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Mobilization of internal Ca^{2+} by vasoactive intestinal polypeptide in endothelial cells

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Abstract

The aims of the present study were to establish whether vasoactive intestinal polypeptide (VIP) could mobilize internally-stored Ca^{2+} and whether Ca^{2+} release could trigger Ca^{2+} influx from the extracellular space. Bovine pulmonary artery endothelial cells from an established cell line were loaded with fura-2/AM and cells were studied in suspension or were imaged in monolayers at 40–80% confluency. In Ca^{2+} imaging studies, VIP evoked Ca^{2+} transients in Ca^{2+}-free medium containing 50 μM EGTA. This was observed in 33 out of 122 cells examined on 29 separate trials. With each cell, the spread of Ca^{2+} appeared to occur from the periphery of the cell to the central core. Cells which did not respond to VIP responded to other stimulants such as bradykinin, endoplasmic reticulum Ca^{2+} pump inhibitors, (cyclopiazonic acid and thapsigargin), and endoplasmic reticulum Ca^{2+} release channel opener, ryanodine. The reintroduction of Ca^{2+} following VIP-induced Ca^{2+} release did not evoke a Ca^{2+} response in 5 cells imaged. Cells in suspension showed typical biphasic responses to bradykinin, thapsigargin or cyclopiazonic acid in the presence of external Ca^{2+}. Stimulation with VIP caused transient Ca^{2+} responses in Ca^{2+}-free physiological saline containing 50 μM EGTA. However, only 1 out of 4 cells tested showed a response to Ca^{2+} when it was reintroduced to the bathing medium. This study provided direct evidence for the first time in these bovine endothelial cells for VIP-mediated elevation of cytosolic concentration of Ca^{2+}. The results also suggested that other mechanisms might prevail preventing capacitative Ca^{2+} entry following the release of internally-stored Ca^{2+}. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+}, internal; VIP (vasoactive intestinal polypeptide); Endothelial cells; Ca^{2+} imaging; Fura-2

1. Introduction

Numerous studies have suggested that accumulation of cAMP mediates the responses to vasoactive intestinal polypeptide (VIP) in various areas of the central nervous systems (Deschodt-Lanckman et al., 1977; Quik et al., 1978; Rostène, 1984; Cholewinski and Wilkin, 1988). Relaxation of vascular smooth muscles by VIP has also been attributed to the accumulation of cAMP (Hirata et al., 1985; Nabika et al., 1985; Sata et al., 1988). So far, only two reports have provided evidence for the release of internally-stored Ca^{2+}, in astrocytes of rat cerebral cortex (Fatatis et al., 1994) and in intestinal smooth muscle (Murthy et al., 1993), suggesting that VIP might perhaps act via a G-protein coupled receptor to release Ca^{2+} and activate constitutive nitric oxide synthase.

In cultured endothelial cells from bovine pulmonary artery of an established cell line, a high density of ^{125}I-VIP binding sites was found with a B_{max} of 534 fmol mg^{-1} protein and a K_D of 1.8 nM (Pasyk et al., 1992). VIP competed for binding against ^{125}I-VIP at high and low affinity binding sites present in a ratio of 1:3.7 (21% and 77% of occupied receptors) with corresponding K_Ds of 12 pM and 4.7 nM, respectively. VIP (10–100 nM) in these cells also inhibited the activation of the inwardly rectifying K^+ currents (I_{KIR}), the most prominent currents manifested by these cells. Subsequent studies showed that the inhibition of I_{KIR} by VIP in isolated membrane patches was not related to cAMP accumulation but to a membrane-bound, cholera toxin-sensitive G-protein (Pasyk et al., 1996). Based on these results, and on an unpublished finding that Ca^{2+} elevation also inhibited these currents, we formed a hypothesis that VIP releases Ca^{2+} from endoplasmic reticulum closely associated with the plasma membrane. It should also be noted that local Ca^{2+} elevation can also activate Ca^{2+} activated K^+ channels to keep membrane polarised sufficient to drive Ca^{2+} entry through other voltage insensitive channels (Inazu et al., 1995).

