OFFICE OF NAVAL RESEARCH

GRANT N00014-91-J-1784

R&T CODE 413v002

Technical Report No. 5

ZONES OF EXOCYTOTIC RELEASE ON BOVINE ADRENAL MEDULLARY CELLS IN CULTURE

by

Timothy J. Schroeder, Jeffrey A. Jankowski, Jan Senshyn, Ronald W. Holz, and R. Mark Wightman

Prepared for Publication in the

Journal of Biological Chemistry

Department of Chemistry
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290

July 5, 1994

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704 0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 5, 1994	Technical - 6/1993 - 6/1994

4. TITLE AND SUBTITLE
Zones of Exocytotic Release on Bovine Adrenal Medullary Cells in Culture

5. FUNDING NUMBERS
N00014-91-J-1784

6. AUTHOR(S)
T.J. Schroeder, J.A. Jankowski, J. Senyshyn, R.W. Holz, and R.M. Wightman

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Chemistry
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290

8. PERFORMING ORGANIZATION REPORT NUMBER
ONR Technical Report V

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5000

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Submitted to Journal of Biological Chemistry

12a. DISTRIBUTION / AVAILABILITY STATEMENT
This document has been approved for public release and sale; its distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Exocytotic secretion of catecholamines from bovine adrenal medullary cells in culture was examined by amperometry with 1-μm radius carbon-fiber electrodes. Secretion is observed as a series of current spikes which originate from a single cell upon exposure to a secretagogue. The small size of the electrodes was exploited to detect and map exocytotic release sites on the surface of bovine adrenal medullary cells. The release sites are spatially stable on a time-scale of 15 minutes. These quantitative studies reveal for the first time that release sites are spatially localized on endocrine cells in culture. Confocal Microscopy experiments which fluorescently monitor the deposition of dopamine-β-hydroxylase (DβH) from the vesicular membrane into the plasma membrane during exocytosis verify the existence of zones of exocytotic activity and inactivity on the surface of the cell. Measurement of coincident spikes with two adjacent electrodes has allowed the spatial resolution of the electrodes to be defined. Microelectrodes with radii of approximately 2.5 μm detect only spikes resulting from exocytotic events occurring within 2 μm of the projected circumference of the electrode on the surface of the cell. In such a small sampling domain, point-source release and diffusional broadening would result in narrow spikes, all with similar $t_{1/2}$ values (Schroeder, et al. 1992), a feature not seen in the data. Thus for bovine adrenal medullary cells, release following exocytotic fusion is not instantaneous, but is a dynamic event occurring over several milliseconds. It therefore follows that the exocytotic release of catecholamine and/or transport to the electrode is governed by a process that is slower than diffusion.

14. SUBJECT TERMS			15. NUMBER OF PAGES
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	

ZONES OF EXOCYTOTIC RELEASE ON BOVINE ADRENAL MEDULLARY CELLS IN CULTURE

Timothy J. Schroeder*, Jeffrey A. Jankowski*,

Jan Senyshyn†, Ronald W. Holz†, and R. Mark Wightman*‡

*Department of Chemistry

University of North Carolina at Chapel Hill

Chapel Hill, North Carolina 27599-3290

†Department of Pharmacology

University of Michigan

Ann Arbor, Michigan 48109

‡ To whom correspondence should be addressed.

RUNNING TITLE: Release zones on adrenal medullary cells

SUMMARY

Exocytotic secretion of catecholamines from bovine adrenal medullary cells in culture was examined by amperometry with 1-μm radius carbon-fiber electrodes. Secretion is observed as a series of current spikes which originate from a single cell upon exposure to a secretagogue. The small size of the electrodes was exploited to detect and map exocytotic release sites on the surface of bovine adrenal medullary cells. The release sites are spatially stable on a time-scale of 15 minutes. These quantitative studies reveal for the first time that release sites are spatially localized on endocrine cells in culture. Confocal Microscopy experiments which fluorescently monitor the deposition of dopamine-β-hydroxylase (DβH) from the vesicular membrane into the plasma membrane during exocytosis verify the existence of zones of exocytotic activity and inactivity on the surface of the cell. Measurement of coincident spikes with two adjacent electrodes has allowed the spatial resolution of the electrodes to be defined. Microelectrodes with radii of approximately 2.5 μm detect only spikes resulting from exocytotic events occurring within 2 μm of the projected circumference of the electrode on the surface of the cell. In such a small sampling domain, point-source release and diffusional broadening would result in narrow spikes, all with similar $t_{1/2}$ values (Schroeder, et al. 1992), a feature not seen in the data. Thus for bovine adrenal medullary cells, release following exocytotic fusion is not instantaneous, but is a dynamic event occurring over several milliseconds. It therefore follows that the exocytotic release of catecholamine and/or transport to the electrode is governed by a process that is slower than diffusion.

INTRODUCTION

Neurons have specialized sites or active zones where exocytosis occurs (Heuser and Reese, 1977). These zones are characterized by a high local concentration of intracellular vesicles and a specialized presynaptic region, as well as specialized postsynaptic regions on adjacent cells (Walrond and Reese, 1985). These regions are of interest because they must contain the biochemical components which are necessary for exocytosis. For example, the density of calcium channels in neurons has been shown to be high at active zones (Augustine et al., 1989; Adler et al., 1991). Less information is available concerning specific release sites in endocrine, exocrine and other cells with a regulated secretory pathway. Secretory cells are often polarized with regulated secretion oriented to a lumen as with pancreatic acinar cells or to blood vessels as with adrenal medullary cells. However, the precise localization of release sites on the cell surface of non-neuronal cells has not been demonstrated. Furthermore, maintenance of primary cells in tissue culture may alter the orientation and localization of release sites in cells. Prior studies using anti-dopamine-β-hydroxylase antibodies to mark exocytotic sites on cultured adrenal chromaffin cells suggest that the sites are randomly located across the surface (Hesketh et al., 1981; Phillips et al., 1983). The present study extends these early findings with two new, high-resolution techniques that demonstrate that release sites of several microns or less exist on bovine adrenal chromaffin cells maintained in primary culture.

Previous work has shown that carbon-fiber microelectrodes can be used as sensors for real time detection of exocytotic release of the catecholamines, epinephrine and norepinephrine, from single bovine adrenal medullary cells (Leszczyszyn et al., 1990, 1991; Wightman et al., 1991; Kawagoe et al., 1991;

Ciolkowski et al., 1991; Chow et al., 1992; Jankowski et al., 1992; Schroeder et al., 1992; Jankowski et al., 1993). Most previous reports have employed carbon fibers with diameters of 10 μm or greater, and thus the size of the electrode approaches the size of the biological cell. However, the development of electrodes with micron (Kawagoe, et al., 1991) or smaller diameters (Strein, et al, 1991) provides the opportunity to probe the cell surface for the existence of localized region from which release occurs. As shown in this work, these electrodes reveal the existence of active and inactive release zones. High resolution confocal microscopic fluorescent images of exocytotically deposited DβH in the cell membrane also reveal zones of release which are of similar dimensions to those revealed by the dynamic electrochemical measurements.

EXPERIMENTAL PROCEDURES

Electrode Construction. A single carbon fiber (5-μm radius, Thornell P-55, Amoco Corp., Greenville, SC) was aspirated into a microfilament capillary (A-M Systems, Inc., Everett, WA). The glass was drawn around the fiber with a micropipet puller (Narishige, Tokyo, Japan). The exposed portion of the carbon fiber was cut with a scalpel leaving a length of approximately 100-500 μm extending beyond the tip. This was passed through the base of an air-methane flame (Strein, et al.; 1992) resulting in a carbon cone of 50-200 μm length with a sub-micron radius at the end of the fiber.

