foot features. In the present case, amperometric experiments were performed on chromaffin cells and their secretion was elicited by the mean of a 2 mM barium solution injection.

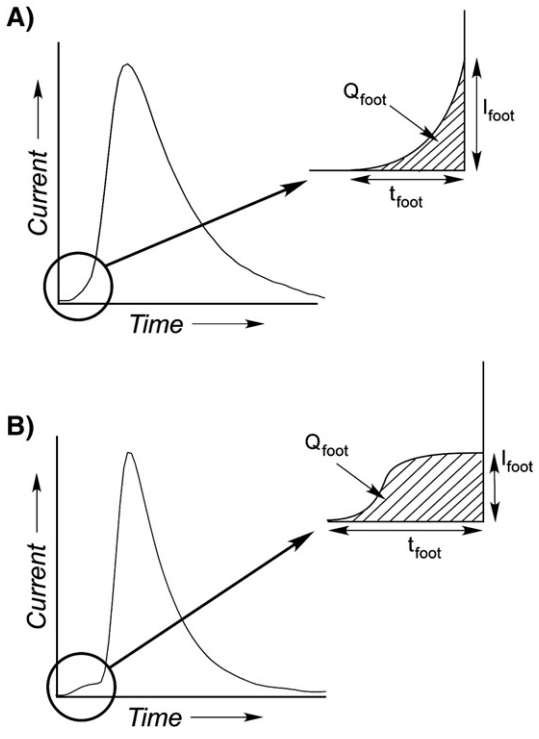

Fig. 2. Representative amperometric events with (30%) a pre-spike feature (PSF). The two shapes of the PSF are depicted: A) when only a ramp is achieved before the spike onsets. B) When the foot current is stabilized as a plateau after its initial rising phase. In both cases, a zoom of a PSF and a representation of the quantitative (Q_{foot}) and kinetic parameters (I_{foot}, t_{foot}) of the foot is provided for clarity.

Table 1
Comparison of the characteristics of the amperometric spike feet in control conditions vs. in the presence of LPC or AA

Conditions	PSF percentage (%)	I_{foot}(pA)	Q_{foot}(fC)	t_{foot}(ms)
Control	32±3	2.5±0.2	9.5±1.3	9.5±0.6
LPC	30±3	5.5±0.8	8.4±2.0	5.8±0.3
AA	31±3	1.2±0.1	9.2±1.4	13.8±1.1

2.2. Influence of the cell membrane curvature on the PSF

Each PSF may be characterized by three main parameters (see Fig. 2: t_{foot}, I_{foot}, Q_{foot}) which describe its main properties, so their variations are expected to reflect those of the pore and of the intravesicular medium. A first study deals with the modification of the ease of mixing and restructuration of the cell and vesicle membranes during the first step of the exocytotic process. It is thus expected to exert a direct influence on the velocity and the efficiency of the fusion, but without affecting the vesicular content. To do so, experiments were conducted on single chromaffin cells briefly bathed (3 min) into lyso-phosphatidylcholine (LPC) or arachidonic acid (AA) solutions (2 and 20 μM, respectively). The composition of the outer cell membrane is therefore affected due to the insertion of one of these exogenous lipids (LPC or AA) [22]. Amperometric analysis has previously established that under these conditions, LPC favors catecholamines release (increases of rate, events frequency, charge released) while arachidonic acid disfavors the exocytotic process. The observed kinetic features are clearly consistent, upon considering the opposite physical constraints applied to the cell membrane by LPC or AA, meaning that the insertion of these exogenous compounds plays a significant role on the membrane curvature without affecting the cell machinery [22]. Experimentally, we observed that the percentage of spikes with a foot remains constant (30%; see Table 1) in both cases compare to control conditions. In contrast, the frequency of the exocytotic events (i.e., the number of spikes per amperogram) is clearly increased in the LPC case and decreased in the AA case [22].