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2. Materials and methods

2.1. Cell cultures

Bovine pulmonary artery endothelial cells were obtained from ATCC (USA) and cultured in MEM (minimum essential medium, GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (GIBCO), 0.1% gentamicin and 0.1% fungizone in a humid atmosphere of 95% air/5% CO_2 at 36°C. These cells were routinely used as a positive control for endothelial-derived nitric oxide synthase and results confirmed their identity as a true population of endothelial cells.

For the experiments, the cells were exposed to 0.5% trypsin to dislodge cells. Cells were then centrifuged at low speed for 5 min and supernatant aspirated. Cells were resuspended in HEPES-buffered saline containing 0.2% bovine serum albumin and, in mM, 126NaCl, 6KCl, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 glucose, 1.5$CaCl_2$ and 0.3$MgCl_2$ with pH adjusted to 7.4 with NaOH. Cells were allowed to recover from the trypsin treatment for 2–3 h prior to loading with the fluorescence dye in cell suspension studies. In single cell studies, the cells were plated on coverslips. Cells were about 40–80% confluent at the time of use. For Ca^{2+}-free HEPES buffer, Ca^{2+} was replaced with 50 μM EGTA. Cells were bathed in Ca^{2+}-free HEPES prior to the commencement of experiments.

2.2. Measurement of cytosolic Ca^{2+}

These studies were routinely conducted in EGTA-containing (50 μM) solution. All containers related to Ca^{2+} studies used were plastic. We had noted in our pilot studies that storage of buffers in glassware had significantly higher contaminating Ca^{2+}. Therefore, our double distilled deionised water from the Nanopure was stored in plastic containers. Control experiments performed comparing nominally Ca^{2+}-free and EGTA-containing solution showed identical responses to bradykinin and CPA, i.e. transient responses. The responses were biphasic when 1 mM Ca^{2+} was present. Also, the addition of 50 μM EGTA in the absence of an agonist did not give noticeable change in ratio between 340 and 380 nm.

2.2.1. Cell suspension studies

Using a previously described method (Low et al., 1996), fura-2/AM was added to 10 ml of cell suspension to a final concentration of 2–6 μM in a tube protected from light. The tube was incubated in a shaking bath at 37°C for 30 min. A similar volume and concentration of cells was removed prior to the addition of fura-2/AM to obtain autofluorescence values. The cells were then spun (1000 rpm × 5 min) down twice, the supernatant aspirated, and the cells resuspended in HEPES buffer. Prior to using the cells for an experiment, the cells were spun down again to remove leaked dye and resuspended in fresh Ca^{2+}-free HEPES saline. Using a Tracor Northern Fluoroplex III (TN-6500), fura-2 fluorescence was recorded from 3 ml aliquot of magnetically-stirred cell suspension in HEPES-buffered saline in a quartz curvette. The curvette was placed in a chamber in the light path of the Xenon lamp (150 W) and photons were detected with a photon multiplier tube (TN-6075) connected to the opposite end of the lamp. The cells were exposed alternately to 340 and 380 nm. Emission was monitored at 510 nm. The experiments were conducted at 22°C in the dark. Due to the limitations of the Fluoroplex, data were collected discontinuously.

2.2.2. Single cell studies

Additional experiments were performed using a Ca^{2+} imaging system (Image-1/FL, Universal Imaging Corporation) with a Zeiss lamp (HBO 100 W/DC) coupled to a Zeiss inverted microscope (Zeiss IM 35) with a 100 × oil immersion lens and a numerical aperture of 1.25, as previously described (Low et al., 1997). Filter wheel held filters at 340 and 380 nm which alternated and images captured on the first and second quadrant of the television monitor. The ratio between these wavelengths was depicted on the third quadrant. The fourth quadrant of the screen graphed the ratio to changes with time and events of selected regions of the cells. Emitted fluorescence was detected with a 540 nm filter. Images were integrated and collected by a Pulnix camera (TM-720), with complete images collected every 3 s. Background values were obtained by defocussing. Images were analysed by assessing the fluorescence intensity of the cells for the course of the experiment using the Image-1/FL software. Ca^{2+} changes in the centre and periphery of the cells can be monitored by selecting windows at the appropriate regions of the cells.

