Simultaneous Measurements of Cytosolic Calcium and Secretion in Single Bovine Adrenal Chromaffin Cells by Fluorescent Imaging of Fura-2 in Cocultured Cells

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Abstract. The cytosolic free calcium concentration ([Ca\(^{2+}\)]_i) and exocytosis of chromaffin granules were measured simultaneously from single, intact bovine adrenal chromaffin cells using a novel technique involving fluorescent imaging of cocultured cells. Chromaffin cell [Ca\(^{2+}\)]_i was monitored with fura-2. To simultaneously follow catecholamine secretion, the cells were cocultured with fura-2-loaded NIH-3T3\(^{+}\) cells, a cell line chosen because of their irresponsiveness to chromaffin cell secretagogues but their large Ca\(^{2+}\) response to ATP, which is coreleased with catecholamine from the chromaffin cells.

In response to the depolarizing stimulus nicotine (a potent secretagogue), chromaffin cell [Ca\(^{2+}\)]_i increased rapidly. At the peak of the response, [Ca\(^{2+}\)]_i was evenly distributed throughout the cell. This elevation in [Ca\(^{2+}\)]_i was followed by a secretory response which originated from the entire surface of the cell.

In response to the inositol 1,4,5-trisphosphate (InsP\(_3\))-mobilizing agonist angiotensin II (a weak secretagogue), three different responses were observed. Approximately 30% of chromaffin cells showed no rise in [Ca\(^{2+}\)]_i and did not secrete. About 45% of the cells responded with a large (>200 nM), transient elevation in [Ca\(^{2+}\)]_i and no detectable secretory response. The rise in [Ca\(^{2+}\)]_i was nonuniform, such that peak [Ca\(^{2+}\)]_i was often recorded only in one pole of the cell. And finally, ~25% of cells responded with a similar Ca\(^{2+}\)-transient to that described above, but also gave a secretory response. In these cases secretion was polarized, being confined to the pole of the cell in which the rise in [Ca\(^{2+}\)]_i was greatest. Exocytosis in response to nicotine occurred over the entire surface of the cell, whereas exocytosis due to angiotensin II was polarized, as was confirmed by immunofluorescent localization of dopamine-B-hydroxylase, a chromaffin granule protein that becomes incorporated into the plasma membrane during fusion.

These results directly demonstrate, for the first time, that intact chromaffin cells can undergo a large, agonist-induced transient rise in [Ca\(^{2+}\)]_i without this stimulating secretion and, furthermore, show that the location of exocytosis around the cell can vary depending on the nature of the stimulus.

EXOCYTOSIS, the process by which intracellular vesicles fuse with the inner surface of the plasma membrane and release their contents into the surrounding medium, is the mechanism underlying the secretion of many physiologically important mediators such as hormones, enzymes, and neurotransmitters. The process is often regulated by an external signal which stimulates release by altering the level of an intracellular second messenger.

The pivotal role that Ca\(^{2+}\) plays in triggering exocytosis was first noted nearly 30 yr ago with the demonstration that depolarized chromaffin cells would not secrete catecholamines in the absence of extracellular Ca\(^{2+}\) (13). The involvement of Ca\(^{2+}\) in exocytosis was advanced by subsequent information obtained from studies with Ca\(^{2+}\)-selective ionophores (16), but it was not until the introduction of two recent technical advances that the nature of the relationship between Ca\(^{2+}\) and secretion has been investigated in greater detail. Thus, the use of permeabilized cells which retain their ability to secrete (15, 22) and membrane-permeable fluorescent Ca\(^{2+}\) dyes to continuously monitor the intracellular concentration of free Ca\(^{2+}\) ([Ca\(^{2+}\)]_i) (10, 35) have resulted in a large body of literature documenting the intracellular environment conducive to exocytosis (3, 14) and the changes in [Ca\(^{2+}\)]_i that occur when cell populations (7) or single cells (26) are stimulated to secrete.

1. Abbreviations used in this paper: [Ca\(^{2+}\)]_i, the intracellular concentration of free calcium ions; DBH, dopamine-B-hydroxylase; InsP\(_3\), inositol 1,4,5-trisphosphate.