Electrical connection with the carbon fiber was obtained by back-filling the glass capillaries with colloidal carbon (Polysciences, Inc., Warrington, PA) and inserting a chromel wire in the open end of the capillary. An insulating layer of poly(oxyphenylene) was electrochemically deposited on the exposed carbon surface (Kawagoe, et al., 1991). This was accomplished by oxidation of 90 mM 2-allylphenol, 90 mM allylamine, and 2% 2-butoxyethanol (V/V) in 1:1 (V:V) water-methanol for 5 to 10 minutes with a potential of +4 volts applied to the carbon fiber. The thickness of the insulating film is estimated to be 1 μm (Strein et al., 1991). The fibers were then immediately rinsed with distilled water and cured at 150° C for 30 - 60 minutes. Insulation was removed from the tip of the etched electrode by polishing for 5 s on a micropipet beveler (Model BV-10, Sutter Instrument Co., Novato, CA) at an angle of approximately 45°. The electrodes were rinsed with distilled water and soaked in isopropanol for 10-30 minutes.

Estimates of the tip radius were made electrochemically. Under steady-state conditions the limiting current (i) at a microelectrode is given by:

$$i = 4nFDCr \quad (1)$$

where n is the number of electrons per mole (n = 2 for catecholamines), F is Faraday's constant (96,485 coulombs/equivalent), D is the free solution diffusion coefficient of catecholamine (6×10^{-6} cm^2/s), C is the concentration of catecholamine in the solution, and r is the radius of the electrode. For an elliptical electrode as in this case the radius determined electrochemically is the average of the major and minor radii (Kelly et al., 1986). Failure to achieve a value of r consistent with measurement by optical microscopy was taken to mean that the polymer insulation was not intact. Such electrodes were discarded.

Amperometry employed a potentiostat (EI-400, Ensman Instrumentation, Bloomington, IN) operated in the three-electrode mode. The reference electrode was a saline saturated calomel electrode (SSCE), and the auxiliary electrode was a 26-gauge chromel wire. The potential of the working electrode was held at 0.65 V versus SSCE. Electrode sensitivity was determined before and after each experiment with 100 μM epinephrine. The output signal was filtered at 16 kHz, digitized with a VCR adaptor (Model PCM-2, Medical Systems Corp., Greenville, NY) and recorded on videotape.

Single-Cell Secretion Experiments. Primary cultures of bovine adrenal medullary cells were prepared from fresh tissue (Leszczyszyn, et al; 1990). The cells were enriched in epinephrine and cultured as previously described at a density of 6×10^5 cells per 35-mm diameter plate (Leszczyszyn, et al; 1990). Experiments were performed at room temperature (23.0° ± 0.1° C) between days 4 and 10 of culture.

For the experiments which employed Ba^{2+} as the secretagogue, the culture

media was replaced with a solution containing 154 mM NaCl, 4.2 mM KCl, 11.2 mM glucose, 0.7 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). Sodium phosphate was not employed to prevent precipitation of $Ba_3(PO_4)_2$. In the pipet solution containing 2 mM Ba^{2+}, the concentration of NaCl in the puffer solution was decreased by 4 mM to maintain a constant ionic strength. For experiments with K^+ or nicotine as secretagogues, the bath solution also contained 1.0 mM Na_2HPO_4 and 2 mM $CaCl_2$. When 60 mM K^+ was used to stimulate the cell, the concentration of NaCl in the puffer solution was decreased to 90 mM. Nicotine puffer solutions contained 100 μM nicotine dissolved in the bath solution.

Secretagogues were applied to the cells with pressure ejection (Picospritzer, General Valve Corp., Fairfield, NJ) from micropipettes (≈10-μm outside tip diameter). The micropipette was positioned 40-50 μm from the cell surface and at an angle of approximately 80° with respect to the flame-etched electrodes. Typical ejection pressures were 5-10 psi.

The culture plates were viewed with an inverted-stage microscope (Axiovert 35, Zeiss, Thornwood, NY). The flame-etched carbon-fiber microelectrodes, with the exposed surface parallel to the cell plate, were positioned above a single cell with a piezoelectric device (PCS-250 Patch Clamp Driver, Burleigh Instruments, Fishers, NY). The electrode-cell spacing was determined by lowering the electrode onto the cell until the cell membrane was visually, elastically deformed. The electrode was then raised and lowered in small increments to determine the position at which the cell membrane just began to be deformed. This location was defined as the cell surface. The electrode was then raised to the desired position above the cell. The two electrodes were positioned at an angle of approximately 160° apart relative to each other (see Figure 1).

Analysis of Coincident Spikes. Recorded data were replayed through an eighth-order, low-pass (400 Hz) filter (CyberAmp 320, Axon Instruments, Foster City, CA), digitized at a rate of 1 ms/point via a commercially available software package (Axotape, Axon Instruments, Foster City, CA), and stored on the hard-drive of a PC compatible computer. Locally written software was used to remove residual 60 Hz noise, locate spikes, and extract their characteristics: peak amplitude, half-width, area, half-rise time, maximum current time, onset time, and ending time. A signal was designated as a spike if the amplitude was greater than five times the value of the rms noise measured over a baseline of 83 ms.

Confocal Microscopy. Chromaffin cells were incubated in physiological salt solution (PSS, 145 mM NaCl, 5.6 mM KCl, 0.5 mM $MgCl_2$, 15 mM HEPES, pH 7.4 and 5 mg/ml BSA) containing either 2.2 mM $CaCl_2$ (FIGURE 7a) or 1 mM $BaCl_2$ (FIGURE 7b, 7c) at 25° C. After 9 minutes, the solutions were removed and replaced with PSS with 2.2 mM $CaCl_2$ at 4° C to stop exocytosis. Cells were incubated for 1.5 hours in the cold with goat anti-DβH antiserum (1:1500 dilution in PSS with 2.2 mM $CaCl_2$), fixed for 10 minutes with 4% paraformaldehyde (in divalent ion-free PBS), quenched with 50 mM NH_4Cl (in divalent ion-free PBS with 0.1% gelatin), and then incubated for 1 hour with swine anti-goat, FITC-IgG (1:50, Boehringer-Mannheim, in divalent ion-free PBS with 0.1% gelatin). The fluorescence was investigated with a BioRad MRC600 Laser Scanning Confocal Microscope with a 100X objective (numerical aperture 1.4). The optical section thickness was estimated to be 0.7 μm from the point spread function of the intensity in the Z axis created by a fluorescent 0.1 μm diameter bead.

Reagents. The culture medium, Dulbecco's Modified Eagle's Medium/Ham's

F12 Medium, was obtained from Gibco Laboratories (Grand Island, NY). Collagenase (Type I) for digestion of glands was obtained from Worthington Chemicals (Freehold, NJ). Renografin-60 was purchased from Squibb Diagnostics (New Brunswick, NJ). All other chemicals were reagent grade from Sigma (St. Louis, MO), and solutions were prepared with doubly distilled water.

RESULTS

Surface Separation of Exocytotic Sites. The small size of flame-etched carbon-fiber microelectrodes (≈1-μm radius) should provide an increased spatial resolution in the detection of release sites at individual biological cells compared to the carbon-fiber microelectrodes (6-μm radius) that have been used predominantly in prior work. In initial experiments, several cells appeared not to release catecholamine upon stimulation when monitored with a flame-etched electrode. Yet, when a larger electrode was placed adjacent to the same cell, release was detected in the form of current spikes. This suggested the existence of active and inactive release zones at the surface of these cells. This phenomena was examined in fourteen cells, in which eight of them displayed zones which did not exhibit exocytosis. In this work Ba^{2+} was employed as a secretagogue since it causes continuous release for several minutes. As shown in FIGURE 1 and FIGURE 2, the detection of release induced by Ba^{2+} was dependent on the specific position of the electrode above the cell surface.

