

Spatial localization of agonist-induced Ca^{2+} entry in bovine adrenal chromaffin cells

Different patterns induced by histamine and angiotensin II, and relationship to catecholamine release

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SUMMARY

The spatial organization of agonist-induced Ca^{2+} entry in single bovine adrenal chromaffin cells has been investigated using video-imaging techniques to visualize fura-2 quenching by the Ca^{2+} surrogate, Mn^{2+} .

The potent secretagogue histamine, in addition to releasing Ca^{2+} from intracellular stores, resulted in a large influx of external Mn^{2+} that occurred over the entire surface of the cell. The influx of Ca^{2+} that this mirrors was found to be an obligatory requirement for the triggering of catecholamine release by histamine, which suggests that such a global influx of Ca^{2+} into the cell probably underlies the ability of this agonist to stimulate a large secretory response. By contrast, the weaker secretagogue angiotensin II, which also acts through the second messenger inositol trisphosphate, produced a localized entry of external Mn^{2+} in 64% of cells. In these

cells, localized Mn^{2+} entry always occurred at the pole of the cell in which the angiotensin II-induced rise in $[\text{Ca}^{2+}]_i$ was largest. Since exocytosis in response to angiotensin II has previously been shown to be restricted to this same pole of the cell (Cheek et al. (1989), *J. Cell Biol.* 109, 1219-1227), these results suggest that localized influx of Ca^{2+} in response to angiotensin II could underlie the polarized exocytotic response observed with this stimulus. These results directly demonstrate that different agonists can induce different patterns of divalent cation influx in the same cells and, furthermore, suggest how these different patterns can have a direct influence on cellular function.

Key words: calcium, fura-2, exocytosis, chromaffin cell

INTRODUCTION

Catecholamine secretion from bovine adrenal chromaffin cells is triggered by the depolarization-induced influx of Ca^{2+} that follows nicotinic receptor stimulation (Baker and Knight, 1981; Burgoyne, 1991). Studies using single cells loaded with the Ca^{2+} -sensitive probes aequorin (Cobbold et al., 1987) and fura-2 (O'Sullivan et al., 1989; Cheek et al., 1989a,b; Neher and Augustine, 1992) have shown that stimuli such as nicotine and electrical depolarization result in a large (>250 nM), transient rise in the average concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and that initially Ca^{2+} invades the entire subplasmalemmal area of the cell, probably elevating $[\text{Ca}^{2+}]_i$ to high (10-100 μM) levels in this localized area, and thereby priming the exocytotic sites (Cheek, 1991; Augustine and Neher, 1992; Neher and Augustine, 1992).

The Ca^{2+} responses to inositol trisphosphate (InsP_3)-

mobilizing stimuli such as muscarinic compounds and bioactive peptides, which are weaker secretagogues, are more variable; some cells respond with large (>250 nM) average elevations in $[\text{Ca}^{2+}]_i$, whereas others are unresponsive (O'Sullivan et al., 1989; Stauderman et al., 1990). Furthermore, in those cells that respond strongly, the Ca^{2+} signal does not necessarily trigger a secretory response (Cheek et al., 1989a,b; Kim and Westhead 1989; Yamagami et al., 1991), probably because any high (μM) levels of Ca^{2+} are spatially restricted to areas remote from the sites of exocytosis (Cheek, 1991; Burgoyne, 1991; Augustine and Neher, 1992).

Histamine and angiotensin II are non-cholinergic InsP_3 -mobilizing agonists that differ in their ability to trigger Ca^{2+} -dependent secretion from these cells. Histamine is a potent secretagogue (Livett and Marley, 1986; Noble et al., 1988), whereas angiotensin II releases only a modest

amount of catecholamine (Bunn and Marley, 1989; O'Sullivan and Burgoyne, 1989; Powis and O'Brien, 1991), probably because secretion is polarized to one area of the plasma membrane (Cheek et al., 1989a).

We have used video-imaging techniques with single fura-2-loaded cells and a modification (Cheek et al., 1991; Robinson et al., 1992) of the Mn^{2+} quench technique introduced by Hallam and Rink (1985) to investigate the Ca^{2+} entry components elicited by these two stimuli. The results suggest that both histamine and angiotensin II are able to induce Ca^{2+} influx into these cells. Histamine induces influx over the entire surface of the cell, whereas influx in response to angiotensin II occurs predominantly at one pole of the cell. Since secretion due to both stimuli is dependent upon the presence of external Ca^{2+} , the spatial organization of Ca^{2+} entry could underlie the different efficacies of these stimuli and the polarized secretion elicited by angiotensin II.

MATERIALS AND METHODS

Materials

fura-2/AM and bisoxonol ($DiSBaC_2$) were from Molecular Probes (Eugene, Oregon, USA), cell culture materials were from Gibco (Paisley, Scotland, UK). All other chemicals were from Sigma (St. Louis, MO).

Methods

Isolation and culture of chromaffin cells and loading with fura-2

Chromaffin cells were isolated from bovine adrenal medullas by enzymic digestion using either the method of Knight and Baker (1983) or a modification (Burgoyne et al., 1989) of the method of Greenberg and Zinder (1982). 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_2PO_4 , 10 mM glucose, 20 mM Hepes, pH 7.4 (buffer A), washed in buffer A and resuspended in DMEM containing 25 mM Hepes, 10% foetal calf serum, 8 μ M fluoro-deoxyuridine, 50 μ g/ml gentamycin, 10 μ M cytosine arabinoside, 2.5 μ g/ml Fungizone, 25 i.u./ml penicillin, 25 μ g/ml streptomycin. The cells were purified by differential plating for 2 h (Waymire et al., 1983), after which time the non-adherent chromaffin cells were resuspended in fresh DMEM and plated onto 22 mm diameter glass coverslips at a density of 1×10^5 cells/ml in 3 ml of medium.

