Volume 289, number 2, 151–154 FEBS 10122 September 1991

ADONIS 001457939100837y

A distinct 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone-sensitive calcium store in bovine adrenal chromaffin cells

Iain M. Robinson and Robert D. Burgoyne

Department of Physiology, University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK

Received 25 June 1991

The fluorescent Ca^{2+} indicator Fura 2 was used to monitor Ca^{2+} release induced by the Ins(1,4,5)P_3-mobilizing agonist angiotensin II (Ag II), caffeine and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tuBHQ), in intact bovine adrenal chromaffin cells. Under low external Ca^{2+} conditions, tuBHQ, Ag II and caffeine elicited Ca^{2+} rises, indicating Ca^{2+} release from internal stores. Prior addition of Ag II had no noticeable effect on the extent of release of Ca^{2+} induced by tuBHQ. Stimulation of the cells with tuBHQ before either Ag II or caffeine, similarly had no effect on Ca^{2+} released by these two agonists. It was concluded, therefore, that there is a third intracellular Ca^{2+} store in bovine adrenal chromaffin cells, distinct and non-overlapping, from those sensitive to caffeine or Ins(1,4,5)P_3-mobilizing agonists.

Caffeine; Chromaffin cell; Inositol 1,4,5-trisphosphate; Intracellular Ca^{2+} store; 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone

1. INTRODUCTION

The importance of changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) in the regulation of many aspects of cell function is now well established. Agonists can elicit rises in $[Ca^{2+}]_i$ in two ways, by either causing entry of external calicium into the cytosol or by releasing calcium form the intracellular calcium stores. The role of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) in releasing Ca^{2+} is well documented [1]. It is also known that many cells also contain a second intracellular Ca^{2+} store that is Ins(1,4,5)P_3-insensitive [1–7]. The potential importance of the Ins(1,4,5)P_3-insensitive store is that it may correspond to the non-muscle cel equivalents of the skeletal and cardiac muscle Ca^{2+}-induced Ca^{2+} release (CICR) stores, and hence have a role in the amplification and propagation of the initial Ca^{2+} signal generated by Ins(1,4,5)P_3 [1,8]. There is much interest at present as to the possible role of these two Ca^{2+} stores in the generation of Ca^{2+} oscillations [1,8–10]. The CICR stores in muscle have been shown to release upon challenge with caffeine, a phenomenon observed in other cell types, including neurons [6,11], bovine [12,13] and rat [7] adrenal chromaffin cells. The localisation of the Ca^{2+} stores in bovine adrenal chromaffin cells is known from video imaging studies [12,13]; these studies suggest that the two stores may be spatially distinct, and in addition may possess distinct Ca^{2+}-ATPase-like proteins [12]. Recently, using permeabilised chromaffin cells, it has been possible to establish that these two stores are in fact distinct [14]; and also to provide further evidence that the stores contain distinct Ca^{2+} ATPase-like proteins, using the Ca^{2+}-ATPase inhibitor thapsigargin [14–16]. In this present study we used another Ca^{2+}-ATPase inhibitor 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tuBHQ) to further characterise the internal stores in chromaffin cells, this compound having previously been used to study Ca^{2+}-ATPase and Ca^{2+} release in rat liver microsomes [17,18] and permeabilised rat hepatocytes [19].

Abbreviations: Ag II, angiotensin II; $[Ca^{2+}]_i$, the concentration of intracellular free calcium; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; CICR, calcium induced calcium release; tuBHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; HEPES, *N'*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid].

Correspondence address: R.D. Burgoyne, Department of Physiology, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, UK. Fax: (44) (51) 794 5372.

2. MATERIALS AND METHODS

Materials were from sources listed in earlier publications [12,14], tuBHQ was a gift from Dr C.W. Taylor, Cambridge.