In the experiment shown in FIGURE 1, two electrodes (radii of 0.9 and 1.1 μm, respectively, for electrodes "A" and "B") were initially located above the top of the cell with the electrodes approximately 2 μm apart. Before stimulation neither electrode exhibited release (see the first portion of FIGURE 1). When the cell was exposed to Ba^{2+} by a 5-s pressure ejection (denoted by the arrow), small amplitude, infrequent spikes were seen at each electrode. Electrode "A" was then moved to a region near the perimeter of the cell. Spikes were still occurring as a result of the prior Ba^{2+} exposure. Reexposure of the cell to Ba^{2+} (second arrows Figure 1) resulted in low amplitude spikes monitored by electrode "B", similar to the first stimulation,

whereas the release from the cell region monitored by electrode "A" increased in amplitude and frequency relative to that obtained at its prior location.

Next, electrode "B" was moved from the top of the cell and positioned near the perimeter of the cell, but on the opposite side from electrode "A". While release monitored by electrode "A" remained vigorous, the frequency of spikes detected with electrode "B" was low (third portion of FIGURE 1). Thus, at this cell, release sites are not uniformly distributed around the perimeter. When electrode "B" was positioned adjacent to electrode "A" and the cell was restimulated with Ba^{2+} (denoted by the arrow in the fourth portion of FIGURE 1), both electrodes monitored robust release characterized by the detection of many spikes from this portion of the cell.

Finally, electrode "A" was moved to a different site on the perimeter of the cell (last portion, FIGURE 1). Although spikes were still recorded at electrode "B" as a result of the prior exposure to barium, at the new position of electrode "A", the activity monitored was negligible. Restimulation of the cell with Ba^{2+} (denoted by the last arrows) yielded large amplitude, high frequency spikes at electrode "B", but little activity at electrode "A". Thus, these experiments reveal that this cell had at least one zone of active release near the perimeter of the cell, while the other regions of the cell surface examined did not exhibit exocytosis.

An experiment with the same electrodes at a different cell is shown in FIGURE 2. Initially both electrodes were positioned directly above the top of the cell with the outside insulation of the two electrodes physically in contact. Neither electrode detected any release from the cell (first portion of FIGURE 2) until a 5-s exposure to Ba^{2+} (arrows in the first portion of FIGURE 2) which caused spikes of relatively large amplitude at both

electrodes. As release continued, electrode "A" was moved to a region near the perimeter of the cell. The spikes detected by electrode "B" continued, but the release detected by electrode "A" decreased (second portion of FIGURE 2), and reexposure to Ba^{2+} did not increase release.

When electrode "A" was moved toward the top of the cell (2 μm from electrode "B") and the cell was restimulated with Ba^{2+} (denoted by the arrow in the third portion of FIGURE 2), release from the cell region monitored by electrode "B" remained robust, while the released spikes detected by electrode "A" were low amplitude and infrequent (third portion of FIGURE 2). However, when electrode "A" was positioned at its initial location (touching the other electrode), restimulation of the cell with Ba^{2+} (arrows in the fourth portion of FIGURE 2), induced vigorous release to be monitored by both electrodes. Thus, this cell displayed an active zone for release near the top of the cell.

Another experiment with similar sized electrodes at a different cell is shown in FIGURE 3. Initially electrode "A" was positioned directly above the top of the cell and electrode "B" was positioned near the perimeter of the cell. Neither electrode detected any release from the cell (not shown) until a 5-s exposure to 100 μM nicotine (arrows in the first portion of FIGURE 3) which caused spikes of relatively large amplitude at electrode "A" while electrode "B" detected infrequent small amplitude spikes.

When electrode "A" was moved toward the perimeter of the cell and electrode "B" was positioned over the top of the cell, the cell was restimulated with 100 μM nicotine (denoted by the arrows in the second portion of FIGURE 3). Release from the cell region monitored by electrode "B" became robust, while the released spikes detected by electrode "A" were low amplitude and infrequent (second portion of FIGURE 3). However, when electrode "A" was

positioned at its initial location (touching the other electrode), restimulation of the cell with 100 μM nicotine (arrows in the third portion of FIGURE 3), induced vigorous release to be monitored by both electrodes. Thus, this cell displayed an active zone for release near the top of the cell.

Simultaneous Detection of the Same Spikes. An estimate of the spatial resolution of an amperometric electrode was obtained by placing two flame-etched electrodes (radii of 2.6 and 2.7 μm, respectively, for electrodes "A" and "B") adjacent to a cell and searching for the detection of the same release event at each electrode while varying the distance between them (the geometry is shown in FIGURE 4a). Larger electrodes were used in this study to make detection of spikes more likely and to eliminate the possibility of inactive zones of release interfering with the experiment. We designate spikes to correspond to the same exocytotic event at the cell surface if they meet the following criteria. First, the difference in onset time of two spikes detected with different electrodes (denoted as Δt_o, FIGURE 5) must be less than the time required for the molecules to diffuse to each electrode. This depends on the difference in distance (Δl) from the release site to each electrode ($\Delta t_o = \Delta l^2/6\ D$). Because the diffusion coefficient of released catecholamines may be less than the value in dilute, aqueous solution (6×10^{-6} cm^2/s, Gerhardt et al., 1982), a value of 30 ms was used with the electrodes adjacent to one another, and this amount was increased by 7 ms for each micron of separation. If the onsets of the spikes were not easily identified due to noise on the baseline, the difference in time between the spike maxima were used (Δt_m, FIGURE 5c). When Δt_m was used rather than Δt_o, an additional 10% of time was allowed, because a spike becomes shorter and wider with an increase in diffusional distance resulting in an increased Δt_m.

The second criterion was that the spike with the later onset time should be shorter and wider than the first because of the greater time for diffusional broadening of the catecholamine packet. Examples of spikes which satisfy these criteria are shown by the overlying spikes in FIGUREs 5c and 6c. Approximately 20% of the spikes which satisfied the first criterion based on spike lag time failed to satisfy the second criterion.

The final criterion to satisfy coincidence was that spikes plotted in normalized coordinates (each spike normalized with respect to its own half-width and maximal current) must have the same shape. An example of a pair of spikes having the same normalized shape is shown by the overlying normalized spikes in FIGURE 6d. A summary of the experimental results is given in Table 1. A significant number of coincident spikes is only ɔn with no spacing or a gap of 2 μm between the electrodes.

Confocal Microscopy. The heterogeneity of release sites was also investigated with confocal microscopy following DβH immunocytochemistry and fluorescence labelling. An unstimulated cell is shown in Figure 7a which exhibited no apparent release sites in any of the images. A quite different appearance is seen from a cell stimulated with Ba^{2+} for 9 minutes (Figure 7b and 7c). The presence of DβH is revealed as 0.3-0.6 μm diameter spots on the surface of the cell when viewed at its upper surface or in cross section. The image in Figure 7B shows many release sites because of the large area of surface membrane visualized. Punctate DβH was apparent at the cell surface in all images of the z-axis series. The images in Figure 7b and c are typical of images obtained from nine cells. All showed similar punctate appearance of surface DβH with some cells showing a greater or lesser frequency of punctate DβH sites across the cell surface.

DISCUSSION

The results shown in this paper reveal, by two independent techniques, the presence of zones on the surface of cultured bovine adrenal medullary cells where exocytosis does not occur. Initially, we demonstrate that the localization of exocytotic sites can be revealed with the use of micron-sized electrodes, a sensor which provides a real time view of exocytosis. Additionally, the use of *DβH* immunocytochemistry with confocal micro reveals regions of the cellular membrane where exocytosis does not oc This second technique provides an integrated view of exocytosis since it is based on the quantity of an exocytosed material which accumulates on the surface during the interval of exposure to a secretagogue.

