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Research Paper

C. Amatore et al.

**Comparison of Apex and Bottom Secretion Efficiency at Chromaffin Cells as Measured
by Amperometry**

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ABSTRACT

In chromaffin cells, the exocytosis of neuromediators involves the fusion between a secretory vesicle and the cell membrane. Many techniques based on electrophysiology, electrochemistry and fluorescence microscopy allow the study of such a complex process at active zones of single immobilized cells. These techniques can provide an effective analysis either at the apex, either at the base of the cell adhering onto a substrate. For instance, patch-clamp (electrophysiology) and amperometry (electrochemistry) deal with detection at the exposed top of the cell, whereas evanescent field microscopy concerns mainly its bottom, i.e., the zone on which the cell rests onto the surface. However, in chromaffin cells, comparison between the two sets of methods remains to be established and whether apex fusion events are comparable or not to those observed at the base of the cell is an open question. In this work, we compare both active zones upon using the same measurement method, viz., by performing electrochemical detection at these both poles (top and bottom) of bovine chromaffin cells. This is performed upon using carbon fiber microelectrodes (apical analysis) and planar ITO transparent (basal analysis) electrodes, respectively. Our results indicate that the processes monitored at each pole differ though the same technique is used.

Keywords: chromaffin cells; electrochemistry; exocytosis; ITO; microelectrodes; TIRFM

INTRODUCTION

Vesicular exocytotic process is one of the main pathways used by higher organisms to release bioactive molecules (neurotransmitters, hormones, proteins ...) in their environment. To do so, in response to an appropriate stimulation[1] (acetylcholine, membrane depolarisation in neuroendocrine cells ...), secretory vesicles are transported to the cell membrane via the actin network.[2-4] Then, a specific series of events leads to the progressive fusion between the cell and the vesicular membranes to the release of molecules initially stored into the fusing vesicle.[5-7] The chromaffin cell, which role is to release neuromediators and mostly catecholamines (epinephrine, norepinephrine, dopamine...)[8] in blood, is usually considered as a suitable model system for the investigation of vesicular exocytosis. Currently, three major techniques are used to investigate this mechanism at the level of a single secretory event: patch-clamp, evanescent wave microscopy and amperometry. These techniques provide different types of kinetic information on the exocytotic process.

Whereas the events detected by amperometry[9] originate from the apex of the cell at ultramicroelectrodes and through patch-clamp measurements (“apical events”),[10, 11] TIRFM[12-14] reports on the phenomena occurring at the bottom of the cell (“basal” events). This essential difference stems from the peculiar geometrical constraints of each type of experiment. Integration of kinetic and quantitative information gathered by the three classes of methods assumes a priori the similarity of secretory properties between “apical” and “basal” events. Such integration is highly desired due to the complementarities of measurements obtained by each method.

Results from patch-clamp measurements and amperometry are globally consistent since they correlate two types of information occurring at the top of the cell.[15, 16] On the one hand, the precise dynamics of the fusion pore which initiates the exocytotic process is available through patch-clamp measurements; on the other hand, the amount of catecholamine molecules released and the dynamics of full fusion which may follow the fusion pore phase are available with amperometry. Yet, both methods “see” exocytosis only when it has already started, viz., after the initial fusion pore formation. Conversely, total internal reflection microscopy (TIRFM) also offers access to data dealing with the status of basal vesicles before and during fusion[12] (displacements, docking kinetics and location, geometric area of fusion). This allows tracking in real-time the traffic of vesicles in the intracellular

medium prior to the release and during the fusion. However, one does not know precisely if the nature of the exocytotic processes occurring at each pole is similar or differ.