2.1 *Isolation and culture of chromaffin cells*

Chromaffin cells were isolated from bovine adrenal medullae by enzymatic digestion as described by Greenberg and Zinder [20] with modifications [21]. Cells were washed in buffer A (145 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM HEPES at pH 7.4), resuspended in culture medium (Dulbecco's modified essential medium with 25 mM HEPES, 10% foetal calf serum, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 U/ml penicillin, and 25 μg/ml streptomycin), plated in 60-mm diameter Petri dishes, for $[Ca^{2+}]_i$ measurements, at a density of $1 \cdot 10^6$ cells/ml ($9 \cdot 10^6$ cells/dish, or for secretion experiments in 16-mm diameter plastic tissue culture wells at $1 \cdot 10^6$ cells/well.

2.2 *Assay of* $[Ca^{2+}]_i$

Intact-cell $[Ca^{2+}]_i$ measurements were made using the fluorescent Ca^{2+} indicator Fura 2. The cells were gently scraped off the plastic Petri dishes and then washed twice in buffer A by centrifugation at

100×g for 10 min. The cells were then incubated with 2 μM Fura 2-AM for 30 min at 20°C in buffer A containing 3 mM $CaCl_2$ and 0.5% bovine serum albumin. The cells were washed and left for 45 min before use. The fluorescence measurements were carried out in buffer A containing 3 mM $CaCl_2$ and 4 mM EGTA (free Ca^{2+} concentration less than $2 \cdot 10^{-7}$M), and calibrated as previously described [22]. Al fluorescence measurements were carried out in a Perkin-Elmer LS-5 luminescence fluorimeter, fitted with a magnetic stirrer.

3. RESULTS

Experiments were carried out in buffers containing less than $2 \cdot 10^{-7}$ M free Ca^{2+}, to elimate Ca^{2+} entry. Under these conditions the basal level of $[Ca^{2+}]_i$ was around 80 nM and tuBHQ (25 μM), angiotensin II (Ag II, 0.3 μM) and caffeine (5 mM), elicited Ca^{2+} rises above basal levels of 77.9±6.7 nM (n=25), 132.1±23.1 nM (n=11) and 168.8±17.8 nM (n=8) respectively due to Ca^{2+} release from internal stores (Figs. 1a,b and 2b). The doses of drugs used were those that gave maximal responses and second additions of the same concentration of drugs elicited no, or only small further rises in $[Ca^{2+}]_i$ (Figs. 1 and 2). The magnitude and overall shape of the responses to Ag II were similar to those already reported for Ag II in chromaffin cells [13,23]. The release caused by tuBHQ elicited a slower, more prolonged response, than that of Ag II. This response is similar in appearance to that reported for thapsigargin (which is also a Ca^{2+}-ATPase inhibitor) in both permeabilised [14] and intact chromaffin cells [16].

In order to investigate whether Ca^{2+} released by tuBHQ came from the agonist-($Ins(1,4,5)P_3$-) sensitive Ca^{2+} store, the effect of prior application of Ag II on the response to tuBHQ was examined. Ca^{2+} release due to Ag II (Fig. 1b) did not significantly affect the time course or the magnitude of the subsequent Ca^{2+} release stimulated by tuBHQ (79.0±11.2% of control values from previously unchallenged cells, n=10) suggesting that most of the Ca^{2+} released by tuBHQ came from an Ag II-insensitive store. A second addition of Ag II did not stimulate further Ca^{2+} release indicating that the agonist-sensitive store was fully emptied before tuBHQ addition (Fig. 1c). Prior addition of tuBHQ reduced Ca^{2+} release due to either Ag II (64.7±11.9% of control, n=9) but this difference was not statistically significant. In addition to emptying the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store prior addition of Ag II would also activate protein kinase C. The tuBHQ response was, however, not modified by treatment with the phorbol ester PMA.