Studies using these two high resolution techniques have resulted in significant new insights into the process of exocytosis. First, we demonstrate that release sites are localized in clusters on these endocrine cells in culture. Although this has long been assumed to be the case *in vivo*, our amperometric results provide the first quantitative experimental data to demonstrate and verify this belief for cultured cells. Second, we demonstrate that these sites are spatially immobilized with respect to the cellular membrane on a time scale of 15 minutes. When compared to the millisecond-time scale for exocytosis, this prolonged heterogeneity shows that the components or structures responsible for localized release must be stably and intimately associated with release sites. Third, we show through spatial localization of the release sites and quantitative analysis of the spike shape that catecholamine release from the cell surface after fusion of the granule membrane is limited by a process other than diffusion. This concept is new, and should instigate new lines of thinking and research concerning the process

of exocytosis. These results are discussed individually in the following paragraphs.

The use of micron-sized electrodes reveals that at some cells the sites of exocytosis are not uniformly distributed across the cell surface. The release zones are found at different locations on the cell surface and vary from cell to cell. The sites of release appear to be stationary on a time scale of several minutes. Thus, an electrode can be moved from a site with no release activity to a release zone, returned at a later time, and the lack of release is still observed. Zones which are inactive with respect to release were characterized by a lack of spikes (or a very low frequency occurrence) upon stimulation. The presence of large amplitude spikes seen in some of the traces indicate that the release events are located directly beneath the electrode surface, whereas small spikes indicate that the location of exocytosis is at a more remote location. The presence of active and inactive zones of release appears to be independent of the secretagogue employed. Experiments employing 100 μM nicotine, 2 mM Ba^{2+}, and 60 mM K^{+} (data not shown) all display evidence of localizations of release zones.

Carbon-fiber electrodes of larger dimensions (6-μm radius) have been used in most prior studies of exocytosis at individual adrenal medullary cells. Because their physical dimensions are similar to those of the cells (8-10 μm radius), the larger electrodes do not have the spatial resolution required to detect the presence of active and inactive release zones on the cell surface. In contrast, the dimensions of the flame-etched electrodes used in this work ($\approx$1-μm radius) provide the ability to monitor much smaller areas of the cell surface. The increased spatial resolution thus allows the first opportunity to observe the localization of release zones on the cell surface

with amperometric electrodes.

The detection of the same exocytotic event with two different electrodes has enabled the spatial resolution of the amperometric electrodes to be defined. When the electrodes are adjacent, so that the thickness of the insulation determines the gap between the electrode, a significant fraction (22%) of the spikes detected by either electrode were coincident. The number of coincident spikes detected by either electrode dramatically decreases when the electrodes are further apart. The experiment can be geometrically approximated with two circular disks (the electrode surfaces) surrounded by 1 μm of insulation (FIGURE 4b, c). The overlapping sampling area (A) can be shown to be:

$$A = 4 \times \int_{r_s}^{R} \sqrt{R^2 - x^2}\, dx \qquad (2)$$

where R is the radius of the circle which defines the sampling region for a single electrode (to be determined) and r_s is the sum of the radius of the carbon surface, the insulation thickness, and one-half of the separation distance between the electrodes. If it is assumed that there is an equal probability of exocytosis anywhere in the region given by πR^2 on the cell surface, then the fraction of coincident spikes detected by an electrode (P) is:

$$P = \frac{A}{\pi R^2} \qquad (3)$$

Solving equation 2 yields:

$$P = \frac{\pi R^2 - 2 r_s \sqrt{R^2 - r_s^2} - 2 R^2 \sin^{-1} (\frac{r_s}{R})}{\pi R^2} \qquad (4)$$

Numerical evaluation of R with the measured values of P shows that an electrode can sample from a region approximately 1.5 to 2 μm beyond its projected circumference on the cell surface (FIGURE 4b, 4c). Thus, the experimental results are consistent with the geometrical expectations, and the spatial resolution is defined.

Since the spikes must originate from a region of the cell surface beneath the electrode, an estimate can be made of the separation between release sites on the cell membrane. In cells with localized release, the sites must be at least 2 μm apart. This estimate is similar to that seen with the fluorescent confocal microscopy. The latter results demonstrate the feasibility of resolving and analyzing release sites visualized by DβH immunocytochemistry. The protocol detects only DβH exposed to the extracellular medium because incubation with the primary antibody precedes fixation.

Evaluation of the spatial resolution of the amperometric probes also enables temporal characteristics of the spikes to be evaluated. Previously we have attempted to describe the exocytotic events as instantaneous point sources of catecholamine packets on the cell surface which are transported to the electrode by diffusion (Schroeder et al., 1992). However, evaluation of the data in this way requires a knowledge of the range of distances that the concentration packet must travel. Since this study shows that spikes must originate within 2 μm of the electrode surface, the diffusion based model predicts that the maximum half-width should be less than 10 ms when the

electrodes are placed 1 μm from the cell surface and the value of the solution diffusion coefficient is employed. Experimentally, more than 50% of the spikes observed with a 1-μm radius electrode have half-widths greater than 20 ms (Jankowski, et al., 1993). Thus, release at the cell surface must not be instantaneous and/or transport of the catecholamine packets to the electrode must be governed by a much slower rate of diffusion. Concurrent appearance of multiple spikes (compound exocytosis) could lead to a broadened measurement. However, our prior work indicates that each spike is the detection of the content of a single vesicle (Wightman, et al., 1991). Thus it appears more likely that the dissociation of the vesicle contents prior to release occurs over a finite period of time as concluded previously (Jankowski, et al., 1993).

TABLE 1. Number of coincident spikes measured at bovine adrenal medullary cells as a function of separation between the tips of the amperometric electrodes. Cells were stimulated by pressure ejection of 100 μM nicotine for 3 s. Two electrodes were placed at the top of the cell, 1 μm from the cell surface with a varying gap distance (d) between them (see FIGURE 3a). Electrodes "A" and "B" had radii of 2.6 and 2.7 μm, respectively. Only cells which did not exhibit localized release phenomena were used in this study. Data were filtered at 400 Hz. The data presented here are from a single cell, however similar behavior was observed at 13 other cells.

Gap distance	0 μm	2 μm	4 μm	6 μm	8μm
Coincident Spikes	50	10	6	2	3
Total Spikes electrode "A"	242	111	127	80	53
Total Spikes electrode "B"	219	130	109	64	60
Average # Spikes at "A" and "B"	230.5	120.5	118	144	56.5
Percent Coincident Spikes	21.7%	8.3%	5.1%	2.8%	5.3%

REFERENCES

Adler, E. M., Augustine, G. J., Duffy, S. N., Charlton, M. P. (1991) *J. Neuroscience*. 11(6), 1496-1507.

Augustine, G. J., Buchanan, J., Charlton, M. P., Osses, L. R., Smith, S. J. (1989) *Secretion and Its Control*, eds. Oxford, G. S. and Armstrong, C. M., pp 203-223. New York: Rockefeller UP.

Chow, R.H., Ruden, R. von. Neher, E. (1992) *Nature*. 356, 60-63.

Ciolkowski, E.L., Cooper, B.R., Jankowski, J.A., Jorgenson, J.W., Wightman, R.M. (1992) *J. Am. Chem. Soc*. 114, 2815-2821.

Gerhardt, G., Adams, R. N. (1982) *Anal. Chem*. 54, 2618-2620.

Hesketh, J.E., Ciesielski-Treska, J. Aunis, D. (1981) *Cell Tissue Res*. 218, 331-343.

Heuser, J.E., Reese, T. S. (1977) *Handbook of Physiology, Section 1: The Nervous System Vol 1: Cellular Biology of Neurons*, ed. Kandel, E. R. (Am. Physiol. Soc., Washington) pp. 261-294.

Jankowski, J.A., Schroeder, T.J., Holz, R.W., Wightman, R.M. (1992) *J. Biol. Chem*. 267, 18329-18335.

Jankowski, J.A., Schroeder, T.J., Ciolkowski, E.L., Wightman, R.M. (1993) *J. Biol. Chem*. 268, 14694-14700.