The fusion between a vesicle and the cell membrane is then facilitated and faster in the LPC case (the opposite being observed in presence of AA). Upon considering that LPC favors the cell membrane curvature required for fusion (AA insertion prevents the fusion event since the AA curvature acts in the opposite sense and disfavors the occurrence of an exocytotic event), the same curvature effects may act also onto the ability of the pore to expand since this decreases the equivalent pore edge energy [24]. For a given event with PSF, the curvature synergistic (LPC) or antisynergistic (AA) effects are expected to affect the foot duration, as well as I_{foot}, whenever the later is related to the fusion pore size [6,28,29]. In the LPC case, PSF kinetics have experimentally been observed to be faster (t_{foot} is reduced and I_{foot} is increased, see Table 1) than under control conditions whereas the PSF kinetics in the AA case resulted slower (t_{foot} is increased and I_{foot} is reduced, see Table 1). It

must be noted that PSF charge (Q_{foot}) remains unchanged in both conditions, in agreement with the non-influence of LPC or AA on the cell internal machinery or onto the vesicle composition. These drastic variations are clearly in contrast with the constant PSF percentage.

Of importance for our present purpose is the observation that the amount of catecholamine released during the spike foot does not vary by modifying the cell membrane curvature (Table 1). Therefore, everything proceeds as if for a given case, a given amount of catecholamine may be released through a PSF feature. This is in agreement with the hypothesis that the molecules which give rise to the PSF amperometric current correspond to an easily releasable amount of catecholamines from the fused vesicle. This is consistent with the presence of a "halo" into the vesicle structure as evidenced by previous observations [8,30]. The presence of a liquid halo into some vesicles or at least a medium in which catecholamines diffusion coefficient is larger than into the dense matrix [7,8,27,31] may explain the observation of a PSF without a correlation with the stability of the fusion pore. In this view, the PSF detection percentage would not depend on the properties of the cell membrane which favor or disfavor the life time of the fusion pore, but only on the percentage of vesicles with a halo [8]. However, the characteristics of each observable PSF should depend on the membrane properties. This is consistent with the fact that LPC, which favors the exocytotic event by establishing the appropriate membrane curvature, allows the catecholamines stored in the halo to diffuse faster through the fusion pore (t_{foot} decreases and I_{foot} increases), while the opposite trend is observed in the AA case. The foot is therefore representative of a catecholamine flux, whose observation would be due to the secretion granule composition and not on the stability of the pore.

In order to sustain additionally the above interpretation, amperometric analysis of exocytotic events was performed on chromaffin cells incubated in a hypotonic (200 mOsm) or hypertonic (750 mOsm) medium in comparison with the control condition (315 mOsm). These modifications affect the dynamics of the events by changing the cell membrane viscosity and surface tension [6,32]. Once again, the number of observed exocytotic events increases under hypotonic conditions and decreases under hypertonic conditions. However, in contrast with these drastic variations, the percentage of spikes with a foot remains identical in both cases (see Table 2) [23,32]. The flux of catecholamines released through the pore (as evidenced by I_{foot}) is increased in the former case and decreased in the latter case but the amount of catecholamines released is quite constant (see Table 2). Accordingly, the time course of PSF decreases (or increases) slightly under the hypotonic condition (or the hypertonic condition). Again, this is consistent with the fact that a given PSF represents the presence of a halo. The release rate of the material contained within the halo correlates with the size of the fusion pore, the latter being function of the local membrane properties. Therefore, increasing indirectly the cell membrane tension by decreasing the ionic external concentration provides two concurrent effects (fusogenic effect through increased membrane tension and a more favorable ionic gradient) which can affect the time duration of an amperometrically observable pore. Hypertonic modifications obviously give the opposite result.

Table 2
Characteristics of the amperometric spike feet in different experimental conditions as a function of the external osmolarity: control (315 mOsm); hypotonic (200 mOsm) or hypertonic (750 mOsm) conditions

Conditions	PSF percentage (%)	I_{foot}(pA)	Q_{foot}(fC)	t_{foot}(ms)
Control	32±3	2.5±0.2	9.5±1.3	9.5±0.6
Hypertonic	29±3	1.5±0.2	9.0±4.1	11.8±1.6
Hypotonic	30±3	3.2±0.3	11.0±1.1	8.5±0.4

All these considerations show that the physical properties of the cell membrane play a dynamic role on PSF features, but have no role on the probability of its statistical PSF observation. The catecholamines stored into the vesicular halo are more or less easily released into the extracellular medium, according to the physical properties of the membrane. In this view, the presence of a PSF is then due to the internal structure of the vesicle that fuses, so that the PSF frequency and the stability of the fusion pore (i.e. the kinetic parameters of the PSF) are most likely not linked. This is consistent with other experiments from Bruns and co-workers who reported studies dealing with the exocytosis of v-SNARE-deficient chromaffin cells. These modifications of SNAREs assemblies affect the PSF features (time duration, charge and current) and thus the fusion pore stability without changing significantly the frequency of amperometric spikes with a foot [33].