In addition to an agonist-sensitive store, chromaffin cells also possess a caffeine-sensitive Ca^{2+} store. The possibility that tuBHQ released Ca^{2+} from this store was examined. A second application of tuBHQ elicited only a small further Ca^{2+} release (Fig. 2a). Following such treatment, however, caffeine was still able to elicit a marked Ca^{2+} release (Fig. 2a). In a series of experiments the rise in $[Ca^{2+}]_i$ due to caffeine was not reduced by a prior single application of tuBHQ being 88±14% of control values (n=4). This result suggests that tuBHQ

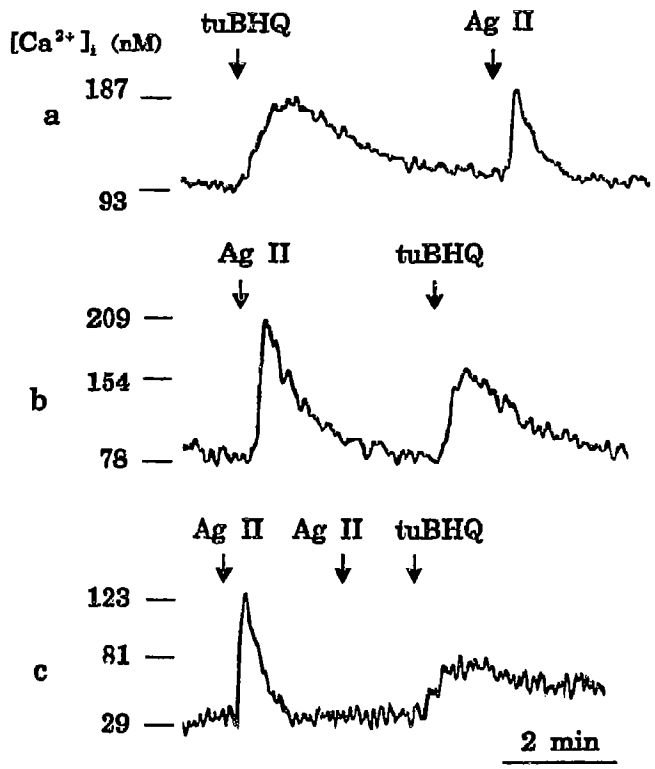

Fig. 1. Effect of tuBHQ and Ag II release in chromaffin cells. The cells were loaded with Fura 2, which was used to monitor $[Ca^{2+}]_i$. The effect tuBHQ (25 μM) followed by Ag II (0.3 μM) is shown in (a). The effect of single or double additions of Ag II prior to tuBHQ are shown in (b) and (c). These two traces show that prior application of tuBHQ does not significantly effect the Ca^{2+} release inducible by Ag II, and vice versa. The effect of two consecutive additions of Ag II shown in (c) indicates that a single addition of 0.3 μM Ag II was sufficient to completely empty the Ag II-sensitive store.

releases Ca^{2+} from a store insensitive to caffeine. In addition, tuBHQ still elicited a release of Ca^{2+} following challenge with caffeine (Fig. 2b). The magnitude of the rise in response to tuBHQ appears smaller in Fig. 2b compared to that in Fig. 2a due to the rise occurring from an elevated baseline and the Fura 2 response being non-linear. The peak rise in $[Ca^{2+}]_i$ due to tuBHQ after a single caffeine addition was 85.2±12.2% (n=6) of control values and after two caffeine additions was 76±9.6% (n=4) of control values from previously unchallenged cells. In addition to releasing Ca^{2+} from the caffeine-sensitive internal stores, caffeine would also raise cAMP levels, however the response to tuBHQ was not modified by treatment with 8-bromo-cAMP.

Release of Ca^{2+} from the agonist- or caffeine-sensitive store is relatively ineffective in stimulating catecholamine secretion from chromaffin cells [13,23]. Similarly tuBHQ elicited catecholamine release that was only marginally above basal release.

