Agonist-dependent patterns of cytosolic Ca^{2+} changes in single bovine adrenal chromaffin cells: relationship to catecholamine release

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The patterns of agonist-induced elevations of cytosolic free Ca^{2+} ([Ca^{2+}]$_i$) were characterized and compared by the use of single adrenal chromaffin cells. Initial histamine- or angiotensin II (A$_{II}$)-induced elevations of [Ca^{2+}]$_i$ were equal in magnitude (peaks 329 ± 20 [SE] and 338 ± 46 nM, respectively). These initial increases of [Ca^{2+}]$_i$ were transient, insensitive to either Gd^{3+} or removing external Ca^{2+}, and were primarily the result of Ca^{2+} release from intracellular stores. After the initial peak(s) of [Ca^{2+}]$_i$, a second phase of moderately elevated [Ca^{2+}]$_i$ was observed, and this response was sensitive to either Gd^{3+} or removing external Ca^{2+}, supporting a role for Ca^{2+} entry. In most cases, the second phase of elevated [Ca^{2+}]$_i$ was sustained during histamine stimulation but transient during A$_{II}$ stimulation. Maintenance of the second phase was a property of the agonist rather than of the particular cell being stimulated. Thus, individual cells exposed sequentially to histamine and A$_{II}$ displayed distinct patterns of [Ca^{2+}]$_i$ changes to each agonist, regardless of the order of addition. Histamine also stimulated twice as much [^{3}H]catecholamine release as A$_{II}$, and release was completely dependent on external Ca^{2+}. Therefore, the ability of histamine and A$_{II}$ to sustain (or promote) Ca^{2+} entry appears to underlie their efficacy as secretagogues. These data provide evidence linking agonist-dependent patterns of [Ca^{2+}]$_i$ changes in single cells with agonist-dependent functional responses.

Introduction

In bovine adrenal chromaffin cells, not all receptor-linked secretagogues are equally efficacious in releasing catecholamines (Noble et al., 1988; Kim and Westhead, 1989; Stauderman and Pruss, 1990). Part of this phenomenon is understandable because some secretagogues,

like nicotine, directly activate a ligand-gated ion channel, whereas others, like angiotensin II (A$_{II}$) and histamine, work indirectly through stimulation of phospholipase C and the production of inositol 1,4,5-trisphosphate (Noble et al., 1986; Plevin and Boarder, 1988; Sasakawa et al., 1989a; Stauderman and Pruss, 1989, 1990; Wan et al., 1989). But even A$_{II}$ and histamine vary at least twofold in their abilities to promote secretion (Noble et al., 1988). The reason for this heterogeneity is unclear.

One possible explanation for the differing capacities of A$_{II}$ and histamine to release catecholamines is that they might stimulate unequal elevations of either inositol 1,4,5-trisphosphate or intracellular free Ca^{2+} concentration ([Ca^{2+}]$_i$). However, we recently showed that high concentrations of A$_{II}$ and histamine produce similar initial increases of both inositol 1,4,5-trisphosphate and [Ca^{2+}]$_i$ in suspensions of chromaffin cells (Stauderman and Pruss, 1990). Although these initial responses were similar, the subsequent inositol phosphate and [Ca^{2+}]$_i$ changes were different for A$_{II}$ and histamine. We hypothesized, therefore, that a relationship might exist between the patterns of inositol phosphate or [Ca^{2+}]$_i$ changes and the ability of A$_{II}$ and histamine to stimulate secretion (Stauderman and Pruss, 1990).

Although [Ca^{2+}]$_i$ measurements in cell suspensions have been quite useful, many events occurring at the single-cell level are undetectable with this technique. Indeed, other groups have now demonstrated that the [Ca^{2+}]$_i$ changes in single chromaffin cells stimulated by inositol phosphate–linked agonists are very heterogeneous (O'Sullivan et al., 1989; Cheek et al., 1989b). These heterogeneous changes of [Ca^{2+}]$_i$ appear to result from both Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry through the plasma membrane, but the specific roles of each process in controlling either the patterns of [Ca^{2+}]$_i$ changes or Ca^{2+}-dependent functional responses have not been clarified. Because it has recently been suggested that

Ca^{2+}-dependent secretion is controlled primarily by Ca^{2+} entry (Cheek *et al.*, 1989b; Kim and Westhead, 1989), it is important to determine the relationship between secretion and the patterns of agonist-induced Ca^{2+} mobilization and Ca^{2+} entry.