2.3. Influence of the vesicular "content" on the PSF

Chromaffin cells store catecholamines in secretory vesicles that possess a high capacity for storage of cationic neuromediators so that catecholamines reach a very high concentration (approximately 0.5 M) [32,34]. The generally accepted view of the catecholamines biosynthetic pathway is that dopamine is formed from DOPA into the cytoplasm. Dopamine is then taken up by chromaffin granules where it is converted into noradrenaline (by dopamine β-hydroxylase) that then leaves the granules in order to become converted into adrenaline in the cytoplasm which is subsequently taken up by granules [35]. The vesicular monoamine transporter (VMAT) can regulate the uptake of catecholamines by single vesicles. Manipulations affecting the loading of vesicles with neurotransmitters thus modulate the number of catecholamines which may be released per exocytotic event.

Based on amperometry and transmission electron microscopy, it was shown that L-DOPA and reserpine treatment on PC12 cells induces alterations on the amount of catecholamines released and on the vesicular volume [9,36]. On the one hand, the catecholamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is expected to bypass the rate-limiting step in catecholamine synthesis (by enhancing formation of cytosolic dopamine) and thus to increase the vesicular content and its size. On the other hand, reserpine has been observed to block the uptake of dopamine into chromaffin granules, thus inducing the inhibition of noradrenaline synthesis and leading

therefore to a decrease of the volume of the vesicles and their catecholamine content [37]. The pharmacological effects of reserpine and L-DOPA on exocytosis have already been investigated [36,38,39]. The more relevant results were obtained on PC12 cells. Because PC12 cells are derived from the rat adrenal gland and the structure as well as the composition of dense core vesicles in these cells resemble those found in chromaffin cells, it is reasonable to assume that catecholamines are stored in much the same way [40,41].

All in all, it was suggested that the changes induced by L-DOPA or reserpine are associated with the swelling and shrinking of vesicles that could occur by stretching and undulating the membrane of the vesicle or through incorporation (or loss) of membrane. Moreover, patch-amperometry studies established that the membrane area of vesicles in chromaffin cells changes markedly in parallel with changes in quantal size following L-DOPA or reserpine treatment [39]. Such alterations of the internal vesicle composition due to a pharmalogical reagent which concomitantly modifies the ability of swelling of its matrix [42,43] should then affect the expansion rate of the pore in the fusion process during exocytosis, whenever this is powered also by the matrix swelling [18,28,44].

In order to determine the effect of vesicles loading alterations on the amperometric foot features, incubation of chromaffin cells were achieved with L-DOPA (1 μM in a DMSO/physioplogical saline solution during 60 min at 37 °C) or reserpine (100 μM in a physiological saline solution during 60 min at 37 °C).

The results gathered in Table 3 show that the reserpine incubation causes a reduction in the PSF percentage, which is in agreement with the expected alteration of the vesicle content (see above). It also induces the decrease of both the amount and the flux of catecholamines released through the fusion pore, which is consistent with a reduced halo. However, the pore duration appears not significantly modified. Conversely, and still according to expectations, incubation with L-DOPA increases the PSF percentage, the amount and the flux of catecholamines released through the fusion pore. It has to be noted that the life time of the pore also increases.

The above series of experiments and their results concerning the features of the amperometric feet are thus clearly consistent with the fact that each PSF occurrence reflects the presence of a vesicular halo (space between the dense core and the vesicular membrane) of liquid that contains a fraction of fast diffusing catecholamines. Indeed, L-DOPA (resp. reserpine) is well known to increase (resp. decrease) the total amount of released species from dense-core vesicles and the vesicular volume [36].

