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# A preferential pole for exocytosis in cultured chromaffin cells revealed by confocal microscopy

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**Abstract Histological studies suggest that adrenal medulla chromaffin cells in situ are polarised, but functional evidence is lacking. We present here the first demonstration for polarisation of exocytosis in isolated, spherical, bovine chromaffin cells. Cells were stimulated with 70 mM $K^+$ to cause a marked enhancement of catecholamine release, monitored amperometrically. FM1-43 and dopamine β-hydroxylase antibodies provided fluorescence confocal pictures that were 2–3-fold more intense in the bottom of the cells, as compared to equatorial or apex planes. This suggests that the solid phase to which the cell attaches serves as a 'trophic' signal for the polarisation of its secretory apparatus.**



*Key words:* Exocytosis; Chromaffin; Confocal microscopy; Polarity; Calcium

## 1. Introduction

Erwin Neher and coworkers [1] recently made a puzzling observation. They were comparing capacitance and amperometric measurements of exocytosis in bovine adrenal chromaffin cells, when secretion was elicited by flash photolysis of caged-calcium or step depolarisations. Their experimental amperometric signals were delayed 107 ms with respect to the simulated amperometric signals derived from the capacitance increase. For the simulation they used a model consisting of a spherical cell, and a secretion-diffusion model that assumed uniform distribution of release sites on the cell surface. The model neglected diffusional restrictions due to chamber walls, patch pipette and particularly the fact that more secretion might occur on those parts of the cell surface oriented toward the chamber bottom (the amperometric carbon fibre electrode was placed 0.5 μm above the apex of the cell).

Inhomogeneous distribution of release sites in bovine chromaffin cells has been detected using various methodological approaches. Earlier studies using anti-dopamine β-hydroxylase (anti-DBH) antibodies to mark exocytotic sites suggested that those sites are randomly located across the entire surface of bovine chromaffin cells [2,3]. Placing 1 μm radius carbon fibre electrodes at different sites of the cell surface, Schroeder et al. [4] mapped exocytotic release sites of 2 μm, alternating with other silent sites on the cell surface of bovine chromaffin cells. 'Hot spots' of $Ca^{2+}$ entry and release were also detected by Robinson et al. [5]. Combining DBH antibodies and confocal microscopy, a punctate appearance of DBH was seen, suggesting once more that exocytosis can occur in specialised patches [6]. However, these experiments are compatible with the currently prevailing idea that although in specialised patches, chromaffin cells in culture are not polarised and hence, exocytosis may occur at random in the entire surface of cells.

By combining confocal microscopy with the exocytotic fluorescent markers FM1-43 and DBH antibodies, we have discovered that spherical bovine chromaffin cells in culture exhibit a strong polarisation to release catecholamines towards their bottom. This finding has important methodological implications, to explain the discrepancies discussed above, and when planning future experiments to measure exocytosis with capacitance and amperometric techniques. It also has relevant physiological implications; even in culture, chromaffin cells maintain the strong polarity of their secretory apparatus that they must have in situ in the intact adrenal medullary gland, as deduced from histological studies of such tissue [7], for an efficient and rapid delivery of catecholamines into the circulation.

## 2. Materials and methods

### 2.1. Isolation and culture of adrenal medulla chromaffin cells

Bovine adrenals were obtained from a local slaughterhouse. Their chromaffin cells were isolated following standard methods [8] with some modifications [9]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were plated on 2.5 cm diameter glass coverslips at a density of $5\times10^4$ cells per ml, and used 2–4 days thereafter.

### 2.2. Amperometric measurements of catecholamine release

The carbon fibre electrodes were prepared by cannulating a 10 μm diameter carbon fibre into polyethylene tubing (diameter: outer, 0.8 mm; inner, 0.4 mm). The tip of the carbon fibre was pulled as described [10] except that the narrow part at the tip was in general longer (200–400 μm). The carbon fibre tip was glued into a glass capillary for mounting on a patch-clamp headstage, and backfilled with 3 M KCl to connect to the Ag/AgCl wire which was held at +650–700 mV. The tip was recut for every new experiment. Amperometric currents were recorded using a home-made amplifier. An Instrutech ITC-16 controlled by a Macintosh Power PC 8200/120 running IGOR 3.02 (Wavemetrics, Lake Oswego, OR, USA), and the Pulse Control XOPs (J. Herrington and R.J. Bookman, University of Miami), were used as acquisition system.

