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Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(t-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells

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We have characterized the effect of the Ca^{2+}-ATPase inhibitors 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBHQ) and thapsigargin on the concentration of cytosolic Ca^{2+} in single bovine adrenal chromaffin cells by video-imaging of fura-2-loaded cells. Addition of either inhibitor released Ca^{2+} from internal stores in the absence of external Ca^{2+}. tBHQ was unable to stimulate further Ca^{2+} release after addition of thapsigargin, but thapsigargin could do so after release by tBHQ, indicating that the tBHQ-sensitive stores are a sub-set of those sensitive to thapsigargin. Angiotensin II was able to elicit Ca^{2+} release after application of tBHQ, indicating that at least part of the tBHQ-sensitive stores were distinct from those discharged by Ins(1,4,5)P_3. In the presence of external Ca^{2+}, both Ca^{2+}-ATPase inhibitors produced a more prolonged rise in cytosolic Ca^{2+} consistent with stimulated Ca^{2+} entry. The ability of the inhibitors to activate a Ca^{2+}-entry pathway was confirmed by monitoring quenching of fura-2 after stimulated entry of the Ca^{2+} surrogate Mn^{2+}. These findings indicate that bovine adrenal chromaffin cells possess a mechanism by which Ca^{2+} entry can be activated, following emptying of certain internal stores, independently of receptor occupation.

INTRODUCTION

Changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) are known to be important in the regulation of many aspects of cellular function. As a result there has been much interest in the mechanisms of release of Ca^{2+} from intracellular stores, and also the mechanisms of receptor-stimulated Ca^{2+} influx into the cell. The release of Ca^{2+} from intracellular stores is particularly well documented. It is known that many cells contain an Ins(1,4,5)P_3-sensitive Ca^{2+} store (for review see Berridge & Irvine, 1989). Many non-muscle cells, including neurons (Lipscombe *et al.*, 1988; Thayer *et al.*, 1990), bovine (Burgoyne *et al.*, 1989; Cheek *et al.*, 1990; Robinson & Burgoyne, 1991*a*; Stauderman *et al.*, 1991*a*) and rat (Malgaroli *et al.*, 1990) adrenal chromaffin cells are now known also to contain another intracellular Ca^{2+} store that is sensitive to caffeine and ryanodine. This store is of particular interest, since it is believed that it may be the non-muscle-cell equivalent of the Ca^{2+}-induced Ca^{2+}-release store of skeletal and cardiac muscle cells.

Less well characterized is the influx of Ca^{2+} into cells after stimulation of Ins(1,4,5)P_3-generating receptors, a topic which has been the subject of recent reviews (Irvine, 1991; Meldolesi *et al.*, 1991). There are two proposed mechanisms for Ca^{2+} influx into cells in response to such stimulation. The first of these is the capacitative theory (Putney, 1986, 1990), which proposes that emptying of the store is the only necessary stimulus to invoke Ca^{2+} influx into the cell. The other route proposes that Ca^{2+} influx follows the opening of receptor- or second-messenger-operated Ca^{2+} channels. These channels may be directly linked to the receptors for various agonists, or may be the so-called second-messenger-operated Ca^{2+} channels, which could be activated by Ins(1,4,5)P_3 or by Ca^{2+} after release of Ca^{2+} from intracellular stores.

Recent work has shown that in bovine adrenal chromaffin cells stimulation by the Ins(1,4,5)P_3-mobilizing agonist angiotensin II, leads to an influx of Ca^{2+} (Stauderman & Pruss, 1989; Cheek *et al.*, 1991). In this present study, two different Ca^{2+}-ATPase inhibitors, thapsigargin (Thastrup *et al.*, 1989) and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBHQ) (Moore *et al.*, 1987), were used. These two ATPase inhibitors mobilize Ca^{2+} from internal stores, and single-cell imaging allowed further characterization of their effect on Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry in bovine adrenal chromaffin cells. Our findings show that Ca^{2+} entry can be elicited independently of receptor occupation following Ca^{2+} release from stores in chromaffin cells.

MATERIALS AND METHODS

Materials

Fura-2/AM (acetoxymethyl ester) was from Molecular Probes (Eugene, OR, U.S.A.), thapsigargin was from LRC Services (Barnet, Herts., U.K.), and tBHQ was kindly given by Dr. C. W. Taylor (Cambridge). All other chemicals were from Sigma (Poole, Dorset, U.K.).