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Complete elucidation of the role of Ca\(^{2+}\) in triggering secretion will only be forthcoming, however, after the quantitative relationship between [Ca\(^{2+}\)]\(i\) and secretion has been explored at the single cell level. In this paper, we have investigated the relationship between [Ca\(^{2+}\)]\(i\) and secretion in single intact bovine adrenal chromaffin cells using fluorescent imaging techniques. Chromaffin cells were loaded with fura-2 to monitor [Ca\(^{2+}\)]\(i\) and cocultured with fura-2-loaded NIH-3T3 cells. These cells were used as markers for the secretion of ATP which is coreleased with catecholamine from chromaffin cells (32). By using this novel coculture technique, we were able to visualize the agonist-induced changes in [Ca\(^{2+}\)]\(i\) in single chromaffin cells and simultaneously monitor any subsequent secretory response after challenge with nicotine, a potent secretagogue (11), or the inositol 1,4,5-trisphosphate (Ins\(P_3\))-mobilizing agonist angiotensin II, which is a weak secretagogue (25).

Materials and Methods

Materials

Fura-2/AM was from Molecular Probes Inc. (Eugene, OR). Second antibodies were from Amersham International Plc (Amerham, U.K.) and were diluted in 0.3% BSA in PBS. All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Anti-dopamine-B-hydroxylase (DBH) was prepared according to the method of Aunis et al. (2).

Culture of NIH-3T3 Cells

NIH-3T3 cells were passaged by trypsinization and plated on 22-mm-diam glass coverslips at a density of 2 × 10^4 cells/ml in 0.8 ml of DME containing 10% fetal calf serum and cultured for 3 d.

Isolation of Chromaffin Cells and Coculture with NIH-3T3 Cells

Chromaffin cells were isolated from bovine adrenal medullas by enzymatic digestion using either the method of Knight and Baker (23) or a modification (6) of the method of Greenberg and Zinder (17). Cells were isolated in Ca\(^{2+}\)-free Krebs-Ringer buffer consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM MgCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 10 mM glucose, 20 mM Hepes, pH 7.4 (buffer A), washed in buffer A, and resuspended in DME containing 25 mM Hepes, 10% fetal calf serum, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 U/ml penicillin, 25 μg/ml streptomycin. The cells were purified by differential plating (36) for 2 h, after which time the nonadherent chromaffin cells were plated onto the NIH-3T3 cells at a density of 3.8 × 10^5 cells/ml in 3 ml of the above chromaffin cell medium. The cells were then cultured overnight.

Loading Cocultured Cells with Fura-2

After overnight incubation the cocultures were washed in buffer A containing 3 mM CaCl\(_2\) and 0.1% BSA and incubated with 2 μM fura-2-acetoxymethyl ester at room temperature for 40 min. These conditions slightly digested the chromaffin cells (26) resulting in prolonged Ca\(^{2+}\) signals in some cells, probably because of inhibition of Ca\(^{2+}\)-channel inactivation (1), but were necessary in order to adequately load the NIH-3T3 cells. The cells were equilibrated to 37°C for 5 min and coverslips were mounted in an aluminum-alloy perfusion chamber for imaging. The cells were perfused at 37°C with just enough buffer A containing 3 mM CaCl\(_2\) and 0.1% BSA to keep them from drying out. The perfusion was shut off and agonist in the same buffer immediately applied via a U-tube positioned to within 2 mm of the field of cells under observation. Experiments with dye solutions showed that, by using this method, all the cells in the field would be challenged virtually simultaneously by the agonists and within 1 s of the onset of application. On termination of agonist application, there was no perfusion of medium over the cells and the cells were submerged in minimal buffer in order to maximize the detection of diffusing ATP.