### 2.3. Confocal microscopy measurements of FM1-43 fluorescence

Images of a field of cells were acquired with a confocal microscope (Bio-Rad MRC1024) with a Nikon Planapo 40×/0.95 dry objective. The dye was excited with a Kr-Ar laser light at 488 nm, in the low-scan mode and 3% power, and emission was detected with the OG515

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nm filter. Cells were placed in an experimental chamber with Krebs-HEPES whose composition was (in mM): NaCl 145, KCl 5.6, $MgCl_2$, 1.2, HEPES 10, $CaCl_2$ 2, and glucose 10. Depolarisation was performed using a 70 mM $K^+$ Krebs-HEPES solution (isosmotic reduction of $Na^+$). Over each field of cells we performed a Z-series (Z step: 2 μm) to determine its height. An optical section was selected to scan and measure the fluorescence. Time 0 was considered the time at which we applied 5 μM FM1-43 in Krebs-HEPES solution. After 9 min the first confocal image was acquired (step 1). The second and third steps consisted in the incubation with Krebs containing 70 mM $K^+$ and FM1-43 during 2 min, and acquisition of two images: 2 s after application, and in the last second before removing the solution. The next steps (4 and 5) consisted in the incubation during 2 min with Krebs and FM1-43, and acquisition of images again 2 s after application and in the last second before removing the solution. As cells were not of the same size, real localisation of a given optical section was calculated cell to cell a posteriori, and grouped into sections (top, middle, bottom). Analysis of fluorescence was performed with NIH Image (Harvey J. Katen, University of California) and IGOR 3.02.

### 2.4. Confocal microscopy measurements of DBH antibodies fluorescence

Cells placed on coverslips were stimulated during 2 min either with 70 mM $K^+$ in Krebs-HEPES, or in normal Krebs-HEPES solution (control cells). The solution was quickly removed and replaced with 4% *p*-formaldehyde in phosphate-buffered saline (PBS, pH 7.4) and maintained for 20 min at 4°C. Fixed cells were rinsed in PBS twice, and then incubated with 0.2% bovine serum albumin for 10 min. After washing, the coverslips were incubated with mouse anti-DBH monoclonal antibody (1:200; Chemicon) for 45 min. Coverslips were then rinsed with PBS and primary antibody was revealed by incubation for 45 min with BODIPY-FL goat anti-mouse IgG conjugate (1:100, Molecular Probes). Following the washout with PBS, coverslips were mounted on PBS 1:1 glycerol (v/v). Images were acquired at 1 μm interval following the *z*-axis of each cell, using a Nikon Planapo 60×/1.4 oil immersion objective. Analysis of fluorescence was performed with NIH Image and IGOR 3.02, in the same way as FM1-43 images.

## 3. Results

### 3.1. Amperometric detection of secretory spikes

To map specialised zones of exocytosis we combined the use of confocal microscopy with two exocytotic fluorescent probes, FM1-43 [11] and DBH [2,6,12]. Before using the fluorophores, initial experiments were performed to define a protocol that provided a high rate of exocytosis. Direct measurement of catecholamine release was achieved using a carbon fibre microelectrode, placed adjacent to the cell being explored. Different stimulation patterns with $K^+$-enriched solutions were attempted. High exocytotic activity was triggered by incubating the cells in a Krebs-HEPES solution containing 70 mM $K^+$ and 2 mM $Ca^{2+}$. Fig. 1 shows the amperometric secretory spikes recorded with a carbon fibre electrode. Every spike indicates the release of the catecholamine contents of a single vesicle; their sizes vary with the position of the electrode with respect to individual exocytotic events [13]. When incubated in Krebs-HEPES, the cell showed no activity. After adding $K^+$, the cell developed a high-frequency pattern of secretory spikes that lasted during the 2-min exposure to $K^+$. After switching the bath solution back to normal Krebs, the secretory response gradually returned to baseline. The cell secretory behaviour was similar in the absence (Fig. 1a) and the presence of 5 μM FM1-43 (Fig. 1b). The integral of secretion spikes in the absence of FM1-43 was 872 pC, and that in its presence amounted to 1.348 pC. Thus, the dye did not interfere with the exocytotic process.

