

## Nicotinic Receptor-mediated Catecholamine Secretion from Individual Chromaffin Cells

CHEMICAL EVIDENCE FOR EXOCYTOSIS\*

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**Nicotinic receptor-mediated secretion of catecholamines from individual cultured bovine adrenal medullary chromaffin cells was measured and characterized with a voltametric microelectrode placed adjacent to the cells. Nicotine-induced secretion is associated with a large increase in chemical spikes that is temporally resolved into the apparent secretion of discrete packets of attomole quantities of easily oxidized molecules. These data are consistent with direct chemical measurement of single exocytotic events.**

Chemical neurotransmission is thought to occur by the process of exocytosis where neurotransmitters, contained in synaptic vesicles, are extruded into the extracellular space following fusion of the vesicle and plasma membranes (1). Considerable evidence exists to support this hypothesis in the case of cholinergic neurons (2). Although acetylcholine secretion has been measured from the growth cones of single neurons with outside-out patches on micropipettes (3, 4), direct chemical measurement of single exocytotic events has not been reported because intrasynaptic measurements would require a transmitter-sensitive probe of very small size (<100 nm). Bovine adrenal chromaffin cells are ideal models to investigate stimulus-secretion coupling of catecholamines because of their neuro-ectodermal origin and close biochemical and functional similarities to postganglionic sympathetic neurons. Evidence that this secretion is an exocytotic process comes from anatomical and biochemical evidence (5-7) and from changes in membrane capacitance (8). Recently, secretion of ATP from single bovine adrenal chromaffin cells in culture has been demonstrated (9). Single cell ATP release was semi-quantitatively measured using a co-culture bioassay that does not have the time resolution to measure individual exocytotic events.

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To investigate catecholamine release from single cells, chromaffin cells were isolated and plated (10) at a density of  $6 \times 10^5$  cells/35-mm culture dish. Following at least 3 days of culture in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) supplemented with 10% fetal bovine serum, the culture medium was replaced with a balanced salt solution (11). Catecholamines were detected with beveled carbon fiber electrodes used in an amperometric mode or with fast-scan cyclic voltametry (12). The electrode tip was positioned under an inverted microscope on a vibration-isolation table with a micromanipulator so that it was  $<5 \mu\text{m}$  from a single cell (Fig. 1). The cell radius is approximately  $8 \mu\text{m}$  which is similar to the dimensions of the tip of the electrode. Chemical agents for stimulation were dissolved in balanced salt solution and introduced with a pulled-glass micropipette positioned approximately  $20 \mu\text{m}$  from the cell (Fig. 1) with a pressure ejection system (Picospritzer, General Valve Corp., Fairfield, NJ).

In order to demonstrate the time course of the chemical stimulus, we ejected norepinephrine from the micropipette. Cyclic voltametry of norepinephrine (Fig. 2A) is similar to that of dopamine and epinephrine, but is distinct from all other known vesicular components. Fig. 2C shows a typical temporal response measured in the cyclic voltametry mode to the ejection of  $20 \mu\text{M}$  norepinephrine in the absence of a cell. This time course simply indicates the concentration profile that will occur at the cell surface for any chemical introduced from the micropipette. Exposure of a cell to  $100 \mu\text{M}$  nicotine results in the trace shown in Fig. 2D. The cyclic voltammogram ensures that the detected substances are catecholamines (Fig. 2B). Exposures of chromaffin cells to this dose of nicotine resulted in catecholamine secretion having a maximal concentration of  $17.9 \pm 1.4 \mu\text{M}$  ( $n = 15$  cells). Similar amplitudes and cyclic voltammograms were observed with electrodes coated with a perfluorinated cation exchange polymer, an electrode that has even greater specificity for catecholamines (13).

Several investigators have shown that the nicotine-induced secretion of catecholamines from cultured adrenal chromaffin cells is sensitive to nicotinic receptor and  $\text{Ca}^{2+}$  channel blockers (5-7, 14-17). Hexamethonium and  $\text{Cd}^{2+}$  also decreased the electrochemical signal induced by nicotine from single chromaffin cells. However, the most unique feature of our results is the large chemical spikes superimposed on the secretion envelope. Cyclic voltammograms recorded during a single nicotine-induced spike show that a concentration packet of catecholamines causes the observed noise. In contrast, spikes are not observed with pressure ejection of nicotine in the absence of a cell or by ejection of a balanced salt solution from the micropipette onto an adjacent cell. Nicotine-induced spikes are still apparent at electrodes that are moved away from the cell by a few micrometers, although their amplitudes are reduced.