In this report, we have characterized the response of single cells to two different agonists. The A_{II}- and histamine-induced patterns of $[Ca^{2+}]_i$ changes were evaluated with respect to the secretory responses elicited by each agonist. The evidence indicates that efficacy to release catecholamines is related to the ability of an agonist to promote and sustain Ca^{2+} entry.

Results

All results reported were obtained from single chromaffin cells prepared, cultured, and identified as described in Materials and methods. An example of a field of cells cultured for 24–48 h is shown in Figure 1.

Basal $[Ca^{2+}]_i$ levels

Our previous results and those of others, using fura-2 loaded cell suspensions at 37°C, indicated that basal $[Ca^{2+}]_i$ levels were 75–140 nM (Pruss and Stauderman, 1988; O'Sullivan and Burgoyne, 1988, 1989; Stauderman and Pruss, 1989, 1990). In a sample of 126 adherent single cells, 20% of the cells (25/126) examined started out with fura-2 ratio signals close to the minimum for our system, indicating that the basal $[Ca^{2+}]_i$ level of these cells was ≤10 nM. However, we believe these values to be an artifact (see Discussion). In the remaining 80% of cells (101/126), the basal $[Ca^{2+}]_i$ averaged 67 ± 5 (SE) nM (n = 101), similar to what others have found (O'Sullivan *et al.*, 1989).

Approximately 25% of the cells displayed low-amplitude, irregular spontaneous oscillations of $[Ca^{2+}]_i$. These low-amplitude oscillations were abolished either by removing external Ca^{2+} or by the addition of an inorganic Ca^{2+} antagonist such as Gd^{3+} (see below).

Responses of histamine in normal buffer

In cells initially stimulated with 1–10 μM histamine, 67/70 cells responded with an elevation of $[Ca^{2+}]_i$. Interestingly, several distinctive patterns of $[Ca^{2+}]_i$ changes were observed (Figure 2A–D), demonstrating a heterogeneity of responses that could not be detected in cell suspensions. For example, responses to histamine in cell suspensions consisted of one prolonged
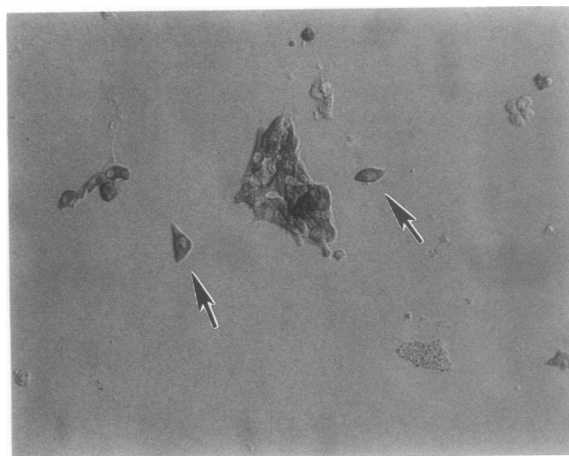
Figure 1. **Photograph of a typical field of cultured chromaffin cells stained with neutral red.** The arrows indicate the shapes of cells that were routinely used for fluorescence measurements (i.e., either a rounded-triangular or an elliptical, "football" shape). Darker cells in the field are those that stained with neutral red, a marker for chromaffin cells. Note that the cells marked by arrows are stained with neutral red, indicating that cells with these shapes were chromaffin cells.
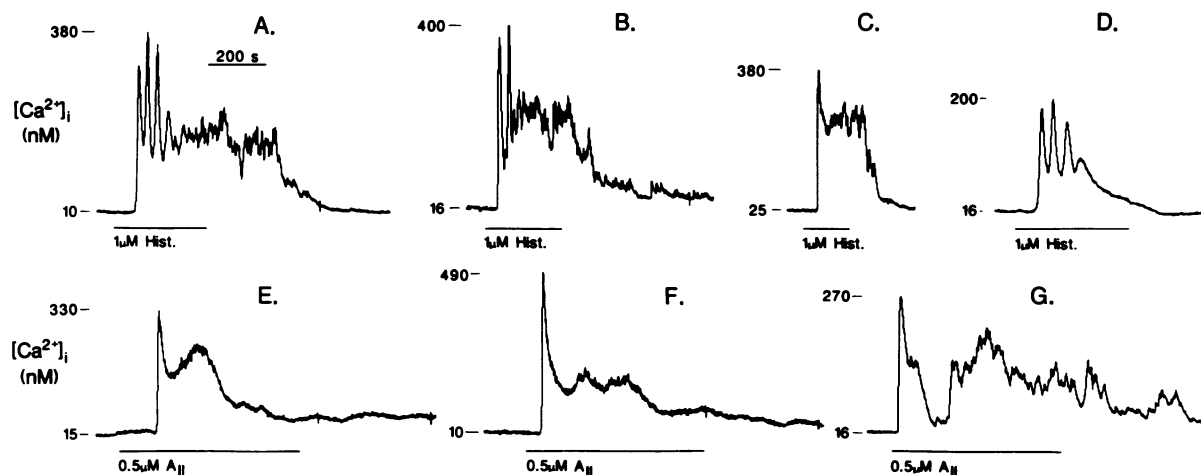
transient of elevated $[Ca^{2+}]_i$ (maximum amplitude 300 nM) (Stauderman and Pruss, 1990); whereas 78% of the single-cell responses commenced with at least one rapid transient of elevated $[Ca^{2+}]_i$ (maximum amplitude 329 ± 20 nM, Table 1). The amplitudes of the initial peaks of $[Ca^{2+}]_i$ after addition of 1 or 10 μM histamine to single cells were not significantly different (Table 1), indicating that 1 μM was at least a maximal concentration for initiating increases of $[Ca^{2+}]_i$.