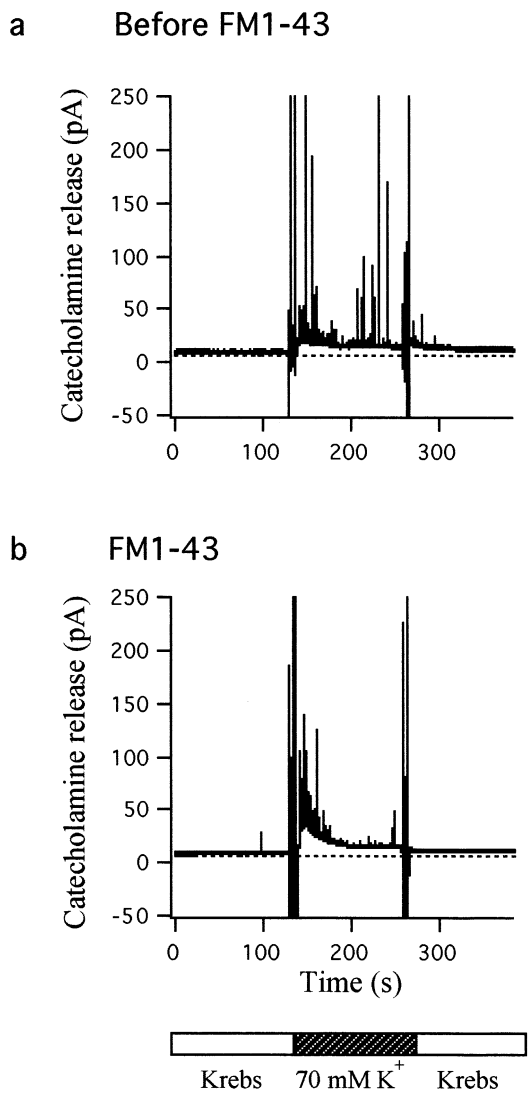


Fig. 1. Catecholamine release in response to 2-min depolarisation with 70 mM $K^+$, measured with a carbon fibre electrode. a: After 2 min recording in Krebs-HEPES solution, this solutions was exchanged with a Pasteur pipette containing 70 mM $K^+$, and recorded for 2 min under this condition. Finally, $K^+$ depolarising solution was removed, and fresh Krebs-HEPES solution was added. Note that the manual exchange of solutions causes a big artefact at the beginning and at the end of the stimulation period, due to the mechanical vibration of the carbon fibre. b: An identical protocol as in a, except that 5 μM FM1-43 was present during the whole experiment, was performed in the same chromaffin cell.

### 3.2. Regional cell distribution of FM1-43 fluorescence during exocytosis

Once the conditions for optimal secretion were settled, exocytosis was measured using 5 μM FM1-43. The styryl dye derivative used here is cell-impermeable and hence accumulates only in the outer leaflet of the plasmalemma of intact cells [14]. The dye fluoresces only when inserted into membranes and therefore it was expected that an increase in fluorescence was produced during exocytosis (note that the dye was present during the entire experiment). This is clearly seen in the cell shown in Fig. 2a. In resting conditions (step 1 of the protocol, see legend to Fig. 2), the cell membrane exhibited a tiny, uniformly distributed fluorescence, which increased gradually at the beginning and the end of the 2-min

