Supplementary Material Available: Tables of average molecular intensities for each camera distance at each temperature, symmetry coordinates, force constants, observed and calculated wavenumbers, and correlation matrices for all refined parameters at the lowest temperature experiments and figures of total intensities with backgrounds for experiments at 388 K and 673 K (DBTF) and 393 K and 473 K (DITF) (16 pages). Ordering information is given on any current masthead page.

Direct Observation of Epinephrine and Norepinephrine Cosecretion from Individual Adrenal Medullary Chromaffin Cells

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Abstract: Cyclic voltammetry at Nafion-coated carbon fiber microelectrodes has been used to monitor directly the release of catecholamines from individual adrenal medullary chromaffin cells and to identify the released catecholamine as epinephrine or norepinephrine. The cultured cells were induced to secrete by exposure to 100 μM nicotine, a recognized secretagogue at these cells. Each cell contains on average 167 fmol of catecholamines, and the secretion event involves only a small percentage of the total stores for a time interval of less than 60 s. Identification of epinephrine and norepinephrine is accomplished because of differences in the rates of intracyclization for the oxidized forms of these compounds which results in differences in the shapes of their voltammograms. Approximately 75% of the cells studied released only epinephrine or norepinephrine in response to a 100 μM nicotine stimulus, while 25% released mixtures of both catecholamines. The ratio of epinephrine:norepinephrine releasing cells is in good agreement with the epinephrine:norepinephrine ratios of total catecholamine stores for the cell populations. Analysis of individual cell catecholamine content by microcolumn liquid chromatography following secretion measurements indicates that the individual cells release catecholamines in the same proportions in which they store catecholamines.

Introduction

Isolated adrenal medullary chromaffin cells in culture are frequently used as a model system for neurosecretion.[1-4] It is generally accepted that at least two subpopulations of adrenal cells exist: those which store and secrete epinephrine (E) and those which store and secrete norepinephrine (NE). This conclusion has been arrived at on the basis of the results of electron microscopy studies which showed two principal types of adrenal cells differing in the size and electron density of their catecholamine storage vesicles,[2,5-8] referred to as granules, and enzyme assays which showed that a subpopulation of cells lacked phenylethanolamine *N*-methyltransferase (PNMT), the enzyme which converts norepinephrine into epinephrine.[9] Some data also suggest that differences in certain aspects of release for these two cell types exist.[10,11] A recent report has shown that these subpopulations can be separately isolated with their secretory machinery still intact.[12]

The majority of studies on adrenal cells have been performed on populations of cells because of experimental limitations. However, techniques are now available which allow for analysis at the level of the single cell. To accomplish this, femtomole and lower detection limits are required. Microcolumn liquid chromatography and capillary zone electrophoresis provide a means to precisely sample the contents of individual cells.[13-16] However, these methods lack sufficient time resolution to monitor a dynamic event such as exocytosis. Recently, microelectrodes have been used to provide spatial and temporal information on local chemical events, a technique referred to as scanning electrochemical microscopy.[17-19] This laboratory has recently used these approaches to monitor secretion of catecholamines from individual adrenal cells in culture with subsecond time resolution.[20,21] Although this technique has provided sufficient time resolution to accurately describe the local changes in catecholamine concentrations at the cell surface, identification of the specific catecholamine secreted has not been possible.

The goal of the present study has been to identify the specific catecholamine secreted, epinephrine or norepinephrine, while the dynamic process of cellular release is monitored. Identification is based on known differences in the rates of intracyclization for

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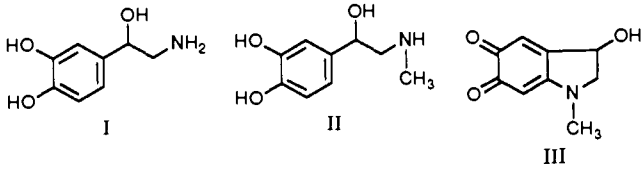
the oxidized forms of the catecholamines.[22] Although norepinephrine (I) and epinephrine (II) are structurally very similar, the rate of cyclization is faster for epinephrine. This leads to the formation of the adrenochrome (III), evidenced by the appearance



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of a new voltammetric wave. With real-time differentiation of secreted catecholamines, a means is provided to further probe the differences between subpopulations of adrenal cells at the level of the single cell.

Experimental Section

Electrodes. Carbon fibers (r = 5 μm, Thornell P-55, Amoco Corp., Greenville, SC) were sealed in glass capillaries to form microelectrodes as previously described.[23,24] The electrodes were polished at a 45° angle on a micropipet beveller (model BV-10, Sutter Instrument Co., Novato, CA) which resulted in electrodes with an elliptical surface. Microelectrodes were coated with Nafion by a dip-coating procedure[25] with a 10% solution (weight/volume in 2-propanol) of Nafion (equivalent weight 1100).

In some experiments a glassy carbon (GC) working electrode (1.5-mm radius, BioAnalytical Systems, West Lafayette, IN) was used. It was polished prior to each experiment with 0.3-μm alumina and sonicated in a 50:50 methanol/water mixture to remove residual alumina. In other experiments this electrode was coated with Nafion by applying 23 μL of a 0.5% solution of Nafion to the electrode and allowing the 2-propanol to evaporate. The polymer film was removed between successive experiments by polishing with 1.0- and 0.3-μm alumina. A new coating was applied for each experiment to ensure that only the analyte of interest was present inside the polymer film. A sodium-saturated calomel reference electrode (SSCE) was used in all experiments.