The initial histamine-induced peak of $[Ca^{2+}]_i$ was sometimes followed (in 30% of cells) by one to three smaller oscillations of $[Ca^{2+}]_i$ (Figure 2, A, B, and D), which then progressed into an irregular but sustained plateau of moderately elevated $[Ca^{2+}]_i$ (Figure 2, A–C). In 81% of the cells responding to 1–10 μM histamine (54/67), the plateau (second phase) of elevated $[Ca^{2+}]_i$ remained as long as histamine was present (Figure 2D shows an exception).

Although the superfusion technique employed in these studies did not enable precise time measurements, it was clear that the response latencies to histamine varied considerably from cell to cell (not shown).

Responses to A_{II} in normal buffer

When the initial agonist exposure was to A_{II} (0.1 or 0.5 μM), 16/18 cells responded with an ele-

Figure 3. The effect of external Ca^{2+} on A_{II}- and histamine-induced changes of $[Ca^{2+}]_i$. Where indicated by bars, the cells were superfused with HBK with no added $CaCl_2$ plus 0.1 mM EGTA (0 Ca^{2+}). (A) A previously unstimulated cell that had low-amplitude spontaneous oscillations before removal of external Ca^{2+}. (A and B) responses, in the absence of external Ca^{2+}, to 0.5 μM A_{II} and 1 μM histamine, respectively. Each panel represents a different cell. These results are representative of those obtained in 12 cells with histamine and 12 cells with A_{II}.