Isolation and culture of chromaffin cells

Chromaffin cells were isolated by a modification (Burgoyne *et al.*, 1988) of the method of Greenberg & Zinder (1982). Cells were isolated in a 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 (Dulbecco's modified Eagle's medium) containing 25 mM-Hepes, 10% foetal-calf serum, 8 μM-fluorodeoxyuridine, 50 μg of gentamycin/ml, 10 μM-cytosine arabinoside, 2.5 μg of fungizone/ml, 25 units of penicillin/ml and 25 μg of streptomycin/ml. 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

Abbreviations used: $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+}; tBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone.
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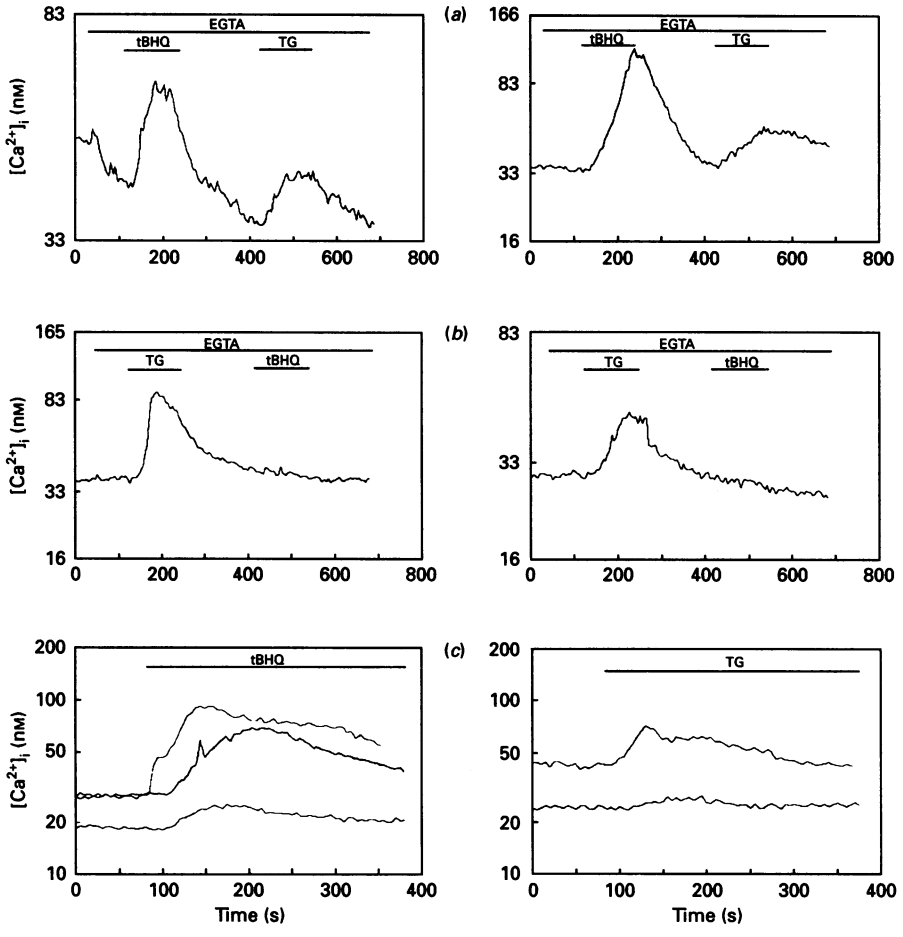

Fig. 1. Effect of tBHQ and thapsigargin (TG) on Ca^{2+} release in chromaffin cells

The effect of tBHQ (25 μM) followed by TG (5 μM) is shown in (*a*); these traces show the typical range of the responses observed. Prior application of tBHQ did not abolish the TG response. TG added before tBHQ greatly attenuated or abolished the tBHQ response (*b*). The traces shown in (*a*) and (*b*) were recorded from cells in a Ca^{2+}-free medium. The traces shown in (*c*) are from cells in a medium containing 3 mM-Ca^{2+}. The responses seen with both tBHQ and TG were more prolonged in the presence of external Ca^{2+}.

medium and plated on 22 mm-diameter glass coverslips at a density of 1×10^5 cells/ml in 3 ml of medium.