To improve temporal resolution, measurements were made in an amperometric mode at a fixed oxidizing potential. The response to  $100 \mu\text{M}$  nicotine was qualitatively similar; however, the spikes became more clearly resolved with random amplitudes and rapid rise and decay times (Fig. 3A). The most rapid half-rise time observed for a single spike was 7 ms, the response time of the instrumentation. In some exper-

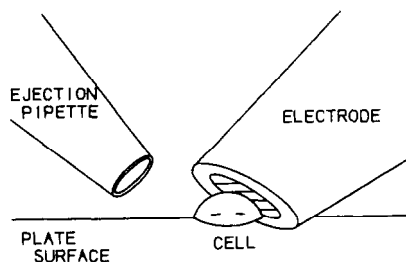


FIG. 1. Experimental arrangement for measuring secretion from single adrenal medullary chromaffin cells. Drawing is approximately to scale.

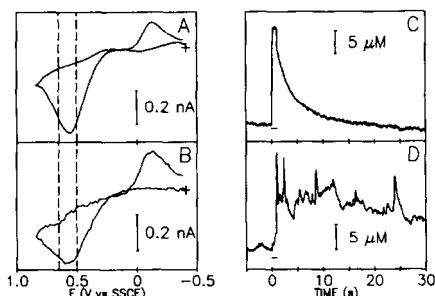


FIG. 2. Cyclic voltammetric response ( $200 \text{ V s}^{-1}$ , repeated at 100-ms intervals) of a carbon fiber electrode to norepinephrine ejection from an adjacent micropipette and to catecholamine secretion from a single chromaffin cell. Data were recorded with an EI-400 potentiostat (Ensmann Instrumentation, Bloomington, IN) interfaced to an XT-style personal computer, and a sodium-saturated calomel electrode (SSCE) was employed. Panels A and B are averaged background-subtracted voltammograms of the substances whose concentration changed during the measurement interval of panels C and D. Each time point in panels C and D is the integrated current recorded from 0.5–0.6 V from individual voltammograms (hatched lines in panels A and B); bars to the right in panels C and D are the conversion of current to catecholamine concentration based on calibration curves constructed with standards. A, C, electrode response to 1-s 3-nl ejection of  $20 \mu\text{M}$  norepinephrine applied at  $t = 0$  with the ejection pipette  $20 \mu\text{m}$  from the electrode. B, D, electrochemical response obtained with the electrode tip adjacent to a single cell; at  $t = 0$ , a 1-s ejection of nicotine ( $100 \mu\text{M}$ ) was made  $20 \mu\text{m}$  away from the cell.

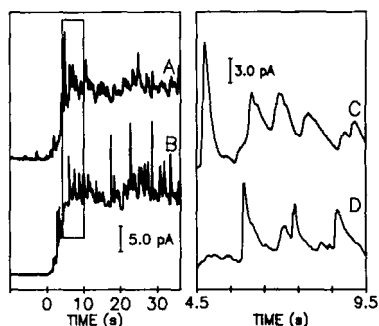


FIG. 3. Current measured from two electrodes ( $E_{\text{appl}} = 0.65 \text{ V}$ ) placed on opposite sides of a single cell. Signals measured following ejection of  $100 \mu\text{M}$  nicotine at  $t = 0$  (A, B). C, D, expanded time and current scale of the boxed area indicated in panels A and B.

iments ( $n = 9$ ) two uncoated electrodes were placed on opposite sides of an individual cell to simultaneously probe the spatial dependence of the spikes. Spikes were observed at either side of the cell (Fig. 3, A and B) but appeared at

different and variable times as seen in the expanded scale (Fig. 3, C and D).

The combined evidence suggests that each fluctuation in chemical concentration measured at single cells following exposure to nicotine represents a direct chemical measure of exocytosis from single, or perhaps multiple, chromaffin granules. Other factors that could cause electrical spikes, such as vibration introduced by the stimulus application or movement of the stimulated cell, would result in coincident noise spikes at each of the electrodes. However, the results in Fig. 3 show a directional specificity of the individual spikes which indicates a single local origin on the cell surface. The variable amplitude of the concentration spikes (Fig. 3) is attributed to the varied distance from the cell surface over which the packets of secreted substances have to diffuse to reach the sensing portion of the electrode. Thus, the secretion process consists of an overall increase in catecholamine concentration around the cell, leading to the observed concentration envelope, on which are superimposed rapid concentration spikes arising from sudden concentration changes originating at the portion of the cell surface nearest the electrode. For exocytotic events which occur at the cell surface adjacent to the electrode, it is likely that all of the easily oxidized substances secreted are detected. This is because the secreted molecules are trapped in the small space between the cell and the electrode. The charge during the larger spikes measured in the amperometric mode is less than 2 picocoulombs, corresponding (by Faraday's law) to 10 attomoles. Each cell contains 20,000–30,000 vesicles (18, 19) and  $\approx 150 \text{ fmol}$  of catecholamine; thus, the average vesicle content is 5–8 attomoles, in close agreement with the value determined from these measurements. Future work with this technique will explore basic questions about stimulus-secretion coupling in a temporal and spatial regime that approaches that of the synapse.

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