Responses to repeated exposures with the same agonist

The data above show that, in contrast to histamine, secondary elevations of $[Ca^{2+}]_i$ declined with prolonged exposure to A_{II} in a majority of cells. Consistent with this observation, in 9 cells
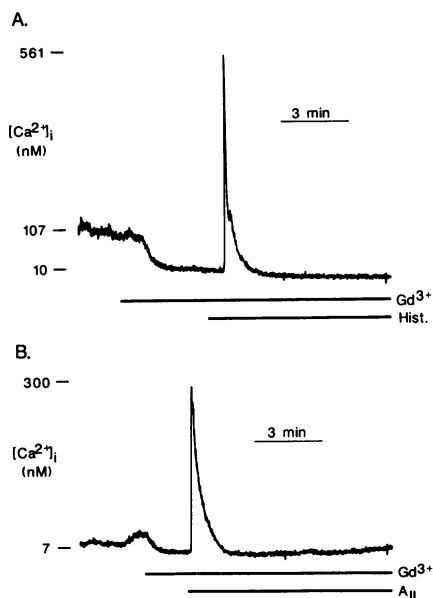
Figure 4. The effect of Gd^{3+} on histamine- and A_{II}-induced changes of $[Ca^{2+}]_i$. Cells were superfused with HBK containing 20 μM Gd^{3+} for 1–2 min before the addition of an agonist. Note that in A, low-amplitude oscillations of $[Ca^{2+}]_i$ were abolished, and the $[Ca^{2+}]_i$ declined, on addition of Gd^{3+}. Shown are $[Ca^{2+}]_i$ responses to 10 μM histamine (A) and 0.5 μM A_{II} (B) in the presence of 20 μM Gd^{3+}. These results are representative of those obtained in four cells with histamine, and three cells with A_{II}.

Figure 5. Effects of repeated exposures to A_{II} and histamine. Each panel is from a different cell. (A) A cell challenged twice with 0.1 μM A_{II} (as indicated by the bars). A total of 9 cells were tested in this manner with 0.1–0.5 μM A_{II}. (B) A cell challenged twice with 1 μM histamine (as indicated by the bars). A total of 19 cells were tested with 1–10 μM histamine. Summarized results for both A_{II} and histamine are presented in Table 2.

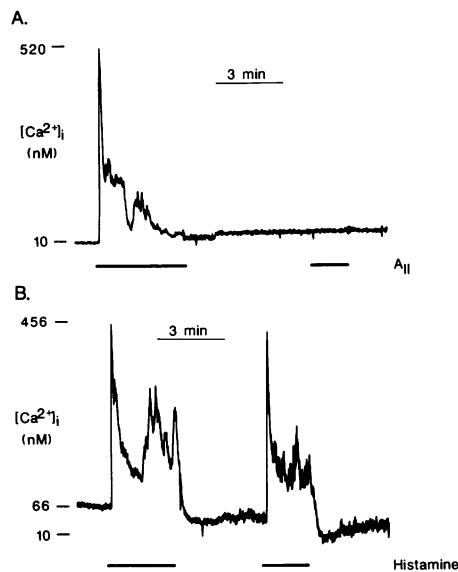previously challenged with A_{II}, a second challenge elicited no response in 4/9 cells (44%) (Figure 5A), a diminished response in 3/9 cells (33%), and a full response in only 2/9 cells (22%) (Table 2). A second challenge with histamine elicited a rise in $[Ca^{2+}]_i$ comparable with the initial response in 11/19 cells (58%) (Figure 5B), and only 2/19 cells (11%) did not respond to

Table 2. Response to a second challenge with the same agonist

Response to second challenge (% of first response)	No. Cells (% of total)	
	A_{II}	Histamine
0%	4 (44%)	2 (11%)
10–50%	3 (33%)	6 (32%)
70–100%	2 (22%)	11 (58%)
Total	9	19

Cells were first challenged for 3–5 min with either 0.1–0.5 μM A_{II} or 1–10 μM histamine. Following a washout period, the cells were challenged a second time with the same concentration of agonist. The peak amplitudes of the $[Ca^{2+}]_i$ increases to the first and second challenges were compared. The second responses were classified as being either 70–100%, 10–50%, or 0% the size of the first response.
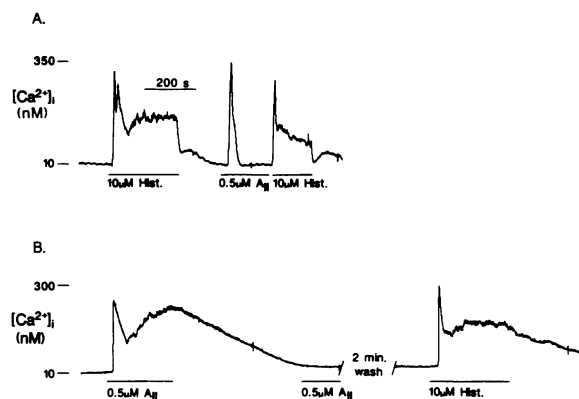
Figure 6. Changes of $[Ca^{2+}]_i$ in response to histamine and A_{II} addition to the same cell. A and B are from different cells. In both panels, note that the patterns of $[Ca^{2+}]_i$ changes are different for each agonist. (B) shows that a cell that had desensitized to the effects of A_{II} still responded to histamine. Out of 21 cells exposed to both histamine and A_{II}, 20 responded to histamine, whereas 18 responded to A_{II}. A small number of cells (10%) were, therefore, sensitive to histamine, but insensitive to A_{II}. This percentage may be an overestimate, however, because we later had cause to suspect the batch of A_{II} that was used on the nonresponding cells.