After overnight incubation, the cultures were washed in buffer A containing 3 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA) and incubated with 2 μ M fura-2-acetoxymethyl ester at room temperature for 30 min. The cells were equilibrated to 37°C for 3 min and coverslips were mounted in a 2 ml capacity aluminium-alloy perfusion chamber for imaging. The cells were perfused at 37°C with buffer A containing 3 mM $CaCl_2$ and 0.1% BSA from a main reservoir and agonist in the same buffer 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 are challenged virtually simultaneously by the agonists and within 1 s of the onset of application. For experiments in Ca^{2+} -free conditions, after loading as described, the cells were perfused at 37°C with buffer A containing 0.1% BSA and 1 mM EGTA for 1 min prior to addition of the agonist prepared in EGTA-containing medium.

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 (Spex Industries Inc., Edison, NJ, USA), and interfaced to a Nikon Diaphot inverted epi-fluorescence microscope. The cultures were all imaged with a UVF $\times 100$ glycerol-immersion objective resulting in a final magnification of $\times 1000$. 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, Robertsbridge, UK). The video signal from this was digitized and stored in an Imagine image processing system (Synoptics Ltd, Cambridge, UK), hosted by a DEC 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. Full details of the this video-imaging system are given by Moreton (1991).

Formation of the ratio image was implemented in a look-up table, computed from the formula given by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d \frac{R - R_{min}}{R_{max} - R} \cdot \frac{S_{f2}}{S_{b2}}$$

where K_d is the dissociation constant for fura-2/ Ca^{2+} (224 nM), R is the intensity ratio for fluorescence at the two chosen wavelengths, R_{min} and R_{max} are ratios at zero and saturating $[Ca^{2+}]_i$, respectively, and S_{f2}/S_{b2} 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 μ M 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 (Grynkiewicz et al., 1985).

Recorded video data were played back through Imagine, using a different programme to give a false-colour representation of image intensities, and to allow individual pictures to be captured on disc. The false-colour images presented depict the ratio image in either resting or stimulated cells.

Agonists

The changes in $[Ca^{2+}]_i$ in individual chromaffin cells were tested using two secretagogues. In each case the concentration was chosen to be close to optimal for inducing catecholamine secretion. Secretagogues used were angiotensin II (3×10^{-7} M) and histamine (1×10^{-5} M).

Determination of catecholamine secretion

After isolation and purification as described above, cells were plated in 24-well trays at a density of 0.7 to 1×10^6 cells/well in DMEM and cultured for 3-5 days. Cells were washed twice with buffer A containing 3 mM $CaCl_2$ and stimulated by addition of secretagogue in this buffer or buffer A containing 1 mM EGTA as appropriate. Catecholamine released over 20 min was determined by removal of buffer from wells, which was then centrifuged for 2 min at 16,000 g in a microcentrifuge before duplicate aliquots were assayed for total catecholamine using the fluorimetric assay of von Euler and Floding (1955). Total catecholamine remaining within the cells was determined after release of catecholamine with 1% Triton X-100.

Blocking L-type voltage-dependent Ca²⁺ channels

To block L-type voltage-dependent Ca²⁺ channels with nifedipine, cells were washed twice with buffer A containing 0.1% BSA and 3 mM CaCl₂, and preincubated for 8 min with 0.1% DMSO or nifedipine in DMSO. Cells were then stimulated with secretagogue in this buffer in the absence or presence of nifedipine. Stimulation with 55 mM KCl was carried out by adding iso-osmotic buffer in which KCl replaced an equal concentration of NaCl. Catecholamine released over 20 min was determined as described above.

Monitoring plasma membrane potential

Oxonols are fluorescent anions whose distribution across a membrane is potential-dependent. The dyes enter depolarized cells where they bind to lipid-rich intracellular components, which enhances their fluorescence. Measurements of the plasma membrane potential can be carried out in the presence of extracellular bisoxonol (Meldolesi et al., 1984; Kitayama et al., 1990). To monitor membrane potential with bisoxonol, chromaffin cells were grown on 60 mm diameter Petri dishes and after 1 day in culture

they were gently scraped from the dishes. Cells (10⁶ in 3 ml buffer A containing 0.1% BSA + 3 mM CaCl₂) were incubated in cuvettes in the presence of 0.15 μM bisoxonol and challenged with 10 μM histamine, 0.3 μM angiotensin II or 10 μM nicotine. Fluorescence was monitored with excitation at 540 nm and emission at 580 nm using a Perkin-Elmer LS-5 fluorimeter.

RESULTS

Changes in [Ca²⁺]_i and secretion in response to histamine

We used video-imaging of fura-2-loaded single cells to examine the source(s) of the histamine-induced rise in [Ca²⁺]_i in adrenal chromaffin cells. In the presence of external Ca²⁺ (Fig. 1 traces 1 and 2), 10 μM histamine resulted in an immediate elevation in [Ca²⁺]_i to 674 ± 22 nM above basal (mean ± s.e.m., n=11). [Ca²⁺]_i then remained at or near the peak level (trace 1) or declined to a new elevated level (trace 2) while histamine was present, but returned to