Cells were used between 2 and 5 days in culture. Before use, the cells were washed in buffer A containing 3 mM-$CaCl_2$ and 0.1% BSA, and incubated with 2 μM-fura-2/AM at room temperature for 30 min. The coverslips on which the cells were grown were then mounted in a 2 ml-capacity aluminium-alloy perfusion chamber for imaging. The cells were perfused at room temperature with buffer A containing 3 mM-$CaCl_2$ and 0.1% BSA from a main reservoir and agonist in the same buffer applied via an U-tube positioned to within 2 mm of the field of cells under observation. For experiments in Ca^{2+}-free conditions, after loading as described, the cells were perfused with buffer A containing 0.1% BSA and 1 mM-EGTA for 1 min before 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, Edison, NJ, U.S.A.), and interfaced to a Nikon Diaphot inverted epi-fluorescence microscope. The cultures were all imaged with an UVF × 100 glycerol-immersion objective, resulting in a final magnification of × 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, Sussex, U.K.). The video signal from this was digitized and stored in an Imagine image-processing system (Synoptics Ltd., Cambridge, U.K.), hosted by a DEC MicroVAX II computer. The excitation source was switched by a rotating-mirror chopper (Glen Creston Instruments, Stanmore, Middx., U.K.) driven by a stepping motor and synchronized with the video timebase to give alternative 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 video tape (SonyUmatic) for subsequent processing. Formation of the ratio image was implemented in a look-up table, computed from the formula given in Grynkiewicz *et al.* (1985) as described previously (O'Sullivan *et al.*, 1989). As a check for equal concentrations of fura-2 in the presence and absence of Ca^{2+}, the ratio of intensities was also measured with excitation at 360 nm, where fluorescence is independent of Ca^{2+} activity (Grynkiewicz *et al.*, 1985).

To study Ca^{2+} influx into the cells after challenge by an agonist, cells were incubated in an essentially Ca^{2+}-free medium (buffer A) containing 1 mM-Mn^{2+} for the times indicated in the Figures. Subsequent quenching of the fluorescent signal after stimulation of the cell was caused by Mn^{2+} acting as a Ca^{2+} surrogate in the influx pathways, and was monitored at excitation and emission wavelengths of 360 nm and 510 nm.

Recorded video data were played back through Imagine, 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.

RESULTS

Changes in $[Ca^{2+}]_i$ due to thapsigargin and tBHQ

Video imaging of single fura-2-loaded cells was used to monitor changes in the $[Ca^{2+}]_i$ in bovine adrenal chromaffin cells induced by maximal doses of thapsigargin and tBHQ. Most of the cells responded to thapsigargin (18 out of 20 cells) and tBHQ (16 out of 20 cells) with an elevation of $[Ca^{2+}]_i$. Figs. 1(*a*) and 1(*b*) show the typical range of responses seen when tBHQ and thapsigargin were applied to bovine adrenal chromaffin cells in the absence of external Ca^{2+}. These traces also show that prior application of tBHQ before thapsigargin did not always abolish the response to thapsigargin. In contrast, if thapsigargin was applied to the cell before tBHQ, then the release elicited by tBHQ was completely abolished in all cells examined. The quantitative data from a series of experiments using both inhibitors are shown in Table 1. The time courses show a transient response with a relatively slow rising and declining phase (Fig. 1), compared with responses seen with an agonist (see Fig. 2). The responses resemble those already published for thapsigargin both in intact cells (Cheek & Thastrup, 1989; Cheek *et al.*, 1989*b*) and in permeabilized cells (Robinson & Burgoyne, 1991*a*); the tBHQ responses are similar to those seen in cell populations (Robinson & Burgoyne, 1991*b*). When the same experiments were carried out in the presence of 3 mM external Ca^{2+} the responses were no longer transient in nature. They had the same slow initial rising phase, but the decline phase was much more prolonged (Fig. 1*c*), for both tBHQ and thapsigargin. These data would be consistent with stimulated Ca^{2+} entry following release from the internal stores. The latter interpretation was supported by the observation that in bovine chromaffin (Cheek & Thastrup, 1989) and other cells (Thastrup *et al.*, 1989) thapsigargin induces the release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} store. In the present experiments the effects of tBHQ and thapsigargin were not reversible over the time courses examined.

From work carried out using populations of chromaffin cells (Robinson & Burgoyne, 1991*b*), it appeared that tBHQ released Ca^{2+} from a store that was insensitive to Ins(1,4,5)P_3, since it had no statistically significant effect on the release elicited by angiotensin II. From the traces shown in Fig. 1, the tBHQ-sensitive store appeared to be a subset of the thapsigargin-sensitive store. To resolve the relationship between the tBHQ- and the Ins(1,4,5)P_3-sensitive store, single cells were challenged with angiotensin II. In controls the naive response of a particular cell was ascertained, before being stimulated for a second time with angiotensin II. After the first stimulation, the angiotensin II was washed away in buffer A containing 3 mM-Ca^{2+}, in order to allow the intracellular Ca^{2+} stores to be replenished, and then the cells were stimulated with angiotensin II in a Ca^{2+}-free medium. The traces in Fig. 2 show the range of responses obtained. The