Prior to each set of experiments, the system was calibrated for maximum in the presence of external Ca^{2+} and thapsigargin (1 μM) and for minimum in the presence of EGTA (5 mM). This was done by indicating which areas are high Ca^{2+} and which are low Ca^{2+} regions. The relationship between the ratios and calculated $[Ca^{2+}]_i$ was defined by a sigmoidal curve and came as an integrated part of the Image-1/FL software package. This procedure is useful to set the range of Ca^{2+} changes we were expecting to see. However, all data are expressed as ratio since fura-2 method has several intrinsic problems in the estimation of absolute cytosolic Ca^{2+} concentration (Shin et al., 1992).

2.3. Drugs

Fura-2 free acid and fura-2/AM were purchased from Molecular Probes Inc. (Eugene, OR). The acid form was dissolved in double distilled deionised H_2O while the AM form was dissolved in dry dimethyl sulphoxide (DMSO). Cyclopiazonic acid (CPA, Sigma), SK and F 96365 (SmithKline Beecham, Frythe, Welwyn, UK) and thapsi-

gargin (Sigma) were dissolved in DMSO. VIP (Sigma) and bradykinin (Sigma) were dissolved in double distilled deionised H_2O. VIP was made up to a stock of 10 μM and frozen at −70°C until used while bradykinin was prepared as a 1 mM stock solution and frozen at −20°C until used. Ryanodine (Research Biochemicals, Nattick, MA) was dissolved in 100% ethanol.

2.4. Statistical analysis

Data are expressed as the mean ± S.E. mean. Differences were analysed for statistical significance using a two-tailed Mann–Whitney tests. One-way analysis of variance was carried out where appropriate. The minimal P value accepted for statistical significance was 0.05.

3. Results

Agents which were capable of modulating cytosolic Ca^{2+} concentration used in this study were: CPA and thapsigargin, endoplasmic reticulum Ca^{2+} pump inhibitors, which are thought to elevate cytosolic Ca^{2+} by preventing Ca^{2+} uptake by the Ca^{2+} store; bradykinin, which acts on specific membrane receptors to elevate cytosolic IP_3 which in turn causes Ca^{2+} release from the endoplasmic reticulum; VIP, which was hypothesised in this study to act like bradykinin; ryanodine, which binds with high specificity to the calcium release channel present on the endoplasmic reticulum to cause the Ca^{2+} channel on the endoplasmic reticulum to open, leaking Ca^{2+} into the cytosolic compartment and SK and F 96365, which is a