Monitoring Fura-2 in Single Cells and Image Processing

Fura-2 fluorescence was excited by twin, high pressure, xenon arc lamps fitted with grating monochromators (Spx Industries Inc., Edison, NJ), and interfaced to a Nikon Inc. (Garden City, NY) Diaphot inverted epifluorescence microscope. The cocultures were all imaged with a UVF 100× glycerol-immersion objective resulting in a final magnification of 1,000×. Excitation wavelengths were set at 340 and 380 nm (10-nm bandwidth). Emitted light was passed through a 400-nm dichroic mirror, filtered at 510 nm (10-nm bandpass), and collected by a single stage intensified CCD camera (Photonic Science, Tunbridge Wells, UK). The video signal from this was digitized and stored in an Imagine image-processing system (Synoptics Ltd., Cambrige, UK), hosted by a Digital Equipment Corp. (Marlboro, MA) MicroVAX II computer. The excitation source was switched by a rotating-mirror chopper (Glen Creston Instruments, Stanmore, UK) driven by a stepping motor and synchronized with the video timebase to give alternate TV frames at each of the two wavelengths. The Imagine video-rate processor was programmed to form from each successive pair of frames a “live” ratio image, which was recursively filtered with a 200-ms time constant (i.e., 5 ratio images/s), and stored on videotape (SonyUmatic) for subsequent processing.

Formation of the ratio image was implemented in a look-up table, computed from the formula (18):

$$
[Ca^{2+}]_i = \frac{R_{max} - R}{R_{max} - R_{min}} \times S_2 - S_1
$$

where $K_d$ is the dissociation constant for fura-2/Ca\(^{2+}\) (224 nM) (18); R is the intensity ratio for fluorescence at the two chosen wavelengths; $R_{min}$ and $R_{max}$ are ratios at zero and saturating [Ca\(^{2+}\)], respectively; and $S_2/S_1$ is the ratio of excitation efficiencies for free and bound fura-2 at the higher of the two wavelengths. All ratios were determined empirically under standard operating conditions, using bulk solutions of CaCl\(_2\) 10 mM EGTA with 5 μM fura-2 added as a penta-sodium salt, and a photomultiplier to measure intensities. As a check for equal concentrations of fura-2 in the presence and absence of calcium, the ratio of intensities was also measured with excitation at 360 nm, where fluorescence is independent of calcium activity (18).

Recorded video data were played back through Imagine, using a different program to give a false-color representation of image intensities, and to allow individual pictures to be captured on disk. Continuous traces of intensity from individual cells were obtained by attaching photo diodes, each mounted behind a collecting lens of ~30-mm focal length, to the screen of a small monochrome TV monitor. Three-dimensional plots were generated from Imagine from the ratio image and in all cases depict the distribution and quantitative rise in [Ca\(^{2+}\)] elicited by the stimulus after subtraction of basal [Ca\(^{2+}\)].

Immunofluorescence Staining with Anti-DBH

After isolation and differential plating, cells were seeded on coverslips in 24-well trays at a density of 10^5 cells/well in chromaffin cell medium and maintained in culture for 2 d. Cells were washed in buffer A containing 3 mM CaCl\(_2\) and 0.1% BSA and incubated with the agonist for 15 min at 37°C in the presence of anti-DBH (1/800). Secretion was terminated by removal of the medium and cells were washed twice and fixed in 4% formaldehyde in PBS overnight. Cells were washed in PBS, sequentially incubated in 0.3% BSA in PBS for 30 min, anti-rabbit biotin (1/100) for 60 min, Texas red-linked streptavidin (1/50) for 30 min, and then mounted and photographed.

Results

Changes in [Ca\(^{2+}\)]\(i\) and Secretion Due to Nicotine

NIH-3T3 cells are a subclone of NIH-3T3 cells that were found not to respond with a rise in [Ca\(^{2+}\)]\(i\) to either nicotine ($n = 31$, data not shown) or angiotensin II ($n = 28$; data not shown), but do respond with a rise in [Ca\(^{2+}\)]\(i\) to ATP and other adenine nucleotides. Because large amounts of ATP are coreleased with catecholamine from stimulated chroma-
Figure 1. Sequential ratio images of \([\text{Ca}^{2+}]_i\) in chromaffin/NIH-3T3 cocultured cells. One chromaffin cell (a, arrow) is surrounded by NIH-3T3 cells. The cells were simultaneously challenged with 10 \(\mu\)M nicotine. The images show the unstimulated field (a) and the field at 7 (b), 18 (c), and 80 s (d) after stimulation with 10 \(\mu\)M nicotine. Only the chromaffin cell responded to the nicotine. The NIH-3T3 cells responded to the ATP released from the stimulated chromatin cell.