The aim of this report is to compare fusion events (kinetics of fusion, amount of catecholamine molecules released...) occurring at each pole of the chromaffin cell with the same method, i.e. the amperometry. This is performed upon using electrochemical measurements under two different configurations at a single chromaffin cell: classical carbon fiber microelectrode positioned at the apical pole vs. amperometry at an ITO planar microelectrode at the bottom of the cell (see Figure 1). Recently, we have demonstrated the feasibility of the electrochemical detection at the base of chromaffin cells by using planar electrodes on which one single cell is allowed to seed.[17] These electrodes consist of a classical glass surface coated with a conducting oxide: ITO (indium tin oxide),[18, 19] which is one of the transparent surfaces used in TIRFM.[20-23] The conducting area, i.e., the electrode surface, is delimited by a circular hole in a thin resin film (Figure 1) owing to photolithographic techniques. Indeed, a high signal/noise ratio of amperometric detection is obtained by restricting the active electrochemical surface to a few times the surface area of a single cell. The non-faradaic information, i.e. the noise from capacitive origin, is thus minimized. In these conditions, adequate amperometric detection of exocytosis could be performed on these ITO microelectrodes.[17]

The present results demonstrate that the frequency, the kinetics and the efficiency of exocytotic events occurring at each pole of the cell are different. We discuss whether this reflects biological roots related to the intrinsic chromaffin cell functions, or differences imposed to the cell membrane by different local conditions imposed by each measurement configuration.

RESULTS AND DISCUSSION

Comparison of exocytosis at the bottom and the apex of chromaffin cells

As described in the scheme of Figure 1, a cell seeded on an ITO electroactive surface well allows either the detection of secretion at the base of the cell, either to study exocytosis at the top of the investigated cell by positioning simultaneously a carbon fiber microelectrode (7 μm diameter). The

present experiments were designed to monitor the amperometric responses at the apex and at the bottom for the group of same cells. The statistical data obtained in both conditions (Bottom: 290 events, 6 cells; Top: 244 events, 4 cells) are gathered in table 1. Globally, our results thus show that each zone of secretion (i.e. base vs. apex) displays a different behavior. This seems to show that chromaffin cells are polarized not only in tissues but also in culture. For simplicity, in the following text, the apex and the base of the cell will be called apical and basal poles, respectively.

Table 1

First of all, concerning the kinetics (time parameters, see Figure 2A) of the events, one observes that the dynamics are systematically slower at the basal pole than at the apical pole (see Figure 2B). Conversely, the maximum oxidation current I_{\max} , is lower (Figure 2C) at the apical pole. In other words, the flux of released catecholamine molecules is significantly larger at the basal pole though with slower kinetics. Furthermore, the measured charge, i.e. the mean amount of catecholamine molecules released during one exocytotic event is ca. 2.5 times larger at the basal pole than at the apical pole of the cell (Figure 2C).

These variations cannot be ascribed to an effect of the electrode surface material since the electrochemical behavior of adrenaline (the main catecholamine released during exocytosis) was shown in vitro to be undistinguishable at ITO and carbon electrodes.[17, 24] Thus, the significant discrepancies evidenced in Table 1 and Figure 2 reflect radical intrinsic differences between vesicular exocytotic events detected at the apical or basal pole of a same group of chromaffin cells.

As previously established, the exocytotic release at chromaffin cells involves the following sequence of events after docking of a vesicle to the cell membrane: (1) a fusion pore forms through vesicular and cell membranes at the end of the docking phase; (2) the fusion pore ruptures under a swelling pressure building up within the vesicle. This is due to the progressive alteration of the compacity of the polyelectrolytic gel contained in the secretion granule owing to the continuous cation exchange occurring during pore release. Our above results showing that exocytotic phenomena are radically different when they occur at the basal or apical pole of the cell, we now wish to discuss the

meaning of these differences according to two separate but complementary points of view: (1) the first one envisions the possibility of different natures and locations of vesicles within the chromaffin cell at each pole; (2) the second one relies on possible effects related to changes of viscous and tension properties of the cell membrane when the cell is just placed into gentle contact with a small ultramicroelectrode (apical) or when it is in adhesive contact with its support over a surface area comparable to that of the whole cell (basal).

