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Quantitation of epinephrine and norepinephrine secretion from individual adrenal medullary cells by microcolumn high-performance liquid chromatography

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Abstract

The cellular response of individual adrenal medullary cells to a prolonged stimulation with carbamylcholine (carbachol) was studied using microcolumn high-performance liquid chromatography (MHPLC) with electrochemical detection. Chromaffin cells will not release all of their catecholamine stores when continuously exposed to a secretagogue. The process of desensitization prevents the continued release of catecholamines after a certain percentage of chemical stores have been depleted. The percentage of a cell's chemical stores released upon stimulation was determined, and whether there is a difference in release between the cell types was examined. A single cell was exposed to a stimulation solution for 5.0 min at 25°C. Approximately 80% of the solution was removed and analyzed on a microcolumn reversed-phase chromatography column. A lysing solution was immediately added to the miniaturized vial which still contained the cell, and this solution was also analyzed chromatographically. The first run was used to determine the amount of catecholamine the cell released upon stimulation. The second run determined the amount of catecholamine that remained in the cell. From these values the percentage of catecholamine released was calculated. Twenty-seven individual cells were studied by this method, and 22 could be classified as norepinephrine or epinephrine dominant. There was no statistical difference in the amount of catecholamine the norepinephrine dominant cells released ($14 \pm 5\%$, $n = 11$) as compared to the epinephrine dominant cells ($12 \pm 4\%$, $n = 11$).

1. Introduction

The natural function of adrenal medullary cells is to release norepinephrine (NE) and epinephrine (E) into the circulatory system when exposed to acetylcholine. Cultured adrenomedullary cells are used as models for the study of neurosecretion. Certain chemical compounds can invoke release from adrenomedullary cells by binding to receptors on the plasma membrane. When stimulated, these receptors activate calcium channels that allow the influx of extracellular calcium to enter the cytoplasm. This in turn causes a vesicular release of catecholamine (CA) by the process of exocytosis. The exact mechanism for this process is not well understood, and it is currently an active area of research [1].

It is commonly believed that adrenal medul-

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lary tissue is composed of two distinct cell types [2,3]. One cell type contains predominantly norepinephrine (NE), while the other type contains predominantly epinephrine (E). Researchers studying CA release have observed what they believe to be a difference in the stimulus–response mechanism between the two cell types [4–6]. A complete separation of cell types into two homogeneous populations has never been accomplished. Most past work comparing cell types has always been performed on large cell populations that are only partially enriched in one cell type or the other. Recent work performed on individual cells taken from monolayer cultures has challenged the belief that there exists only two cell types. Results from studies examining cellular secretion [7] as well as total stores [8] of individual bovine adrenomedullary cells have shown that approximately 20% of the cultured cells contain significant amounts of both catecholamines. In light of these new findings, conclusions drawn in earlier work that assumed the existence of only two cell types might require reexamination.

This paper describes work undertaken to determine the percentage of a cell's stores which are secreted upon prolonged contact with a chemical agonist. Adrenal cells are known to become "desensitized" when continuously exposed to a secretagogue. After prolonged exposure to a chemical agonist, desensitization occurs when the cell no longer releases any of its stores, even when still in contact with the stimulating agent. Cell desensitization has been shown to be a complex phenomena. The exact mechanism is not understood. It is not solely caused by such obvious factors as nicotinic receptor desensitization [9], a lack of oxidative energy sources [10], or the depletion of releasable storage vesicles [9,11]. Processes that may be involved are the inactivation of Na^+ and Ca^{2+} channels [12], the inactivation of intracellular Ca^{2+} processes [13,14], or the inactivation of intracellular secondary messengers [9]. It is becoming increasingly evident that desensitization is not the result of any one of these processes, but is believed to be the result of several of these processes acting in concert [9]. This work was undertaken to determine if the percentage of total cellular CA released before desensitization was a function of cell type.

Since the absolute separation of adrenomedullary cells by cell type is currently not possible, the best approach to obtaining information relative to the different cell types is to study a population of cells individually. Recent work in our laboratory involved the quantitation of the total amount of NE and E in individual adrenomedullary cells using MHPLC with electrochemical detection [8]. All the single cell work performed in our laboratory in the past has involved isolating a single cell in a miniaturized vial, followed by its immediate lysis in order to determine total cellular storage content [8,15,16]. The study described herein increases the utility of our past work by enabling the determination of how a single cell responds to environmental changes once isolated. The usefulness of our single cell technique is greatly increased by this ability. In this way, the response of a single cell to changes in its environment, both chemical or physical, can be better studied.