4. DISCUSSION

The present experiments were carried out in buffers containing low concentrations of Ca^{2+}, which obviated

the possibility of extracellular Ca^{2+} entry into the cells. The results show that tuBHQ released Ca^{2+} from internal stores in chromaffin cells. When the experiments were carried out under nominally Ca^{2+}-free conditions, tuBHQ was still able to elicit a release of Ca^{2+} (n=4) (not shown). The results also show that prior application of tuBHQ resulted in only small, non-significant reductions in the Ca^{2+} responses elicited by either Ag II or caffeine. Challenge by Ag II or caffeine prior to tuBHQ did not reduce the tuBHQ Ca^{2+} response even though second applications of either of the two latter drugs failed to elicit significant further Ca^{2+} release, indicating that the stores sensitive to Ag II or caffeine were fully emptied. We conclude from these results that chromaffin cells contain a third intracellular Ca^{2+} store, distinct from the Ins(1,4,5)P_3-sensitive and caffeine-sensitive stores. We cannot, however, rule out minor overlap between these three stores. TuBHQ has been reported to be a Ca^{2+}-ATPase inhibitor which would explain its action on Ca^{2+} release. The extent of its specificity is unclear. Nevertheless, the present and previous data [12,14] suggest that the three internal Ca^{2+} stores in chromaffin cells may posses distinct Ca^{2+}-ATPases, sensitive to tuBHQ or thapsigargin, or insensitive to both. Previous work on hepatocytes suggests that tuBHQ is able to elicit Ca^{2+} release from an Ins(1,4,5)P_3-insensitive store but its sensitivity to caffeine was not determined [19]. The tuBHQ-sensitive store in chromaffin cells does not require a proton gradient: experiments using the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) in permeabilised chromaffin cells, showed that the tuBHQ response was not inhibited (unpublished observations). This result also shows that the tuBHQ store is distinct from either mitochondrial or secretory vesicle Ca^{2+} stores, which both require proton gradients to sequester Ca^{2+}.

The role of the various Ca^{2+} stores is at present of interest in providing an explanation for the intracellular Ca^{2+} oscillations observed in many cell types. It has been suggested that production of Ins(1,4,5)P_3 causes a release of small amounts of Ca^{2+} which serve to prime the release of Ca^{2+} from an Ins(1,4,5)P_3-insensitive store [24]. Ca^{2+} waves are then believed to propagate across the cell by CICR and Ca^{2+} would then be pumped across the plasma membrane or sequestered, before the process is repeated in a cyclical manner, giving rise to $[Ca^{2+}]_i$ oscillations. Ca^{2+} oscillations have also been induced by stimulation of Ins(1,4,5)P_3-sensitive Ca^{2+} stores, without the concurrent production of Ins(1,4,5)P_3 [25]. Foskett et al. [25], using pancreatic acinar cells described Ca^{2+} oscillations that were induced by thapsigargin. These oscillations were similar to those seen following carbachol stimulation of these cells. It was also reported that Ca^{2+} oscillations were inducible in these cells by tuBHQ [25]. If tuBHQ-sensitive stores are generally distributed, then it is possible that this third store may, in some way, be involved in the production

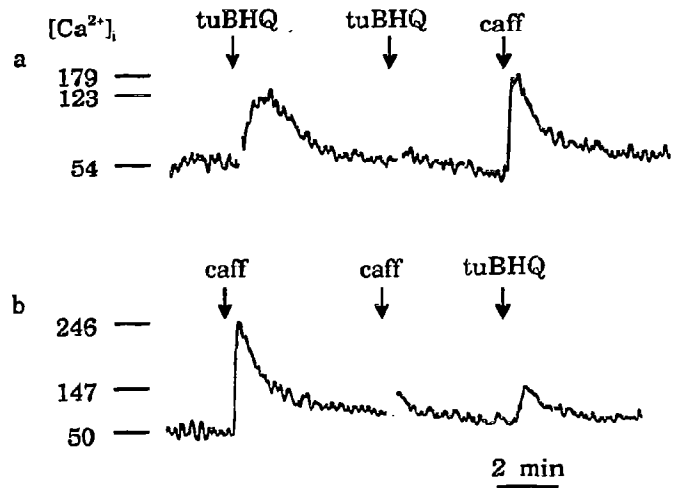

Fig. 2. Effect of tuBHQ and caffeine on Ca^{2+} release in chromaffin cells. TuBHQ (25 μM) was added either before (a) or after caffeine (5 mM) (b). Two prior additions of tuBHQ indicated that the tuBHQ-sensitive store could be fully emptied but that this did not effect the magnitude of the Ca^{2+} response elicited by caffeine. Prior additions of caffeine did not block the ability of tuBHQ to release Ca^{2+}.