Kawagoe, K.T., Jankowski, J.A., Wightman, R.M. (1991) *Anal. Chem*. 63, 1589-1594.

Kelly, R., Wightman, R.M. (1986) *Anal. Chim. Acta*. 187, 79-87.

Leszczyszyn, D.L., Jankowski, J.A., Viveros, O.H., Diliberto, E.J. Jr., Near, J.A., Wightman, R.M. (1990) *J. Biol. Chem*. 265, 14736-14737.

Leszczyszyn, D.L., Jankowski, J.A., Viveros, O.H., Diliberto, E.J. Jr., Near, J.A., Wightman, R.M. (1990) *J. Neurochem*. 56, 1855-1863.

Phillips, J. H. Burridge, K. Wilson, S. P., Kirshner, N. (1983) *J. Cell Biol.* 97, 1906-1917.

Schroeder, T.J., Jankowski, J.A., Kawagoe, K.T., Wightman, R.M., Lefrou, C., Amatore, C. (1992) *Anal. Chem.* 64, 3077-3083.

Strein, T.G., Ewing, A.G. (1992). *Anal. Chem.* 64, 1368-1373.

Walrond, J.P., Reese, T. S. (1985) *J. Neurosci.* 5, 1118-1131.

Wightman, R.M., Jankowski, J.A., Kennedy, R.T., Kawagoe, K.T., Schroeder, T.J., Leszczyszyn, D.L., Near, J.A., Diliberto, E.J. Jr., Viveros, O.H. (1991) *Proc. Natl. Acad. Sci. USA* 88, 10754-10758.

FIGURE LEGENDS

FIGURE 1. Simultaneous amperometric measurements with flame-etched electrodes (≈1-μm radius), positioned 1 μm from the surface of individual bovine adrenal medullary cells. The breaks in the traces indicate the times when one of the electrodes was repositioned. The arrows below the traces indicate 5-s stimulations with 2 mM Ba^{2+}. Traces "A" and "B" correspond to the currents from the respective electrodes shown in the lower diagrams. The diagrams show a top view of the placement of the electrodes at the cell surface. Solid ellipses in the diagrams represent the active carbon surface of the microelectrodes.

FIGURE 2. Simultaneous amperometric measurements as in Figure 1 but at a different cell.

FIGURE 3. Simultaneous amperometric measurements with flame-etched electrodes (≈1-μm radius), positioned 1 μm from the surface of individual bovine adrenal medullary cells (different electrodes than presented in FIGURES 1 and 2). The breaks in the traces indicate the times when one of the electrodes was repositioned. The arrows below the traces indicate 5-s stimulations with 100 μM Nicotine. Other conditions as is FIGURE 1.

FIGURE 4. A) Schematic representation of two flame-etched electrodes positioned at a bovine adrenal medullary cell for experiments involving the change in the number of coincident spikes with a changing gap distance (d) between the electrodes. B) and C) Concentric circle representation of a top view of the overlapping sampling regions of the two electrodes with gap distances (d) of 0 and 2 μm respectively, for B) and C). Darkest inner circle represents the active carbon surface of radius r. Next darkest circle represents the insulation around the electrodes. Dashed circle represents the

experimentally determined sampling region of the electrode with radius R. The overlapping area between the two dashed circles represents the area where the coincident spikes were originating.

FIGURE 5. Simultaneous amperometric current traces measured with flame-etched electrodes (≈2.5-μm radius), positioned 1-μm from the surface of an adrenal medullary cell. The spacing between the two electrode was approximately 0 μm. Trace "A" corresponds to the current detected by electrode "A". Trace "B" corresponds to the current detected by electrode "B". The spikes in trace "A" and trace "B" indicated by the asterisks are superimposed in trace "C". Δt_o is the difference between the onsets of the two spikes. Δt_m is the difference between the peak maxima of the two spikes.

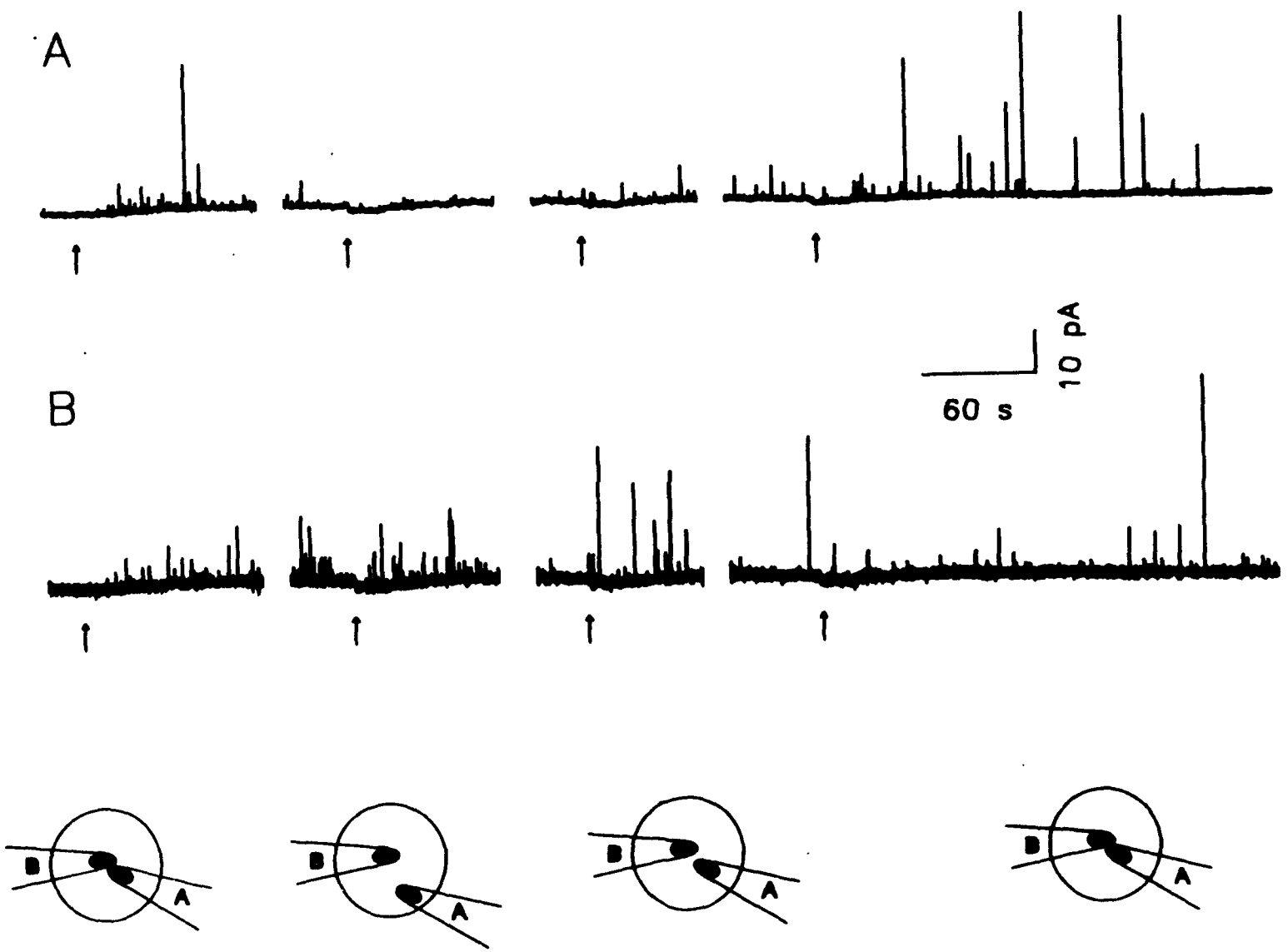
FIGURE 6. Simultaneous amperometric current traces measured with electrodes (≈2.5-μm radius) positioned 1 μm from the surface of an adrenal medullary cell. The spacing between the two electrode was approximately 0 μm. Traces A and B correspond to the currents detected by electrodes "A" and "B", respectively. C: The spikes marked by the asterisks in traces A and B superimposed. D: The same spikes as in C plotted with their axis normalized to their respective width at half height and maximal current.