2. Experimental

2.1. Chemicals and solutions

All chemicals were purchased from Sigma (St. Louis, MO, USA), except DHNE which was purchased from Aldrich (Milwaukee, WI, USA). Chemicals were used as received. Solutions were made using deionized water (Barnstead, Dubuque, IA, USA).

A balanced salt solution consisted of 150 m*M* NaCl, 11.2 m*M* glucose, 4.2 m*M* KCl, 2.0 m*M* $CaCl_2$, 1.0 m*M* NaH_2PO_4, 0.7 m*M* $MgCl_2$, 10.0 m*M* HEPES, and was adjusted to pH 7.4 with 1 *M* NaOH. The chromatographic mobile phase was a 0.1 *M* phosphate buffer that was prepared from 85% phosphoric acid, that also contained 1 m*M* EDTA. The pH was adjusted to 3.0 with concentrated NaOH solution. The stimulation solution was prepared by making the balanced salt solution up in 1.0 m*M* carbamylcholine

(CCh), $1.00 \cdot 10^{-5}$ *M* 3,4-dihyroxybenzylamine (DHBA), and 1 m*M* glutathione (GSH). The lysing solution was composed of $1.00 \cdot 10^{-5}$ *M* 3,4-dihydroxynorephedrine (DHNE), 0.1 *M* Na_2SO_3, made up in 0.3 *M* $HClO_4$.

2.2. Chromatography

The chromatographic columns used were fused-silica capillaries (50 cm × 42 μm I.D.) slurry packed in our laboratory with YMC ODS-AQ (YMC, Wilmington, NC, USA) 5-μm spherical C_{18} particles. The packing procedure is described elsewhere [17]. Briefly, a frit was fabricated 1 mm from one end of a fused silica capillary by sintering a 100-μm wide band of 5-μm bare silica particles. The 1-mm gap is left to allow placement of the working (detection) electrode. The capillary was placed in a high pressure slurry reservoir containing a 10% (w/v) slurry of packing material and hexane. Columns were packed at 20.7 MPa.

2.3. Detection

The detector used has been described before [18–22]. In brief, a carbon fiber was used as the working electrode. The carbon fiber, having a diameter of 9 μm and a length of 1 mm, was located inside a glass detector cell. The electrochemical cell was constructed to surround the fiber with supporting electrolyte solution and contained a reference counterelectrode. The detector cell was mounted on a positioning stage (Oriel Corp., Stratford, CT, USA) for precise movement. The detection end of the capillary column was inserted into the electrochemical cell and the movement of the column was independent of the positioning stage. The column and carbon fiber were visible with the aid of a microscope (Carolina Biological Supply, Burlington, NC, USA). By adjusting the positioning stage, the carbon fiber was inserted directly into the end of the capillary column. Amperometric detection was obtained by applying +0.63 V to the working electrode *versus* a Ag/AgCl reference electrode. Currents produced were measured with a Keithley 427 current-to-voltage converter (Cleveland, OH, USA), and the output voltage was fed directly to a microcomputer with 16-bit analog-to-digital converter. Data acquisition and manipulation were both performed using an IBM XT personal computer (Boca Raton, FL, USA). All software used was written in-house. The data acquisition rate was 2.0 Hz.

In order to achieve high run-to-run reproducibility, the working electrode was electrochemically cleaned before each run by applying a triangular waveform from 0 to +1.8 V for 30 s at a rate of 1 V/s [19].

2.4. Sample preparation and injection

The bovine adrenomedullary cells used were cultured following a previously described procedure [7]. The cell preparations involved a procedure to selectively enrich some of the culture plates in NE- and some in E-storing cells [22]. Only cells which had been in culture for three to seven days were used. The cell growth medium in the culture plates was rinsed with and replaced by the balanced salt solution prior to each analysis. The procedure for the isolation and analysis of a single cell involves removing a cell from a culture plate, and transferring it to a miniaturized container called a microvial. A microvial is fabricated by heat sealing the end of a 0.7-mm I.D. borosilicate glass capillary. The capillary is then cut to a length of 1 to 2 mm. Although the total volume of the microvial is 250 nl, only the very bottom of it is used.