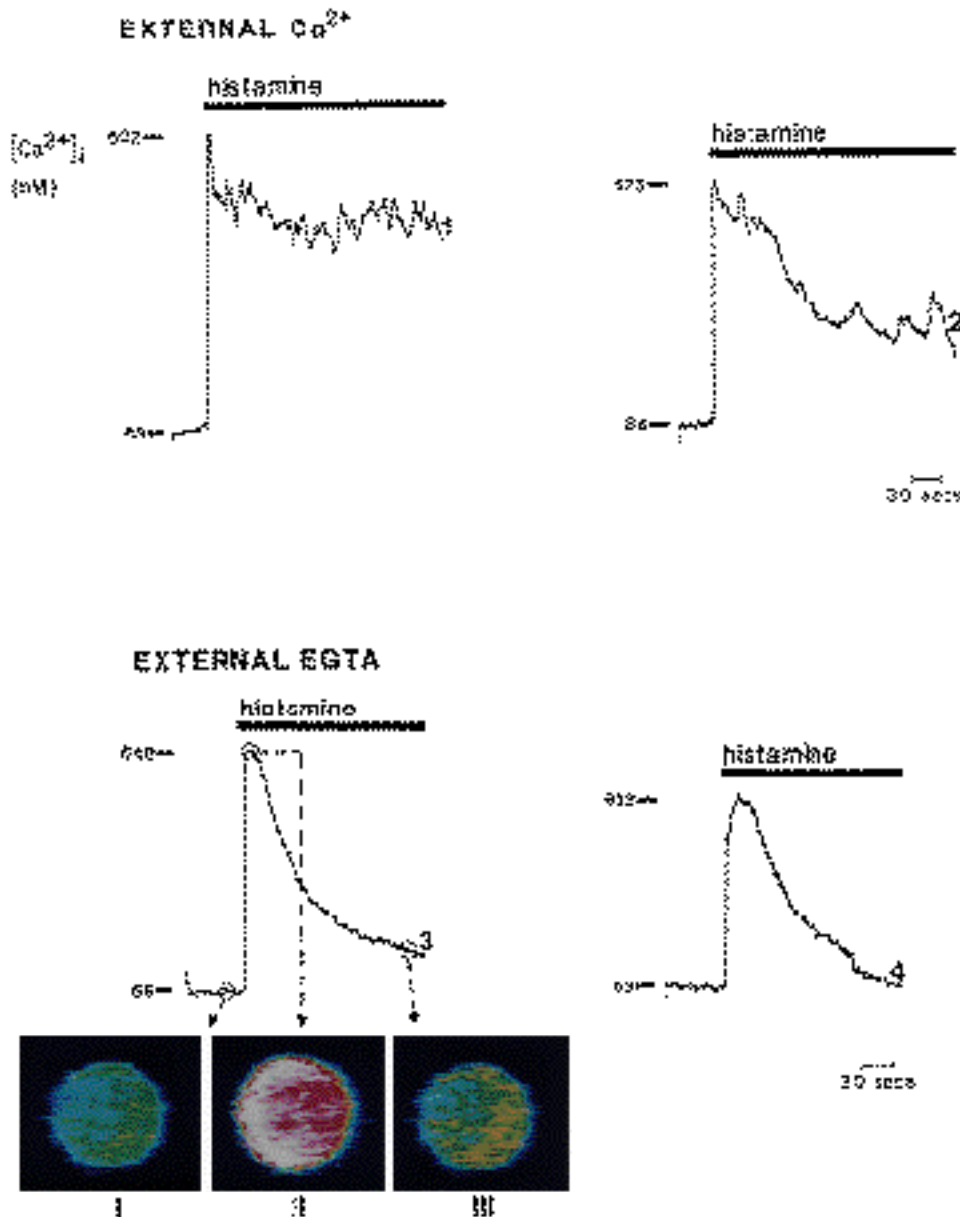


Fig. 1. Representative traces illustrating the time-course of changes in [Ca²⁺]_i in single fura-2-loaded chromaffin cells in response to histamine in the presence and absence of external Ca²⁺. Changes in [Ca²⁺]_i in 4 single cells in response to 10 μM histamine either in the presence of 3 mM external Ca²⁺ (traces 1 and 2) or in the presence of nominally Ca²⁺-free medium containing 1 mM EGTA (traces 3 and 4). In the presence of external Ca²⁺, 8 out of 11 (73%) of cells responded as typified by trace 2, whereas the remaining cells all responded as typified by trace 1. For Ca²⁺-free conditions, cells were incubated in EGTA medium for 1 min prior to the addition of histamine. The false-colour images show the subcellular localization of [Ca²⁺]_i at the times indicated on trace 3.

resting levels on removal of the agonist (data not shown). In the absence of external Ca^{2+} (Fig. 1, traces 3 and 4), 10 μM histamine elevated $[\text{Ca}^{2+}]_i$ to 570 ± 43 nM (mean \pm s.e.m., $n=13$). This was followed by a decay to resting levels that was usually complete within 4 min. These results show that there are two components to the histamine-induced rise in $[\text{Ca}^{2+}]_i$; a release of Ca^{2+} from internal stores and some form of receptor-mediated Ca^{2+} entry across the plasma membrane. The histamine-induced change in $[\text{Ca}^{2+}]_i$ in the cell shown in Fig. 1, trace 3, was visualized using video-imaging techniques (Fig. 1, trace 3, images I-III).

To establish whether secretion in response to histamine was triggered by internal Ca^{2+} release or by the influx component, assays of catecholamine released from cell populations were undertaken. Histamine-induced release of catecholamine was completely dependent upon the presence of external Ca^{2+} (Fig. 2); 10 μM histamine released $5.3 \pm 0.6\%$ of total cellular catecholamine above basal in the presence of 3 mM external Ca^{2+} , whereas in the absence of external Ca^{2+} there was no additional release above basal levels.