Table 3
Effect of L-DOPA or reserpine incubation on the characteristics of the amperometric spike feet detected during exocytosis of chromaffin cells

Conditions	PSF percentage (%)	I_{foot} (pA)	Q_{foot} (fC)	t_{foot} (ms)	100 (Q_{foot}/Q_{peak})
Control	32±3	2.5±0.2	9.5±1.3	9.5±0.6	2.13±0.18
Reserpine	18±3	1.8±0.2	5.5±1.6	8.8±0.8	1.65±0.18
L-DOPA	41±3	3.2±0.2	16.0±2.1	15.4±1.0	2.23±0.19

The last column deals with the comparison of the proportion of the foot charge vs. the whole charge event.

Moreover, in the L-DOPA case, most of the vesicular volume increase is due to the halo [36], whereas the increase of the dense-core volume appears limited. The catecholamines amount contained into the halo thus increases, a fact which is observed experimentally by an increased Q_{foot} value (Table 3). Accordingly, I_{foot} also increases, though less than Q_{foot} so that t_{foot} also increases [9]. In the reserpine case, since the vesicular halo volume decreases, less fast diffusing catecholamines may be released through the fusion pore, as evidenced by the reduced values of I_{foot} and Q_{foot}.

The PSF percentage varies in the appropriate directions which is additional support for the "halo" hypothesis. Indeed, if the observation of a given PSF depends on the secretion granule composition, the vesicular volume variations (and more particularly the changes concerning the volume of the halo) have to influence the relative number of PSF detected. Thus, after reserpine incubation, some vesicles that fuse would lose their halo or at least, have a halo in which free catecholamines amount is too weak to be detected. Conversely, after L-DOPA incubation, enrichment of catecholamines into the halo would favor the detection of PSF that would be "silent" under control conditions. Finally, it is well known that reserpine (or L-DOPA) induces the decrease (or the increase) of the vesicular volume [36]. This would mean that the PSF percentage increases with the vesicular size. This is fully consistent with one of our previous studies, which evidenced that amperometric spikes displaying a foot are events giving rise to a larger charge [8].

It is necessary to note that the same trends are observed in the works dealing with the reserpine or L-DOPA treatment on PC12 [9] and chromaffin cells (our present work), excepted the results concerning the PSF percentage. Indeed, the frequency of spikes with a foot has been shown above to be decreased (or increased) because of the reserpine (or L-DOPA) treatment when the studies are performed on chromaffin cells. The converse trend is observed on PC12 cells [9]. This discrepancy has already been noticed recently elsewhere [45]. We ascribe these differences to the experimental PSF detection since small PSF will be retained or not, depending on the rules of selection filter (see experimental procedure). These depend on the experimental accuracy available within a given laboratory. For instance, in contrast with the above conclusion [9], the PSF percentage has been previously reported to increase with the L-DOPA treatment in the PC12 case [46].

The modification of the vesicular internal composition thus appears to play a key role on the dynamics of the phenomenon and on the percentage of exocytotic events providing an amperometric foot. It must be emphasized that in the L-DOPA incubation case, the enrichment in catecholamines seems to be achieved equally between the dense core and the halo since Q_{foot}/Q_{peak} remains constant, compared to control conditions (Table 3), in agreement with the uniform increase of catecholamines into the vesicle [47]. In the reserpine case, the lack in catecholamines is slightly more elevated in the halo (Q_{foot}/Q_{peak} slightly decreases, compared to control conditions, Table 3), showing that the loss of vesicular catecholamines mainly occurs from the halo, which is in accordance with the decrease of the relative number of spikes displaying a foot.

These results contrast with those obtained when only the physical properties of the cell membrane have been modified (see above), since then the charge detected in the amperometric foot remains unchanged. Only the kinetic parameters of the foot were then affected, which is assigned to the stability and the dynamics of the fusion pore rupture. In other words, the stability of the fusion pore and its dynamics strongly depend on the membrane features but the amount of catecholamines released during the phase of the fusion pore would be controlled by the amount of neurotransmitters available in the halo. In so far as the PSF observation requires the detection of a minimum of neurotransmitters molecules, the volume of a minimal sized halo would condition the observation of a PSF during a given vesicular exocytotic event.

2.4. Influence of the temperature on the PSF

Influence of the temperature on exocytosis at chromaffin cells has already been investigated. For instance, upon using electrophysiological measurements, Neher and co-workers evidenced that the vesicle maturation significantly accelerated at 37 °C [48]. We identified only three studies performed by the mean of amperometry. However, two of these studies are not helpful to our purpose here since in one only a few spike parameters are available [49] while the other one reports mainly on the total integration of the current per trace [50]. A third more quantitative work has been performed by Wightman and colleagues [51], who studied comprehensively the exocytosis of chromaffin cells at room temperature and 37 °C by amperometry, but without providing the data that deal with the PSF.