Before the cell transfer, exactly 10.0 nl of the stimulation solution was added to the microvial with the aid of an in-house constructed microdispenser [23]. A single cell was then removed from a culture plate and transferred into the microvial by using a micropipette with an I.D. of 20 μm. The average diameter of the adrenomedullary cells was 16 μm. This micropipette was connected to a mercury filled syringe. The mercury acted as a hydraulic fluid, so that when the plunger of the syringe was moved, fine control of the mercury in the tip of the pipette was obtained. Approximately 1 nl of the balanced salt solution from the culture plate was transferred

along with the cell. The microvial was capped with a piece of parafilm to prevent evaporation. After an incubation period of exactly 5.0 min at 26°C, approximately 8 nl of this solution was removed from the microvial with a micropipette, with care being taken to leave the cell behind. This solution, representing what the cell released upon prolonged stimulation, was injected directly onto the capillary column. Next, exactly 10.0 nl of the cell lysing solution was added to the microvial. The microvial was then capped with a small piece of parafilm, and centrifuged at 12 000 *g* for 8 min. The supernatant from the microvial was removed with another micropipette and also underwent chromatographic analysis.

The procedure used to inject low nanoliter sample volumes directly onto a capillary separation column has been described before [23]. On-column microinjections were performed by fitting a pneumatic microinjector with a pipette that had an O.D. at its tip of approximately 35 μm. This enabled it to be inserted directly into the 42 μm I.D. separation column. The procedure involved inserting the pipette into the microvial and allowing capillary action to fill the pipette with sample. Pressure was then taken off of the separation column, and it was removed from the mobile phase delivery system. The injection end of the column was held firmly underneath a microscope. The pipette containing the sample was inserted into the end of the separation column, and 207 kPa of He pressure was used to inject the sample onto the column. All of the sample manipulations and injections were performed with the aid of a Wolfe Selectra II stereomicroscope (Carolina Biological Supply), and a Narishige MM-33 micromanipulator (Medical Systems, Greenvale, NY, USA).

3. Results and discussion

3.1. Viability of an isolated cell

All the single cell work previously conducted in our laboratory involved transferring a cell to a microvial, followed by immediately lysing it by either physical or chemical means [8,16]. A modification to past procedures was necessary in order to ensure that the isolated cell would be viable after the cell transfer while it was in contact with the stimulation solution. It was determined that a cell would frequently lyse if transferred to a microvial, followed by the addition of the stimulation solution with the microdispenser (five 207-kPa pulses of 2 nl each). In order to test whether the cell lysis was due to the stimulation solution specifically, or to the impact of the pressurized solution on the cell, the stimulation solution was replaced with the balanced salt solution. Cells would lyse also under these conditions. Initially, it was unknown whether the cell was being damaged during the transfer process, or by the force of the liquid added by the microdispenser after the transfer. It was discovered that if 10.0 nl of the stimulation solution was dispensed into the microvial first, followed by the addition of the cell, that the cell would remain intact and functionally active after the transfer. It appears that the force caused by the addition of any solution to the microvial with the microdispenser is sufficient to lyse the cell.

3.2. Solution additions

Carbamylcholine (CCh), or carbachol, was chosen as the chemical secretagogue. CCh is an analog to acetylcholine (ACh), the latter not being used because of reports that cultured bovine adrenomedullary cells release acetylcholinesterase [24]. Whereas 0.1 m*M* ACh yields maximum CA secretion, it is necessary to use 1.0 m*M* CCh to obtain a similar level of effectiveness [25]. The stimulation solution also contained DHBA for use as an internal standard and the antioxidant glutathione.

The cell was exposed to the stimulation solution for 5.0 min. It has been reported that CCh-induced CA secretion ceases after 5 min [12,26]. The lysing solution consisted of perchloric acid to lyse the cell, DHNE as a second internal standard, and Na_2SO_3 as an antioxidant.