Ca^{2+} entry in response to histamine

In view of the fact that Ca^{2+} entry is necessary to trigger secretion, we studied the entry component stimulated by histamine in more detail. A maximal dose of histamine did not appear to depolarize chromaffin cells and induce substantial Ca^{2+} entry through voltage-dependent Ca^{2+} channels, as is the case for nicotinic stimuli. The potential-sensitive fluorescent dye bisoxonol showed no depolarizing signal after a population of chromaffin cells were exposed to 10 μM histamine, but showed a large depolarization in response to a subsequent application of 10 μM nicotine (Fig. 3, trace 1). Indeed, histamine evoked a small but reproducible reversible decrease in fluorescence, possibly reflecting a transient hyperpolarization of the membrane due to activation of Ca^{2+} -activated K^+ channels (Fasolato

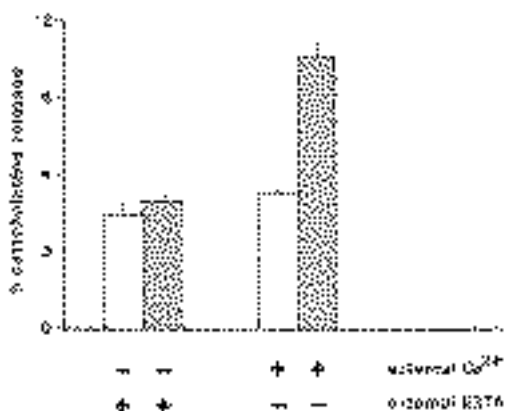


Fig. 2. The effect of removing external Ca^{2+} on resting and histamine-induced catecholamine secretion from chromaffin cell cultures. Cells were challenged with (■) or without (□) 10 μM histamine in the presence of either 3 mM external Ca^{2+} or 1 mM external EGTA (with no added Ca^{2+}). Catecholamine released over 20 min was measured and is expressed as a percentage of the total catecholamine in the cell. Data show mean values \pm s.e.m., $n=3$. Histamine-induced secretion was totally dependent upon the presence of external Ca^{2+} .

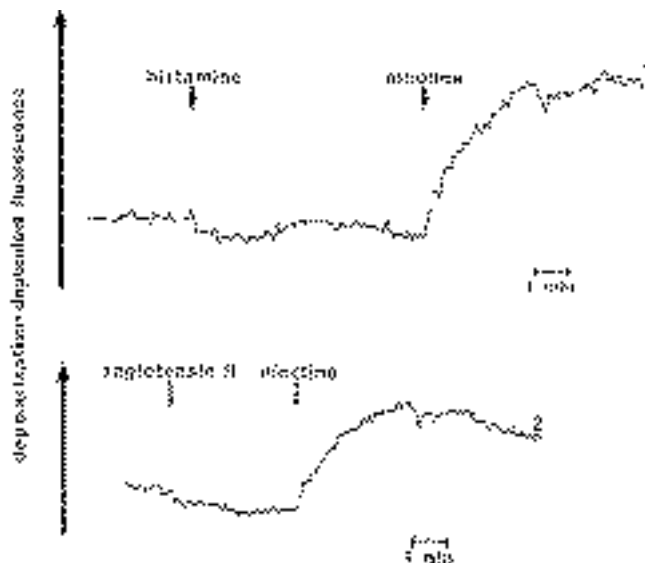


Fig. 3. Time-courses showing changes in membrane potential in a population of chromaffin cells responding to histamine, angiotensin II and nicotine. Cells were incubated with bisoxonol (see Materials and Methods) and then challenged with 10 μM histamine followed by 10 μM nicotine (trace 1), or 0.3 μM angiotensin II followed by 10 μM nicotine (trace 2). Traces are representative of the results observed in three independent experiments. Nicotine, but not histamine or angiotensin II, resulted in depolarization of the cells.

et al., 1989). Also, 0.1 to 1 μM nifedipine, a dihydropyridine antagonist that inhibits L-type voltage-dependent Ca^{2+} channels (the predominant voltage-dependent Ca^{2+} channel in chromaffin cells; Cena et al., 1989) inhibited K^+ -induced secretion by 63-73% over a 20 min period but inhibited histamine-induced secretion by only 2-13% over the same period (Table 1).

Monitoring divalent cation entry in response to histamine

In order to monitor the influx of Ca^{2+} induced by histamine we used Mn^{2+} as an indicator of divalent cation entry. Mn^{2+} is particularly well suited to studying the nature of agonist-stimulated Ca^{2+} entry because it quenches the fura-2 fluorescence on entering the cytosol. Furthermore, cells do not possess endogenous agonist-releaseable Mn^{2+} stores, so any quench of the fluorescence unequivocally indicates that Mn^{2+} has entered the cell from the external medium. This is most commonly observed using the isosbestic excitation wavelength of 360 nm, at which fura-2 fluoresces independently of $[\text{Ca}^{2+}]_i$ (e.g. see Jacob, 1990). In the following experiments, however, we have excited the fura-2 at the wavelengths normally associated with monitoring $[\text{Ca}^{2+}]_i$ (340 and 380 nm). Although this results in contamination of the fluorescence quench induced by Mn^{2+} with an increase in fluorescence that indicates a rise in $[\text{Ca}^{2+}]_i$ (Cheek et al., 1991; Robinson et al., 1992) it was necessary in order to monitor any spatial organization of the Mn^{2+} quench because the ratio method accounts for any uneven distribution of fura-2. It is not possible, however,

Table 1. The effect of nifedipine on catecholamine release due to high (55 mM) K⁺ and 10 μM histamine

[Nifedipine] (μM)	K ⁺ -induced secretion % of control (n)	Histamine-induced secretion % of control (n)
0.1	37.3±2.5 (6)	87.4±5.0 (6)
0.3	31.3±2.0 (6)	96.0±1.7 (6)
1.0	26.7±0.4 (4)	98.4±9.0 (6)

Chromaffin cells were preincubated for 8 min with 0.1% DMSO or nifedipine in DMSO and stimulated in the absence or presence of nifedipine. Catecholamine released over 20 min was measured as described in Materials and Methods. Data are mean ± s.e.m. Control secretion triggered by 55 mM K⁺ was 7.5% of total cellular catecholamine, and 10 μM histamine triggered the release of 5.6% of total cellular catecholamine. Concentrations of nifedipine that inhibited secretion due to high K⁺ were relatively ineffective at inhibiting histamine-induced secretion.

to use these data to study the temporal relationship between the rise in [Ca²⁺]_i and the stimulated Mn²⁺ entry. Whether the decrease in fluorescence observed is due to the fact that Mn²⁺ quenches fura-2 fluorescence at different rates at different excitation wavelengths, or because sufficient fura-2 is quenched to cause the fluorescence signal to collapse completely is under investigation.