the second challenge (Table 2). Thus, cells tended to desensitize to the effects of A_{II} but not histamine. However, cells desensitized to A_{II} could still respond to histamine (see below, Figure 6B).

It is also noteworthy that the first and second exposures to histamine did not elicit identical patterns of $[Ca^{2+}]_i$ changes, although they were similar. Thus, Ca^{2+} "fingerprints" (Prentki et al., 1988) may not be occurring in these cells, although the recovery time may not have been sufficient in between histamine challenges to observe this phenomenon.

Responses to A_{II} and histamine in the same cell

In 18/21 individual cells tested with both A_{II} (0.1–0.5 μM) and histamine (1–10 μM), an increase of $[Ca^{2+}]_i$ was elicited by both agonists regardless of the order of addition, as long as there was a sufficient recovery period between additions. One cell did not respond to either A_{II} or histamine, and 2 cells responded to histamine but not A_{II}. More importantly, in cells responding to both agonists, the patterns of $[Ca^{2+}]_i$ changes to A_{II} were distinguishable from those of histamine (Figure 6). It should be noted, however, that although the overall patterns of $[Ca^{2+}]_i$ changes were different for A_{II} and histamine,

the maximum amplitudes of the agonist-induced peaks of $[Ca^{2+}]_i$ were equivalent in each cell tested with both agonists.

A_{II}- and histamine-stimulated catecholamine release

The patterns of $[Ca^{2+}]_i$ changes described above indicated that histamine and A_{II} were equally effective at causing the initial mobilization of intracellular Ca^{2+} but that histamine was much better at maintaining subsequent Ca^{2+} entry. To test whether these patterns translated into functional variations, we examined [³H]catecholamine release in response to each agonist in the presence and absence of external Ca^{2+}. The data in Table 3 illustrate that, over a 6-min period in the presence of 1.1 mM external Ca^{2+}, 10 μM histamine stimulated about twice as much release as 0.5 μM A_{II}. Histamine, in turn, was two to three times less effective than depolarization with 40 mM KCl, which will increase $[Ca^{2+}]_i$ to ~630 nM (Pruss and Stauderman, 1988). In the absence of external Ca^{2+}, however, none of these agents stimulated [³H]-catecholamine release (Table 3).

Discussion

We and others have reported previously on $[Ca^{2+}]_i$ changes in suspensions of fura-2–loaded chromaffin cells (O'Sullivan and Burgoyne, 1988, 1989; Pruss and Stauderman, 1988; Cheek and Thastrup, 1989; Sasakawa et al., 1989b; Stauderman and Pruss, 1989, 1990). However, the determinations of $[Ca^{2+}]_i$ in single cells appear

Table 3. Role of external Ca^{2+} on catecholamine release stimulated by histamine, A_{II}, and KCl

Treatment	[³H]Catecholamine release (% of stores)		
	1.1 mM Ca^{2+}	0 Ca^{2+}	Ca^{2+}-dependent
Control	3.15 ± 0.26	2.15 ± 0.28	1.00
50 mM KCl	16.9 ± 1.88*	2.60 ± 0.37	14.3
0.5 μM A_{II}	4.18 ± 0.40	2.03 ± 0.18	2.15
10 μM histamine	8.05 ± 1.22†	2.93 ± 0.33	5.12

Release was measured over a 6-min period as described in Methods. For comparison, cells were also stimulated by depolarization with 50 mM KCl. All treatments were performed in either the presence or absence of 1.1 mM external Ca^{2+}. The data are presented as means ± SE (n = 4).
* p < 0.01 versus control.
† p < 0.05 versus control.

to be superior in both sensitivity and ability to resolve small changes of $[Ca^{2+}]_i$.