Unlike a conventional HPLC injector that transfers a precise volume onto the chromatographic column, the microinjection process described here does not transfer the same amount

of sample onto the column each time [8]. An internal standard was used with each injection solution to account for this variability, making the volume injected onto the column unnecessary for quantitation.

3.3. Quantitation

Sample linearity was demonstrated between 0.100 and 300 fmol for both NE and E. Once linearity was determined, calibration curves were generated using 1.00, 10.0, and 100 fmol of each catecholamine.

Calibration curves were generated for (1) NE and E *vs.* DHBA, (2) NE and E *vs.* DHNE, and (3) DHBA *vs.* DHNE. In the first case, 100 fmol of DHBA, NE, and E were microdispensed into a microvial, and then microinjected onto the column as previously described. This procedure was repeated using 1.00 and 10.0 fmol of both NE and E with 100 fmol DHBA. A calibration curve was constructed by plotting the ratios of the peak areas of NE and E to the peak area of DHBA for that run *vs.* the number of fmol of NE and E originally added to the microvial. This procedure was repeated in case (2) by substituting DHNE for DHBA, and in case (3) by substituting DHBA for NE and E. The number of moles of catecholamine present in a microvial was determined by ratioing the peak area of each catecholamine to the peak area of the internal standard in that run and interpreting from the calibration curves the number of moles present.

Fig. 1A is a chromatogram of an aliquot of stimulation solution after having been exposed to an individual cell for 5 min. Fig. 1B is a chromatogram of the acid lysing solution after it was in contact with the same cell as in Fig. 1A. The percentage of NE and E that a single cell released due to a prolonged exposure to CCh was determined by the following procedure:

Step 1. The amount of catecholamine released after stimulation can be obtained directly from the peak areas seen in Fig. 1A.

Step 2. The peak areas seen in Fig. 1B are the sum of what was not released from the cell during the cell stimulation as well as what stimulation solution was not removed from the microvial by the injection pipette. The area under these peaks was interpolated from calibration curves to give an "uncorrected" acid release value.

Step 3. The chromatogram of the lysing solution always displayed a peak for DHBA, the internal standard from the first run. The amount of DHBA was quantified and used to determine the percentage of stimulation solution removed from (or conversely what was left in) the microvial by the injection pipette.

Step 4. From the percentage of stimulation

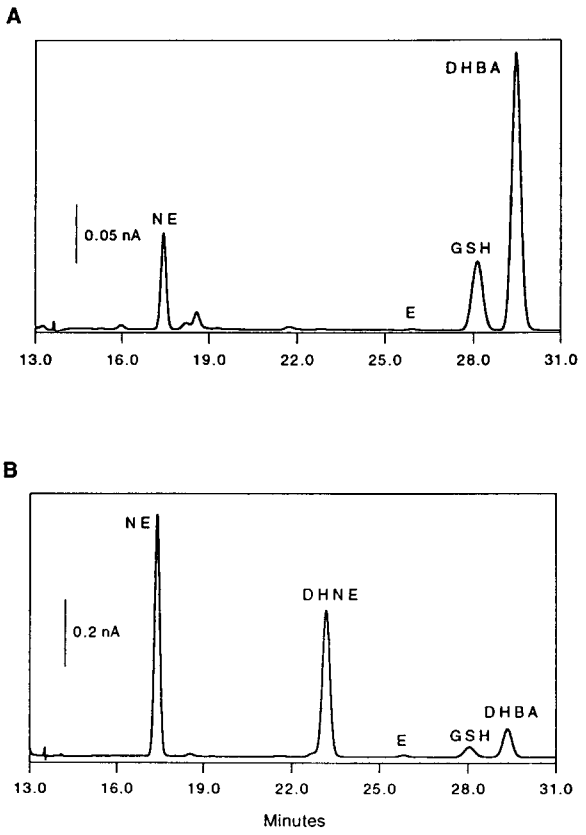

Fig. 1. (A) Chromatogram obtained of stimulation solution after being exposed to an individual adrenal medullary cell for 5.0 min. (B) Chromatogram obtained of lysing solution after being exposed to the same cell as in (A). Separations were performed on a 50 cm × 42 μm I.D. capillary column packed with 5-μm C_{18} particles. The peak labels are: (NE) norepinephrine, (DHNE) 3,4-dihydroxynorephedrine, (E) epinephrine, (GSH) glutathione, and (DHBA) 3,4-dihydroxybenzylamine. All unlabeled peaks are unknowns. See Experimental for details.

solution that remained in the microvial (step 3) and from the exact amount of NE and E released upon stimulation (step 1), it was possible to determine how much extracellular NE and E remained in the microvial as a result of not being removed by the injection pipette.