False-colour images of a cell challenged with 10 μM histamine in nominally Ca²⁺-free medium containing 1 mM MnCl₂ are shown in Fig. 4. This time-course shows that, on stimulation with histamine, fura-2 fluorescence initially increased as Ca²⁺ was released from internal stores and [Ca²⁺]_i increased. After 20 s the influx pathway began to predominate as the fluorescence became progressively quenched by Mn²⁺ entering the cytosol. The sequence of images in Fig. 4 also shows the subcellular localization of the Mn²⁺ quench. In response to histamine, the Mn²⁺-induced quench clearly originated at the cell periphery and, from 20–120 s after stimulation, the fluorescence became progressively and uniformly quenched throughout the entire cell. Note that the cell had an apparent 'shrunken' appearance at 120 s. This was due to quenching of fura-2 in the cortical region of the cell by the influx of Mn²⁺ because a similar 'shrunken' appearance was not observed when cells were stimulated with histamine in the absence of external Mn²⁺ (Fig. 1, trace 3, image III). All 12 cells that responded with a histamine-induced rise in [Ca²⁺]_i in the presence of external Mn²⁺ showed a quenching phenomenon that was similarly localized. The same subcellular localizations of both the initial rise in [Ca²⁺]_i and the subsequent Mn²⁺ quench were obtained when cells were challenged in medium containing 3 mM external Ca²⁺ and then subsequently perfused with nominally Ca²⁺-free medium containing histamine and 1 mM MnCl₂ (data not shown).

These data indicates that, in addition to mobilizing internally stored Ca²⁺, histamine is capable of gating Ca²⁺ entry over the entire surface of the plasma membrane.

Changes in [Ca²⁺]_i in response to angiotensin II

In the presence of external Ca²⁺ (Fig. 5, trace 1), 0.3 μM angiotensin II resulted in an immediate elevation in [Ca²⁺]_i to 582 ± 34 nM (mean ± s.e.m., n=16) above the basal level, which then declined to a maintained elevated plateau. In the

absence of external Ca²⁺ (Fig. 5, trace 2) the transient peak was still present but there was no significant elevated plateau phase. This biphasic pattern of response has been reported in these (Stauderman and Pruss, 1989; Stauderman et al., 1990) and other (e.g. see Jacob, 1990) cell types responding to InsP₃-mobilizing stimuli and has been attributed to a release of internal Ca²⁺ followed by Ca²⁺ entry at the plasma membrane (Berridge and Irvine, 1989; Putney, 1990). As for histamine, the angiotensin II-induced entry of external Ca²⁺ into chromaffin cells does not appear to involve voltage-dependent Ca²⁺ channels because 0.3 μM angiotensin II did not depolarize a population of cells, as monitored using the membrane potential-sensitive dye bisoxonol, whereas nicotine was fully active (Fig. 3, trace 2).

Monitoring divalent cation entry in response to angiotensin II

It has previously been shown that angiotensin II triggers a polarized secretory response in chromaffin cells (Cheek et al., 1989a) and that secretion is dependent upon the presence of external Ca²⁺ (Bunn and Marley, 1989; O'Sullivan and Burgoyne, 1989; Powis and O'Brien, 1991). We therefore used the Mn²⁺ quench technique outlined above to monitor the spatial organization of the entry component stimulated by angiotensin II.

False-colour images of a cell challenged with 0.3 μM angiotensin II in nominally Ca²⁺-free medium containing 1 mM MnCl₂ are shown in Fig. 6. On stimulation with angiotensin II, fura-2 fluorescence initially increased as Ca²⁺ was released from internal stores. At the peak of the response (20 s), peak [Ca²⁺]_i was recorded in one pole of the cell (area A, Fig. 6). After 45 s, the influx pathway began to predominate as the fluorescence quenched due to Mn²⁺ entering the cytosol. In contrast to histamine, however, Mn²⁺ influx triggered by angiotensin II displayed a clear polarity such that influx occurred predominantly at the pole of the cell in which the initial rise in [Ca²⁺]_i was greatest (area A, indicated by the arrows on the images at 45, 55 and 90 s in Fig. 6). Area A was clearly the first part of the cell to decay back to, and beyond, its resting fluorescence value (Fig. 6). The same spatially organized Mn²⁺ entry was seen in experiments in which cells were challenged in medium containing 3 mM external Ca²⁺ and then subsequently perfused with nominally Ca²⁺-free medium containing angiotensin II and 1 mM MnCl₂ (data not shown). Of the 11 cells that responded with a rise in [Ca²⁺]_i to angiotensin II, 7 (64%) showed Mn²⁺ entry that was spatially organized in this way.

The decay of the Ca²⁺ response to angiotensin II

We were concerned that the spatially organized decay of the fluorescence signal induced by angiotensin II in the presence of external Mn²⁺ (Fig. 6) may have represented the normal decay of the angiotensin II-induced Ca²⁺ signal, rather than the decay resulting from Mn²⁺ entering the cell and quenching fura-2 fluorescence.