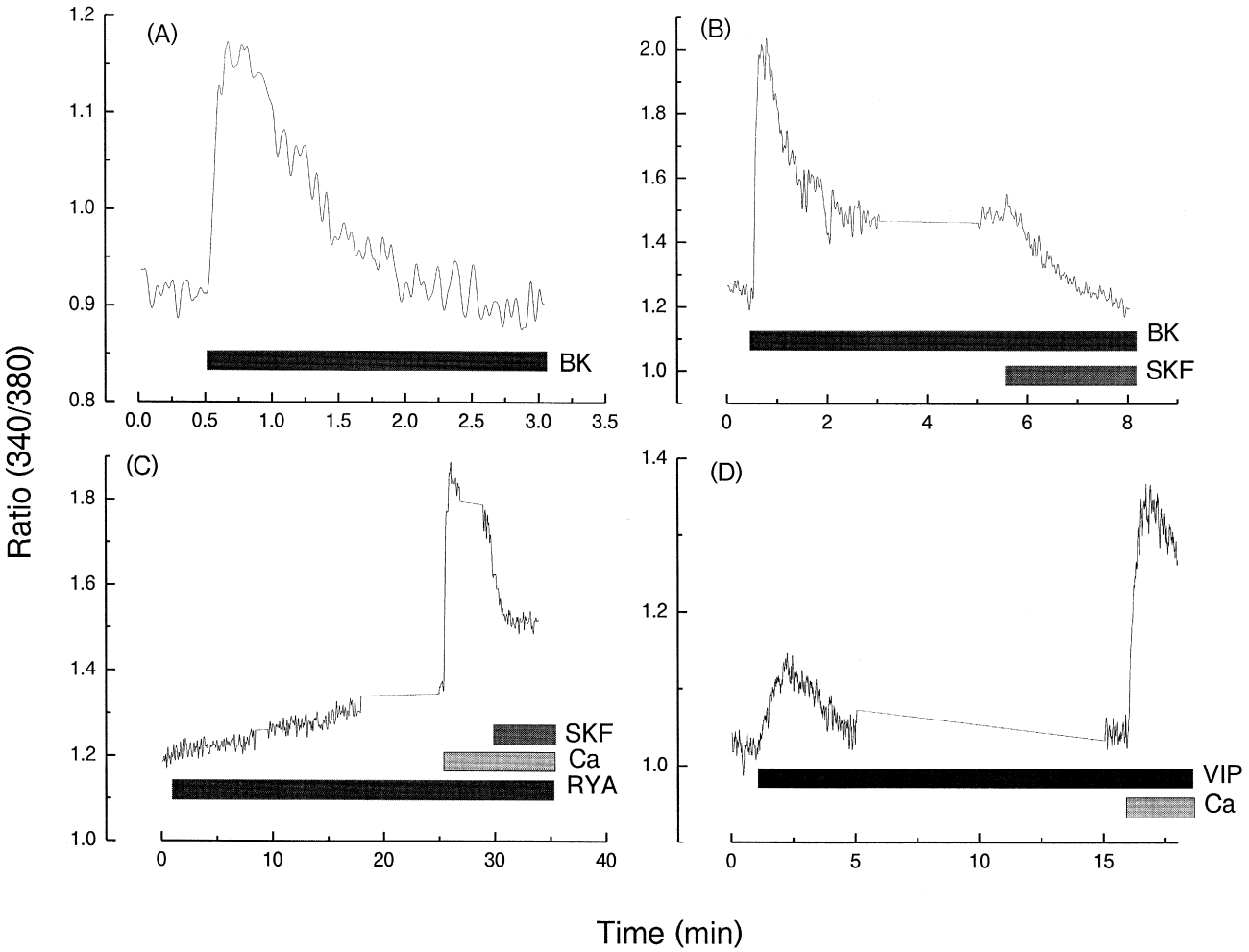
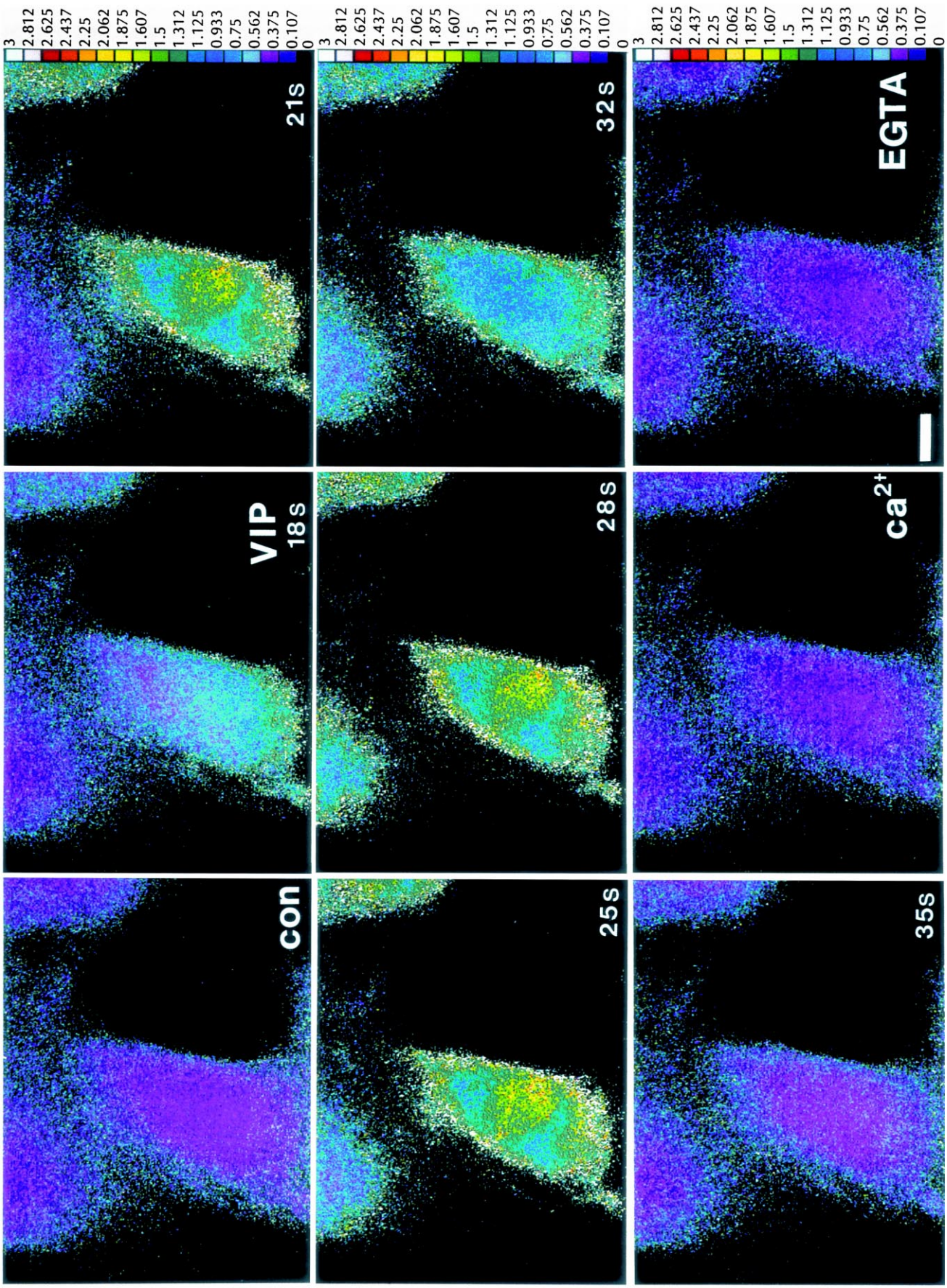