Analysis of the events frequencies

The number of exocytotic events measured per unit of time at the basal pole of the cell is greater at the apical pole (see Table 1). However, this reflects in part an intrinsic artefact due to the change of the cell active surface probed by the electrode surface under each configuration. A chromaffin cell resting on a planar surface is roughly hemispherical with a mean diameter of about 20 μm . [25] The cell-ITO contact surface is then a disk whose diameter is roughly equal to 20 μm . Therefore,

$$S_{\text{contact}}^{\text{ITO}} = \pi \cdot R^2 = 3.14 \times (10)^2 \approx 315 \mu\text{m}^2$$

showing that the ITO probes more than 300 μm^2 of the cell basal surface.

In amperometry at the apical pole, i.e., using a carbon fiber microelectrode (7 μm diameter), the active surface is only ca 90 μm^2 , since it is now limited by the electrode active surface area. As a consequence, upon assuming that the intrinsic events frequency would be similar per unit of surface probed at the apical and basal poles, one expects the relationship between the frequency (F) detected at ITO electrode vs. the one at a carbon electrode surface, to be given by the following relationship:

$$\frac{F(\text{basal})}{F(\text{apical})} = \frac{S_{\text{contact}}^{\text{ITO}}}{S_{\text{contact}}^{\text{Carbon}}} = \frac{315}{90} \approx 3.5$$

This ratio contrasts with the experimental mean frequencies of events. Indeed, $F \sim 1.1$ Hz is detected at the apical pole (carbon fiber electrode) while $F \sim 2.3$ Hz is detected at the basal pole (ITO). Therefore, the experimental ratio $F(\text{basal})/F(\text{apical})$ is only 2.1 under our conditions, being significantly smaller than the above expected 3.5 value. A more meaningful measure is therefore

obtained upon renormalizing the experimental frequencies vs. the surface, viz., upon comparing the number of events detected on each pole in terms of frequency per unit of surface area. Thus:

$$F_S(\text{basal}) = \frac{F^{\text{Base}}}{S_{\text{contact}}^{\text{ITO}}} = 7.3(\pm 1.6) \times 10^{-3} \text{ Hz}\mu\text{m}^{-2}$$

$$F_S(\text{apical}) = \frac{F^{\text{Apex}}}{S_{\text{contact}}^{\text{Carbon}}} = 12.2(\pm 2.5) \times 10^{-3} \text{ Hz}\mu\text{m}^{-2}$$

Despite the fact that both values are quite numerically close together, the frequencies per unit of surface (for apical and basal poles) are statistically different (considering a deviation of at most 20 % and 3 % for the surface of contact between the cell and the electrode and the mean frequency, respectively). This shows that, for the same given surface area, the basal pole is less active than the apical pole, evidencing that exocytotic events observed in TIRFM, i.e., at the base of the cell, cannot be compared directly to those usually monitored at the apical pole by a carbon disk microelectrode. This may reflect a strong heterogeneity in the membrane releasing ability, or an intrinsic difference.

Kinetics of the events at the base of the cell

As summarized in table 1, the exocytotic phenomena are significantly slower at the basal pole of the chromaffin cell. This appears to be consistent with the experiments performed using evanescent wave microscopy[12, 26], in which the time scale of the exocytotic events reported is often larger than those usually observed in amperometry, though a precise comparison is difficult due the lower signal-to-noise ratio of TIRFM compared to amperometry.

A first simple explanation assumes a constitutive difference of the cell, viz., that exocytosis at basal and apical poles implies different vesicular populations.[27, 28] Notably, it has been reported that the kinetics of the exocytotic events could depend on the size and the amount of catecholamines stored in each vesicle.[29]