A control experiment was therefore carried out in which the pattern of fluorescence decay in a cell challenged with angiotensin II in the presence of external Mn²⁺ was compared with the pattern of decay observed after a challenge with angiotensin II in the absence of external Mn²⁺ (Fig. 7).

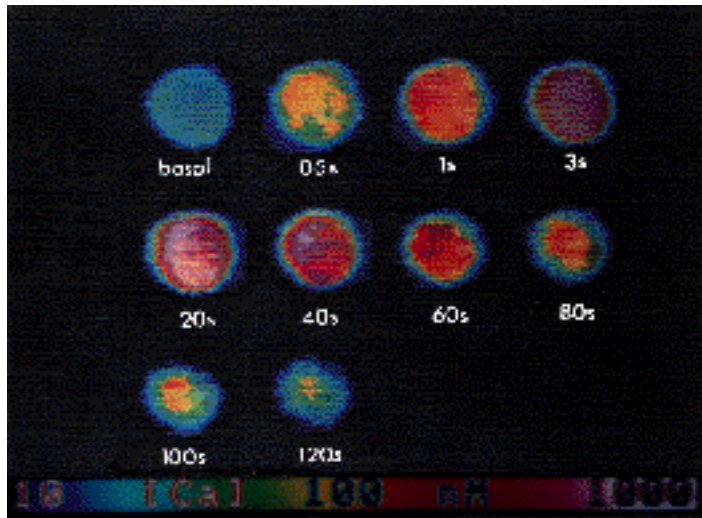


Fig. 4. Visualization of both the rise in $[Ca^{2+}]_i$ and Mn^{2+} entry in a single chromaffin cell in response to histamine. A fura-2-loaded cell was challenged with $10 \mu M$ histamine in the presence of $1 mM$ external Mn^{2+} in nominally Ca^{2+} -free medium. The false-colour images show the cell at rest (basal) and at the times (s) indicated after stimulation. Initially, Ca^{2+} was released from internal stores and, at the peak of the response ($3-20 s$), appeared evenly distributed throughout the cell. From $20 s$ onwards the influx pathway began to predominate as the fura-2 was progressively quenched by Mn^{2+} entering the cell from the external medium. This response was observed in $12/12$ cells. Note that the quench occurred evenly over the entire surface of the cell.

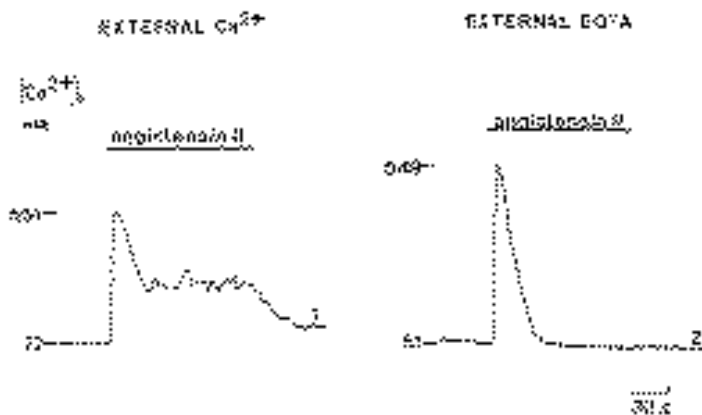


Fig. 5. Representative traces illustrating time-courses of changes in $[Ca^{2+}]_i$ in single fura-2-loaded chromaffin cells in response to angiotensin II. Changes in $[Ca^{2+}]_i$ in 2 single cells in response to $0.3 \mu M$ angiotensin II either in the presence of $3 mM$ external Ca^{2+} (trace 1) or in the presence of nominally Ca^{2+} -free medium containing $1 mM$ EGTA (trace 2). For Ca^{2+} -free conditions, cells were incubated in EGTA medium for $1 min$ prior to the addition of agonist. Note the biphasic Ca^{2+} transient in the presence, but not absence, of external Ca^{2+} .

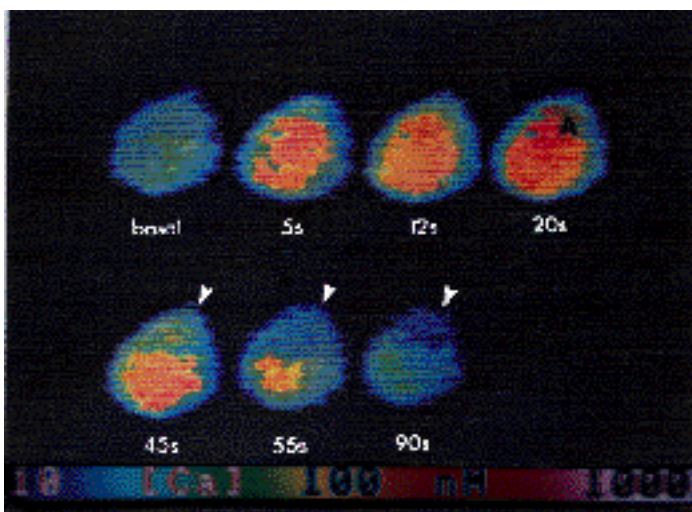


Fig. 6. Visualization of both the rise in $[Ca^{2+}]_i$ and Mn^{2+} entry in a single chromaffin cell in response to angiotensin II. A fura-2-loaded cell was challenged with $0.3 \mu M$ angiotensin II in the presence of $1 mM$ external Mn^{2+} in nominally Ca^{2+} -free medium. The false-colour images show the cell at rest (basal) and at the times (s) indicated after stimulation. Initially, Ca^{2+} was released from internal stores and, at the peak of the response ($20 s$), appeared as a continuous gradient with maximum $[Ca^{2+}]_i$ (the red colour) being recorded in area A of the cell (area A, $20 s$). From $45 s$ onwards the influx pathway began to predominate as the fura-2 was progressively quenched by Mn^{2+} entering the cell from the external medium. Note that the Mn^{2+} -induced quench of fura-2 was not uniform, but seemed to occur predominantly into area A of the cell (arrowheads). This response was observed in $7/11$ cells, suggesting that Mn^{2+} influx in response to angiotensin II in these cells was localized to one pole of the cell.