Resting $[Ca^{2+}]_i$ levels in cell populations at 37°C are reported to be 75–173 nM (O'Sullivan and Burgoyne, 1988, 1989; Pruss and Stauderman, 1988; Sasakawa et al., 1989b; Stauderman and Pruss, 1989), but they were 67 nM in single cells at 22°C. The latter values are similar to those previously reported by O'Sullivan et al., (1989) for single chromaffin cells at 37°C (79nM).

The unusually low basal $[Ca^{2+}]_i$ levels (<10 nM) observed in 20% of the cells were probably an artifact attributable either to incompletely deesterified forms of fura-2 acetoxymethyl ester (fura-2/AM) (Scanlon et al., 1987; Oakes et al., 1988) or to an increase of fluorescence intensity at longer wavelengths due to cellular viscosity (Poenie, 1990), or both. Indeed, by adding Mn^{2+} and ionomycin to quench intracellular fura-2, we determined that Mn^{2+}-insensitive fluorescence contributed as much as 30% of the total fluorescent signal in some cells (data not shown). In spite of this problem, however, there was no indication of an abnormal response pattern in any of the cells with low basal $[Ca^{2+}]_i$ levels.

One of the aims of this study was to establish whether single-cell $[Ca^{2+}]_i$ responses to A_{II} and histamine reflect those observed in cell populations. It was found that measurements in single cells were quantitatively but not qualitatively similar to those from cell populations. For example, in our hands, histamine and A_{II} initially increased $[Ca^{2+}]_i$ to similar levels (~300 nM) in both suspensions and single cells (Stauderman and Pruss, 1990). However, in single cells, the responses to A_{II} and histamine usually commenced with a clearly defined oscillation of $[Ca^{2+}]_i$, which was not observed in the cell suspensions (Stauderman and Pruss, 1990). Asynchronous elevations of $[Ca^{2+}]_i$, due to different response latencies between cells, probably accounts for the inability to detect these initial transients in cell suspensions (Rooney et al., 1989).

Another interesting result was that, unlike histamine, A_{II} was unable to elicit multiple transients of elevated $[Ca^{2+}]_i$ during the beginning of a response. The reason for this difference is unknown, but may relate to spatial restrictions of A_{II}-induced elevations of $[Ca^{2+}]_i$ (Burgoyne et al., 1989; Cheek et al., 1989a; O'Sullivan et al., 1989).

The initial agonist-induced $[Ca^{2+}]_i$ transient(s) in single cells were apparently due to Ca^{2+} mobilization from internal stores, because they

were insensitive to either a Ca^{2+} antagonist (Gd^{3+}) or removal of external Ca^{2+}. We have recently shown that both A_{II} and histamine stimulate the rapid formation of inositol 1,4,5-trisphosphate in chromaffin cells (Stauderman and Pruss, 1989, 1990). Therefore, it is likely that the initial $[Ca^{2+}]_i$ transients are triggered by inositol 1,4,5-trisphosphate-induced Ca^{2+} release from internal stores. On the other hand, the secondary $[Ca^{2+}]_i$ elevations after the initial transients appear to be supported more by Ca^{2+} entry, because they were blocked by Gd^{3+} and removing external Ca^{2+}.

It has recently been suggested that catecholamine release from chromaffin cells is stimulated by Ca^{2+} entry and not internal Ca^{2+} mobilization (Cheek et al., 1989b; Kim and Westhead, 1989). Our data confirm this suggestion. First, as expected, depolarization-induced release of catecholamines was completely dependent on external Ca^{2+}. Second, although A_{II} and histamine elicited the same Ca^{2+} mobilization-dependent increase of $[Ca^{2+}]_i$ in the presence of external Ca^{2+}, histamine stimulated twice as much catecholamine release. Third, and most important, under conditions where both agonist-induced Ca^{2+} entry and the release of catecholamines were blocked, i.e., in the absence of external Ca^{2+}, both A_{II} and histamine stimulated equivalent peaks of $[Ca^{2+}]_i$ (because of internal Ca^{2+} mobilization), and the magnitude of these peaks was identical to those observed in the presence of external Ca^{2+}. Entry of Ca^{2+}, perhaps in restricted domains of the chromaffin cell (Burgoyne et al., 1989; Cheek et al., 1989a,b; O'Sullivan et al., 1989), rather than simple elevation of $[Ca^{2+}]_i$, must therefore be necessary for catecholamine release to occur.