Electrochemistry. Voltammetry with microelectrodes employed an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN) in two-electrode mode. The potential was controlled by an IBM PC-XT (Boca Raton, FL) using locally written software and a commercial interface (Labmaster, Scientific Solutions, Solon, OH). Background subtraction[26] was used for all microelectrode experiments. For epinephrine and norepinephrine differentiation, the voltage was scanned from −500 to +900 mV and back at 10 V/s. Successive scans were initiated every 1.025 s. This sampling interval was chosen such that the diffusion layer would have sufficient time to relax and successive scans would occur 180° out of phase with respect to line frequency. Because the measured currents are very small, interferences from line noise can distort the voltammograms. Collection of voltammograms at a repetition time that is out of phase with respect to line frequency was used to reduce this interference.[27-29]

Chronoamperometry and voltammetry at the glassy carbon electrode were performed in a single-compartment cell with a Princeton Applied Research (PAR) Model 174A polarographic analyzer and a platinum counter electrode. Voltammetric data were acquired on a Houston Instruments Model 2000 X-Y recorder or a Nicolet 310 digital oscilloscope. Solutions were purged with nitrogen for at least 15 min to prevent air-oxidation of the catecholamines. For experiments at the Nafion-coated GC electrode, the polymer film was allowed to equilibrate with the analyte solution for at least 30 min. Chronoamperometry was performed at 23 ± 1 °C.

Cell Culture. Bovine adrenal medullary chromaffin cells were isolated as previously described.[30] Cell populations were enriched in either epinephrine or norepinephrine by centrifugation using a single-step Renografin density gradient (10–12 min at 7700g and 20 °C in a Sorvall SS-34 fixed-angle rotor).[9,31] In contrast to these reports, this procedure resulted in two bands of cells separated by approximately 1–1.5 cm. Each band was separately collected by aspiration, and the cells from the respective fractions were plated at a density of 6×10^5 per 35-mm-diameter tissue culture plate (Becton-Dickinson, Oxnard, CA). The cells were kept in an incubator with 95% air/5% CO_2. The initial culture medium consisted of 1:1 DMEM/F12 with 15 mM HEPES (pH 7.4), antibiotics, and 10% fetal bovine serum. After 3 days this medium was replaced with an identical medium minus the fetal bovine serum. The medium was then changed every 2 days. Experiments were performed between days 4 and 8 of culture.

Enrichment of Epinephrine and Norepinephrine Containing Cell Populations. To estimate the degree of enrichment achieved by the Renografin centrifugation, cell populations were lysed with 0.1 N perchloric acid and the total catecholamine content of the fractions was assayed by standard reversed-phase HPLC with electrochemical detection. The less dense (upper) fraction was enriched in norepinephrine (E:NE = 0.54 ± 0.26, mean ± sd, n = 3 preparations) while the more dense (lower) fraction was enriched in epinephrine (E:NE = 6.1 ± 2.9).

Voltammetry Adjacent to Single Cells. Cell culture plates were placed on the stage of an inverted microscope. The working electrode was positioned approximately 5 μm from an individual cell using a piezoelectric driver (PCS-250 patch-clamp driver, Burleigh Instruments, Fishers, NY). Single cells were stimulated by pressure ejection (picospritzer, General Valve Corp., Fairfield, NJ) of 100 μM nicotine, and release was monitored by background-subtracted cyclic voltammetry.[20,21] The nicotine was contained in one barrel of a triple-barrel micropipet while the other two barrels contained standard solutions of epinephrine and norepinephrine. Typical ejection volumes were approximately 10 nL. Calibrations were performed immediately after each stimulation by moving the working electrode away from the cell and ejecting each of the standard solutions at the electrode. In this manner, any changes in electrode response with time were taken into account.

Microscale Liquid Chromatography. The procedure for microscale liquid chromatography on individual cells has been described elsewhere.[32] For these experiments, a cell was stimulated as above and the release was monitored voltammetrically. The bottom of the culture plate was scratched with the micropipet tip to identify the cell. The same cell was then removed from the culture plate with a glass pipet (i.d. = 20 μm) and transferred to a 250-nL microvial. In two cases the marked area of the plate was found to contain two adjacent cells. These results were discarded. Exactly 10.0 nL of a perchloric acid solution containing an internal standard and an antioxidant was added to the microvial. The microvial was centrifuged at 12000g for 8 min, and the supernatant was injected directly onto a reversed-phase packed capillary column (l = 50 cm, i.d. = 42 μm). Catecholamines were detected by amperometry.

Solutions. Chronoamperometry was performed in pH 7.4, 150 mM Na^+ phosphate buffer (60 mM Na_2HPO_4, 30 mM NaH_2PO_4). For studies of cyclization rates in Nafion films, a pH 7.4, 1.15 M Na^+ (437 mM Na_2HPO_4, 275 mM NaH_2PO_4) buffer, a pH 6.5, 150 mM Na^+ (22 mM Na_2HPO_4, 106 mM NaH_2PO_4) buffer, and a pH 8.3, 150 mM Na^+ (72 mM Na_2HPO_4, 6 mM NaH_2PO_4) buffer were used. The balanced salt solution used in the cell experiments consisted of 150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM glucose, 0.7 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES at pH 7.4. All buffers were adjusted to their final pH values with 4 M NaOH.