Fig. 1. The results from experiments performed on cell populations are shown here. (A) In Ca^{2+}-free medium, bradykinin (BK, 10 μM) produced a Ca^{2+} transient. The intracellular release of Ca^{2+} was rapid, peaking immediately following addition. Ca^{2+} levels were gradually reduced, returning to the baseline. Responses are expressed as the ratio of excitation wavelengths 340 and 380 nm versus time (min). (B) In the presence of extracellular Ca^{2+} (2.5 mM), bradykinin (BK, 10 μM) produced a biphasic response. Internal Ca^{2+} release occurred immediately following the addition of bradykinin and the Ca^{2+} response remained sustained after peaking at 1 min. Subsequent application of SK and F 96365 (SKF, 50 μM) reduced the Ca^{2+} response to the basal level. Responses from two successive collections were separated by 2 min (dotted straight line). (C) Ryanodine (RYA, 33 μM) in a Ca^{2+}-free medium steadily increased cytosolic concentration of Ca^{2+}, progressing for the first 18 min. Addition of Ca^{2+} (Ca, 2.5 mM) to the medium increased the Ca^{2+} signal which was reversed by SK and F 96365 (SKF, 50 μM). Responses from four successive collections were separated by 2, 6 and 2 min (dotted straight lines). (D) The addition of VIP (1 μM) to a suspension of cells in Ca^{2+}-free medium caused a rapid transient elevation of the Ca^{2+} signal. Subsequent addition of Ca^{2+} (Ca, 2.5 mM) caused a marked increase in cytosolic Ca^{2+} but only in one out of four cells tested. Discontinuous data collection over 10 min was represented by a dotted straight line.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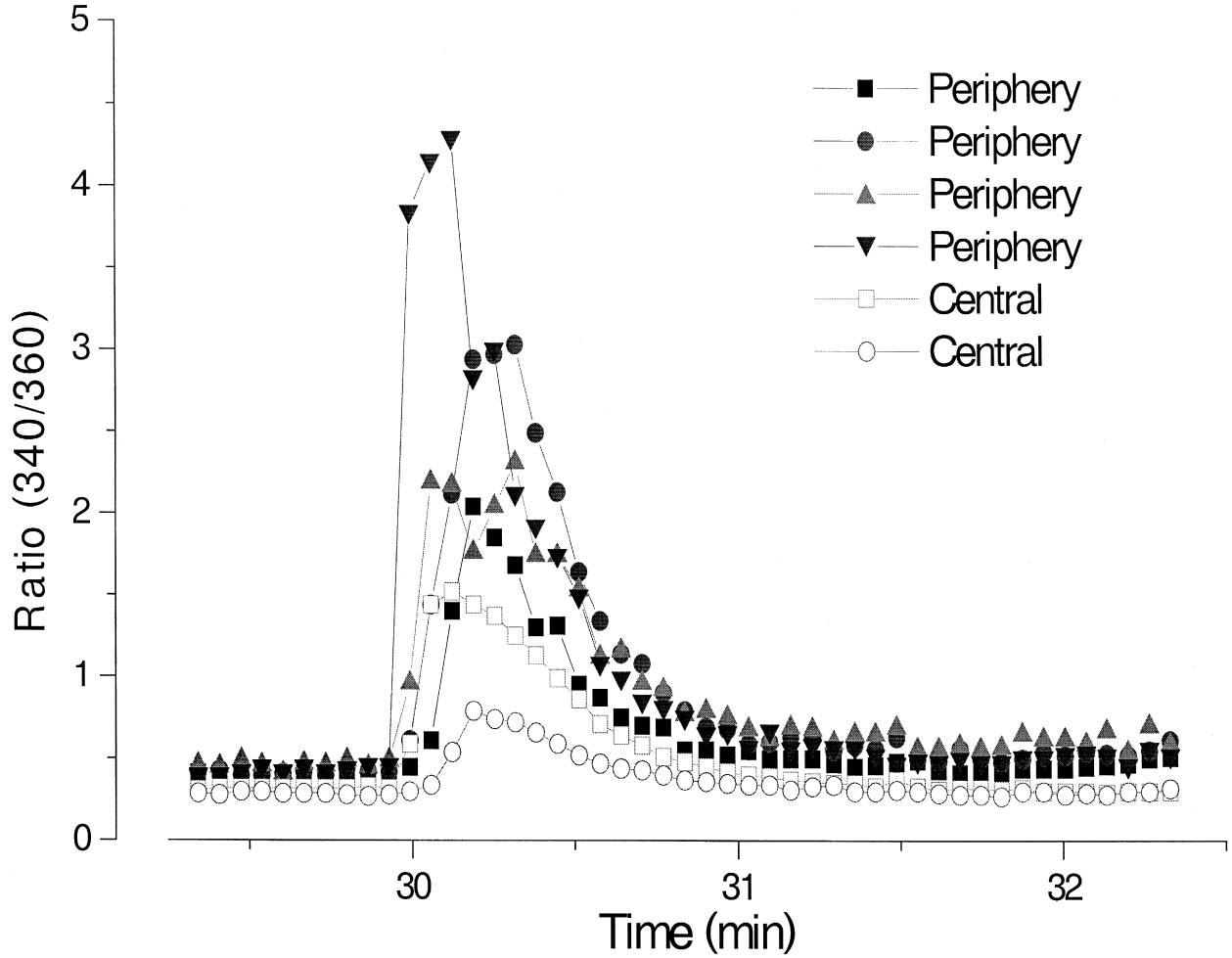