As discussed above, both A_{II} and histamine initially produced similarly sized transients of elevated $[Ca^{2+}]_i$. However, the subsequent responses differed markedly. Histamine promoted a secondary elevation of $[Ca^{2+}]_i$ that, in a majority of cells, was maintained as long as histamine was present. In contrast, the secondary elevation of $[Ca^{2+}]_i$ after A_{II} decayed with continued exposure in a majority of cells. The explanation of these results appears to be that most cells rapidly desensitize to the effects of A_{II} but not histamine, as shown by the lack of responsiveness to a second challenge with A_{II}.

Although unlikely, the different patterns of A_{II}-versus histamine-induced changes of $[Ca^{2+}]_i$ could have resulted simply from cell-to-cell variability, rather than genuine agonist-depen-

dent patterning of $[Ca^{2+}]_i$. An important finding, therefore, was that cells exposed to both agonists also displayed distinctive patterns of $[Ca^{2+}]_i$ changes in response to each agent. This result demonstrates that the pattern of $[Ca^{2+}]_i$ change is a specific property of the receptor system being stimulated. Furthermore, the agonist-dependent patterns of $[Ca^{2+}]_i$, like the patterns of inositol phosphate formation (Stauderman and Pruss, 1990), might encode information that permits cells to respond differently to each agonist (Woods et al., 1987; Berridge et al., 1988; Goldbeter et al., 1990).

To determine whether a variation in a functional response could be associated with different agonist-induced patterns of $[Ca^{2+}]_i$ changes, we examined catecholamine release. Because we had already established that Ca^{2+} entry was necessary for catecholamine release, it was apparent that the secondary elevations of $[Ca^{2+}]_i$ in response to A_{II} or histamine (i.e., those supported by Ca^{2+} entry) would be important factors in determining the amount of catecholamine released by each agonist. At supramaximal concentrations, histamine stimulated more catecholamine release than A_{II}, in agreement with previous findings (Noble et al., 1988). At these same agonist concentrations, and over a similar time period, A_{II}, unlike histamine, did not sustain the Ca^{2+} entry-dependent component of the $[Ca^{2+}]_i$ response. These data indicate that an association exists between the strength of catecholamine release and the capacity to maintain Ca^{2+} entry.

In conclusion, the data we have presented indicates that, in addition to other potential regulatory factors (Pocette et al., 1985; Bader et al., 1989), the ability of A_{II} and histamine to promote and sustain Ca^{2+} entry plays a major role in determining their efficacy as secretagogues in bovine adrenal chromaffin cells. This information provides evidence that variations of agonist-induced $[Ca^{2+}]_i$ entry may influence cellular functions in an agonist-dependent manner.

Materials and methods

Materials

Histamine and cytosine arabinofuranoside were purchased from Sigma (St. Louis, MO). A_{II} was from Peninsula Labs (Belmont, CA). Fura-2/AM and fura-2 free acid were products of Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin, and streptomycin were GIBCO (Grand Island, NY) products. Culture dishes (6-well, 35-mm diam) were made by Costar (Cambridge, MA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT) or Advanced Biotechnologies (Silver

Spring, MD). Other chemicals, if not specifically mentioned, were of the highest purity obtainable from Fluka Biochemicals (Buchs, Switzerland). Glass coverslips were purchased from Biophysica Technologies (Baltimore, MD). Suppliers of other pieces of equipment are mentioned in the text.

Preparation of chromaffin cells

Bovine adrenal glands were obtained from a local slaughterhouse (Kleuner's, Cincinnati, OH). The procedure for preparing adrenal medullary chromaffin cells has been described previously (Pruss et al., 1985). This procedure yields a population of cells that is ≥85% authentic chromaffin cells, as judged by staining with neutral red.