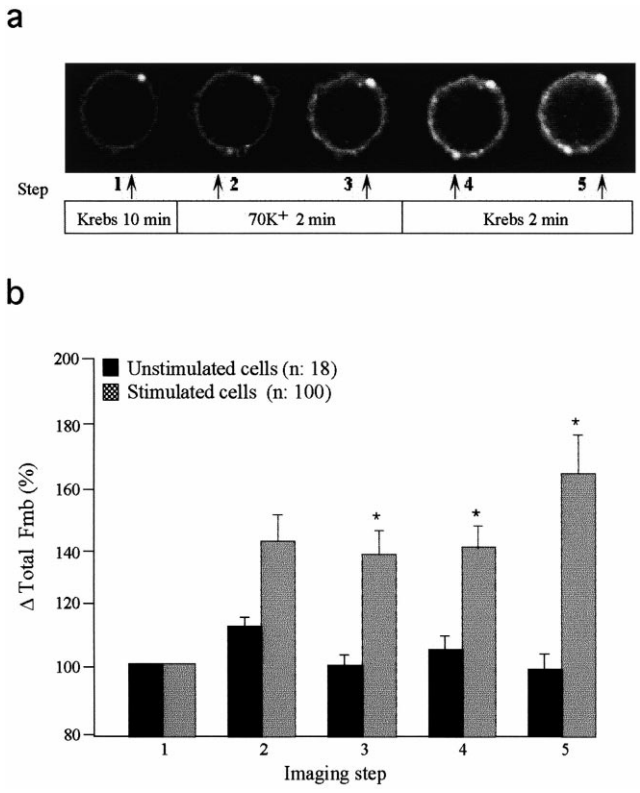


Fig. 2. Increase of FM1-43 fluorescence in a chromaffin cell selected from a field of cells that were stimulated with a high $K^+$ solution. The dye FM1-43 (5 μM) was always present in the external solution. During step 1 cells were bathed with Krebs-HEPES for a 10-min period. The membrane fluorescence at this moment (arrow) was considered basal. Images 2 and 3 were acquired at the beginning and at the end of the incubation with 70 mM $K^+$ (arrows). Finally, cells were incubated back in Krebs-HEPES solution and two confocal images were taken again (arrows). Panel b shows averaged data for the fluorescence obtained in different experiments following the protocol shown in panel a. The fluorescence found in each cell during the first incubation (step 1) with FM1-43 without stimulation was considered basal (100%); the increase of fluorescence found in steps 2–5 of the protocol was expressed as per cent of basal. Data are means ± S.E.M. of 18 unstimulated cells and 100 cells stimulated with $K^+$. $*P < 0.05$ with respect to unstimulated cells.

$K^+$ stimulation period (steps 2 and 3). Fluorescence continued to increase during the post-$K^+$ incubation period (steps 4 and 5), according to the exocytotic pattern observed using the carbon fibre electrode (Fig. 1). The persistence of secretion after the end of the stimulus has been shown in chromaffin cells, for short depolarising pulses [15,16], or trains of action potentials [17]. Therefore, it is reasonable to get a delayed exocytosis after the long stimulus protocol used in our experiments, where prolonged entry of $Ca^{2+}$ will allow a slow decay of its cytosolic concentration. Also, the FM1-43 fluorescence is voltage-dependent, being lower under depolarisation (the period of incubation with 70 mM $K^+$) than under resting conditions [11]. The increasing fluorescence after the end of the stimulus (steps 4 and 5) also indicates that massive exocytosis occurring under our experimental conditions seemed not to be totally counteracted by the different mechanisms of endocytosis present in chromaffin cells [18–21].

Cell membrane FM1-43 fluorescence (Fmb) was measured in 18 unstimulated cells and 100 $K^+$-stimulated cells, from various cultures. A confocal image was taken for each of the five steps of the protocol in a given field of cells (see Fig. 2a). The membrane fluorescence in each cell of every step was averaged, to obtain an approximation of the increase of fluorescence in each of the five steps of the protocol. The