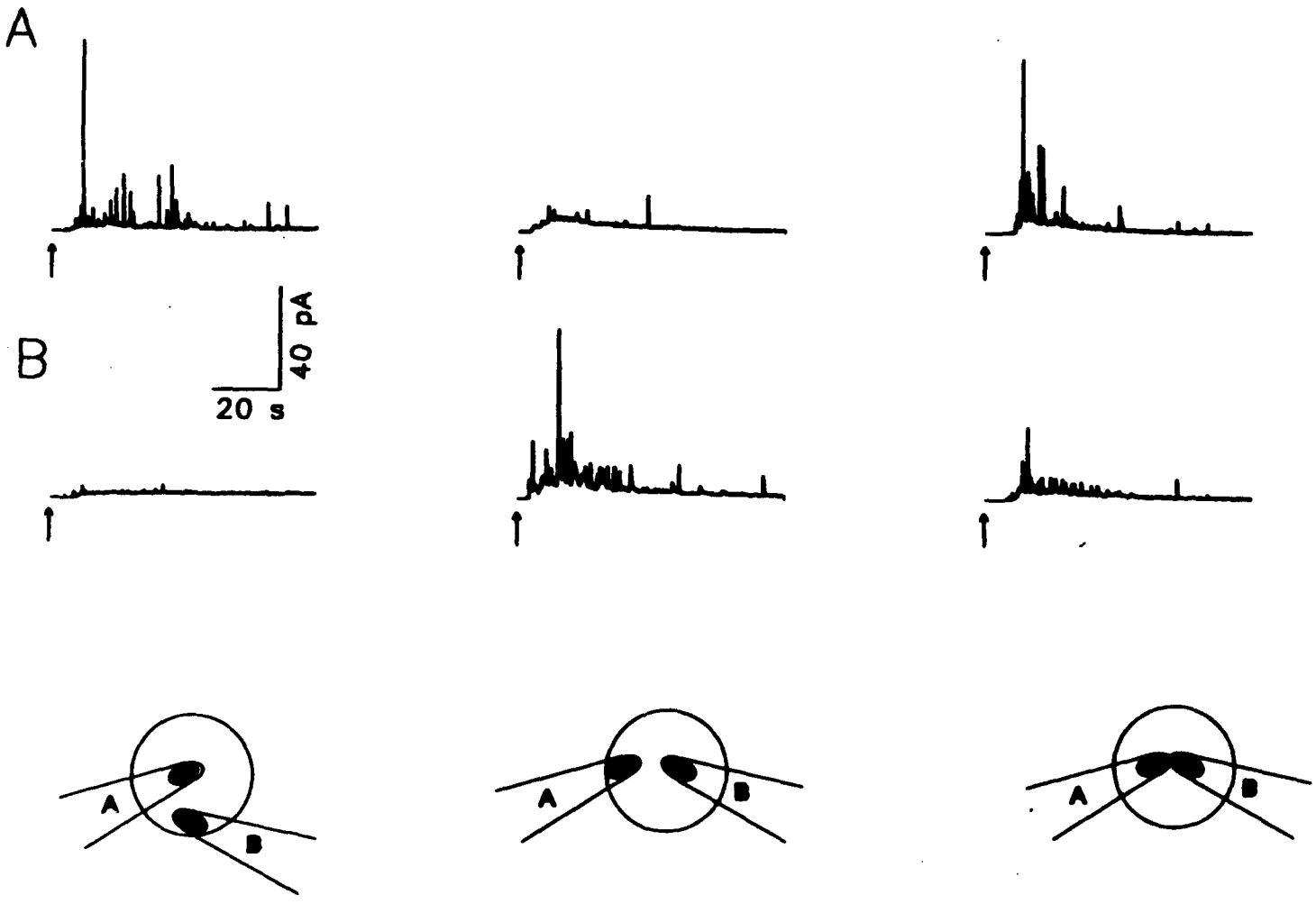
FIGURE 7. Results from fluorescent confocal microscopy after DβH immunocytochemistry. A: Results from an unstimulated cell. B,C: 2 of 7 images perpendicular to the Z-axis of a cell stimulated with Ba^{2+}. Panel B shows the top-most image that demonstrated DβH when the plane of focus was lowered from above the cell. It represents an image of the top surface of the cell. Because the Z-axis resolution was approximately 0.7 μm, the cell surface is not necessarily exactly parallel to the XY plane. Panel C is an image 1.5 μm deeper into the cell. It demonstrates punctate DβH in the

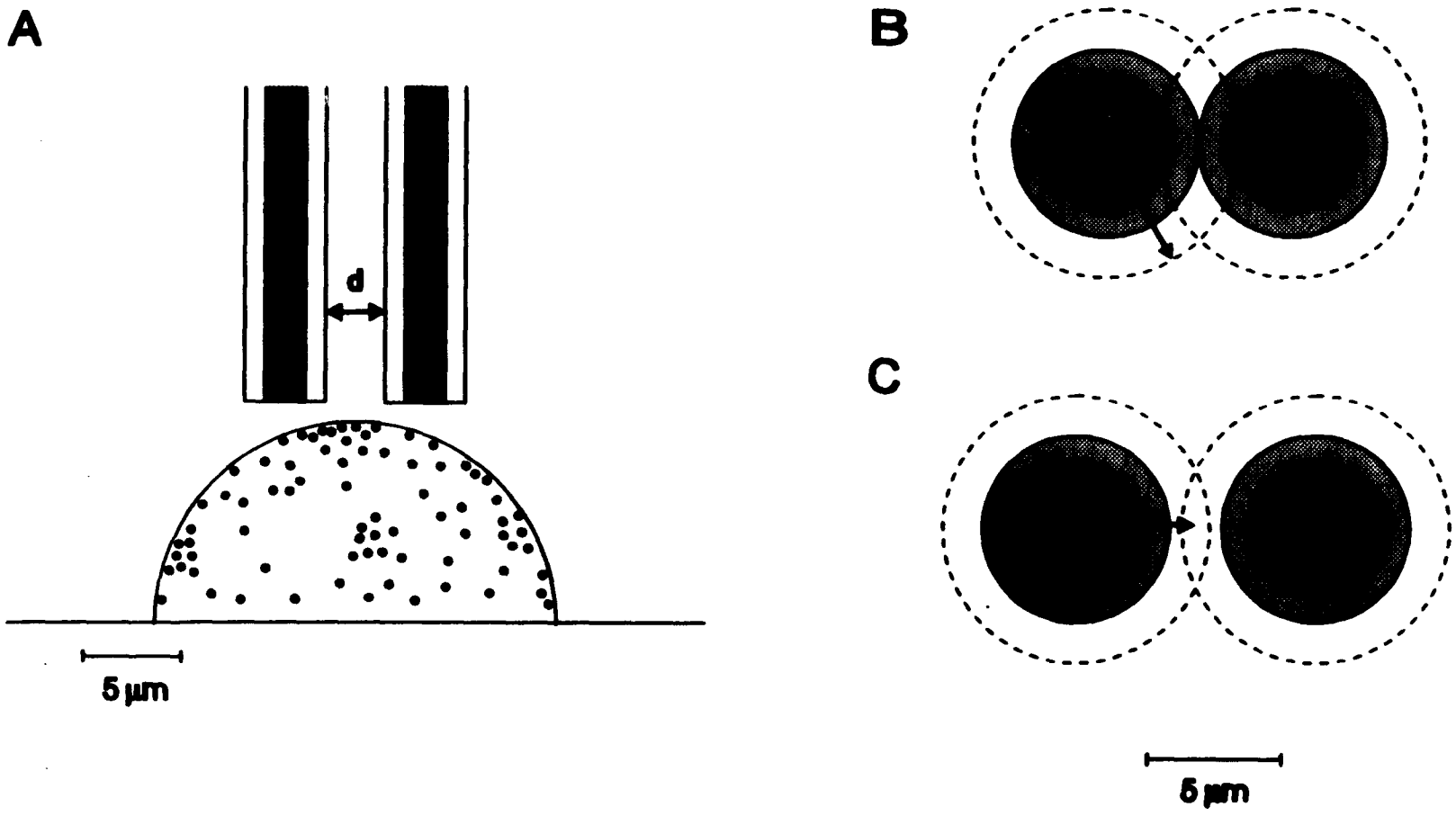
periphery where the section crosses the plasma membrane. Punctate DβH was apparent in all sections of the cell. The box in panel B has dimensions of 2.6 × 1.1 μm.