Chemicals. All chemicals were used as obtained from commercial sources. Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 Medium (F12) were obtained from Gibco Laboratories (Grand Island, NY). Collagenase (type I) was acquired from Worthington Chemicals (Freehold, NJ). Renografin was obtained from Squibb Diagnostics (New Brunswick, NJ). All other chemicals were from Sigma (St. Louis, MO). Stock solutions of catecholamines were prepared in 0.1 N perchloric acid, stored at 4 °C, and diluted with buffer to the appropriate concentration just prior to use. Solutions were prepared with doubly distilled water.

Results

Intracyclization in pH 7.4 Solutions. Values for the rates of intramolecular cyclization for the catecholamines were determined from the results of chronoamperometric experiments at glassy carbon electrodes plotted in the form of n_{app} vs log t.[33,34] The data were consistent with a DISP1 mechanism[22,35] with apparent

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rate constants for dopamine, 0.13 ± 0.05 s^{-1}, norepinephrine, 0.98 ± 0.52 s^{-1}, and epinephrine, 87 ± 10 s^{-1} (mean ± sd, n = 3). The theory of DISP1 reactions for cyclic voltammetry[36] and the derived rate constants allow prediction of a scan rate which allows optimal resolution of the catecholamines by the shape of their cyclic voltammograms. This exists when cyclization occurs completely for one compound, but only partially for the other. For the case of epinephrine and norepinephrine, the two catecholamines of interest in this work, this scan rate is calculated to be 10 V/s. However, at this scan rate convergent diffusion predominates at microelectrodes and sigmoidal voltammograms are obtained. Contributions from convergent diffusion can be lessened by using a higher scan rate. At 50 V/s sufficient time exists for some epinephrine cyclization to occur while norepinephrine cyclization does not. However, when uncoated microelectrodes were used to detect catecholamine release from single cells, slight fouling of the electrode obscured the reduction wave of the adrenochrome and precluded identification.

Intracyclization in Nafion Films. The perfluorinated ionomer Nafion has been used to protect electrode surfaces from protein adsorption.[25] Therefore, the voltammetry of the catecholamines at Nafion-coated and uncoated electrodes was investigated. At glassy carbon electrodes in pH 7.4 phosphate buffer, the rates of cyclization at Nafion-coated electrodes are decreased approximately 6-fold with respect to uncoated electrodes. The pH inside the Nafion film can be calculated by[37]

$$[H^+]_p = [H^+]_s[SO_3^-]_p/(K[C^+]_s + [H^+]_s) \quad (1)$$

where $[H^+]_p$ is the concentration of protons in the polymer film, $[H^+]_s$ is the concentration of protons in solution, $[SO_3^-]_p$ is the concentration of sulfonate sites in the polymer film, $[C^+]_s$ is the concentration of supporting electrolyte cations in solution, and K, the ion-exchange selectivity coefficient, is defined as

$$K = [H^+]_s[C^+]_p/[H^+]_p[C^+]_s \quad (2)$$

where $[C^+]_p$ is the concentration of supporting electrolyte cations in the polymer film. With K = 1.22[38] and $[SO_3^-]_p$ = 1.44 M,[39,40] the pH inside a Nafion film immersed in a pH 7.4, 150 mM Na^+ solution is calculated to be 6.52. Voltammograms of norepinephrine recorded at an uncoated GC electrode in pH 6.5, 150 mM Na^+ buffer reveal a 4-fold decrease in rate relative to that at pH 7.4; however, the observed cyclization is faster than at Nafion-coated electrodes in pH 7.4 solutions.

To further explore the effect of pH, voltammograms of norepinephrine at Nafion-coated GC electrodes were recorded in solutions that were pH 7.4 containing 1.15 M Na^+, and pH 8.3 containing 150 mM Na^+. According to eq 1, the pH inside the film under both of these conditions should be 7.4. In both cases the apparent rate of cyclization was accelerated relative to the results with Nafion coatings in solutions containing 0.15 M Na^+, pH 7.4, but were still slower than the rates at an uncoated electrode in pH 7.4 buffer.

Characterization of Nafion-Coated Carbon Fiber Microelectrodes. Since diffusion coefficients for catecholamines in Nafion are low ($\sim 1 \times 10^{-9}$ cm^2 s^{-1}),[25,41] the onset of convergent diffusion in cyclic voltammograms at electrodes with thick Nafion coatings is not seen until lower scan rates than at uncoated electrodes. Experimentally it was found that epinephrine and norepinephrine both gave peak-shaped voltammograms at a scan rate of 10 V/s at microelectrodes with thick coatings, and that the degree of cyclization, evidenced by a peak at −425 mV, was sufficiently different that the two compounds could be distinguished (Figure 1). Since epinephrine has a greater affinity for Nafion, the current amplitude is 2.5 times larger than for norepinephrine.