However, since full fusion kinetics are linked to membrane dynamics,[6] a second explanation can deal with differences in viscous dissipation properties. Indeed, full fusion release requires the vesicular membrane to be readily shuttled into the cell membrane so that it can be rapidly evacuated from the expanding pore area to let a matrix exposed to the extracellular medium. Whenever the cell membrane

experiences a strong viscous adhesion to the substrate, the excess membrane should not be evacuated at sufficient rate.[30] This should induce the creation of ripples (Figure 4) around the fusion zone in agreement with direct EM observations. In the basal configuration, the active cell membrane area is maintained in contact with the ITO planar electrode due to the cell adhesion.[31] Conversely, probing the apical pole involves only a limited area submitted to a gentle contact pressure without adhesion. This important difference may affect the whole topology of the cell membrane dynamics through imposing non-isotropic surface tension and viscosity. Both factors are expected to slow down the lateral translocation of incorporated vesicle membrane during the full fusion process. Thus, on the one hand, it may affect the release kinetics (viscosity) and on the other hand, it may decrease the local membrane surface tension at the release point. The ITO configuration due to the strong adhesion is expected to favour such an increase of the viscosity and a decrease of the tension surface energy, therefore possibly slowing the release during exocytosis.

Amounts and flux of catecholamines released at the basal pole

Contrastingly with their slower kinetics at the basal pole, the mean released charge is 2.5 times larger than at the apical pole. This means that the exocytotic events observed in an equivalent TIRFM configuration correspond to the release of higher amounts of catecholamines per vesicular event. Based on recent literature, several hypotheses may help to rationalize these results.

Once again, one can explain the present data by assuming that different vesicular populations are implied in “basal” and “apical” exocytotic processes. Assuming that the composition of the cytoskeleton as well as the nature of vesicle pools near the cell membrane depend on the pole due to the cell polarization, our experiments show that vesicles that fuse at basal pole in chromaffin cells are larger than at the apical pole since they release a larger catecholamines content than those that are implied at the apical cell pole. Indeed, it is not expected that the vesicular concentration of catecholamines may vary in the required proportion.[29, 32-34] Yet, in TIRFM studies, previous observations reported by Almers and coll. suggested that vesicles may reseal frequently before full completion of fusion.[14, 26] Based on patch-clamp measurements, Artalejo and coll. reported that in chromaffin cells, 60 % of the fusion events which occur at the apical pole stop before completion.[11]

Considering that this result remains valid when investigated by amperometry at ultramicroelectrodes, exocytotic processes occurring at apical pole of the cell under our conditions ought to be similarly regulated. In our experiments, the higher average charge detected at basal pole may then well correspond to a larger proportion of events undergoing full release without significant releasing. This could explain the present results, without requiring any vesicle-size dependence.

Nevertheless, it can not be excluded that two-vesicle fusions [5] occur more often at the basal pole by the mean of pre-fusion of vesicles before fusion with the cell membrane. This kind of vesicles, formed apart from the docking loci, [35, 36] would store more catecholamines, which could explain the increase of charge detected at the basal pole. This hypothesis is reinforced upon considering recent works dealing with the study of exocytosis in PC12 cells using TEPIQ (Two-photon Extracellular Polar-tracer Imaging-based Quantification) analysis.[37] They have shown that 76% of large dense-core vesicles follow sequential compound exocytosis at the base of the cell.

So, it is seen that this matter cannot be solved unambiguously at this stage. Figure 3 compares both mean spikes shapes obtained in each series of measurements after normalizing them to the same amplitude (viz., I/I_{\max} is plotted instead of I and t/t_1 instead of t ; see figure 2A for definitions) and stresses the large differences in kinetics at the apical and basal poles.. It can be seen that the spikes at the basal pole are systematically more long-tailed than those at the apical one. This is clear evidence that at the basal pole, release becomes more and more sluggish while time proceeds.

Such a visible difference may reflect several differences between the experimental configurations. The two main effects that one may envision are: (1) a difference of local pH in the extracellular cleft created by each configuration; (2) local changes of viscosity and surface tensions of membranes.