Fig. 3. Regional changes within the endothelial cells shown in Fig. 2. Central and peripheral regions were marked and responses were graphed as shown. The regions differed in their peak responses, with regions along the edge of the cell responding more rapidly.

blocker of Ca^{2+} channels on the plasma membrane, the route by which Ca^{2+} from the extracellular space can gain entry into the cytosol to affect cytosolic Ca^{2+} concentration.

3.1. Fura-2 cell suspension experiments

In fura-2-loaded endothelial cells, Ca^{2+} transients were seen when bradykinin (10 μM, $n = 6$, Fig. 1A) and CPA (10 μM, $n = 6$) when applied in the absence of extracellular Ca^{2+} while ryanodine (10 μM, $n = 4$, Fig. 1C) caused a slow increase in the concentration of cytosolic Ca^{2+}. The kinetics of Ca^{2+} release was bradykinin > CPA $\ggg$ ryanodine. When extracellular Ca^{2+} was restored, a large increase in the Ca^{2+} signal was observed suggesting Ca^{2+} entry into cytoplasm. SK and F 96365 (50 μM) lowered the plateau of Ca^{2+} entry induced by bradykinin ($n = 6$, Fig. 1B), CPA ($n = 5$) and ryanodine ($n = 2$, Fig. 1C) in Ca^{2+}-containing medium.