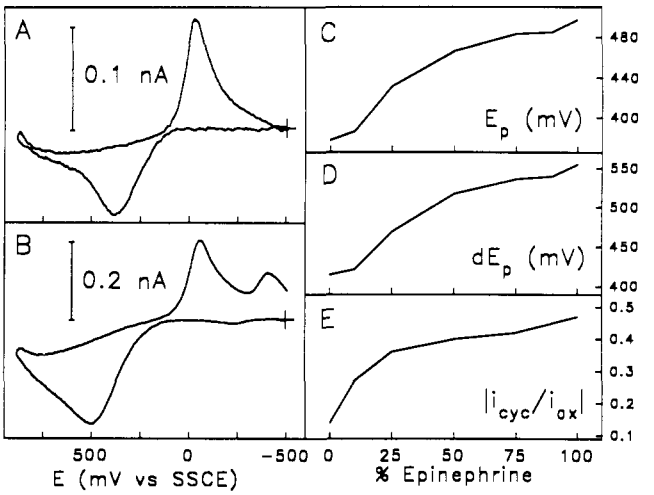

Figure 1. Electrode response to mixtures of epinephrine and norepinephrine. (A) and (B) are the background-subtracted cyclic voltammograms for norepinephrine and epinephrine, respectively, at a Nafion-coated carbon fiber microelectrode in pH 7.4 balanced salt solution (see Experimental Section) (scan rate 10 V/s). (C), (D), and (E) are E_p, ΔE_p, and i_{cyc}/i_{ox} versus percent epinephrine for mixtures of epinephrine and norepinephrine at a single electrode, respectively. Total catecholamine concentration was 20 μM.

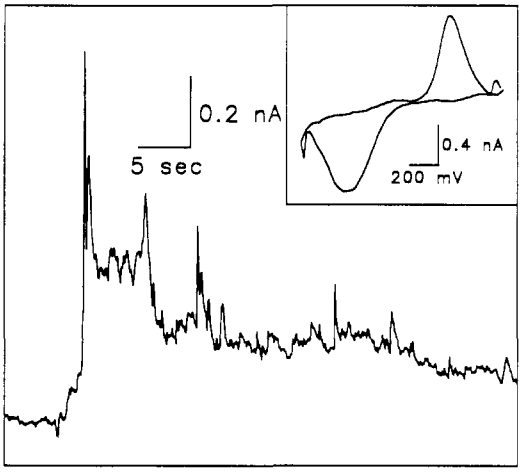

Figure 2. Current versus time profile of the response of a single cell to 100 μM nicotine. Detection was performed with a bare carbon fiber microelectrode scanned at 300 V/s. Inset shows the background-subtracted cyclic voltammogram obtained at the maximal response.

In addition to the presence of a voltammetric wave for the cyclization of epinephrine (expressed as the ratio to the oxidative wave, i_{cyc}/i_{ox}), two other criteria serve to distinguish these compounds: the oxidative peak potential (E_p) and the separation between the oxidative and reductive peaks (ΔE_p). The values for these parameters are shown in Figure 1 for mixtures of the two catecholamines. Since the changes are nonlinear, quantitative determination of the amount of each catecholamine in a mixture is not possible. However, the parameters do provide a way to distinguish five categories: epinephrine or norepinephrine individually, primarily epinephrine or norepinephrine, or mixtures in which the predominant catecholamine cannot be distinguished.

Detection of Stimulated Catecholamine Release from Single Cells. Voltammetric detection of release of catecholamines from individual bovine adrenal medullary cells with carbon fiber microelectrodes placed adjacent to the cell has been reported.[20,21] Data obtained with an uncoated electrode during release induced by pressure ejection of 100 μM nicotine are shown in Figure 2.

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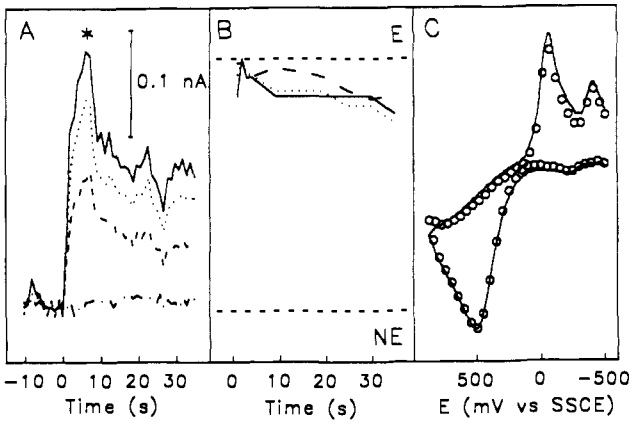

Figure 3. Secretion of epinephrine from an individual adrenal cell. (A) Current versus time profile of single cell response to 100 μM nicotine introduced at 0 s: oxidative current (solid line), reductive current (dotted line), adrenochrome current (dashed line), current at 0 V (dashed–dotted line). (B) E_p (solid line), ΔE_p (dotted line), and i_{cyc}/i_{ox} (dashed line) versus time. The scale for each of these parameters is adjusted so that the values will fall between the limits of pure epinephrine (upper dashed line) and pure norepinephrine (lower dashed line) as determined by the postcalibrations. (C) Background-subtracted cyclic voltammogram (solid line) taken at the asterisk in (A). Circles represent the background-subtracted cyclic voltammogram of the epinephrine postcalibration. Voltammograms have been normalized to the oxidative peak currents. Data are taken from cell no. 6 of Table I.