The pH of the extracellular fluid is necessarily an important point since this is one of the driving thermodynamic forces which provoke release out of the vesicular matrix. In fact, Wightman and coll. reported experiments confirming this point.[38, 39] Here, the initial extracellular buffered solution pH is identical at each pole. Yet, electrochemical oxidation releases $2H^+$ per catecholamine detected. This necessarily forces the pH within the electrode/cell cleft to decrease drastically if extra protons cannot

be evacuated (diffusion) or neutralized (buffer) sufficiently fast. Even accounting for the relatively fast diffusion of protons towards the external cleft circular edge, one may estimate that the pH may in average drop down locally to ca. 4.5 after release, i.e., the artificial synaptic cleft may become more acidic than the matrix itself. However, proton escape occurs by cylindrical diffusion, which varies logarithmically with distance.[40] This feature is expected to impose similar diffusion rates under our two arrangements, since the radius of the probed areas does not vary sufficiently in logarithmic units between the basal and apical configurations. Such pH-based explanation thus cannot be retained to elucidate the change of behaviour. Nevertheless, it can not be excluded that the distance between the cell membrane and the electrode surface plays a role. Indeed, the latter is probably shorter in the basal configuration, due to the cell adhesion, than in the apical one. This would lead to increase the concentration of the species released into the cleft.

A second explanation which may be envisioned deals with a possible change of local mechanical properties of the cell membrane due to the change of the experimental arrangement. Indeed the mechanical movement of the vesicle membrane pouring into the cell membrane during fusion occurs at a much slower rate than the above proton diffusion because of a large viscosity and also because it involves a whole membrane area[30] rather than a small ion. Under such circumstance, evacuation of the membrane excess cannot reach its cylindrical diffusion pseudo steady-state[40] during the time range of interest here. It ensues that the size of the contact disc area between the cell and the electrode matters a lot. A ratio of about 3 between the ultramicroelectrode (7 μm) and the ITO-cell contact (ca. 20 μm diameter) implies a change in equivalent diffusion rates by ca. 9, since diffusional times vary as the square of the distance. This difference only may explain qualitatively why the release spikes are more sluggish at the basal pole than at the apical pole. Indeed, if the evacuation of membrane excess is slowed down, this may result into two effects and any of their combinations. The excess membrane released which cannot be evacuated as fast as requested by the vesicle unmasking may either create a series of ripples around the exposed fraction. Such ripples have actually been observed by electron microscopy[41] although the authors did not insist on this peculiarity in their published work. Eventually, the energy stored by these ripples may bounce back or

even exceed that released by the unfolding vesicle membrane, so that the full fusion should slow down or may possibly stop and begin a resealing movement. Such phenomenon would be more severe for larger vesicles and when the rate of membrane evacuation is lesser, that is in full agreement with our present observations.

CONCLUSION

Our studies show that the geometrical constraints due to the intrinsic measurement nature play a significant role on the exocytotic events by imposing different conditions at basal and apical poles. The different exocytotic features observed at both poles may be ascribed to the role of induced differences in the local membrane dynamics. Yet, it is also likely that different vesicle pools are implied at both poles, which would be in agreement with the polarized nature of the chromaffin cell. Beyond the real underlying causes, these behavioural differences between events occurring at each pole show that one must be extremely careful when comparing results obtained by TIRFM or by amperometry at carbon fiber electrodes. We wish to stress that this caveat does not probably stem from the different physicochemical basis of the two techniques, but rather from the differences in their geometrical requirements.

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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.[42] They were used on days 3-10 after culture.

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[43] and back-filled with mercury for electric of contact. Electrode tips were polished at a 45° angle on a micropipette beveling wheel (Model EG-4, Narishige Co., London, UK) for 20-30 min. before experiments. Only electrodes with a very stable amperometric baseline current were used for cell measurements.

Concerning the ITO planar microelectrode, the whole procedure was described previously.[17] Briefly, glass slides (75mm x 25 mm x 1 mm) were coated with a film of Indium Thin Oxide (ITO, 500 nm thickness; ACM, Villiers Saint Frédéric, France). Micrometric disk surfaces of ITO were delimited by photolithography by using an insulating resin (SPR220, MicroChem Corp., Newton, MA, USA) in which 40 to 300 μm diameters wells were obtained. Slabs of poly-dimethyl-siloxane (PDMS, 5 mm thickness) with holes punched through (10 mm diameter) were used as wells positioned over the ITO microelectrodes for solution, microcapillary and reference electrode positioning.