Changes in \([\text{Ca}^{2+}]_i\) and Secretion Due to Angiotensin II

The response of chromaffin cells to angiotensin II was much more variable. 7/24 chromaffin cells showed no rise in \([\text{Ca}^{2+}]_i\) in response to 0.3 \(\mu\)M angiotensin II (optimum
Figure 2. Time course of changes in \([\text{Ca}^{2+}]_i\) due to nicotine and then ATP in the cocultured cells shown in Fig. 1. (a) Time courses are photo diode recordings of video images and show responses to cells challenged with 10 \(\mu\)M nicotine followed by 100 \(\mu\)M ATP. Event markers show duration of agonist perfusion. Cell 1 was the chromaffin cell, cells 2–10 the NIH-3T3 cells. The three-dimensional plot shows the distribution of \([\text{Ca}^{2+}]_i\) in the chromaffin cell at the peak of the response to nicotine. (b) Cell map indicating position of cells from which data in a were collected (cell 10 was out of the field of view in Fig. 1). Mean \([\text{Ca}^{2+}]_i\) indicates average \([\text{Ca}^{2+}]_i\) throughout the entire cell. Max \([\text{Ca}^{2+}]_i\) indicates the maximum \([\text{Ca}^{2+}]_i\) achieved at any one point within the cell. Note the oscillations in cell 3.

Table I. The Distance of the NIH-3T3 Cells from the Chromaffin Cell and the Latency Before the Onset of Their \([\text{Ca}^{2+}]_i\) Response to Nicotine and ATP

<table>
<thead>
<tr>
<th>NIH-3T3 Cells</th>
<th>Distance from Chromaffin Cell ((\mu m))</th>
<th>Latency (s)</th>
<th>Nicotine (10 (\mu)M)</th>
<th>ATP (100 (\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>2</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>8</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>8</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>8</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17.5</td>
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<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>12</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>14</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

The NIH-3T3 cells farthest from the chromaffin cell generally had a longer latency than those nearer the chromaffin cell after perfusion with nicotine, but all responded after equal latency on perfusion with ATP.

* As shown in the field of cocultured cells in Figs. 1 and 2 b.

dose, see reference 26) and no secretion (data not shown). Fig. 3 shows the result observed in 6/24 chromaffin cell/NIH-3T3 cocultures. In response to 0.3 \(\mu\)M angiotensin II, there was a transient elevation of \([\text{Ca}^{2+}]_i\) in the chromaffin cell. In contrast to the situation with nicotine, however, maximal \([\text{Ca}^{2+}]_i\) (252 nM) was only achieved in one pole of the cell (Fig. 3 a; three-dimensional plot). Although 4/5 of the surrounding NIH-3T3 cells showed a subsequent rise in \([\text{Ca}^{2+}]_i\), indicating secretion from the chromaffin cell, the correlation between delay in NIH-3T3 response and distance from the chromaffin cell, seen in response to nicotine, was lost (Table II). Furthermore, the NIH-3T3 cells which detected the least amount of secretion (i.e., whose \([\text{Ca}^{2+}]_i\) response was <50% of the control ATP response) were cell 6 (no response) and cell 3 (38% of the ATP response). The fact that these two cells lie within close proximity of one another (Fig. 3 b) indicated that the secretory response could have been polarized with a bias towards area A of the cell (Fig. 3, a and b, three-dimensional plot).

Fig. 4 shows a typical result obtained from 11/24 chro-
maffin cell/NIH-3T3 cocultures after a challenge with 0.3 μM angiotensin II. The chromaffin cell (cell 1) responded with a large, transient elevation in \([\text{Ca}^{2+}]_i\) with peak \([\text{Ca}^{2+}]_i\) (287 nM) again being recorded in one pole of the cell (Fig. 4 a; three-dimensional plot). No subsequent secretion was detected by a rise in \([\text{Ca}^{2+}]_i\) in any of the five neighboring NIH-3T3 cells. Similar results were seen in other experiments in which the chromaffin cell was completely surrounded by NIH-3T3 cells. The cells were then perfused with nicotine with the result that \([\text{Ca}^{2+}]_i\) in the chromaffin cell again increased rapidly. As in Fig. 2, peak \([\text{Ca}^{2+}]_i\) (266 nM) was recorded uniformly throughout the cell (Fig. 4 a; three-dimensional plot). In this case, a subsequent secretory response was detected by the NIH-3T3 cells, with the NIH-3T3 cells furthest away from the chromaffin cell having a longer latency before the onset of their response (Table III). This delay was genuine, as was the lack of response after perfusion with angiotensin II, since all the NIH-3T3 cells responded virtually simultaneously to ATP when applied through the U-tube (Fig. 4 a and Table III).