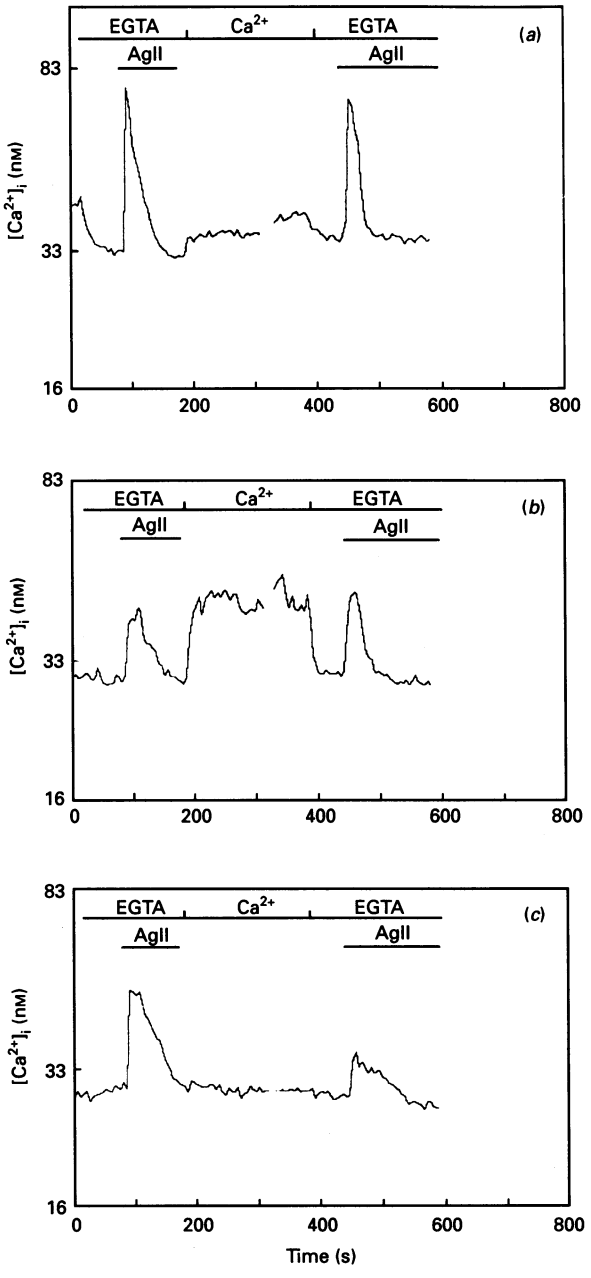

Fig. 2. Effect of two additions of angiotensin II (AgII; 0.3 μM) on $[Ca^{2+}]$ in chromaffin cells

The cells were challenged in a Ca^{2+}-free medium. The agonist was then washed away in a medium containing 3 mM-Ca^{2+} in order to allow the intracellular Ca^{2+} stores to replenish before a second stimulation. In (*a*) and (*b*) there is no attenuation of the second AgII response, compared with the naive response of the cell; (*c*) shows results for a cell in which the response was partially attenuated.

Table 1. Effect of tBHQ and thapsigargin on $[Ca^{2+}]_i$ in adrenal chromaffin cells

Fura-2-loaded chromaffin cells were challenged with either tBHQ (25 μM) or thapsigargin (TG; 5 μM) and then with the second inhibitor in the absence of external Ca^{2+} and with 1 mM-EGTA present. The number of cells responding with a rise in $[Ca^{2+}]_i$ is shown as well as the mean increase in $[Ca^{2+}]_i$ over basal (ΔCa^{2+}) and the time from challenge to the peak of the response for those cells that responded.

Treatment	No. of cells responding	ΔCa^{2+} (nM)	Time to peak response (s)
tBHQ	9/11	30.1±7.1	97.4±10.1
tBHQ after TG	0/11	–	–
TG	7/11	33.8±6.0	104.3±8.0
TG after BHQ	5/10	14.5±1.8	134.4±9.0

