Refilling of endothelial calcium stores without bypassing the cytosol

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The present study was undertaken to define the route of Ca^{2+} used for refilling of intracellular Ca^{2+} stores in endothelial cells. Ca^{2+} stores, after emptying with bradykinin in Ca^{2+} free solution and termination of the stimulation with the bradykinin antagonist, Hoe 140, were allowed to refill by addition of Ca^{2+}. Refilling was prevented by 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (BuBHQ), an inhibitor of microsomal Ca^{2+} sequestration. BuBHQ induced large increases in the cytosolic Ca^{2+} concentration during the refilling phase. This finding is not compatible with a model proposing Ca^{2+} uptake into the stores directly from the extracellular space but provides evidence for uptake from the cytosolic compartment.

Endothelial cell; Intracellular Ca^{2+} store; Calcium uptake inhibition; 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone

1. INTRODUCTION

Sustained elevations in the cytosolic free calcium concentration in non-excitable cells require the presence of extracellular Ca^{2+} but the mechanisms by which $Ca^{2+}{}_e$ enters the cytosol are not clear. It has been proposed that $Ca^{2+}{}_e$ is used for continuous refilling of intracellular Ca^{2+} stores via a route bypassing the cytosol and raises $[Ca^{2+}]_c$ indirectly, after discharge from the stores by second messengers [1]. Tentative evidence for this view has been presented in parotid acinar cells [2] and appears possible in endothelial cells [3,4]. In the latter studies, Mn^{2+} was used as a probe for Ca^{2+} entry. Although the cellular handling of Mn^{2+} was similar to that of Ca^{2+}, it was not identical [4]. Thus the experimental limitations prohibited using the data to resolve the route by which Ca^{2+} enters the cell.

In the present study, we followed a different approach. We applied BuBHQ, an inhibitor of microsomal Ca^{2+} sequestration [5], to endothelial cells and measured the cytosolic Ca^{2+} concentration during the refilling phase of intracellular Ca^{2+} stores, after the end of a stimulation with the Ca^{2+} mobilizing agonist bradykinin. If Ca^{2+} uptake occurred directly from the extracellular space, no effect of BuBHQ on the Ca^{2+} concentration within the cytosolic compartment would be expected. However, we observed large increases in $[Ca^{2+}]_c$, indicating that refilling of endothelial Ca^{2+} stores is accomplished by uptake of Ca^{2+} from the cytosol.

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Abbreviations: $[Ca^{2+}]_c$, cytosolic free calcium concentration; $[Ca^{2+}]_e$, extracellular Ca^{2+}; BuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate

2. EXPERIMENTAL

Endothelial cells from porcine aorta were kept in culture and grown on quartz coverslips. They were loaded with the fluorescent $[Ca^{2+}]_c$ indicator indo-1 by incubation (37°C, 90 min) with the pentaacetoxymethylester of indo-1 (1 μM) in the presence of the detergent pluronic F-127 (0.025% w/v). Fluorescence was measured at 35°C in a fluorometer (Schoeffel RRS 1000). The signals were digitized and stored in a computer at a rate of 4/s. $[Ca^{2+}]_c$ was calculated from the ratio of the fluorescence intensities emitted at 400 nm and 450 nm (excitation 350 nm) [6]. Further details of the method have been recently described [7,8].

3. RESULTS AND DISCUSSION

Effects of BuBHQ on the cytosolic free calcium concentration in endothelial cells are shown in Fig. 1. It evoked increases in $[Ca^{2+}]_c$ preferentially in cells prestimulated with receptor-dependent agonists such as bradykinin but also in resting cells and even in the absence of extracellular Ca^{2+}. It did not accelerate the quenching of cytosolic indo-1 by entry of extracellular Mn^{2+} by which transmembrane influx of Ca^{2+} can be assessed [9]. These results indicate that BuBHQ elevates $[Ca^{2+}]_c$ as the consequence of a redistribution of Ca^{2+} from internal stores to the cytosol. Therefore we designed a protocol (Fig. 2) to test the hypothesis that BuBHQ specifically interferes with the uptake of Ca^{2+} into internal stores in endothelial cells and that BuBHQ may be used to define the route by which Ca^{2+} reaches these stores.