In the presence of external Mn^{2+} , the area of highest $[Ca^{2+}]_i$ within the cell was the first area of the cell to decay (area A, Fig. 6). In the absence of external Mn^{2+} , however, the opposite pattern of decay was seen (Fig. 7). The area of highest $[Ca^{2+}]_i$ at the peak of the response (area B, Fig. 7) was the last area of the cell to decay. Furthermore, in the presence of external Mn^{2+} , the fluorescence decayed beyond its resting level ($90 s$, Fig. 6), whereas in the

absence of external Mn^{2+} the fluorescence decayed only back to resting levels ($65 s$, Fig. 7). This result confirmed that the fluorescence decay in the presence of external Mn^{2+} was due to Mn^{2+} influx, rather than simply representing the decay pattern of the normal angiotensin II-induced Ca^{2+} signal, and supports the notion that angiotensin II-stimulated Mn^{2+} entry into these cells can be spatially organized.

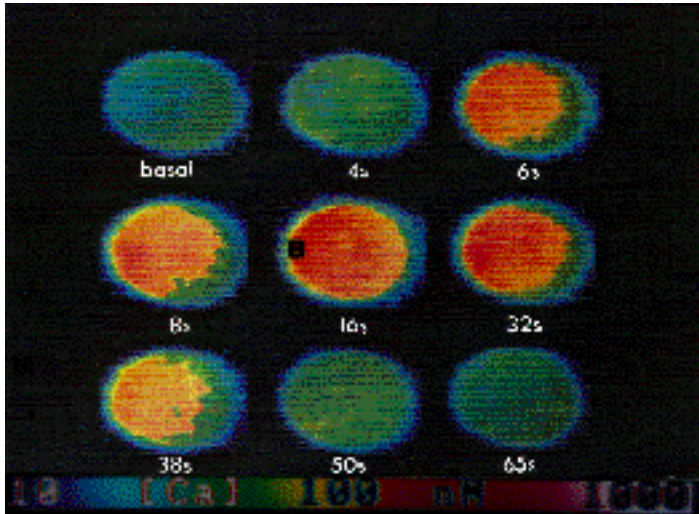


Fig. 7. Visualization of the change in $[Ca^{2+}]_i$ in a single chromaffin cell in response to angiotensin II. A fura-2-loaded cell was challenged with $0.3 \mu\text{M}$ angiotensin II in the absence of external Ca^{2+} . The false-colour images show the cell at rest (basal) and at the times (s) indicated after stimulation. At the peak of the response (16 s), $[Ca^{2+}]_i$ existed as a continuous gradient with maximum $[Ca^{2+}]_i$ (the red colour) being recorded predominantly in area B of the cell (area B, 16 s). From 32 s onwards the Ca^{2+} signal decayed to basal levels. Note that the area of highest $[Ca^{2+}]_i$ at the peak of the response (area B, 16 s) was the last area of the cell to decay to its resting level (65 s), whereas it was the first area to decay in the presence of external Mn^{2+} (Fig. 6, area A). This response was observed in 16/16 cells and confirmed that the localized decay of the fluorescence signal observed in the presence of external Mn^{2+} (Fig. 6, arrowheads) was due to a localized entry of external Mn^{2+} , and was not simply the normal decay of the peak Ca^{2+} response.

DISCUSSION

As a continuation of our studies on the relationship between $[Ca^{2+}]_i$ and secretion in bovine chromaffin cells, we have studied the influx of external Ca^{2+} elicited in single cells by the potent secretagogue histamine and compared this with the influx invoked by the weaker stimulus angiotensin II. In order to visualize divalent cation entry in response to these agonists, we used video-imaging and a modification (Cheek et al., 1991; Robinson et al., 1992) of the Mn^{2+} quench technique introduced by Hallam and Rink (1985) that enabled us to monitor any spatial organization associated with the entry component. The results indicate that histamine and angiotensin II stimulate different subcellular patterns of Ca^{2+} influx and suggest that such spatial differences in Ca^{2+} influx could underlie the differing abilities of these stimuli to trigger secretion.

The entry of external Ca^{2+} , rather than the release of intracellularly stored Ca^{2+} , is a vital requirement for the triggering of a secretory response from intact bovine chromaffin cells (Cheek et al., 1989a,b; Kim and Westhead, 1989; Stauderman et al., 1990; Yamagami et al., 1991). The use of video-imaging techniques to visualize stimulus-induced changes in $[Ca^{2+}]_i$ (reviews: Cheek, 1991; Burgoyne, 1991), and whole-cell patch clamp (Augustine and Neher, 1992; Neher and Augustine, 1992) and flash photolysis (Neher and Zucker, 1993) techniques to directly manipulate $[Ca^{2+}]_i$ at the single cell level, have suggested that this is likely to be because only entry of Ca^{2+} from the external medium delivers Ca^{2+} in sufficient magnitude to the subplasmalemmal exocytotic sites to activate fusion. Paradoxically, however, histamine releases intracellular Ca^{2+} via mobilization of $InsP_3$ (Plevin and Boarder, 1988; Stauderman et al., 1990) and is also a potent secretagogue (Noble et al., 1988; this study Fig. 2). Our results show that the histamine-induced secretory response is dependent upon the presence of external Ca^{2+} (Fig. 2), suggesting that, in addition to mobilizing intracellular Ca^{2+} , histamine is able to induce considerable Ca^{2+} influx into these cells. This influx is revealed in Fig. 1.