Step 5. The number of moles left in the microvial from the stimulated release (step 4), subtracted from the uncorrected amount of catecholamine released after addition of lysing solution (step 2), yields the actual amount of catecholamine released from the cell after the addition of the lysing solution.

Step 6. The sum of the values obtained in steps 1 and 5 yields the total number of moles of catecholamine in the cell prior to being placed in the microvial.

Step 7. The percentage of catecholamine released from a cell due to stimulation with CCh relative to the cell's total stores prior to release is obtained from the values calculated in steps 1 and 6.

The results of performing this method on 27 individual adrenomedullary cells can be seen in Table 1. The two chromatograms displayed in Fig. 1 correspond to cell No. 5 in Table 1. Cells 1–11 predominately store NE, and cells 12–22 are dominant in E. In Table 1, the percentage released from the dominant CA is presented in bold text. As seen in prior single cell studies,

Table 1
Percentage of norepinephrine and epinephrine released from individual adrenomedullary cells by prolonged exposure to carbamylcholine

Cell No.	Norepinephrine			Epinephrine		
	Release (fmol)	Total (fmol)	Release (%)	Release (fmol)	Total (fmol)	Release (%)
1	24.1	119	**20.2**	4.04	5.86	68.9
2	33.3	168	**19.8**	4.46	11.9	37.5
3	25.4	129	**19.6**	1.83	12.3	14.8
4	22.7	137	**16.6**	0.59	1.78	33.1
5	20.4	128	**15.9**	0.33	1.56	21.1
6	10.5	69.2	**15.2**	0.93	1.85	50.3
7	21.4	158	**13.5**	1.19	13.0	9.2
8	14.8	157	**9.4**	0.75	5.73	13.1
9	8.01	103	**7.8**	0.59	2.29	25.8
10	13.1	168	**7.8**	0.01	11.3	0.1
11	11.5	189	**6.1**	3.78	20.4	18.5
12	4.42	12.5	35.4	12.9	64.1	**20.1**
13	0.54	2.36	22.9	30.0	164	**18.3**
14	3.20	34.0	9.4	34.7	228	**15.2**
15	0.75	2.50	30.0	14.3	99.8	**14.3**
16	1.02	6.75	15.1	27.4	191	**14.3**
17	1.32	9.99	13.2	25.6	201	**12.7**
18	0.95	3.40	27.9	15.2	138	**11.0**
19	2.12	7.68	27.6	9.22	96.8	**9.5**
20	5.82	19.1	30.5	14.2	159	**8.9**
21	3.56	8.96	39.7	5.50	84.5	**6.5**
22	4.37	35.6	12.3	14.8	231	**6.4**
23	18.4	104	17.7	15.0	53.7	28.0
24	28.8	140	20.6	4.64	55.4	8.4
25	14.4	68.1	21.1	11.5	219	5.3
26	10.5	62.6	16.8	19.6	148	13.2
27	21.9	141	15.5	4.65	46.7	9.9

Table 2
Basal catecholamine levels found in 2 nl of culture plate bathing solution

Plate No.	Plate type[a]	NE (fmol)	E (fmol)
1	NE	0.45	0.10
2	NE	0.43	0.02
3	E	N.D.[b]	0.77
4	E	0.12	0.69

[a] The culture plate was enriched in this cell type.
[b] N.D. is none detected.

approximately 20% of the cells examined contained comparable amounts of both catecholamines [7,8]. These cells are indicated in Table 1 as 23–27. It is unclear whether cells 23–27 represent a third cell type *in vivo*, or if they are artifacts of the culturing process.