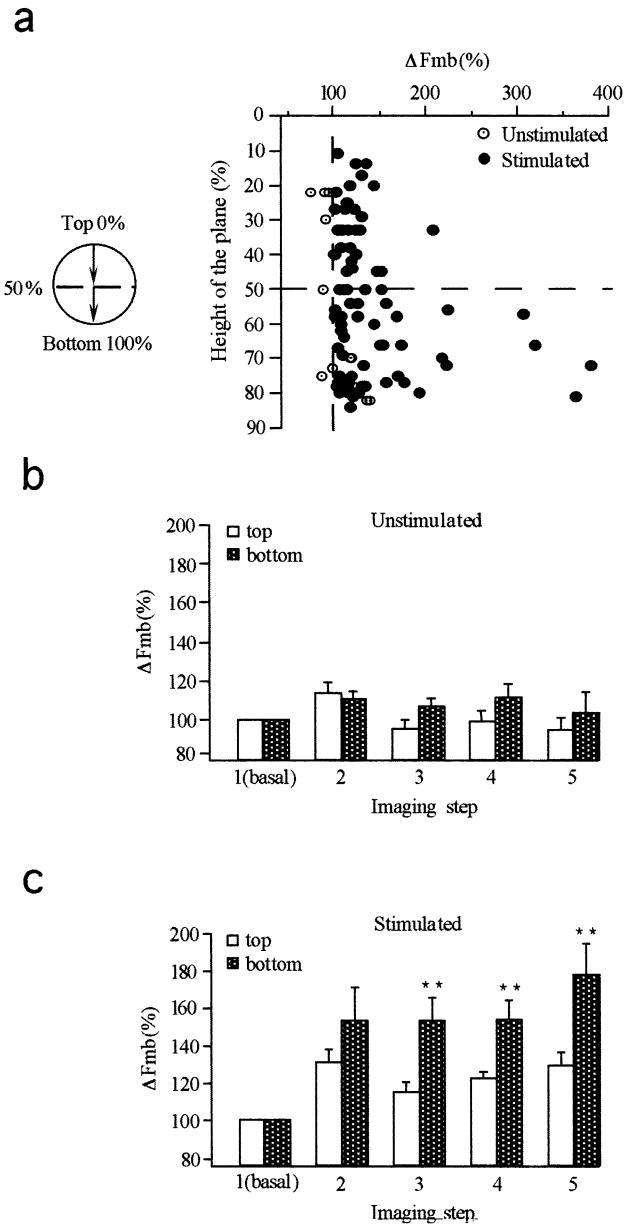


Fig. 3. Uneven distribution of FM1-43 fluorescence in stimulated chromaffin cells. Each dot in panel a represents the fluorescence in a given plane of a cell, according to its position in the $z$-axis (ordinate; 0–100% from top to bottom of the cell shown in the inset to the left). The confocal image plotted here was taken at step 4 of the protocol, in unstimulated cells and in cells stimulated with $K^+$. Panels b and c show averaged fluorescence obtained in cells whose confocal images were taken above an equatorial plane in the $z$-axis (top half) or below this plane (bottom half). The first confocal image was taken in resting conditions (step 1, basal fluorescence), and was normalised to 100% (ordinate, membrane fluorescence, Fmb). Fluorescence signals obtained in subsequent steps were expressed as % increments above basal. Panel b shows the variation of fluorescence in steps 2–5 of 18 unstimulated cells, and panel c shows the increments in fluorescence during steps 2–5 in 78 $K^+$-stimulated cells. Data are means ± S.E.M. $**P < 0.01$ with respect to the fluorescence values seen in the top half of each cell.

basal fluorescence of step 1 was normalised to 100%, and the increment of fluorescence in subsequent steps was expressed in per cent of basal fluorescence. In unstimulated cells the total fluorescence remained within basal levels through the different steps of the protocol (note that these cells were bathed the whole time in normal Krebs-HEPES solution containing FM1-43). In contrast, the fluorescence of stimulated cells significantly increased between 40% and 65% over steps 2–5 of the protocol (Fig. 2b).