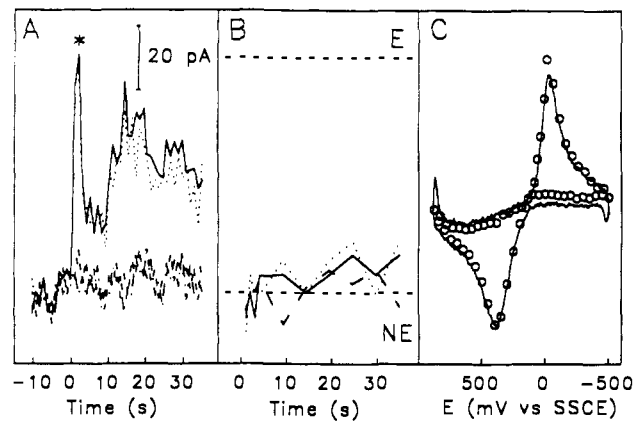

Figure 4. Secretion of norepinephrine from an individual adrenal cell. The parameters for (A) and (B) are the same as those for Figure 3. (C) Same as Figure 3, but circles represent the background-subtracted cyclic voltammogram of the norepinephrine post-calibration. Data are taken from cell no. 11 of Table I.

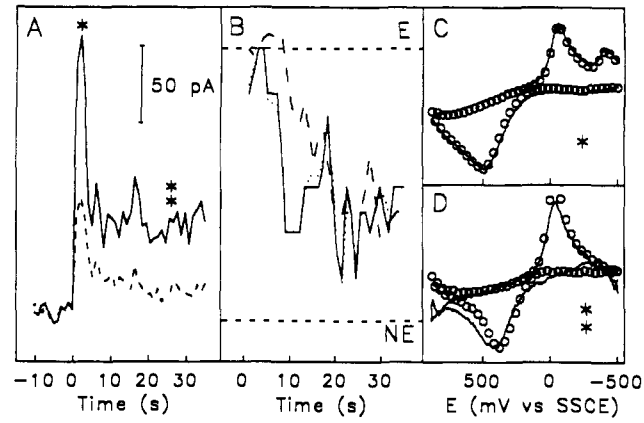

Figure 5. Cosecretion of epinephrine and norepinephrine. (A) Oxidative current (solid line) and adrenochrome current (dashed line) versus time. (B) Same as Figures 3 and 4. (C) Background-subtracted cyclic voltammogram (solid line) taken at the asterisk in (A). Circles represent the background-subtracted cyclic voltammogram of the epinephrine postcalibration. Voltammograms have been normalized to the oxidative peak currents. (D) Background-subtracted cyclic voltammogram (solid line) taken 25 s after the stimulus. Circles represent the background-subtracted cyclic voltammogram of the norepinephrine postcalibration. Data are taken from cell no. 5 of Table I.

When sampled with a scan rate of 200 V/s, catecholamine release can be measured with background-subtracted cyclic voltammetry, but the individual catecholamines cannot be distinguished.[21] The release monitored at the potential for catecholamine oxidation has a broad envelope with sharp spikes superimposed on it. The spikes have been identified as the result of vesicular release, while the envelope results from the merger of the concentration spikes as they diffuse away from the cell.[42]

Identification of Catecholamines Released from Single Cells. Voltammograms acquired at 10 V/s were used to distinguish between the catecholamines secreted from these cells. Cyclic voltammetric data recorded at a single cell on an epinephrine-enriched plate is shown in Figure 3. The background-subtracted voltammogram recorded 7 s after exposure to nicotine clearly shows that epinephrine is released from this cell at that instant (Figure 3C). The three voltammetric parameters (Figure 3B) described above are normalized so that their time-dependent values will indicate the catecholamine secreted. Because the parameters do not vary linearly with increasing percent epinephrine, the vertical scale cannot be viewed as a linear representation of the percent epinephrine in a mixture. For this cell, the parameters indicate that epinephrine is the released substance over the entire time interval. The currents recorded at the potentials for the oxidative, reductive, and adrenochrome waves all follow the same time course, which indicates that the observed current is due only to catecholamine. Additionally, when the current is monitored at 0 V, a potential where catecholamines are not electroactive, no change is seen during release. The average maximal concentration observed during secretion at epinephrine-releasing cells was 15 ± 12 μM (mean ± sd, n = 28 cells).

Figure 4 shows the results obtained at a single cell which released norepinephrine following stimulation with 100 μM nicotine. Notice in Figure 4A that no current is observed at the potential for the adrenochrome wave and that the other parameters all indicate that norepinephrine is released throughout the entire period (Figure 4B). Background-subtracted cyclic voltammograms for the release and the norepinephrine calibration immediately after the stimulation are shown in Figure 4C. The average maximal concentration during secretion from cells which release norepinephrine was 22 ± 17 μM (n = 18 cells).

Corelease of epinephrine and norepinephrine has been observed in approximately 25% of the cells analyzed. The data from one such cell are shown in Figure 5. The oxidative current plateaus after the initial spike, but the current due to the adrenochrome decreases with time (Figure 5). The values for E_p, ΔE_p, and i_{cyc}/i_{ox} indicate that epinephrine was released initially, but that at the end of the collection period a mixture of epinephrine and norepinephrine was being detected. A background-subtracted cyclic voltammogram taken at the asterisk in Figure 5A clearly shows that the initial spike is due to the release of epinephrine. However, a cyclic voltammogram taken 25 s after the stimulation does not fit either the epinephrine or the norepinephrine calibration. The absence of the adrenochrome wave, though, is a strong indication that norepinephrine is the predominant catecholamine present at this time.