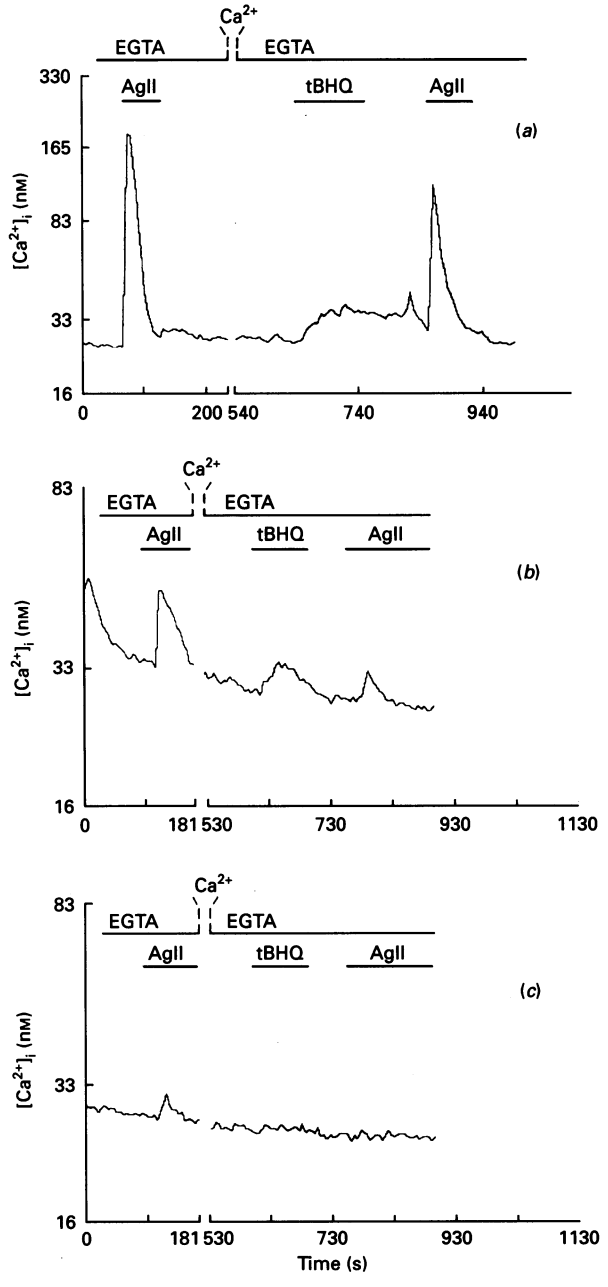

Fig. 3. Effect of tBHQ (25 μM) on the release of Ca^{2+} from stores induced by angiotensin II (AgII; 0.3 μM)

The trace shown in (*a*) depicts a cell in which there was a slight attenuation of the AgII response. In (*b*) there is a greater degree of attenuation of the AgII response. Fig. (*c*) shows a cell in which the initially small AgII response was abolished.

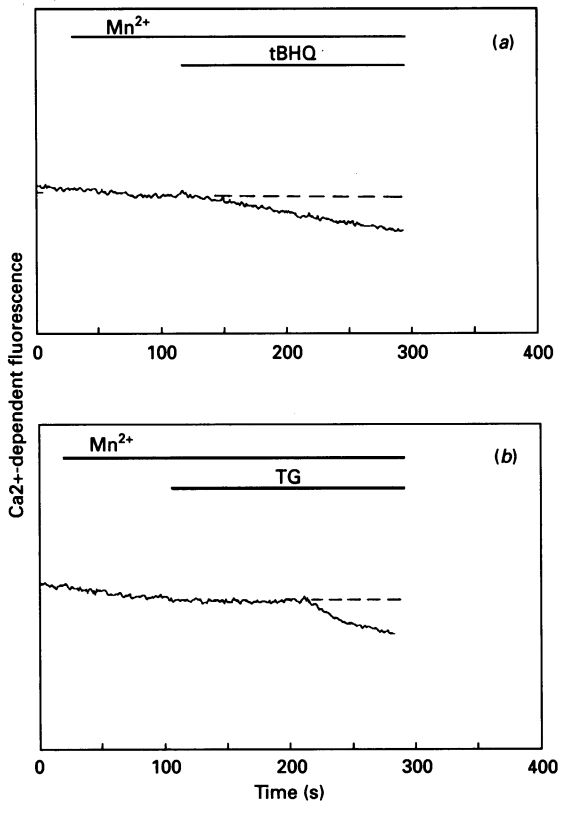

Fig. 4. Effect of tBHQ (25 μM) and thapsigargin (TG; 5 μM) on Ca^{2+} entry in chromaffin cells

Ca^{2+} was replaced in the extracellular medium by 1 mM-Mn^{2+}. Fura 2 fluorescence was recorded by excitation at 360 nm and emission at 510 nm. The quenching, and thus entry, induced by tBHQ is shown in (*a*), and that stimulated by TG is shown in (*b*).

second addition of angiotensin II showed no attenuation (Figs. 2*a* and 2*b*) when compared with the naive response of the same cell, or was partially attenuated (Fig. 2*c*). Fig. 3 depicts the effect of tBHQ on the release of Ca^{2+} by a second stimulation with angiotensin II, and shows that there was again a heterogeneous response to angiotensin II. Even though we would expect the second response to angiotensin II to be attenuated (see Fig. 2), a response persisted in most (5 out of 6) cells after Ca^{2+} release by tBHQ (Fig. 3). The mean change in $[Ca^{2+}]_i$ in response to angiotensin II after tBHQ treatment was 31.6 ± 10.2 nM ($n = 5$), compared with a rise caused by angiotensin II in virgin cells of 49.7 ± 10.4 nM ($n = 11$). These results show that at most there is no more than a partial overlap of the tBHQ-and angiotensin II-sensitive Ca^{2+} stores. With the knowledge that thapsigargin and tBHQ emptied intracellular Ca^{2+} stores, we aimed to determine whether Ca^{2+} entry was indeed activated after release from stores in the absence of receptor agonists.