VIP (1 μM) caused Ca^{2+} transients in Ca^{2+}-free medium in 4 out of 17 aliquots of cells associated with a marked reversal of the 340 and 380 nm signal monitored using the dual wavelength Fluoroplex. Fig. 1D shows the transient Ca^{2+} signal evoked when VIP was added to the

Fig. 2. Ca^{2+} imaging studies on endothelial cells in culture at about 40% confluency. This micrograph shows one central cell and two other cells located on either side. Bathed in Ca^{2+}-free physiological saline solution, cells loaded with fura-2/AM showed greater Ca^{2+} levels in the periphery at rest. When stimulated with VIP (1 μM), high Ca^{2+} level regions started after 18 s of addition. The change in intracellular Ca^{2+} spread throughout the cell peaking around the 25 s and Ca^{2+} distribution decreased to basal levels. Restoring Ca^{2+} did not evoke a response. The image was taken after about 15 s following the addition of Ca^{2+}. Images (not shown) over the next 27 s also did not show any change in cytosolic Ca^{2+} in response to Ca^{2+} addition in the presence of VIP. EGTA (5 mM) caused no additional changes. Note that not all the cells responded to VIP all at the same time. While the central cell had reached a peak response, the top left cell was just about to respond to VIP. Calibration bar represents 10 μm. Purple to wide pseudocolor scale represents low to high concentration of cytosolic Ca^{2+}. Corresponding numbers represent ratio values.

aliquot of these cells. Restoring Ca^{2+} did not consistently produce a response in the presence of VIP. In fact, only one aliquot of cells (1 out of 4) showed a Ca^{2+} signal when Ca^{2+} was restored into the bathing medium. In contrast to bradykinin which caused a Ca^{2+} transient each time the cells were stimulated, a Ca^{2+} transient occurred in one of four VIP applications. In those experiments which VIP did not evoke a response, CPA or thapsigargin was used to verify that cells were indeed viable and were capable of Ca^{2+} release.

3.2. Fura-2 single cell studies

In single cell studies, 1 μM VIP evoked Ca^{2+} transients in 33 out of 122 cells examined on 29 separate trials. Those cells which did not respond to VIP could produce Ca^{2+} transients in response to a variety of other stimulants such as bradykinin, ryanodine, CPA or thapsigargin. These results showed that the lack of VIP response could not be attributed to poor dye loading or inadequate uptake of Ca^{2+} into the stores. There was a gradient of high to low Ca^{2+} from periphery to central regions of the cells, respectively, as seen in Fig. 2. Sequential cytosolic Ca^{2+} changes due to VIP (1 μM) were observed beginning from the periphery and spreading to the central regions. Bradykinin and CPA also showed similar patterns of Ca^{2+} changes within the cell interior loaded with fura 2/AM.

Fig. 3 shows peripheral and central regions of all the cells in Fig. 2. The region of the cells were marked to show four periphery regions from the 3 cells shown, and two central regions from the centre cell, to illustrate regional Ca^{2+} changes. The responses from the central regions of the cells were typically slower and less marked than those changes occurring in the peripheral regions of the cells. When the ratio of peripheral to central Ca^{2+} was measured in 9 cells, a ratio of greater than 1 ($P < 0.05$) and these ratios were maintained at peak VIP (2.07 ± 0.15) or EGTA (1.77 ± 0.12) responses compared with basal levels (1.98 ± 0.13).

Bradykinin, CPA, ryanodine or thapsigargin given immediately after VIP administration was also capable of evoking a Ca^{2+} transient suggesting an efficient recycling