In one cell the corelease of epinephrine and norepinephrine has been time resolved. Plots of the peak oxidation current and peak adrenochrome current versus time are shown in Figure 6. Note that the current for the adrenochrome increases dramatically 15 s after the stimulation. At the same time, all three of the parameters for identification indicate a transition from norepinephrine to epinephrine with a return to norepinephrine soon afterward. A background-subtracted cyclic voltammogram of the

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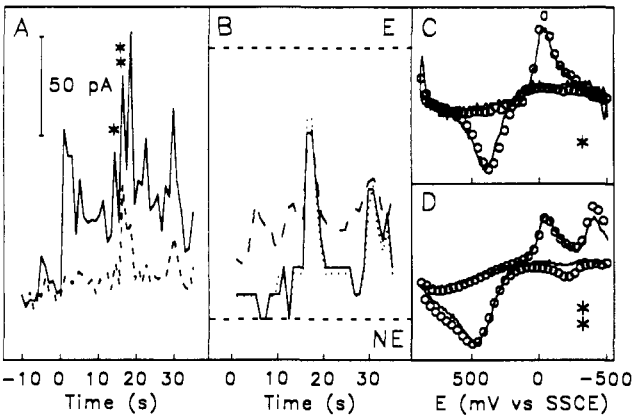

Figure 6. Cosecretion of epinephrine and norepinephrine. The parameters for (A) and (B) are the same as those for Figure 5. (C) Background-subtracted cyclic voltammogram (solid line) obtained at the single asterisk in (A). Circles are the norepinephrine postcalibration. (D) Background-subtracted cyclic voltammogram (solid line) obtained at the double asterisk in (A). Circles are the epinephrine postcalibration. Background voltammograms were obtained 12 s after the stimulation. All voltammograms have been normalized to the oxidative peak currents. Data are taken from cell no. 15 of Table I.

concentration spike at 13 s shows that the catecholamine present is norepinephrine. A voltammogram of the concentration spike at 15 s, however, is significantly different and suggests that epinephrine is the predominant catecholamine present at that time.

Comparison of Released Catecholamine to Population Total Stores. Because the majority (~75%) of the cells were found to be dominant in one of the catecholamines, the ratio of epinephrine to norepinephrine cells from a given fraction is expected to be similar to the epinephrine:norepinephrine ratio of total catecholamine content for that fraction. In one preparation, release from 18 cells from an epinephrine-enriched fraction was measured, and release from 22 cells from a norepinephrine-enriched fraction was measured (Table I). For the purpose of this comparison, cells which released both catecholamines were counted as if they only contained the catecholamine that was predominantly released. Only one cell (no. 25) was ambiguous, and was counted as $^1/_2$ cell for each subtype. The number of epinephrine cells was divided by the number of norepinephrine cells in each fraction, and the values were in good agreement to the epinephrine:norepinephrine values obtained from LC analysis of the total plate contents.

Comparison of Released Catecholamine to Individual Cell Content. To evaluate whether the catecholamine content correlates with catecholamine secreted, catecholamines in individual cells were determined with microscale liquid chromatography after they had been stimulated. Of the 22 cells studied, 18 released catecholamines in the same approximate proportions that were contained within the cell (Table II).

Discussion

The difference in rates of intracyclization of the oxidized catecholamines, norepinephrine and epinephrine, allows for distinction between these two compounds at a scan rate determined by their relative rates. The rates found in physiological buffer agree with a recent report.[43] Uncoated carbon fiber microelectrodes are susceptible to fouling when used in biological systems. Therefore, Nafion was used to eliminate this problem. However, the coated electrodes show a slower rate of cyclization for both of the catecholamines.

The catecholamine cyclization reaction is known to be pH dependent,[22] and it was hypothesized that a lower pH inside the Nafion film was the determining factor in the observed rate decrease. Although the experimental data are partially consistent with this hypothesis, the data suggest that an additional factor may serve to slow the rates. One possibility is that the protonated amine side chains of the catecholamines electrostatically interact with the anionic sulfonate sites inside the Nafion film. The

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Table I. Voltammetric Identification of Released Catecholamine from Individual Bovine Adrenal Medullary Cells

epinephrine-enriched fraction		norepinephrine-enriched fraction	
cell no.	voltammetric identification	cell no.	voltammetric identification
1	E	19	E
2	E	20	NE
3	E	21	NE (mix.)[b]
4	NE	22	E
5	E (mix.)[a]	23	NE
6	E	24	NE
7	E	25	mix
8	E (mix.)[a]	26	NE
9	E (mix.)[a]	27	NE (mix.)[b]
10	E	28	E
11	NE	29	E (mix.)[a]
12	E	30	NE
13	E	31	NE
14	E (mix.)[a]	32	E
15	NE (mix.)[b]	33	NE
16	E	34	NE
17	E	35	NE
18	E	36	NE
		37	E
		38	E
		39	NE (mix.)[b]
		40	E

	epinephrine-enriched fraction	norepinephrine-enriched fraction
no. E Cells	15	8.5
no. NE Cells	3	13.5
E:NE	5.0	0.63
LC E:NE[c]	4.7	0.55

[a] Indicates that both catecholamines were detected, but that epinephrine was dominant. [b] Indicates that both catecholamines were detected, but that norepinephrine was dominant. [c] Ratios as determined from HPLC analysis of total catecholamine content of entire cell culture plates.