The pathways of bivalent-cation (and thus Ca^{2+}) entry can be monitored by replacing extracellular Ca^{2+} with Mn^{2+}, and observing quenching of the fura-2 fluorescent signal. The traces shown in Fig. 4 show the results obtained from experiments of this type. Fig. 4(*a*) shows quenching of the fura-2 signal after challenge of the cell with thapsigargin (16 cells). Fig. 4(*b*) is a typical response observed when the cells were challenged with tBHQ (16 cells). It is noteworthy that with thapsigargin there is a longer lag between application of the drug and quenching of the fluorescence, whereas upon challenge of the cell with tBHQ the influx of Mn^{2+} is much more rapid. The mean lag times were 36.4 ± 8.5 s and 73.0 ± 12.5 s for tBHQ and thapsigargin respectively ($n = 16$ in both cases).

Fig. 5 shows false colour images of the response of a chromaffin cell to tBHQ, at several time points after stimulation in the presence of Mn^{2+}. These images show that in response to tBHQ an initial rise in $[Ca^{2+}]_i$ occurs, presumably owing to release of internal Ca^{2+}. This reached a peak at around 20 s after tBHQ addition, when the response became dominated by fluorescence quenching due to Mn^{2+} entry. This latter response was manifested as a decrease in the size of the cell image, owing to loss of fluorescence. Fluorescence quenching and thus Mn^{2+} entry occurred around the entire plasma membrane, with no indication

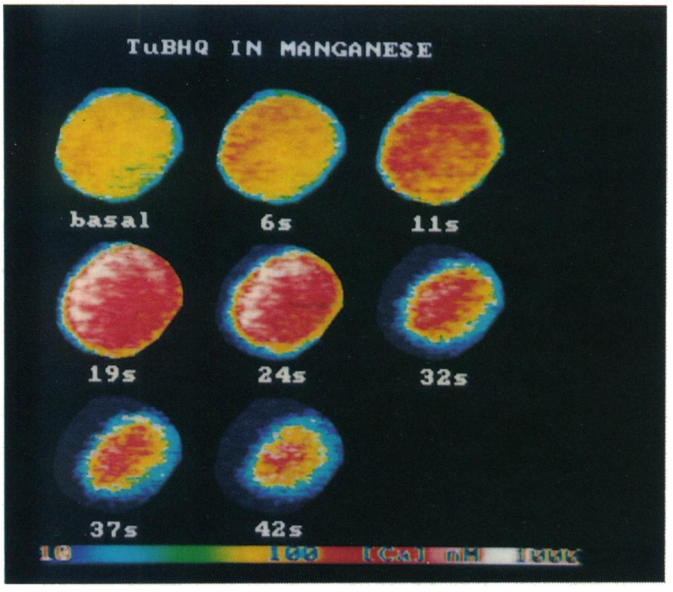

Fig. 5. Video-imaging of Ca^{2+} release from internal stores and Mn^{2+} quenching in single chromaffin cells

The cells were treated with 25 μM-tBHQ ('TuBHQ') in the presence of 1 mM-Mn^{2+} which replaced extracellular Ca^{2+}. The initial response to tBHQ (over the first 20 s) is a rise in $[Ca^{2+}]_i$ due to release from internal stores. The response then became dominated by fluorescence quenching around the entire plasma membrane from 32 s due to Mn^{2+} entry. Note that the blue ring of apparent low $[Ca^{2+}]_i$ at the extreme edge of the cells before quenching is an artefact and is outside of the cell boundary. In this cell the mean basal and peak stimulation values for $[Ca^{2+}]_i$ were 93 and 343 nM respectively.

of a spatial localization of this effect ($n = 11$). In the example in Fig. 5, Mn^{2+} quenching had occurred around the whole cell by 32 s after tBHQ addition. This is in contrast with findings with angiotensin II, where polarized quenching due to Mn^{2+} entry was clearly observed (Cheek *et al.*, 1991). A similar response to that in Fig. 5 was seen when the cells were challenged with thapsigargin (results not shown).