Because of its importance in triggering secretion, we used video-imaging techniques and the Ca^{2+} surrogate Mn^{2+} to

visualize the sub-cellular organization of the histamine-induced entry of divalent cations. The results indicate that histamine is able to trigger a continual influx of cations over the entire surface of the plasma membrane (Fig. 4). An unusual characteristic of H_1 receptors on these cells is that they show little desensitization with time (Noble et al., 1988). The additional ability to trigger Ca^{2+} influx may explain how prolonged exposure (hours) of these cells to histamine results in a greater secretory response than that seen after activation of nicotinic receptors, which also trigger Ca^{2+} entry but which do desensitize (Fenwick et al., 1982). Furthermore, the observation that influx occurs uniformly over the surface of the cell is consistent with the recent finding that exocytosis in response to histamine has also been reported to occur over the entire cell surface (Pender and Burgoyne, 1992).

The peptide angiotensin II is known to trigger a transient elevation in $[Ca^{2+}]_i$ in chromaffin cell populations (O'Sullivan and Burgoyne, 1989; Stauderman and Pruss, 1989) and a small secretory response that is dependent upon external Ca^{2+} (Bunn and Marley, 1989; O'Sullivan and Burgoyne, 1989; Powis and O'Brien, 1991). The rise in $[Ca^{2+}]_i$ in response to angiotensin II is spatially restricted such that peak $[Ca^{2+}]_i$ is often recorded in one pole of the cell (Cheek et al., 1989a,b; this study, Figs 6 and 7). There is evidence to suggest that it is to this same pole of the cell that exocytosis is localized in response to angiotensin II (Cheek et al., 1989a). The observations using Mn^{2+} in the present study implicate a pivotal role for localized Ca^{2+} entry in triggering this polarized secretion. The results show that, not only is there influx of divalent cations induced by angiotensin II, but also that the influx occurs predominantly into the pole of the cell that is responsible for the polarized secretion. The finding that Mn^{2+} influx in response to angiotensin II is localized to a specific subcellular area in chromaffin cells, rather than being localized uniformly over the cell surface, would also be consistent with the relatively small magnitude of the angiotensin II-induced Mn^{2+} quench recorded using chromaffin cell populations (Stauderman and Pruss, 1989).

Polarized Cl^- secretion, also triggered by a localized Ca^{2+} signal, has been reported in exocrine pancreas (Kasai and

Augustine, 1990) and polarized exocytotic secretion has previously been reported in non-neuronal secretory tissues such as mast cells (Lawson et al., 1978). Just how the exocytotic machinery can be locally activated is not fully understood, but the present results suggest that one possibility is to activate localized entry of external Ca^{2+} . A similar situation may exist in pituitary gonadotropes, where Ca^{2+} influx has also been reported to be polarized (Rawlings et al., 1991) and at some synapses, where transmitter release occurs at specialized active zones. In addition to containing synaptic vesicles and cytoskeletal elements, active zones are thought to contain hot-spots of Ca^{2+} channels (Smith and Augustine, 1988). Indeed, localized Ca^{2+} influx through spatially restricted plasma membrane Ca^{2+} channels has been reported in cells of the squid giant synapse (Smith and Augustine, 1988; Llinas et al., 1992) in addition to other cell types such as pituitary gonadotropes (Rawlings et al., 1991), N1E-115 neuroblastoma cells (Silver et al., 1990), sympathetic neurons (Lipscombe et al., 1988) and cerebellar Purkinje cells (Hockberger et al., 1989).

The mechanisms by which histamine and angiotensin II induce Ca^{2+} entry into chromaffin cells are unknown, although significant depolarization via activation of L-type voltage-dependent Ca^{2+} channels can be excluded (Fig. 3 and Table 1). Mn^{2+} entry has been reported to be activated by Ca^{2+} -ATPase inhibitors such as thapsigargin in the absence of surface receptor activation (Robinson et al., 1992), indicating the existence of a capacitance entry mechanism in these cells (Putney, 1990). Whether or not such a mechanism is responsible for all of the entry observed after receptor activation, however, remains to be elucidated. Ca^{2+} entry in response to angiotensin II has been observed after prior depletion of the internal store with ionomycin, suggesting that even with depleted stores, entry could still be stimulated by receptor activation (Stauderman and Pruss, 1989). One possibility is that hormones directly open a receptor-operated channel in the plasma membrane, as does ADP in platelets (Sage et al., 1989) and ATP in PC12 cells (Raha et al., 1993). An alternative possibility is that an intracellular messenger, such as $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$, may promote Ca^{2+} entry through a second messenger-operated channel (Irvine, 1992). With reference to this point, $\text{Ins}(1,4,5)\text{P}_3$ has been reported to directly open a plasma membrane channel that conducts Ca^{2+} in chromaffin cells (Mochizuki-oda et al., 1991) and there is evidence suggesting that both angiotensin II and histamine elevate the levels of $\text{Ins}(1,3,4,5)\text{P}_4$ in these cells (Stauderman and Pruss, 1990). Other possible intracellular messenger activators of plasma membrane Ca^{2+} channels, yet to be explored in chromaffin cells, include Ca^{2+} itself (von Tscharner et al., 1986) and GTP (Mullaney et al., 1988).

In summary, these results directly demonstrate that different InsP_3 -mobilizing stimuli can induce different patterns of divalent cation influx in the same cells and, furthermore, suggest how these different patterns can have a direct influence on cellular function. Given its importance in triggering secretion, establishing the mechanisms underlying the stimulus-induced influx will be central to elucidating the stimulus-secretion coupling pathway evoked by angiotensin II and histamine in chromaffin cells.

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