As noted above, in each experiment with FM1-43 the variations in fluorescence seen during the five protocol steps were followed in a single horizontal confocal plane. But not all cells of a given field were captured at the same height on the *z*-axis, since their sizes were different and their positions in the coverslip also varied. FM1-43 experiments were performed in a field of cells selected at random and visualised by confocal microscopy in a plane selected at random, too. In order to localise the confocal plane into each cell where Fmb was measured, the height of each cell was calculated at the end of the experiment. A Z-series over the field of the cells was performed and individual height for each cell was calculated and normalised (0% top, 50% equatorial plane, 100% bottom). After this, the selected confocal plane tested was defined. In this manner we could group the cells according to the height of the plane on the *z*-axis, on which their fluorescence was measured. Fig. 3a shows that unstimulated cells had a fluorescence near basal (103%) along the *z*-axis. However, the fluorescence of $K^+$-stimulated cells increased from top to bottom of the cell. The fluorescence obtained in the confocal sections of the top and bottom halves of all cells was averaged and plotted. Fig. 3b shows the averaged fluorescence of unstimulated cells, which was similar in the top and bottom halves. However, in stimulated cells the fluorescence was significantly higher (53–78%) in the bottom, as compared to the top half (15–31%) (Fig. 3c). These differences were greater at the last steps of the protocol (steps 3–5).

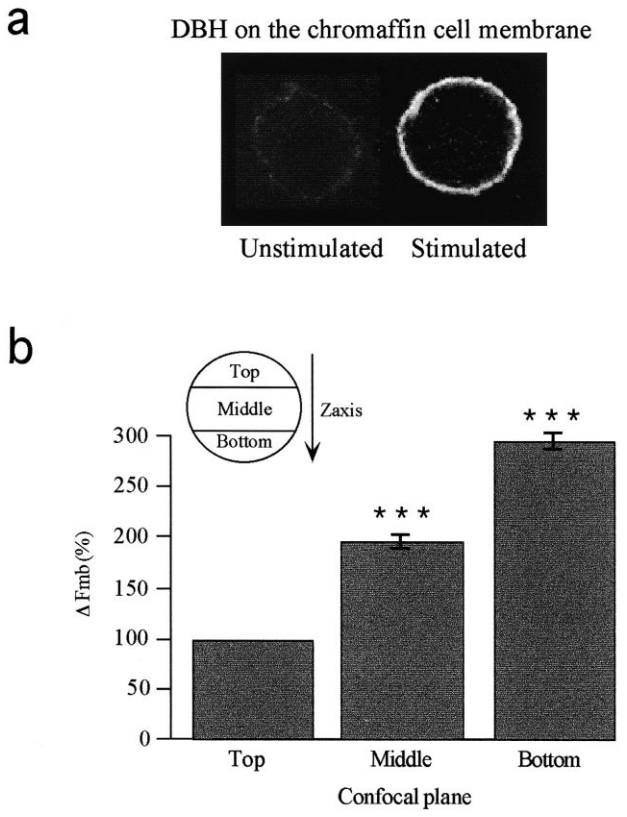


Fig. 4. Localised exocytosis measured with DBH antibodies. Panel a shows the fluorescence obtained in an equatorial plane in an unstimulated cell, and in a cell stimulated for 2 min with 70 mM $K^+$; cells were fixed and exposed to DBH antibodies (see Section 2). Panel b shows the averaged regional distribution of fluorescence, along the top, middle and bottom parts of $K^+$-stimulated cells, following the *z*-axis. A Z-series at 1-μM intervals was performed in each individual cell. The cell was divided into three thirds and the fluorescence integrated for each cell region (top, middle, bottom). The fluorescence of the top part in each individual cell was normalised to 100%, and those of the middle and bottom parts were expressed as % increment above top fluorescence. Data are means ± S.E.M. of 10 cells from different cultures. $***P < 0.001$ with respect to top fluorescence, and also between them.

### 3.3. *Regional distribution of DBH antibodies fluorescence during exocytosis*

A more detailed confocal study of the exocytotic activity of the different planes of chromaffin cells, upon $K^+$ stimulation, could be achieved using DBH antibodies. The experiment to stimulate exocytosis was performed as above; but after the $K^+$ stimulation period ended (2 min), cells were quickly fixed following a non-permeant protocol (see Section 2). Then, cells were exposed to DBH antibodies that bind to DBH of chromaffin vesicle membranes fixed while they were suffering exocytosis. A secondary fluorescent antibody decorates the cell surface, the fluorescence being proportional to the rate of exocytosis [6]. See in Fig. 4a the lack of membrane fluorescence of an unstimulated cell, and the drastic increase in fluorescence of a cell exposed to 70 mM $K^+$ during 2 min. The confocal image in this cell was taken at an equatorial plane.