DISCUSSION

The results presented in this study show that two structurally unrelated inhibitors of the endoplasmic-reticulum Ca^{2+}-ATPase lead to mobilization of intracellular Ca^{2+} and activation of Ca^{2+} influx in bovine adrenal chromaffin cells, thereby providing evidence for the concept of regulation of Ca^{2+} influx by the filling state of the intracellular stores in these cells. In addition, these results have allowed further characterization of the effect of tBHQ in releasing Ca^{2+} from internal stores in chromaffin cells. In a previous study using populations of cells (Robinson & Burgoyne, 1991*b*), it was seen that prior addition of tBHQ appeared not to attenuate significantly any subsequent responses that were stimulated by either angiotensin II or caffeine. In agreement with these findings, Fig. 3 shows that in single cells tBHQ did not abolish the release of Ca^{2+} induced by angiotensin II. It is known from previous studies that thapsigargin empties the Ins(1,4,5)P_3-sensitive store in chromaffin cells (Cheek & Thastrup, 1989; Cheek *et al.*, 1989*b*; Robinson & Burgoyne, 1991*a*). The traces in Fig. 1 show that the tBHQ-sensitive Ca^{2+} store appeared to be a subset of the thapsigargin-sensitive store, since prior application of thapsigargin completely abolished the tBHQ response. The tBHQ-sensitive store was, at least in part, distinct from the Ins(1,4,5)P_3-sensitive store, since angiotensin II could still elicit a response after prior application of tBHQ to most cells. These results also showed that it was not a subpopulation of cells that were responding to tBHQ and not to angiotensin II.

As in many other cell types, agonist-induced Ca^{2+} signals in bovine chromaffin cells involve both the release of internally stored Ca^{2+} and the influx of external Ca^{2+}. Although it is clear that hormones such as angiotensin II and histamine release intracellular Ca^{2+} (Stauderman & Pruss, 1989; Cheek *et al.*, 1991) via mobilization of Ins(1,4,5)P_3 (Bunn & Marley, 1989; Wan *et al.*, 1989; Stauderman & Pruss, 1990; Challiss *et al.*, 1991) how these stimuli regulate Ca^{2+} influx is still poorly understood. Ca^{2+} entry could be activated by a receptor-linked channel, a second-messenger-operated channel controlled by Ca^{2+}, Ins(1,4,5)P_3 or Ins(1,3,4,5)P_3, or by capacitative Ca^{2+} entry (Putney, 1986, 1990). Both Ins(1,4,5)P_3 (Kuno & Gardner, 1987; Penner *et al.*, 1988; Mochizuki *et al.*, 1991) and Ins(1,3,4,5)P_4 (Irvine & Moore, 1987; Morris *et al.*, 1987; Changya *et al.*, 1989; Petersen, 1989) have been implicated as having a role in Ca^{2+} entry after receptor activation. There is some debate as to whether or nor Ins(1,3,4,5)P_4 is essential for Ca^{2+} entry (Bird *et al.*, 1991), or whether it facilitates Ca^{2+} entry induced by some other mechanism (Luckhoff & Clapham, 1992). The capacitative model proposed by Putney (1986, 1990) argued that emptying of the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores was sufficient to cause Ca^{2+} entry. In this present study, we have tried to address the question as to whether emptying of intracellular Ca^{2+} stores in bovine adrenal chromaffin cells was sufficient to elicit Ca^{2+} entry in the absence of receptor activation. In order to examine this question we have used two Ca^{2+}-ATPase inhibitors, thapsigargin and tBHQ.

When the responses to thapsigargin and tBHQ were examined in the presence of extracellular Ca^{2+}, the decline phase of the response was very much prolonged, compared with that observed in a Ca^{2+}-free medium. These results suggested indirectly that emptying of the tBHQ- or thapsigargin-sensitive Ca^{2+} stores stimulated Ca^{2+} entry. Further indication of this came from the finding that if these two Ca^{2+}-ATPase inhibitors were used to stimulate the cells in a Ca^{2+}-free medium, and then external Ca^{2+} was reintroduced after the initial Ca^{2+} transient, a second, more prolonged, transient could be observed, which resembled that seen when cells were stimulated in the presence of extracellular Ca^{2+} (results not shown).

A more direct way in which to follow the bivalent-cation influx pathway in cells after stimulation was introduced by Hallam & Rink (1985). This method involved the use of extracellular Mn^{2+}, which enters the cell as a Ca^{2+} surrogate. Mn^{2+} is known to displace Ca^{2+} from fura-2, and as it does so the fluorescence signal of the dye is quenched. The traces in Fig. 4 show that both thapsigargin and tBHQ stimulated the entry of Mn^{2+} into chromaffin cells, as shown by the decrease in the levels of fluorescence in the cells. By using a modification of the method of Hallam & Rink (1985), as described by Cheek *et al.* (1991), Mn^{2+} entry into chromaffin cells, after stimulation by thapsigargin and tBHQ, was studied by using imaging techniques. Fig. 5 shows that, in response to tBHQ, Mn^{2+} quenching occurred around the entire plasma membranes, indicating that the influx mechanisms had been triggered over the entire surface of the cell. These results argue against the involvement of receptor-operated channels ('ROC') being needed for Ca^{2+} influx, unlike the case in platelets, in which ADP stimulated Ca^{2+} influx through such a pathway (Sage *et al.*, 1989). It was of interest that the lag times for the release of internal Ca^{2+} were similar for tBHQ and thapsigargin, but that Mn^{2+} entry showed a much longer lag with thapsigargin. The reasons for this are not clear.