of Ca^{2+} oscillations; though it is not yet known if Ca^{2+} is released from this store in response to cell stimulation. It is possible that tuBHQ may be of use in further characterisation of intracellular Ca^{2+} stores in other cell types, and may also provide a useful tool for the further elucidation of the mechanisms behind the generation of Ca^{2+} oscillations.

Acknowledgement: I.M.R. was supported by a Medical Research Council Studentship.

REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.
[2] Ghosh, T.K., Mullaney, J.M., Tarazi, F.I. and Gill, D.L. (1989) Nature 340, 236–239.
[3] Thevenod, F., Dhelinger-Kremer, M., Kremmer, T.P., Christian, A.-L., Potter, B.V.L. and Schulz, I. (1989) J. Membrane Biol. 109, 173–186.
[4] Henne, V., Piper, A. and Soling, H.-D. (1987) FEBS Lett. 218, 153–158.
[5] Gill, D.L., Ghosh, T.K. and Mullaney, J.M. (1989) Cell Calcium 10, 363–374.
[6] Thayer, S.A., Perney, T.M. and Miller, R.J. (1990) J. Neurosci. 8, 4089–4097.
[7] Malgaroli, A., Fesce, R. and Meldolesi, J. (1990) J. Biol. Chem. 265, 3005–3008.
[8] Berridge, M.J. and Galioni, A. (1988) FASEB J. 2, 3074–3082.
[9] Opsichuk, Y.V., Wakui, M., Yule, D., Gallacher, D.V. and Petersen, O.H. (1990) EMBO J. 9, 585–602.
[10] Rooney, T.A., Sass, E. and Thomas, A.P. (1989) J. Biol. Chem. 264, 17131–17141.
[11] Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) Neuron 1, 355–365.
[12] Burgoyne, R.D., Cheek, T.R., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Mata, A.M., Colyer, J., Lee, A.G. and East, J.M. (1989) Nature 342, 72–74.
[13] Cheek, T.R., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. (1990) FEBS Lett. 266, 91–95.

[14] Robinson, I.M. and Burgoyne, R.D. (1991) J. Neurochem. 56, 1587–1593.
[15] Thastrup, O., Dawson, A.P., Scharff, O., Foder, B. Cullen, P.J., Drobak, B.K., Bjerrum, P.J., Christesen, S.B. and Hanley, M.R. (1989) Agents Actions 27, 17–23.
[16] Cheek, T.R. and Thastrup, O. (1989) Cell Calcium 10, 213–221.
[17] Moore, G.A., McKonkey, D.J., Kass, G.E.N., O'Brien, P.J. and Orrenius, S. (1990) FEBS Lett. 274, 214–216.
[18] Kass, G.E.N., Duddy, S.K., Moore, G.A. and Orrenius, O. (1989) J. Biol. Chem. 264, 15192–15198.
[19] Oldershaw, K.A. and Taylor, C.W. (1990) FEBS Lett. 274, 214–216.
[20] Greenberg, A. and Zinder, O. (1982) Cell Tiss. Res. 226, 655–665.
[21] Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. (1988) FEBS Lett. 238, 151–155.
[22] O'Sullivan, A.J. and Burgoyne, R.D. (1988) Biochim. Biophys. Acta 969, 211–216.
[23] O'Sullivan, A.J. and Burgoyne, R.D. (1989) Bioscience Reports 9, 243–252.
[24] Berridge, M.J. (1991) Cell Calcium 12, 63–72.
[25] Foskett, K.J., Roifman, C.M. and Wong, D. (1991) J. Biol. Chem. 266, 2778–2782.