We thus studied exocytosis of chromaffin cells at three different temperatures (15 °C, 22 °C and 37 °C). Overall our observations on the spikes agree with those reported by Wightman et al. [51] The results concerning the PSF properties are gathered in Table 4.

On the one hand, it is noted that no significant evolution of the PSF percentage with the temperature occurs, except may be for a slight increase at 37 °C. On the other hand, quantitative features of the amperometric foot are significantly affected. Thus, the time-duration of the foot is decreased when the temperature is raised. If the increase of the temperature from 22 °C to 37 °C does not induce any significant modification of the catecholamines flux or of its charge during the foot, these last two parameters decrease meaningfully when the temperature decreases from 22 to 15 °C.

Temperature is expected to play a significant role on the physico-chemical parameters of the cell membrane, particularly on its viscosity and on its tension. As observed above when other physical parameters of the membrane are changed, the PSF percentage does not depend significantly on the membrane properties but rather on the vesicular content, so these experiments are clearly consistent with our above observations. Moreover, since during the time-scale of our experiments, the vesicular composition is not expected to be affected by the temperature change (as previously reported [51], the reversibility of the effects of a higher temperature on exocytosis temperature evidences that the basic cell functions are not altered by the temperature), the invariance of the PSF percentage reinforces the hypothesis that PSF presence is linked to that of a halo.

Changing the temperature has been shown to not affect the biosynthesis of catecholamines but rather the rate of dissociation of the vesicular content [51]. Within the framework of our model [18, 28], this should favour the pore rupture at higher temperature. It is then not surprising that an increase in temperature forces the time duration of the pore to be decreased whereas the catecholamines flux and charge remains constant, since these reflect only the halo characteristics.

At low temperature (15 °C), all the kinetics of the fusion pore are decelerated (the catecholamines flux is decreased while the time duration of the foot is increased). Moreover, the number of catecholamines released through the fusion pore is also decreased. At the present stage, the reasons for such a result remain speculative. For instance, it can be due to the diffusion coefficient of catecholamines into the halo. The latter is expected to be lower at 15 °C than under control conditions (22 °C). As a consequence, the catecholamines flux during the pore phase is expected to decrease when the temperature decreases. If the corresponding increase in PSF duration is such as the full release from the halo exceeds the intrinsic pore stability, the whole spike current will be observed before full halo release has occurred. This would decrease Q_{foot}, as observed experimentally. In this connection, it must be recalled that studies achieved on mast cells suggest that the opening and the closure of the fusion pore are regulated by lipids that come phase separated at 13 °C [52]. Such effect is then consistent with the fact that at 15 °C the intrinsic fusion pore duration may limit the PSF release.

Once again, it appears that if the vesicular content is not directly affected under the experimental conditions, the PSF frequency remains constant, reinforcing our above hypotheses.

Table 4
Effect of temperature on the characteristics of the amperometric spike feet detected during exocytosis of chromaffin cells

Conditions	PSF percentage (%)	I_{foot}(pA)	Q_{foot}(fC)	t_{foot}(ms)
Control (22 °C)	32±3	2.5±0.2	9.5±1.3	9.5±0.6
15 °C	34±3	1.1±0.1	5.6±0.6	13.3±1.1
37 °C	37±3	2.5±0.2	8.1±1.3	7.1±0.4

3. Conclusion

The amperometric foot or pre-spike feature (PSF) detected during an exocytotic event reveals a flux of catecholamines occurring during the phase of the initial fusion pore opening. The present experimental observations suggest that this PSF flux does not only correspond to a pre-release of catecholamines contained in the vesicular matrix. Our experimental data are fully consistent with the presence of a halo in some vesicles. Only these halo-equipped vesicles give rise to an amperometric PSF. Experimental modifications of the properties of the cell membrane only play on the size and further dynamics of the pore but do not affect the PSF frequency, which is an intrinsic property of the fusing vesicles. It ensues that the percentage of spikes with a foot and the stability of the fusion pore are two