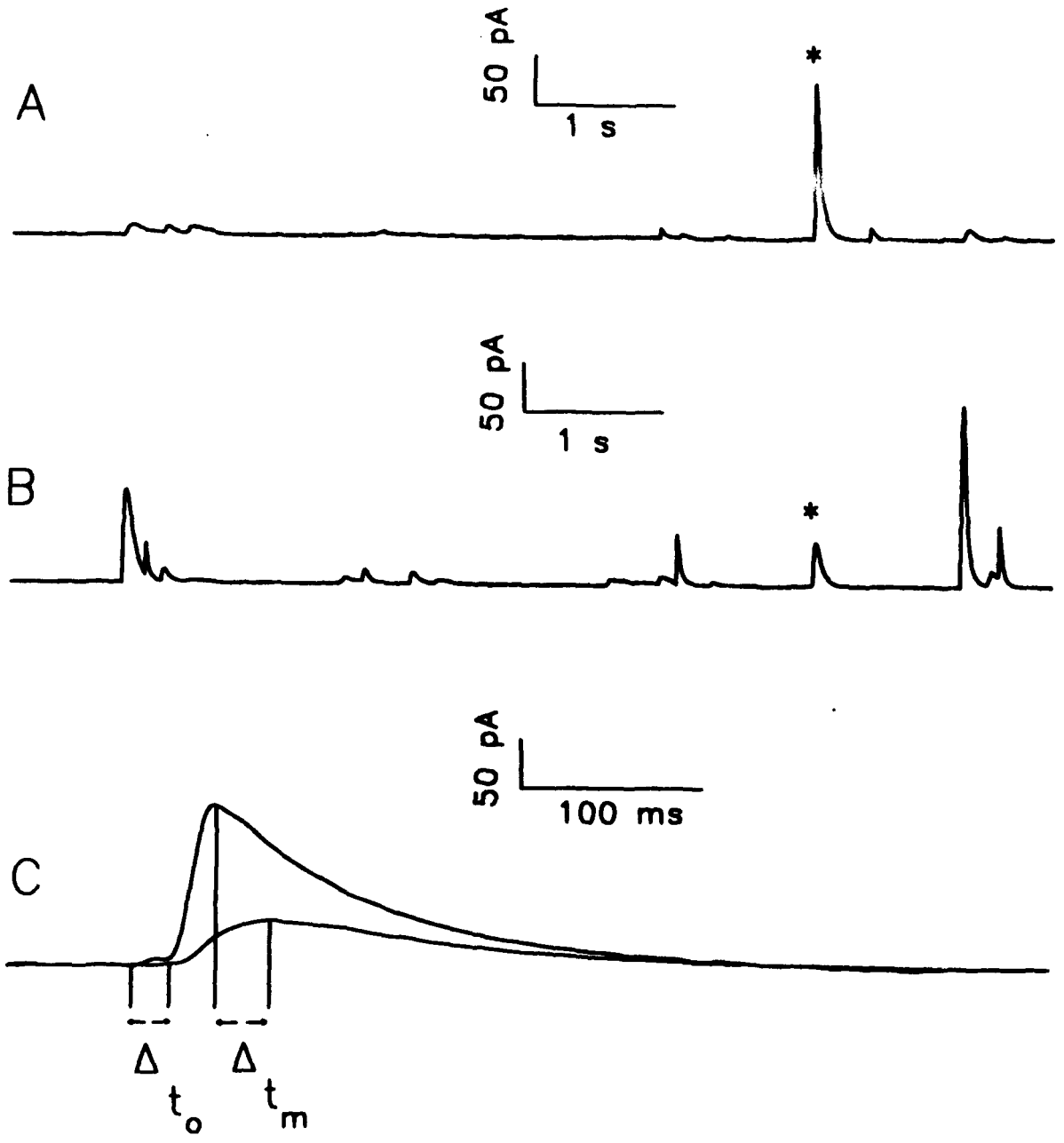
ACKNOWLEDGEMENT

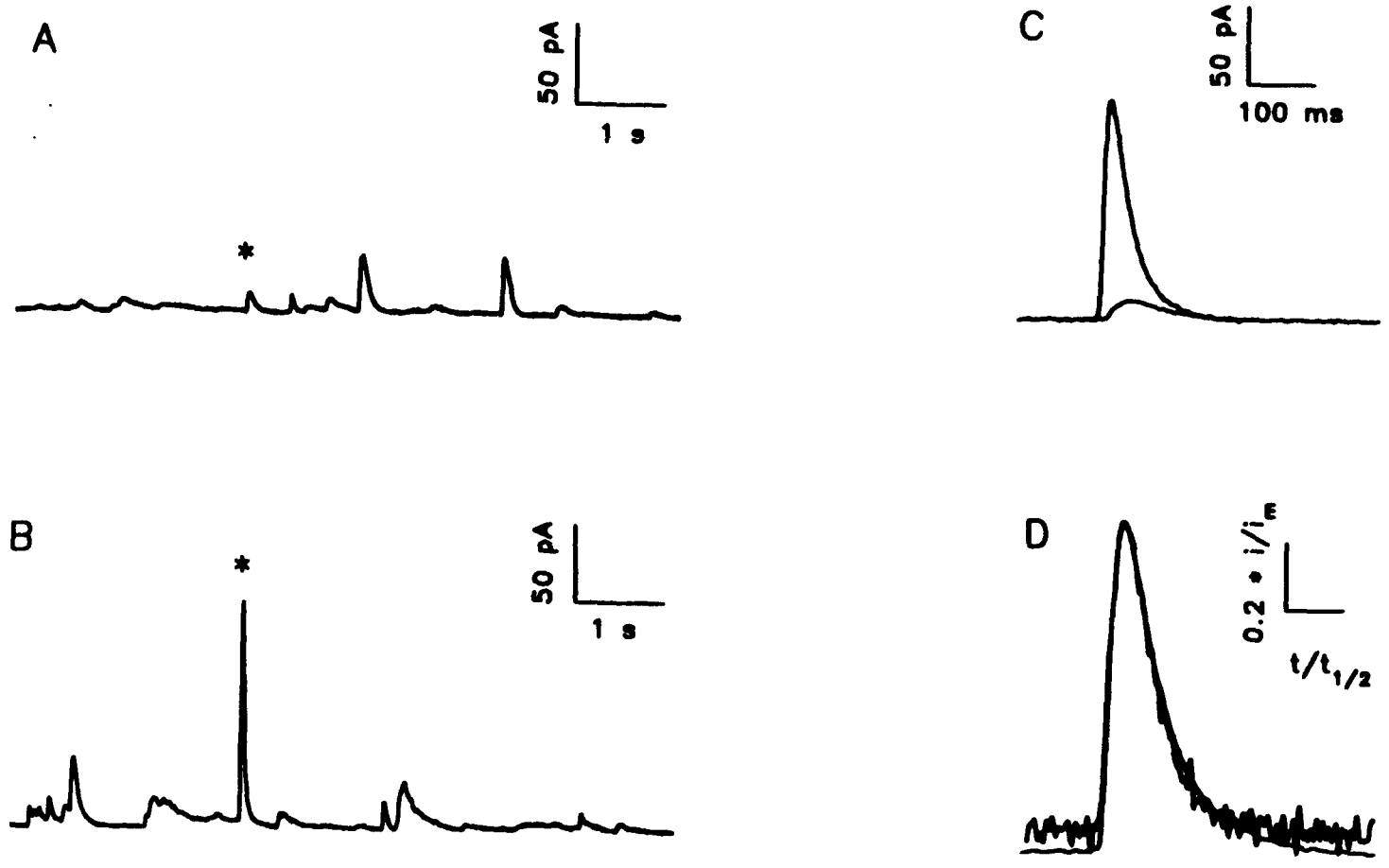
The electrochemical research was supported by a grant from the Office of Naval Research. TJS and JAJ received DOE fellowships. The confocal microscopic research was supported by a National Institutes of Health Grant RO1-DK 27959, a National Science Foundation Grant IBN-9112191 and an American Heart Association of Michigan Grant (to RWH). We are grateful to Dr. Brian Athey of the Department of Anatomy and Cell Biology and the Cell Biology Laboratory for help with the confocal microscopy.