Internal Ca^{2+} stores were emptied by bradykinin in the absence of $Ca^{2+}{}_e$. Stimulation with bradykinin was terminated by addition of Hoe 140, a competitive antagonist of bradykinin B_2 receptors that inhibits the effects of bradykinin (10 nM) on $[Ca^{2+}]_c$ and EDRF for-

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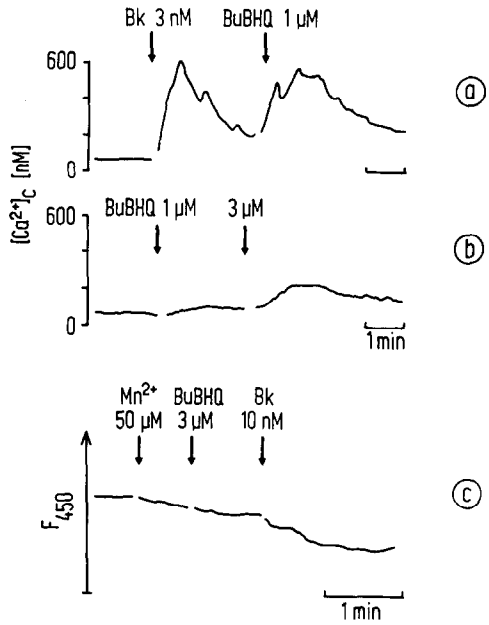

Fig. 1. Effects of BuBHQ on cytosolic free calcium in endothelial cells. (a and b) Effects on $[Ca^{2+}]_c$ in cells prestimulated with bradykinin (Bk) and in resting cells (c) Effects on the quenching of fluorescence (at 450 nm, the isosbestic wavelength for Ca^{2+}) of intracellular indo-1 by extracellular Mn^{2+} (50 μM), an indirect measure of Ca^{2+} entry. The experiments were performed in the presence of 1 mM extracellular Ca^{2+}. Drugs were added to the cells at the time indicated by the arrows.

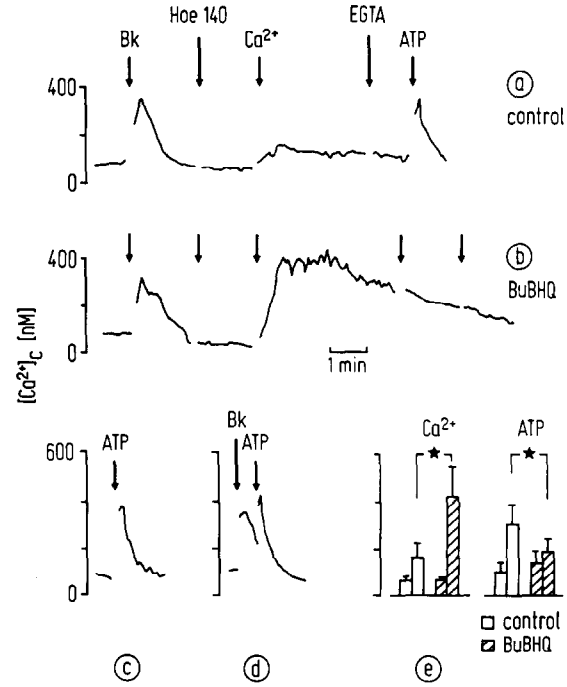

Fig. 2. Effects of BuBHQ on refilling of endothelial Ca^{2+} stores (a) Control. The protocol involved three steps. (i) Emptying of the internal Ca^{2+} stores by stimulation with bradykinin (Bk, 10 nM, 90 s) in Ca^{2+} free solution. Bk stimulation was terminated by application of the competitive Bk-antagonist, Hoe 140 (1 μM). (ii) Refilling of Ca^{2+} stores by addition of Ca^{2+} (1 mM). (iii) Assessment of the fullness of the stores by application of ATP (10 μM) after chelation of $Ca^{2+}{}_e$ with EGTA (1.5 mM). (b) Same protocol as in (a) but in the presence of BuBHQ (3 μM, preincubation 90 s). (c and d) Effects of ATP (10 μM) on $[Ca^{2+}]_c$ in control cells and in cells prestimulated (40 s) with bradykinin (10 nM), in Ca^{2+} free medium. (e) Summary of experiments performed with the protocol of (a) and (b) (n = 12 for each protocol). Left: $[Ca^{2+}]_c$ before and maximal $[Ca^{2+}]_c$ (± SD) during the refilling period (addition of Ca^{2+}), in the absence (control, open colums) and presence (hatched colums) and presence (hatched columns) of BuBHQ. Right: $[Ca^{2+}]_c$ before and peak $[Ca^{2+}]_c$ during stimulation with ATP, after refilling of Ca^{2+} stores had been allowed. The asterisks denote significant ($P<0.01$, Wilcoxon matched-pair signed rank test) differences.