independent phenomena which are not directly correlated, in agreement with previous observations by us [22, 23] and others [53]. However, such a conclusion rests on the fact that except in special cases where the PSF duration may be too long (as noted here at 15 °C), the fusion pore is ruptured within a millisecond or less, i.e., as soon as the inner matrix core starts to swell [18,28,44]. The presence and duration of a PSF under such normal conditions is then only function of the presence of a halo and of its volume. However, under other specifically altered circumstances, PSF presence may also reflect other phenomena which originate from other effects pertaining to the vesicle matrix and/or the membrane. For instance, Xu and Tse showed that treatment of chromaffin cells by brefeldin A (that disassembles Golgi apparatus) induces the increase of the number of PSF events although it had a low effect on the Ca^{2+}-dependent exocytosis [54]. Conversely, Grabner and Fox recently evidenced that alterations in Ca^{2+} at release sites on chromaffin cells may also produce significant changes of the number of PSF [45].

4. Experimental section

4.1. 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 [55]. They were then plated (4×10^4 cells/cm^2) on 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.

4.2. 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 20–30 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) 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 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 to inject (Femtojet injector, Eppendorf Inc., Hamburg, Germany) for 10 s a stimulating solution ($BaCl_2$ 2 mM in Locke buffer supplemented with 0.7 mM $MgCl_2$, without carbonates) towards the cell surface. 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.

During the works reported in this article, four kinds of experiments have been performed. The experimental procedure is briefly described for each one below.

4.2.1. Influence of the membrane curvature by incubation with LPC and AA

Lysophosphatidylcholine (egg, chicken) and arachidonic acid were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Cells were placed on a coverslip located into a plastic dish (35 mm) filled with the isotonic physiological solution described above supplemented with LPC (2 μM) or AA (20 μM). After equilibrium between exogenous lipids and cell membrane is reached (3 min), amperometric experiments were achieved. The measurements in presence of exogenous lipids (LPC or AA) have been achieved on one cell per dish.

4.2.2. Influence of the external osmolarity

Experiments with hypotonic (200 mOsm) or hypertonic (750 Osm) external environment were performed with the physiological solution described above in which the NaCl concentration has been adjusted accordingly.

4.2.3. Influence of the cell content by incubation with reserpine and L-DOPA

Reserpine stock solution was prepared at 1 mM in dimethyl sulfoxide (DMSO). The chromaffin cells have been treated in bath solution with reserpine 1 μM (solution prepared in a 1000:1 mixture of the physiological saline solution described elsewhere and DMSO) for 60 min at 37 °C. The control was treated with the same conditions (excepted reserpine). We have evidenced that DMSO itself (i.e. the vehicle used for reserpine administration) had no significant effect on any of the spikes characteristics. After the treatment, the cells were bathed in a physiological saline solution without any additive and were allowed to recover for 10 min before being stimulated for exocytosis.

The protocol for L-DOPA incubation was quite similar to the reserpine treatment. The cells have been incubated with a 100 μM L-DOPA solution for 60 min at 37 °C. The cells were thus transferred in a bathing physiological solution (without any additive) and were then allowed to recover 10 min before doing experiments.

4.2.4. Influence of the temperature

In order to control the temperature, the solutions are heated by using a plate which was placed on the microscope stage. The temperature was controlled by a TRZ 3700 unit or cooled by a water bath. The temperature of the solution which bathed the cells is continuously checked by a thermocouple-controlled resistance heater.

4.3. 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 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. Furthermore, the minimum accuracy of the PSF determination is set at a current equal to 2.5× the above noise level [56]. Additionally, two shapes are considered as an amperometric foot: ramp or ramp-plus-plateau. An amperometric foot whose current slightly decreases before the onset of the main amperometric spike is excluded 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 total maximum amplitude of the spike ($I_{foot}/I_{max} > 1/3$) will be also considered as a superimposition of two independent exocytotic events and therefore excluded from the analysis [27]. Indeed, assuming that the pore radii is reflected by the foot current [18,28,29,44], a spike whose foot current would be comparable to the spike current itself would not make physical sense.

The amperometric foot (or pre-spike feature) 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 σ is the standard deviation and n the number of values considered for the mean). All plots were created using Sigma Plot 9.0 software (Systat Software Inc., Richmond, CA, USA).

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