Cell culture

Cells used for $[Ca^{2+}]_i$ measurements were plated on collagen-coated glass coverslips (31-mm diam, 0.07 mm thick) and cultured at 37°C for 1–3 d in DMEM containing dialyzed (24 h) 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10^{-5} M cytosine arabinofuranoside. The cells used for catecholamine release assays were plated into collagen-coated 24-well dishes at a density of 300 000 cells per well in 1 ml of medium. Cells were used for release experiments after 1 wk in culture.

Measurement of $[Ca^{2+}]_i$

The culturing medium was removed and the cells washed twice in a N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Krebs buffer (HBK; 188 mM NaCl, 4.6 mM KCl, 10 mM glucose, 25 mM sodium-HEPES, 1.2 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4). The cells were then incubated for 30 min at 37°C in 2 ml HBK containing 2 μM fura-2/AM. At the end of this period, the cells were washed twice with HBK and then stored in 1 ml HBK at room temperature before use. For $[Ca^{2+}]_i$ measurements, coverslips were placed in a low-volume chamber (Biophysica Technologies) on the stage of a Zeiss (Thornwood, NY) IM-35 inverted microscope equipped with a 100× Nikon fluorite objective. Excitation light was from an dual-excitation spectrophotometer (Deltascan I; Photon Technology International, Princeton, NJ) and alternated between 350 and 390 nm 50 times a second. Fluorescence emission passed through a bandpass filter (480-nm cutoff) and was monitored by photon counting via a photomultiplier tube (Thorn, EMI). Using a pinhole coupler to the photomultiplier, we were able to restrict the detection field to just the cytoplasmic region of a cell, avoiding the nucleus which was more fluorescent than the rest of the cell (Burgoyne et al., 1989). The temperature of the cells during these experiments was 22°C. Agonists were introduced via a superfusion system that kept the cells bathed at a rate of 0.9 ml/min in a volume of ~0.9 ml. $[Ca^{2+}]_i$ levels were calibrated from a standard curve constructed with the use of 10 μM fura-2 free acid with known amounts of free Ca^{2+} (Grynkiewicz et al., 1985). Data were compared by a randomized analysis of variance.

Catecholamine release

The release of preloaded [³H]norepinephrine from chromaffin cells was performed by a modification of the method of Boksa and Livett (1984). Cells were loaded for 2 h in serum-free medium containing 10^{-7} M [³H]norepinephrine (10 Ci/mmol) and 10^{-3} M ascorbic acid. Cells were then washed and incubated in fresh medium for 2 h. Medium was replaced

with 400 μl HBK and [^{3}H]catecholamine release initiated by addition of 100 μl of a 5× solution of the secretogogue in HBK. When release was measured in the absence of calcium, calcium-free HBK containing 0.1 mM ethylene glycol-bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was used in place of HBK. After 6 min, a portion of the HBK was collected and the cells solubilized by addition of sodium dodecyl sulfate. An aliquot of the cell extract was collected for determination of the total radioactivity. The percent release was calculated by the use of the following formula:

$$100 \times \frac{(\text{dpm in HBK aliquot/volume of HBK counted})}{\text{total dpm}} \times \text{total volume of HBK}$$

Where total dpm = dpm in HBK aliquot + [(dpm in cell aliquot/volume of cells counted) × total cell volume]. Data were compared to control by a Student's t test.

Identification of chromaffin cells

The choice of cells for [Ca^{2+}]$_i$ measurements was based on the following criteria. First, the cell had to be sufficiently isolated from other cells to ensure uncontaminated fluorescence measurements. Second, the cell had to have either a rounded-triangular or an oval shape (Figure 1), as these cell shapes tended to respond more consistently to agonists. Cells with these shapes stained with neutral red, a dye that identifies chromaffin cells, and so were most likely genuine chromaffin cells (Figure 1). Additionally, virtually all of the cells examined responded to histamine (see Results), and histamine responses have been positively correlated with genuine chromaffin cells (Plevin and Boarder, 1988).

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