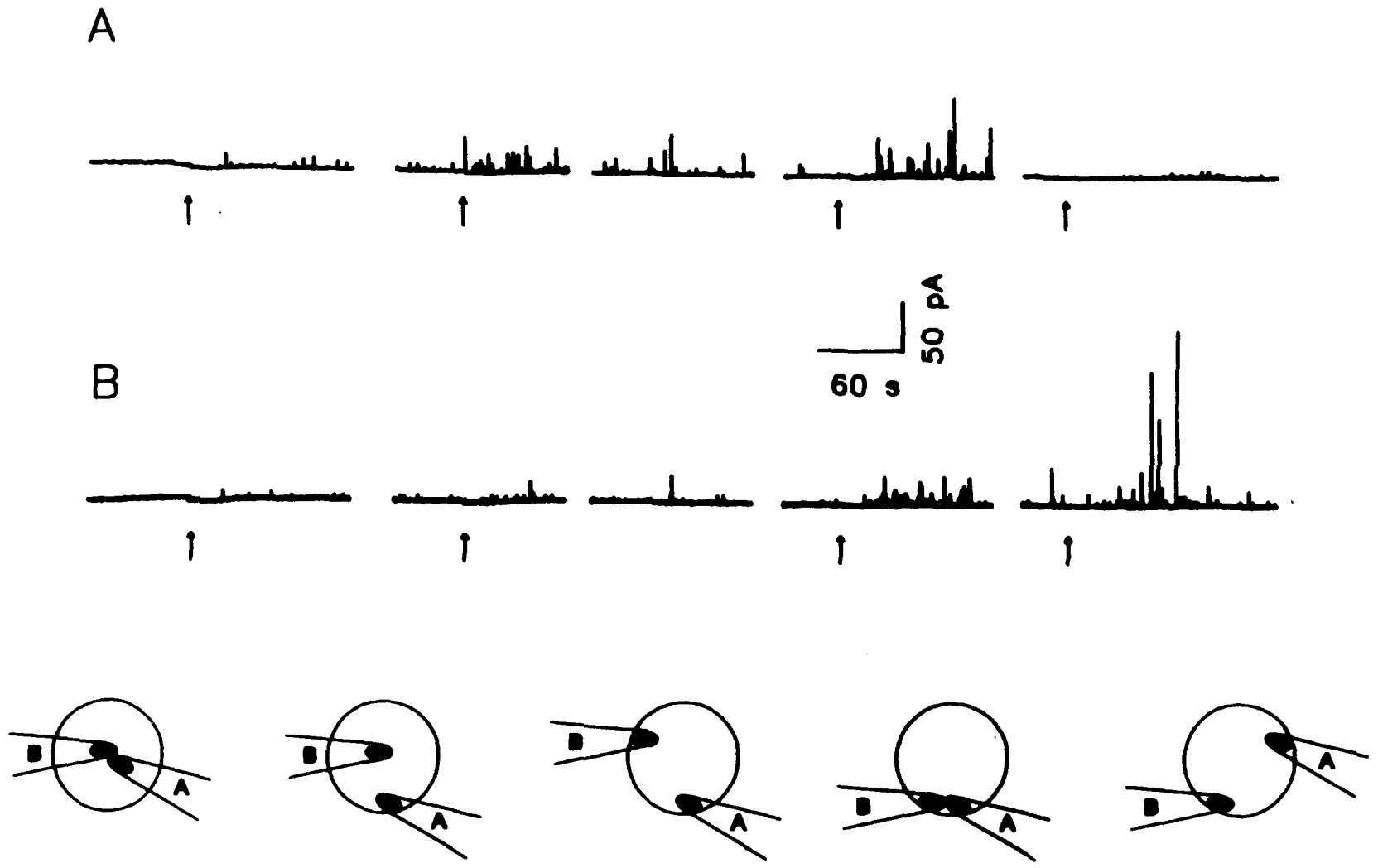

A
B
10 pA
60 s
B
A

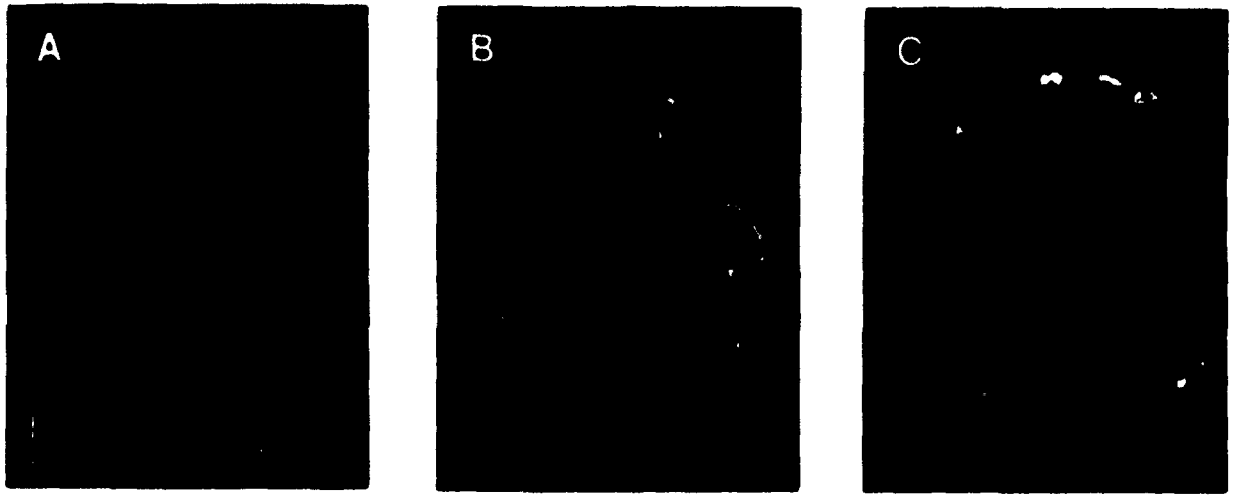

A
B
40 pA
20 s
A
B
A
B
A
B


A
d
5 µm
B
C
5 µm


A
50 pA
1 s
*
B
50 pA
1 s
*
C
50 pA
100 ms
Δ
t
o
Δ
t
m


A
50 pA
1 s
*
B
50 pA
1 s
*
C
50 pA
100 ms
D
0.2 • i/i_m
t/t_1/2


A
B
50 pA
60 s
B
A
B
A
B
A
B
A
B
A


A
B
C