About 1 milliliter of an isotonic physiological saline solution (154 mM NaCl, 4.2 mM KCl, 0.7 mM MgCl₂, 11.2 mM glucose, 10 mM HEPES, pH 7.4) containing cells (at most $3 \cdot 10^5$) was injected into a well of PDMS lying on an ITO microelectrode. The cells were allowed to seed for 20 to 30 min. at room temperature. In general, four or five cells adhered to the surface of each ITO well. A glass microcapillary (10-20 μm diameter) was then positioned with a micromanipulator (MHW-103, Narishige, Tokyo, Japan) at a distance of 20 to 30 μm from the single cell located in the well and used to inject (Femtojet injector, Eppendorf, Hamburg, Germany) a stimulating solution (BaCl₂ 2mM in Locke buffer supplemented with 0.7 mM MgCl₂, without carbonates) towards the cell surface for 10 s. This configuration allowed to stimulate one cell at a time and to detect its response at the ITO surface

(placed at the bottom of the cell) or at a carbon fiber microelectrode (positioned at the top of the cell). All chemicals were from Sigma-Aldrich (St-Louis, MO, USA). Experiments were conducted at room temperature ($22 \pm 1^\circ\text{C}$).

Data Acquisition and Data Analysis

Electrodes were held at + 0.65 V vs. a silver/silver chloride reference electrode using a commercially available picoamperometer (model AMU-130, Radiometer Analytical Instruments, Copenhagen, DK), for which the adjustable time-response was 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 if 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 signal. Special attention was applied to verify 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. Each spike characteristics, i.e., the maximum oxidation current I_{max} (pA), the total electrical charge Q (fC), the half-width $t_{1/2}$ (ms), and the rise time $t_{20/90}$ (the delay between $I = 20\%$ of I_{max} and $I = 90\%$ of I_{max} in ms), were determined using home made software. All values are reported as the mean \pm SEM considering Gaussian-type distributions of the data (I_{max} , Q , $t_{1/2}$, $t_{20/90}$) or of its logarithm (Q) and all plots were created using Sigma Plot 9.0 software (Systat Software Inc., Richmond, CA, USA).

FIGURE LEGENDS**Figure 1.**

Sketch of the electrochemical detection of vesicular exocytosis by a chromaffin cell as performed at the apical pole (carbon fiber microelectrode) or at the basal pole (ITO planar electrode). Note that the planar ITO microelectrode is used both as support for cell adhesion and to detect exocytotic events and that the chromaffin cell is located into a well delimited ITO-exposed by a resin layer in order to minimize the non-faradic information.

Figure 2.

(a) Graphical definition of the relevant parameters (Q , I_{\max} , $t_{20/90}$, t_1 , t_2) measured on each exocytotic event extracted from the amperometric trace. (b) Kinetic parameters ($t_{20/90}$, t_1 , t_2 , $t_1 + t_2$) of the exocytotic events detected at both ITO (black) and carbon (gray) surface (apical and basal pole of the cell, respectively). (c) Comparison of additional spikes features between apical (carbon; gray) and basal (ITO; black) detection: normalized frequencies (vs. the electrode surface); maximum flux of catecholamine molecules released (I_{\max}); amount of catecholamines (Q) released during the exocytotic event at basal and apical pole of the cell. All values are given as mean \pm standard error (see text).

Figure 3.

Superimposition of mean apical (dotted line) and basal spike (thick line) shapes (30 spikes averaged for each condition). The average spikes have been rescaled (I/I_{\max} ; t/t_1) to help their comparison.