**Table II.** The Distance of the NIH-3T3 Cells from the Chromaffin Cell and the Latency Before the Onset of Their \([\text{Ca}^{2+}]_i\) Response to Angiotensin II and ATP

<table>
<thead>
<tr>
<th>NIH-3T3 Cells</th>
<th>Distance from Chromaffin Cell (μm)</th>
<th>Angiotensin II (0.3 μM)</th>
<th>ATP (100 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
<td>No response</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>4</td>
<td>20.0</td>
<td>11</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

The latencies after perfusion with angiotensin II were variable (cell 6 showed no rise in \([\text{Ca}^{2+}]_i\)) and there was no correlation with distance from the chromaffin cell (cf. Table I). After perfusion with ATP the latencies were equal.* As shown in the field of cocultured cells in Fig. 3.

**Immunofluorescent Localization of Exocytotic Sites**

The notion that secretion from chromaffin cells occurs over the entire cell surface in response to nicotine, but is polarized to one area of the cell in response to angiotensin II, was confirmed by an independent immunofluorescence technique. Fig. 5 shows the result of challenging chromaffin cells with 10 μM nicotine and 0.3 μM angiotensin II for 15 min in the presence of an antibody to DBH, which was subsequently detected by Texas red fluorescence. DBH is a major constituent of the chromaffin granule membrane (30) and its presence in the plasma membrane after stimulation of the cell therefore indicates where exocytosis has occurred. After a challenge with nicotine, the plasma membrane of chromaffin cells is completely illuminated by a ring of Texas red fluorescence (Fig. 5 d). By contrast, in response to angioten-
Figure 4. Time course of changes in $[\text{Ca}^{2+}]_i$ in chromaffin/NH-3T3 cocultured cells in response to angiotensin II, nicotine, and then ATP. (a) Time courses are photo diode recordings of ratio images and show responses to cells challenged successively with 0.3 µM angiotensin II, 10 µM nicotine, and then 100 µM ATP. Event markers show duration of agonist perfusion. Cell 1 was the chromaffin cell, cells 2-6 the NH-3T3 cells. The three-dimensional plots show the distribution of $[\text{Ca}^{2+}]_i$ in the chromaffin cell at the peak of the responses to angiotensin II and nicotine: max $[\text{Ca}^{2+}]_i$ was localized to one area of the cell ($a$ and $b$, asterisk) in response to angiotensin II, but was uniformly recorded throughout the cell in response to nicotine. (b) Cell map indicating position of cells from which data in $a$ were collected. The position of the asterisk on cell 1 (chromaffin cell) corresponds to the asterisk on the three-dimensional plots in $a$. Mean $[\text{Ca}^{2+}]_i$ indicates average $[\text{Ca}^{2+}]_i$ throughout the entire cell. Max $[\text{Ca}^{2+}]_i$ indicates the maximum $[\text{Ca}^{2+}]_i$ achieved at any one point within the cell.

Discussion

As a continuation of our studies on the relationship between $[\text{Ca}^{2+}]_i$ and secretion in chromaffin cells, we have compared the $\text{Ca}^{2+}$ and secretory responses elicited by a depolarizing stimulus (nicotine) with the corresponding responses elicited by an InsP$_3$-mobilizing agonist (angiotensin II) at the level of the single cell. In other experimental systems (27, 28) the problem of simultaneously monitoring $[\text{Ca}^{2+}]_i$ and measuring secretion from the same cell has been overcome by using plasma membrane capacitance measurements on cells loaded with fura-2. Such cells can be dialyzed with second messengers to trigger release directly, but rapidly lose their ability to secrete in response to external stimuli due to washout of key cytoplasmic constituents (29). By using intact cells, the coculture system presented here circumvents this constraint and has the added advantage of allowing not only visualization of the initial $\text{Ca}^{2+}$ signal but also the spatial organization of the subsequent secretory response.