Table II. Comparison of Released Catecholamine to Total Catecholamine Content of Individual Bovine Adrenal Medullary Cells

cell no.	voltammetric identification of released catecholamine	LC quantification of individual cell contents: NE (fmol)	E (fmol)	% E[a]
41	NE	26	4.6	15
42	NE	341	2.0	1
43	NE (mix.)	48	24	33
44	E	13	176	93
45	E	4.2	251	98
46[b]	NE (mix.)	71	1.3	2
47	NE	253	2.1	1
48	mix	65	93	59
49	E	3.8	157	98
50	E	3.2	155	98
51	NE	65	4.6	7
52	NE	72	0.9	1
53	E	30	156	84
54[b]	E	77	157	67
55	E (mix.)	83	120	59
56	E	9.4	217	96
57	E	1.1	13	92
58	E	21	69	77
59	NE (mix.)	110	57	34
60[b]	E	97	164	63
61	NE	301	1.1	<1
62[b]	mix	121	24	17

[a] Cells with <20% epinephrine are considered norepinephrine dominant, while those with >80% epinephrine are considered epinephrine dominant. [b] Cells which showed a discrepancy between voltammetric identification of release and cellular catecholamine content.

mechanism of the intracyclization reaction is a nucleophilic attack of the electron-deficient *o*-quinone by the amine side chain, which could be hindered by electrostatic binding to the polymer's sulfonate groups.

Since the rates of cyclization are decreased in Nafion, a relatively slow scan rate of 10 V/s must be used for optimum discrimination. In order to maintain linear diffusion at the scan rates employed, relatively thick (>350 μm) films were coated onto the microelectrodes to maintain the majority of the diffusion layer within the film. With this approach three different criteria for distinguishing epinephrine from norepinephrine exist. The ratio of adrenochrome peak current to oxidative peak current (i_{cyc}/i_{ox}) differs for these two catecholamines because the rate constants for their cyclization reactions are different. The other two criteria appear to be a consequence of the Nafion coating, since the differences are not seen at uncoated electrodes. Such shifts in E_p and ΔE_p also have been reported for $Ru(bpy)_3^{2+/3+}$ in both Nafion[44] and poly(styrenesulfonate)[45] films.

Another consequence of the Nafion coating is that these electrodes are nearly 2.5 times more sensitive to epinephrine than to norepinephrine. Because epinephrine is more readily detected, it is easy to observe small traces of epinephrine in the presence of norepinephrine; however, small proportions of norepinephrine are almost completely obscured by the epinephrine signal. Due to the difference in sensitivities for these two catecholamines, quantitative analysis of mixtures is not possible. However, differences between in vitro voltammograms and calibrations readily indicate when both catecholamines are present, and the parameters used for identifying the catecholamine allow approximate determination of the relative proportions of epinephrine and norepinephrine present in these mixtures, especially when norepinephrine is the dominant catecholamine.

A disadvantage of the thick Nafion film is the diffusional distortion of the individual vesicular events.[21,46] The spikes are rapid events which occur at an average rate of one per second. Diffusional distortion as well as the relatively slow rate of data acquisition leads to the absence of well-resolved spikes seen with fast acquisition at uncoated electrodes (Figure 2).

The average amount of catecholamine contained in a single cell is approximately 160 fmol,[21,32,47] but the variance is quite large (see Table II). Because the amount released is only a fractional portion of the total catecholamine content,[10,48,49] considerable sensitivity is required to measure these small amounts. The design of these experiments with the electrode placed very near the cell surface reduces the sampling volume and effectively maintains a high concentration at the cell surface as catecholamines are ejected into the gap between the cell and the electrode. The sensitivity of the voltammetric detection of release is particularly evident for cells 41, 43, 46, 51, 52, 57, and 58 which contained less than half of the average amount of catecholamine. In these cells the maximal concentration during secretion ranged from 4.4 to 37 μM.

The agreement between the ratio of epinephrine- to norepinephrine-releasing cells as determined by voltammetry and the relative concentrations on the culture plate (Table I) demonstrates a straightforward but previously undetermined principle: on a statistical basis an individual cell secretes what it contains. The results with microscale liquid chromatography confirm this, as the correlation between released catecholamine and stored catecholamine is reasonable (Table II). In only four of twenty-two cells was a discrepancy noted. However, in each of these four cells, the voltammetric detection indicated more epinephrine present than indicated by the chromatography. This is most probably because of the higher sensitivity of these electrodes toward epinephrine. Alternatively, some norepinephrine may be stored in a nonreleasable pool to be used for synthesis of epinephrine.

The majority of the cells studied secreted primarily one of the catecholamines. Identification of the secreted species is made with all of the pieces of information available. Note in Figures 3B and 4B that the information from plots of E_p, ΔE_p, and i_{cyc}/i_{ox} as a function of time shows that the identity of the catecholamine does not change during the course of release. At any single time point the background-subtracted cyclic voltammograms provide conclusive evidence of the identity of the released catecholamine. These data, which are representative of that obtained for 75% of the cells, show that bovine adrenal medullary cells in culture are indeed specialized to release one catecholamine or the other.