mation, with an IC_{50} of 0.9 nM (unpublished results from this laboratory). Thereafter, Ca^{2+} stores were reloaded by addition of $Ca^{2+}{}_e$. Finally, after chelation of $Ca^{2+}{}_e$ with EGTA, the fullness of the stores was assessed by application of ATP that, like bradykinin [10], stimulates phospholipase C and the production of inositol phosphates [11].

In the absence of BuBHQ (control, Fig. 2a), there was only a small increase in $[Ca^{2+}]_c$ during the refilling phase and refilling appeared complete because the response to ATP was not different from that in other cell preparations not previously exposed to bradykinin (Fig. 2c). After preincubation with BuBHQ (Fig. 2b), restitution of $Ca^{2+}{}_e$ after emptying the stores induced large elevations of $[Ca^{2+}]_c$ but the effects of ATP were almost completely abolished. A summary of these experiments is presented in Fig. 2e. The level of $[Ca^{2+}]_c$ before ATP stimulation was higher in some BuBHQ-treated than in control cells, yet Fig. 2d shows that ATP induces increases in $[Ca^{2+}]_c$ from such a level, provided the internal Ca^{2+} stores have not been emptied.

These experiments demonstrate that BuBHQ prevents the refilling of Ca^{2+} stores, most likely located within the endoplasmic reticulum. The marked accumulation of Ca^{2+} within the cytosol during inhibition of re-uptake provides evidence for a model explaining refilling of Ca^{2+} stores by transmembrane influx of Ca^{2+} into the cytosol and simultaneous uptake from this cellular compartment. In contrast, the results argue strongly against the hypothesis that Ca^{2+} stores are refilled via direct communication between the extracellular space and the endoplasmic reticulum.

For this interpretation, it is crucial to exclude an $InsP_3$-like action of BuBHQ, i.e. enhancement of Ca^{2+} efflux from internal stores. Fig. 3a shows that after exposure to BuBHQ for 8 min in Ca^{2+} free solution, the endothelial $[Ca^{2+}]_c$-response to ATP was well preserved. In further experiments with the same protocol, ATP induced increases in $[Ca^{2+}]_c$ from 64 ± 19 (SD) nM to 360 ± 30 nM in the absence of BuBHQ (n = 7) and to 340 ± 45 nM in BuBHQ-treated cell preparations (n = 7).

Therefore, mobilization of Ca^{2+} does not account for the effects of BuBHQ. The small increase in $[Ca^{2+}]_c$ in response to BuBHQ may be explained by an interrup-

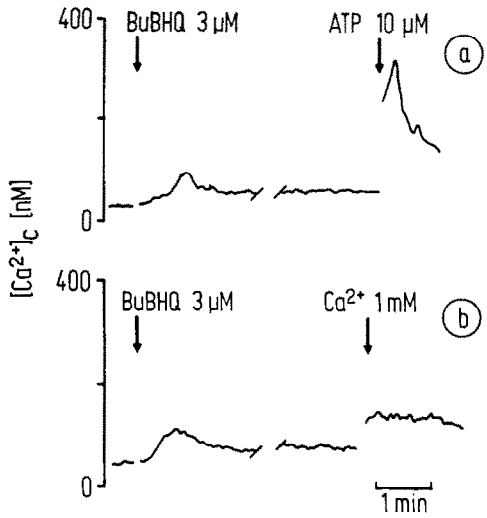

Fig. 3. Effects of BuBHQ on mobilization of Ca^{2+} from intracellular stores. Endothelial cells preincubated with BuBHQ for 8 min in Ca^{2+} free solution were either (a) stimulated with ATP (10 μM) or (b) exposed to Ca^{2+} (1 mM).

tion of the continuous cycling of Ca^{2+} between the stores and the cytosol. The rate of this cycling appears small in comparison to the Ca^{2+} movements in the presence of bradykinin.