3.4. Error analysis

We are mindful of two factors that introduce an error into the stimulated release values seen in Table 1. The first involves the cellular bathing solution from the culture plate that is transferred to the microvial. Cultured cells are known to secrete NE and E, even without a stimulus, in order to maintain a basal concentration of these two catecholamines in the surrounding cell bathing fluid [27]. When a cell was removed from the culture plate and transferred to a microvial, approximately 1 nl of the bathing solution was transferred as well. In order to determine approximately how much extraneous catecholamine was being transferred with the cell, 2.0 nl of bathing solution was removed from four different culture plates. Each aliquot was analyzed for catecholamine content. The results are shown in Table 2. The volume of bathing solution that was transferred along with each cell varied and was not determined, although it was always less than 2 nl. The highest basal levels of catecholamine detected were in the E-enriched plates, which averaged 0.75 fmol E per 2.0 nl.

The second factor that introduces error into the stimulated release values involves release of CA from the cell of interest that was not caused by the secretagogue. As a control, four cells were examined using the same method outlined above, the only exception being that no secretagogue was present. The results from this experiment are shown in Table 3. It is clear that initial CA release levels without a secretagogue are much higher than can be accounted for by the carryover of cellular bathing solution as shown in Table 2. This can be caused by two processes. The first process is that a cell will release a small fraction of its chemical stores when physically touched. The fraction is much smaller than what is released when the cell is chemically stimulated [27]. This phenomenon has been referred to as a "mechanical stimulation" [28]. During the cell transfer process, the transfer pipette made contact with the cell in order to dislodge it from the culture plate. Our method of cell transfer may cause a mechanical stimulation to occur. The second process is that while a cell resided for 5.0 min in the microvial without any secretagogue, it released basal levels

Table 3
Catecholamine release from individually isolated adrenomedullary cells without the presence of a secretagogue

Cell No.	Norepinephrine			Epinephrine		
	Release (fmol)	Total (fmol)	Release (%)	Release (fmol)	Total (fmol)	Release (%)
1	4.15	112	**3.7**	0.11	0.87	13
2	1.25	13.9	9.0	7.88	226	**3.5**
3	0.30	4.36	6.9	2.57	218	**1.2**
4	2.03	29.8	6.8	2.91	105	2.8

of CA. Reports have shown that, using entire culture plates, within 5 min basal levels of CA range from 15–20% of the choline stimulated release values [25,26,29].

A third internal standard could have been used to account for any mechanically stimulated release if it could have been added it to the microvial after the addition of the cell. As discussed earlier, this could not have been performed without compromising the integrity of the cell, therefore this release, if any, went uncorrected. It would have been possible to compensate for the levels of CA transferred to the microvial with the cell bathing solution by simply adding another internal standard to the culture plate prior to the cell transfer. However, since the values seen in Table 2 are much less than the release values shown in Table 3, it was deemed unnecessary. All the uncompensated errors discussed would affect the stimulated release values reported in Table 2 by making them appear slightly larger than they should be. This would consequently result in an increase in the release percentages, also.

It is important to stress that the emphasis in Table 1 is placed on the values obtained for the major component in a cell. As far as percentage released, these larger values will be affected by the uncorrected error mentioned above.

3.5. Accuracy

The average amount of total catecholamine present in a single cell was calculated, using all 27 cells from Table 1, to be 165 ± 56.0 fmol (all values reported are mean ± standard deviation). This number is in close agreement with the previously determined value of 145 ± 48.7 fmol per cell determined using a more direct approach [8]. Upon prolonged CCh stimulation, NE-dominant cells, numbers 1–11, were found to release 13.9 ± 5.3% of NE. E-dominant cells, numbers 12–22, were found to release 12.4 ± 4.4% of E. Other researchers have reported a CA release of 13% from cultured bovine adrenal cells after a 5-min exposure to 0.3 m*M* CCh at 25°C [12]. Still others have exposed cultured bovine cells to 0.1 m*M* CCh at 20°C and determined a release of approximately 10% [30]. Both of these referenced studies were performed on cultured cells that did not involve an enrichment process, so the results are for an average of all cell types.

Different secretagogues can induce stimulation through different mechanisms. If an alternate mechanism is used, a different percentage of total cellular CA is often released. It has been shown that K^+ induced secretion releases up to 55–60% of the CA levels seen relative to the release caused by stimulation with ACh [25,29,31]. It was decided, therefore, to stimulate some individual cells with K^+. If a reduction in the percentage of CA released was seen, it would increase our confidence in the methodology. The procedure followed was identical to before, with the exception that 1.0 m*M* CCh was replaced with 56 m*M* K^+. The results are shown in Table 4. Note that the percentage release values are greater than the percentage released seen without a secretagogue, but approximately half what was seen when CCh was present.