In response to the potent secretagogue nicotine, 95% of cells examined gave a strong (>200 nM) rise in $[\text{Ca}^{2+}]_i$ which originated at the cell periphery (data not shown) and then infilled such that peak $\text{Ca}^{2+}$ was recorded uniformly throughout the cell, as previously described (9, 26). This in-

Table III. The Distance of the NH-3T3 Cells from the Chromaffin Cell and the Latency Before the Onset of Their $\text{Ca}^{2+}$ Response to Angiotensin II, Nicotine, and ATP*

<table>
<thead>
<tr>
<th>NIH-3T3 cell</th>
<th>Distance from chromaffin cell (µm)</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 2</td>
<td>4.5</td>
<td>No response</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>No response</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>No response</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>No response</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>No response</td>
</tr>
</tbody>
</table>

After perfusion with angiotensin II, no rise in $[\text{Ca}^{2+}]_i$ was detected in any of the NIH-3T3 cells. The NIH-3T3 cells furthest from the chromaffin cell generally had a longer latency than those nearer the chromaffin cell after perfusion with nicotine, but all responded with comparable latencies after perfusion with ATP.

* As shown in the field of cocultured cells in Fig. 4.
Figure 5. Anti-DBH staining of chromaflin cells to reveal sites of exocytosis in response to nicotine or angiotensin II. 2-d-old chromaflin cell cultures were challenged in the presence of anti-DBH with no agonist (a), 10 μM nicotine (c), or 0.3 μM angiotensin II (e) for 15 min. The anti-DBH was localized by subsequent staining with Texas red. This revealed no fluorescence in the absence of agonist (b), a continuous ring of fluorescence around the plasma membrane of most cells after stimulation with nicotine (d), and highly localized fluorescence in a minority of cells in response to angiotensin II (f). Bar, 10 μm.
crease in Ca\(^{2+}\) was always followed by a strong secretory response, as indicated by the Ca\(^{2+}\) responses elicited in the NIH-3T3 cells which were adjacent to the central chromaffin cell (Figs. 2 and 4). There are a number of reasons for supposing that this Ca\(^{2+}\) response of the NIH-3T3 cells was evoked by ATP coreleased with catecholamine from the chromafin granules. Firstly, the NIH-3T3 cells do not have a nicotinic receptor linked to Ca\(^{2+}\) influx. Secondly, the NIH-3T3 cells nearer the chromaffin cell responded quicker than those furthest away after the chromafin cell had been stimulated to release its ATP. When the field was directly perfused with ATP from the U-tube, all cells responded simultaneously (Tables I and III). Thirdly, when the experiment was carried out in the presence of hexokinase (which in combination with glucose removes ATP [33]) the NIH-3T3 Ca\(^{2+}\) response was abolished. Although some diffusion of material released from one pole of the cell to the opposing pole cannot be discounted, the most likely explanation for the fact that ATP release was detected all around the chromaffin cell after a challenge with nicotine is that secretion in response to this stimulus occurs over the entire cell surface. This was confirmed by an independent immunofluorescence technique in which the exocytotic sites were revealed using an antibody to DBH (Fig. 5 d). This protein is a component of the chromaffin granule membrane and becomes incorporated into the plasma membrane during the fusion process, thereby highlighting the sites of exocytosis. This result is consistent with earlier electron microscopic evidence which also showed that these cells are capable of supporting exocytosis over their entire surface (19).

The transient nature of the initial NIH-3T3 cell Ca\(^{2+}\) response, which lasts for \(~40-60\) s (Figs. 2 a and 4 a), probably reflects desensitization of the nicotine-induced secretory response. This is suggested since the NIH-3T3 cells showed a strong Ca\(^{2+}\) signal in response to control ATP \(~160\) s after they had been stimulated by ATP released from the chromaffin cell indicating that the ATP receptor on the NIH-3T3 cells had not desensitized. This implied that chromaffin cell secretion had probably finished some time earlier. Interestingly, luciferin/luciferase detection of ATP released from chromaffin cell populations also showed secretion to terminate at \(~40-60\) s (32). Our results also demonstrate that secretion can terminate despite [Ca\(^{2+}\)] in the chromaffin cell remaining elevated. This is consistent with previous suggestions (20, 21) and is likely to be due to desensitization of some aspect of the exocytotic process; for example, a reformation of the cytoskeletal barrier at the cell periphery (6, 8).