Furthermore, Fig. 3b shows that addition of $Ca^{2+}{}_e$ during incubation with BuBHQ alone induced far smaller elevations in $[Ca^{2+}]_c$ (by 130±23 nM) than observed after preincubation with BuBHQ and a transient stimulation with bradykinin (increase in $[Ca^{2+}]_c$ by 264±110 nM; Fig. 2). Hence, effects of BuBHQ cannot be attributed to inhibition of transmembrane Ca^{2+} extrusion. Moreover, the findings confirm previous reports that Ca^{2+} influx is determined by the status of internal Ca^{2+} stores [3,12–14] and does not necessarily require the presence of agonists. The question arises how the endoplasmic reticulum controls Ca^{2+} influx, particularly under experimental conditions where levels of second messengers, formed after occupation of membrane receptors, are presumably not elevated. The simplest explanation [15], that the $[Ca^{2+}]$ inside the Ca^{2+} stores controls a direct Ca^{2+} pathway into the store is in clear contradiction with the present study. Plausible is a recently proposed model [16]: in response to $[Ca^{2+}]$ within the endoplasmic reticulum, an endoplasmic protein (probably the $InsP_3$ receptor) would bind to, or dissociate from, a membrane Ca^{2+} pore or carrier (additionally being the $InsP_4$ receptor), thereby inhibiting, or allowing, Ca^{2+} entry.

Previously, the route of Ca^{2+} during refilling of stores has been analyzed either by introduction of Ca^{2+} chelators into the cytosol [17] or by quenching of cytosolic fluorochromes with Mn^{2+} [3,4,17]. One may argue that Ca^{2+} chelators are a Ca^{2+} sink capable of enhancing Ca^{2+} efflux from the endoplasmic reticulum, whereas the cellular handling of Mn^{2+} appears similar but not identical to that of Ca^{2+} [4]. BuBHQ, on the other hand, allows the demonstration of the central importance of Ca^{2+} uptake from the cytosol for refilling of Ca^{2+} stores in endothelial cells, which may be a general model for other non-excitable cell types.

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REFERENCES

[1] Putney, J.W. (1986) Cell Calcium 7, 1–12.
[2] Merritt, J.E. and Rink, T.J. (1987) J. Biol. Chem. 262, 17362–17369.
[3] Hallam, T.J. Jacob, R. and Merritt, J.E. (1989) Biochem. J. 259, 125–129.
[4] Jacob, R. (1990) J. Physiol. 421, 55–77.
[5] Moore, G.A., McConkey, D.J., Kass, G.E.N., O'Brien, P.J. and Orrenius, S. (1987) FEBS Lett. 224, 331–336.
[6] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[7] Lückhoff, A. and Busse, R. (1990) Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 94–99.
[8] Lückhoff, A. and Busse, R. (1990) Pflügers Arch. 416, 305–311.
[9] Hallam, T.J., Jacob, R. and Merrit, J.E. (1988) Biochem. J. 255,179–184.
[10] Derian, C.K. and Moskowitz, M.A. (1986) J. Biol. Chem. 261, 3831–3837.
[11] Pirotton, S., Raspe, E., Demolle, D., Erneux, C. and Boeynaems, J.M. (1987) J. Biol. Chem. 262, 17461–17466.
[12] Kass, G.E.N., Duddy, S.K., Moore, G.A. and Orrenius, S. (1989) J. Biol. Chem. 264, 15192–15198.
[13] Sage, S.O., Reast, R. and Rink, T.J. (1990) Biochem. J. 265, 675–680.
[14] Staudermann, K.A. and Pruss, R.M. (1989) J. Biol. Chem. 264, 18349–18355.
[15] Rink, T.J. and Hallam, T.J. (1989) Cell Calcium 10, 385–395.
[16] Irvine, R.F. (1990) FEBS Lett. 263, 5–9.
[17] Muallem, S., Khademazad, M. and Sachs, G. (1990) J. Biol. Chem. 265, 2011–2016.