However, a minority of the cells show much different behavior. Some cells (n = 10 out of 62 cells) release a mixture of the two catecholamines that remains uniform during the entire release interval (data not shown). The single-cell chromatographic analysis confirms that the composition of the catecholamines in these cells is mixed. These results can be interpreted in view of the known biochemistry of catecholamine synthesis.[50] The enzyme which synthesizes norepinephrine from dopamine, dopamine β-hydroxylase, is located in the catecholamine storage granules. Conversion of norepinephrine to epinephrine is catalyzed by PNMT, an enzyme associated with the outer surface of the vesicles.[51-53] Therefore, cosecretion of both catecholamines suggests that PNMT is heterogeneously distributed or that its activity is low in these cells. Low enzyme activity is likely an artifact of the culture procedure since PNMT activity is known to decrease in cultured cells because of the absence of regulation by the adrenal cortex.[54] The second minority behavior (n = 5 out of 62 cells), shown in Figure 5, was unexpected. In these cells, secretion is initially of epinephrine, and then evolves into a mixture of the two catecholamines. This supports the hypothesis of a heterogeneous distribution of PNMT within a single cell. Cosecretion of substances from adrenal cells[55-57] and biological cells in general[58] is an area of considerable interest because of its potential importance in chemical communication between cells. However, this is the first report to our knowledge of a real time observation of cosecretion.

Although observed only once (Figure 6), the rapid change in secreted species during a short time interval is striking. The concentration spike 13 s after stimulation is clearly due to the release of norepinephrine, while the next concentration spike is due to the release of epinephrine. Both substances are known to be coming from the same region of the cell surface because of the nature of the electrode placement.[20] Although observed with poor time resolution, the rapid change in identity of the catecholamines suggests that the two catecholamines are stored in separate granules. This result is also consistent with a heterogeneous distribution of PNMT within a single cell.

Conclusions

These results show that analysis at the level of the single cell is an important step forward toward our understanding of the

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fundamental events of neurosecretion. Prior work has demonstrated that two subpopulations of adrenal cells which are significantly enhanced in their content of either epinephrine or norepinephrine can be isolated with their secretory machinery intact. This work has shown that adrenal cells isolated in this manner can be classified according to the catecholamine that they release upon stimulation because the catecholamines are released in the same relative proportions that they are stored in the cell. Verification of the voltammetric technique, which provides information on transient secretion, is possible with microscale chromatographic analysis. Because voltammetry is a nondestructive technique, any method of single-cell analysis may be subsequently performed to obtain complementary information. With this information, the nature of the differences between these subpopulations of adrenal cells may be more completely explored.

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Detection of a Paramagnetic Intermediate in the S_1 State of the Photosynthetic Oxygen-Evolving Complex†

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Abstract: We report the detection of a new electron paramagnetic resonance (EPR) signal that demonstrates the presence of a paramagnetic intermediate in the resting (S_1) state of the photosynthetic oxygen-evolving complex. The signal was detected using the method of parallel polarization EPR, which is sensitive to $\Delta m = 0$ transitions in high spin systems. The properties of the parallel polarization EPR signal in the S_1 state are consistent with an $S = 1$ spin state of an exchange-coupled manganese center that corresponds to the reduced form of the species giving rise to the multiline EPR signal in the light-induced S_2 state. The implications for the electronic structure of the oxygen-evolving complex are discussed.

Water is the terminal electron donor for the electron transfer processes that constitute the light reactions of plant photosynthesis. The splitting of water to produce molecular oxygen, four hydrogen ions, and four electrons takes place in photosystem (PS) II of green plants and cyanobacteria and is mediated by the oxygen-evolving complex, which contains redox-active manganese ions. Electron transfer in PS II begins with the photoexcitation of the primary donor followed by transfer of the photoexcited electron to the iron–quinone acceptor complex via an intermediate pheophytin species. Transfer of an electron from the oxygen-evolving complex via an intermediate tyrosine species reduces the photooxidized primary donor, allowing repetition of the photochemical cycle.[2] Since the oxidation of water to molecular oxygen is a four-electron process, while the reduction of the photooxidized primary donor is a single-electron process, the oxygen-evolving complex must couple the four-electron oxidation of water to the single-electron photochemistry of the rest of the reaction center. This function has been described in terms of an S-state model[3] in which the oxygen-evolving complex cycles through a series of states, S_0–S_4, as it transfers electrons to reduce the primary donor while accumulating oxidizing equivalents for water oxidation. When the complex reaches the state S_4, molecular oxygen is released and the complex reverts to the S_0 state. The resting state of the complex is the S_1 state.

The chemical identity and electronic structure of the species that constitute the S states and the relation of this structure to the function of the oxygen-evolving complex have been the subject of continued study and speculation.[4] Manganese is an essential part of the oxygen-evolving complex and is thought to form the binding site for water in the water oxidation process. Quantitation procedures have estimated a stoichiometry for four functional manganese ions per PS II unit,[5] but the structural organization and oxidation states of the manganese ions throughout the S-state cycle have not yet been established. Although the system has been thoroughly studied with conventional electron paramagnetic resonance (EPR) spectroscopy, only two signals attributed to manganese in the native enzyme have been reported, and both occur in the S_2 state. The multiline signal, comprised of approximately 19 hyperfine components centered near $g = 2$, is consistent with an $S = {}^1/_2$ spin state of an exchange-coupled mixed-valence manganese cluster.[6] A second signal appears at an effective g value of 4.1,[7-9] consistent with an $S = {}^3/_2$ species of nearly axial symmetry, and the correlation of the generation of this signal with an increase in the manganese X-ray absorption energy suggests that the signal originates from manganese.[10]

In this paper, we present a new EPR signal associated with the S_1 state of the oxygen-evolving complex and discuss its implications for the structure of the redox-active manganese centers. The presence of half-integral spin EPR signals in the S_2 state, together

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