A Z-series of confocal images were taken at 1-μm interval in 10 cells (209 confocal sections) stimulated with $K^+$ and stained with DBH antibodies. Cells were divided in three thirds, and the image fluorescence grouped and averaged. The top third images in each individual cell were normalised to 100%, and the fluorescence obtained in the middle and bottom thirds expressed as per cent of the top third. Fig. 4b shows that the middle confocal planes had doubled the fluorescence (197%), and the planes of the bottom third tripled the DBH fluorescence with respect to the top third (297%). Thus, these results corroborate those obtained with FM1-43 and support the view that exocytosis evoked by $K^+$ depolarisation occurs more intensely in the pole through which chromaffin cells attach to the coverslip. In a recent study [6] DBH antibodies and confocal microscopy were used. However, Z-series were not practised and thus, conclusions on regional distribution of specialised sites for exocytosis and polarisation could not be established.

## 4. Discussion

It is interesting that chromaffin cells in culture, in spite of being spherical, maintain a strong polarity to secrete their catecholamines. And it is puzzling why cells decided to develop their polarity towards their attachment pole in the coverslip cell surface. One possibility is that endothelial cells, which grow with chromaffin cells in culture [22], might send a signal to address the secretory pole near them. This makes sense, if we consider the likely situation in the intact adrenal gland.

Contrary to their spherical shape in culture, chromaffin cells in situ adopt a columnar or polygonal shape. Morphological evidence suggests that the distribution of the various organelles is not completely random and so, chromaffin cells seem to be polarised within the adrenal medulla. The polarisation appears to take place with respect to nerve endings and the nucleus on one side of the cell, and blood vessels, typically sinusoids, on the other side of the cell [7]. In fact, secretory vesicles have been reported to be more numerous near the sinusoids and endothelial cells, and to be located more densely in one pole of the cell, opposite to the nucleus [23]. Since the discovery of EDRF-nitric oxide [24], endothelial cells have become a rich source of regulatory factors; some of these factors might exert 'trophism' for the exocytotic machinery so that it becomes polarised in chromaffin cells. This organisation is highly suitable for rapid exocytosis during stress, with nervous stimulation approaching one end of the cell and the catecholamines discharged at the other, near the vessels. Before this study, there was no functional evidence for discernible polarity within these cells. We have therefore provided the first functional evidence for such polarisation that, interestingly, is also expressed in spherical chromaffin cells maintained in primary cultures.

In addition to these physiological implications, our study also has important methodological relevance. Chromaffin cells in culture are widely used in many laboratories all over the world as models to study at the single-cell level the regulation of their voltage-dependent $Ca^{2+}$ channels [25], $Ca^{2+}$ gradients [26] and exocytosis measured either with capacitance [27] or amperometric techniques [28]. In fact, due to their easy preparation and durability in primary cultures [8], and their nice spherical shape, they have served as excellent models for pioneering studies of mammalian neurosecretory cell currents using patch-clamp techniques [29,30]. Since bovine chromaffin cells express L, N, and P/Q subtypes of voltage-dependent $Ca^{2+}$ channels, the possibility exists that some of these channels and the $[Ca^{2+}]_i$ local signals generated by their activation might also be highly polarised. Our findings will strengthen the motivation for novel studies on $Ca^{2+}$ and exocytotic microdomains in polarised chromaffin cells, as well as the search for the factors that make chromaffin cells to become polarised to secrete their catecholamines. The methodological relevance of our findings is illustrated with the example of the puzzling findings of Neher's laboratory commented in Section 1. This suggests that from now onwards, all single-cell studies on $Ca^{2+}$ gradients and exocytosis in chromaffin cells must take into consideration the strong polarity that these cells exhibit when they are maintained in primary cultures.

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