Just how thapsigargin and tBHQ stimulate Ca^{2+} influx into

cells is unknown. In bovine chromaffin cells, ionomycin (Stauderman & Pruss, 1990) and sub-maximal doses of caffeine (Cheek *et al.*, 1990) did not appear to trigger sustained Ca^{2+} entry, since, even in the presence of external Ca^{2+}, they gave only a transient rise in $[Ca^{2+}]_i$. These findings appear to rule out the possibility that thapsigargin or tBHQ leads to the opening of second-messenger-operated Ca^{2+} channels ('SMOC') directly through the elevation of Ca^{2+} or via Ins(1,4,5)P_3 generated by the Ca^{2+}-dependent phospholipase C in these cells (Sasakawa *et al.*, 1987; Eberhard & Holz, 1987). The involvement of voltage-sensitive Ca^{2+} channels ('VSCC') can also apparently be ruled out, since measurements made with the voltage-sensitive plasma-membrane dye bisoxonol showed that neither thapsigargin nor tBHQ depolarized the cells (I. M. Robinson & R. D. Burgoyne, unpublished work). The data would therefore be most consistent with Ca^{2+} entry occurring as a consequence of the emptying of the thapsigargin- or tBHQ-sensitive stores (the capacitative model), but we cannot rule out a role for a second messenger. It should be noted that one study, using ionomycin to release Ca^{2+} from stores, has been interpreted as showing that store emptying does not lead to Ca^{2+} entry (Stauderman & Pruss, 1990). In that study, however, the entry pathway was not directly examined by Mn^{2+} quenching in response to ionomycin, and in the traces shown, ionomycin gave a more sustained rise in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} than in its absence, consistent with Ca^{2+} entry.

Entry of Ca^{2+}, following the challenge of cells with both thapsigargin (Takemura *et al.*, 1989) and tBHQ, has been observed in other cell types. Acinar cells (Takemura *et al.*, 1989), endothelial cells (Dolor *et al.*, 1992), HL60 cells and human leukaemic T cells (Demaurex *et al.*, 1992) all behave in a similar manner to chromaffin cells as reported here. In addition, Foskett *et al.* (1991), showed that both of these Ca^{2+}-ATPase inhibitors could induce Ca^{2+} oscillations in rat parotid acinar cells that were dependent on the presence of extracellular Ca^{2+} for their maintenance. In contrast, thapsigargin and tBHQ mobilized intracellular Ca^{2+}, but failed to stimulate Mn^{2+} influx in hepatocytes (Llopis *et al.*, 1991), even though vasopressin and angiotensin II could do so by an alternative route (Llopis *et al.*, 1992). Thus from these results it would seem that there are different mechanisms controlling Ca^{2+} entry into different cell types, but that emptying the intracellular Ca^{2+} stores has some role to play in this process. Such a link was suggested by the finding that the ability of both histamine and thapsigargin to stimulate Ca^{2+} influx varied with the cell cycle in HeLa cells (Preston *et al.*, 1991).

Ca^{2+} entry into bovine adrenal chromaffin cells has been shown by a number of reports (Kim & Westhead, 1989; O'Sullivan *et al.*, 1989; O'Sullivan & Burgoyne, 1989; Cheek *et al.*, 1989*a*,*c*; Stauderman *et al.*, 1991*b*) to be a vital requirement for the triggering of a secretory response. Also a localized Ca^{2+} entry elicited by angiotensin II has been implicated as being the trigger for a localized secretory response after angiotensin II stimulation (Cheek *et al.*, 1989*b*). The Ca^{2+}-entry pathways activated by agonists such as angiotensin II are unknown. The present findings show that Ca^{2+} entry in response to angiotensin II need not involve receptor-operated Ca^{2+} channels, since adrenal chromaffin cells possess a mechanism whereby Ca^{2+} entry is activated after emptying of Ins(1,4,5)P_3-sensitive Ca^{2+} stores. Further work is necessary to determine if this mechanism is responsible for entry due to receptor activation by angiotensin II and also histamine, which activates a less spatially restricted entry pathway (Cheek *et al.*, 1991).

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