There have been reports that different secretagogues cause different ratios of NE and E to

Table 4
Percentage of norepinephrine and epinephrine released from individual adrenomedullary cells by prolonged exposure to 65 m*M* K^+

Cell No.	Norepinephrine			Epinephrine		
	Release (fmol)	Total (fmol)	Release (%)	Release (fmol)	Total (fmol)	Release (%)
1	0.86	10.2	8.4	15.7	215	**7.3**
2	0.76	13.9	5.5	10.7	187	**5.7**
3	1.14	20.3	5.6	10.4	207	**5.0**

be secreted into the surrounding cellular fluid. Researchers have used this data to conclude that the two cell types must react differently under the same stimulation conditions. One report determined that the secretagogue veratridine caused a preferential release of E, while a stimulation with K^+ caused a preferential release of NE [31].

Livett and Marley exposed cultured bovine cells to the stimulant nicotine [4]. After a thorough rinse with balanced salt solution, the same culture plates were exposed to K^+. Twice as much E was released by the K^+ as in control plates that were only exposed to K^+, without previously undergoing nicotine stimulation. In contrast, 33% less NE was released by the K^+ in plates where the cells had previously been exposed to nicotine compared to cells in plates that had no previous nicotine exposure. It appeared that the nicotine sensitized the E-dominant cells to K^+ release, while desensitizing the NE-dominant cells. These researchers concluded that there appears to be a difference in the stimulus–secretion coupling mechanism between E and NE cell types.

The two referenced works mentioned above were performed on culture plates that had not been enriched in a particular cell type. The researchers assumed that there were two and only two cell types. They concluded that all the E seen originated from E-dominant cells and that all the NE seen originated from NE-dominant cells. From our work discussed so far, this is a false assumption.

Better results can be obtained using plates enriched in one cell type or the other. Unfortunately, little work using this approach for desensitization studies has been published. Lemaire *et al.* [6] demonstrated an adrenal cell enrichment procedure utilizing sedimentation through a stepwise bovine serum albumin gradient. It is reported to produce a cell population with an E to NE cell ratio of 0.8, and another with a ratio of 3.5. When stimulated with 50 μM ACh at 37°C for 5 min, a total catecholamine release of 20% was seen for the NE-enriched population, and of 5% for the E-enriched population. The data presented here, using CCh, do not show a statistical difference in the percentage of catecholamine released as a function of cell type.

Of the 22 cells that can be categorized as either NE- or E-dominant, the amount of CA released of the minor substituent was always less than 6 fmol. The average amount released was *ca.* 2 fmol. It is interesting to note that in the indeterminant cells, numbers 23–27, two were in this range while three were not. These three (numbers 23, 25 and 26) had release values for their minor substituents that were much higher than the average. This may be a unique property characteristic of the indeterminant cell type.

4. Conclusions

The cellular response of individual adrenal medullary cells to a prolonged stimulation with carbamylcholine was studied using MHPLC. Twenty-seven individual cells were studied by this method, and 22 could be classified as NE- or E-dominant. There was no statistical difference in the amount the NE-dominant cells released ($14 \pm 5\%$, $n = 11$) as compared to the E-dominant cells ($12 \pm 4\%$, $n = 11$).

We feel that our approach of examining cells individually is the most direct method for determining densitization differences between the cell types. Past work has always been conducted on culture plates that were at best only enriched in one cell type. We feel that the conclusions drawn herein are more accurate than analyzing culture plates containing a mixture of cell types, particularly when 20% of the cultured cells cannot be considered NE- or E-dominant.

It is possible that if a different secretagogue other than CCh is used, differences in the release percentages between the cell types may emerge. Since many different secretagogues can initiate CA release in different ways, each can be used to answer specific questions regarding release mechanisms. To better understand and define the mechanisms of cellular desensitization, further studies with different secretagogues at the single cell level would prove useful. Finally, densitization is just one cellular function that can

be better examined using this method of single cell analysis.

5. Acknowledgement

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