In response to the InsP\(_3\)-mobilizing agonist angiotensin II, three different chromaffin cell responses were observed. Approximately 30% of the cells showed no Ca\(^{2+}\) response and did not secrete ATP. Approximately 45% responded with a large Ca\(^{2+}\) transient, with peak [Ca\(^{2+}\)]i observed only in one pole of the cell, and no detectable secretory response. And \(~25\%) of cells responded with a similar Ca\(^{2+}\) transient, and also gave a subsequent secretory response. By directly demonstrating that these cells are capable of undergoing a large, agonist-induced transient rise in [Ca\(^{2+}\)]i without being stimulated to secrete, these results reiterate the paradoxical observations that although chromaffin cell populations (25) and a high proportion of single cells (26) showed large elevations in [Ca\(^{2+}\)]i due to angiotensin II, the drug stimulated very little secretion from cells in culture (24, 25).

We have previously reported similar large Ca\(^{2+}\) transients in these cells in response to muscarinic drugs (9, 26), which also do not support secretion from cell populations (7, 9).

The unifying factor linking all of these results is their strong indication that Ca\(^{2+}\) influx, and not mobilization of internal Ca\(^{2+}\), is the most effective trigger for secretion from these cells. Evidence obtained in the early 1980's by other researchers also pointed towards secretion being better correlated with the event of Ca\(^{2+}\) influx rather than maximal [Ca\(^{2+}\)]i attained (for review see reference 31). The reasons underlying this phenomenon are still not clear. We have previously shown that depolarizing stimuli give rise to initial Ca\(^{2+}\)-activation of the entire subplasmalemmal area of the cell, whereas InsP\(_3\)-mobilizing stimuli result in Ca\(^{2+}\) being released from a discrete part of one pole of the cell (9, 26). It could be that initial Ca\(^{2+}\)-activation of the exocytotic sites at the plasma membrane is a necessary prerequisite for a full secretory response and this is only achieved with stimuli which promote Ca\(^{2+}\) influx. In support of this notion, disassembly of the cortical cytoskeleton (4, 8), translocation of protein kinase C to the plasma membrane (34), and recruitment of key cytosolic proteins to the granule membrane (4, 5) are all Ca\(^{2+}\)-requiring subplasmalemmal events that have been proposed to precede the fusion event.

The second major result to emerge from this study is that the morphology of the secretory response due to angiotensin II differs from that observed in response to nicotine. Both the coculture experiments and the immunofluorescence studies showed that in those cases where angiotensin II resulted in secretion, the release of granule contents was polarized; whereas release due to nicotine occurred over the entire surface of the cell. It is also significant that the area of the plasma membrane at which exocytosis occurred in response to angiotensin II correlated with the area of the cell which recorded the peak [Ca\(^{2+}\)]i (Fig. 3, a and b), though why an apparently similar rise in [Ca\(^{2+}\)]i in other cells (Fig. 4, a and b) did not trigger secretion remains unknown. It cannot be argued that cells, such as that in Fig. 4, are in fact secretting but that the amount of material released is so small that it is undetectable by the coculture method, since the immunofluorescence experiments confirmed that only a minority of cells secreted in response to angiotensin II. A more likely possibility is that after Ca\(^{2+}\) mobilization angiotensin II in some cells results in a small amount of Ca\(^{2+}\) influx which acts locally to trigger polarized release, since secretion, but not peak [Ca\(^{2+}\)]i, in response to angiotensin II is attenuated by the removal of external Ca\(^{2+}\) (24, 25).

In conclusion, we have used a novel technique to directly demonstrate that influx of external Ca\(^{2+}\), and not release of internally stored Ca\(^{2+}\), is a vital requirement for the triggering of a full secretory response from these cells. Furthermore, we have shown that in these cells the location of the sites of exocytosis depends on the nature of the stimulus. With reference to this point, it remains to be seen whether or not the area of the plasma membrane responsible for the polarized release corresponds to the area exposed to the bloodstream in the intact gland.

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