TABLES

	Frequency (Hz)	t ₁ (ms)	t ₂ (ms)	t ₂₀₋₉₀ (ms)	I _{max} (pA)	Q (fC)
Apical (Carbon fiber)	1.1 ± 0.2	16.7 ± 0.5	43.4 ± 1.1	15.6 ± 0.6	11.1 ± 0.8	800 ± 55
Basal (ITO)	2.3 ± 0.1	18.7 ± 0.7	53.8 ± 2.0	19.3 ± 0.9	19.8 ± 0.8	1750 ± 105

Table 1. Comparison of vesicular exocytotic events occurring at the apical and basal poles as monitored by amperometry.

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Figure 1

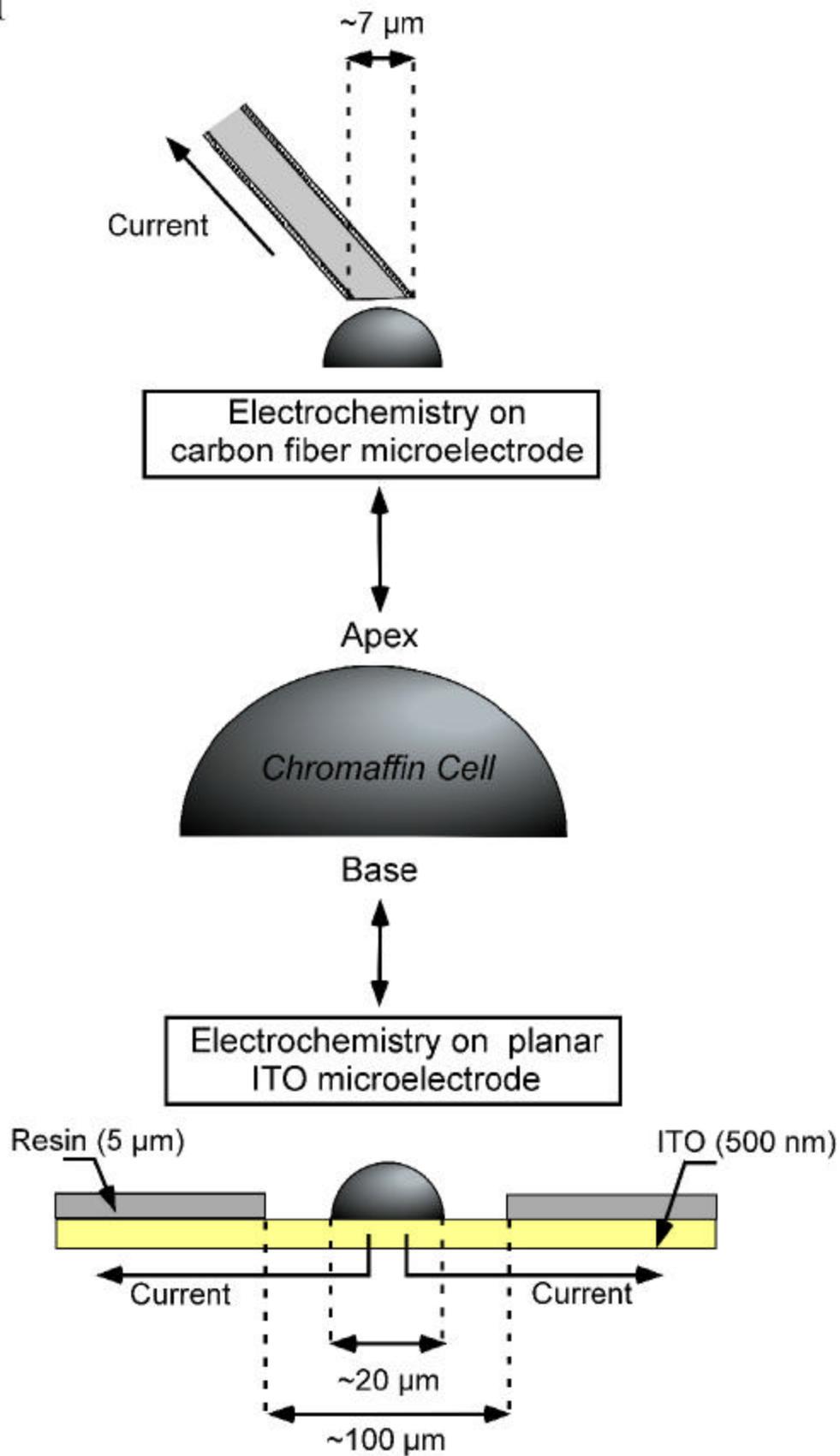


Figure 2

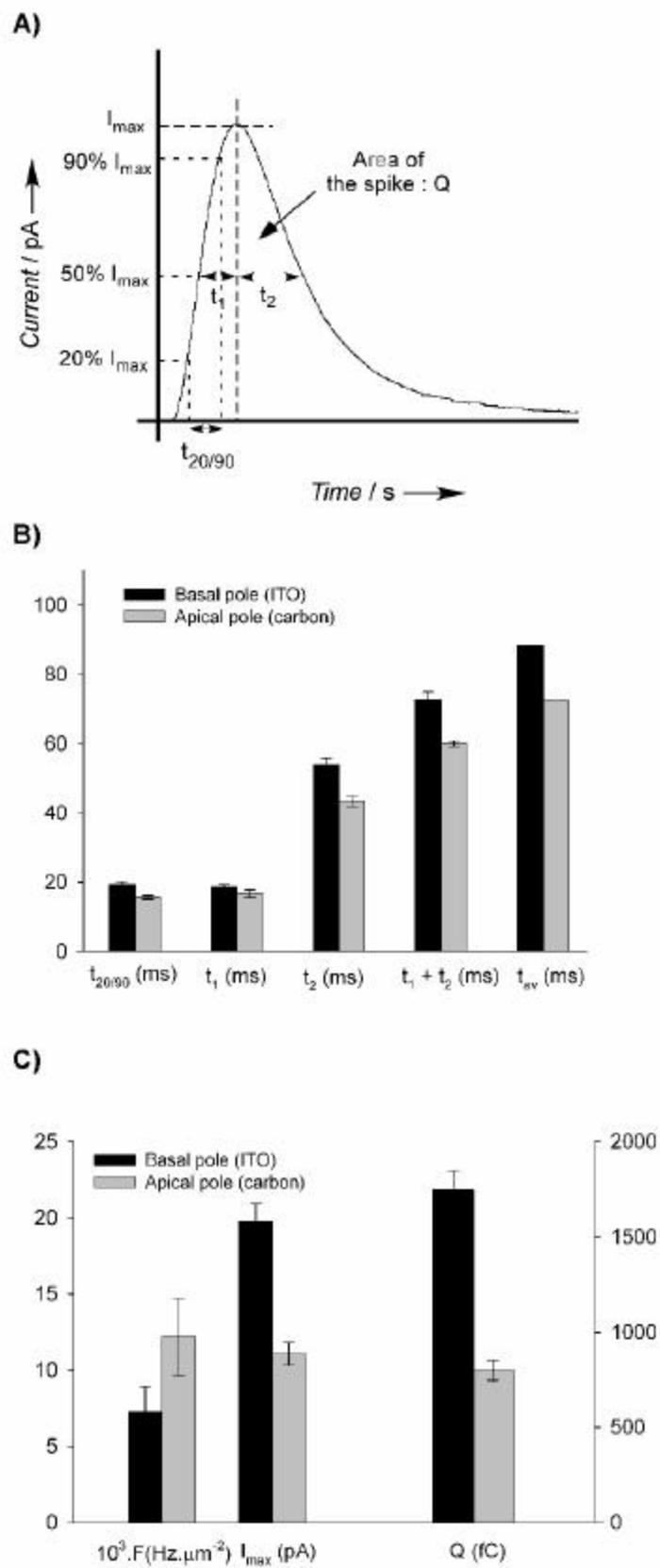


Figure 3