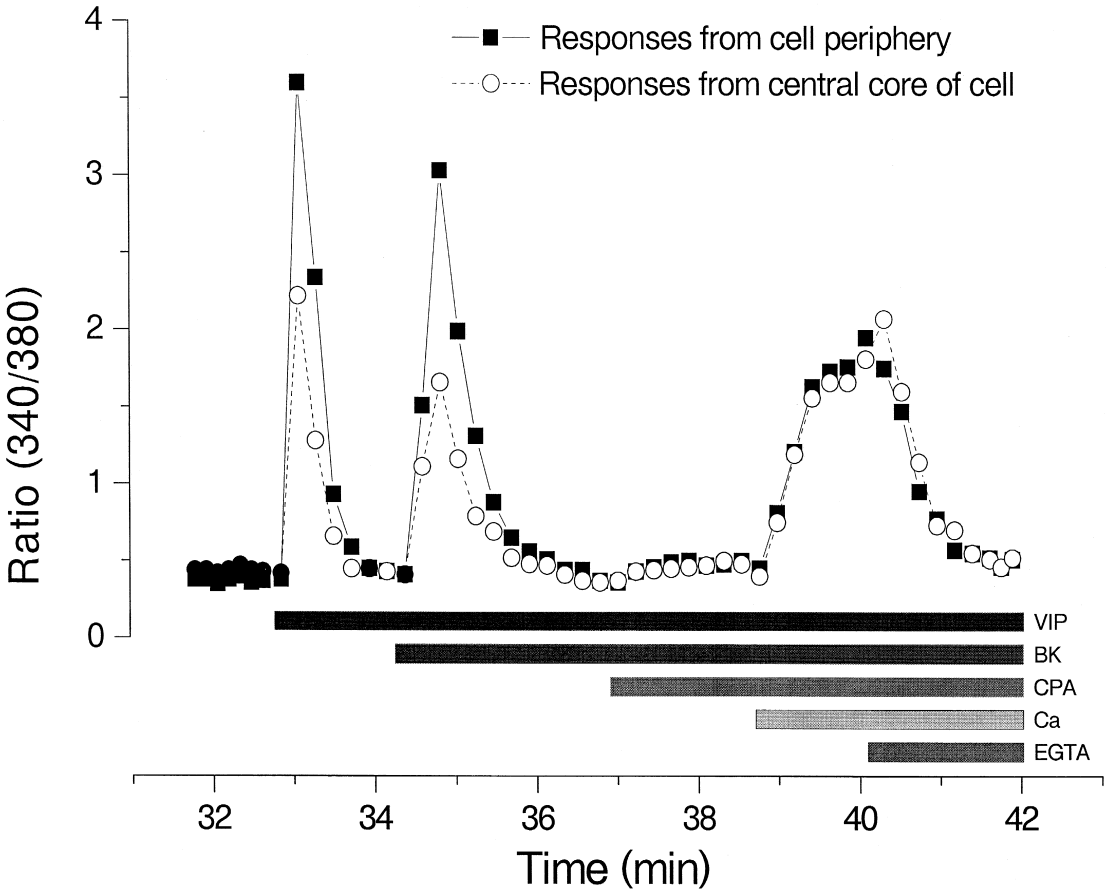

Fig. 4. Effect of stimulation of endothelial cells with VIP (1 μM) and bradykinin (BK, 1 μM) in succession in nominally Ca^{2+}-free HEPES. Periphery of the cell showed greater peaks than the central regions of the cell. CPA (10 μM) responses were abolished by the prior stimulations with VIP or bradykinin. Restoring Ca^{2+} (1 mM) caused an increase in the Ca^{2+} signal which was reversed with EGTA (5 mM), a Ca^{2+} chelator. Little difference Ca^{2+} levels between the periphery of the cell and central core was observed when external medium contained Ca^{2+}.

of cytosolic Ca^{2+} or the existence of two Ca^{2+} stores, one coupled to VIP receptors and the other uncoupled. Another possibility is that VIP could not completely empty the internal Ca^{2+} store (Fig. 4). Responses from the peripheral regions of the cells showed larger Ca^{2+} transient than the central regions, whereas little difference was seen when Ca^{2+} was re-admitted into the medium in the presence of the receptor agonists. In the presence of VIP alone, the endothelial cells did not respond to the reintroduction of external Ca^{2+} (before Ca^{2+} addition, 0.53 ± 0.05 (periphery) and 0.34 ± 0.02 (central), versus, after Ca^{2+} addition, 0.52 ± 0.07 (periphery) and 0.31 ± 0.02 (central), $n = 5$, not significantly different).

Fig. 5 shows changes associated with windows selected from the central and peripheral regions of a cell. The cell was first stimulated with VIP (1 μM), then with CPA (30 μM). Although VIP did not cause an increase in cytosolic concentration of Ca^{2+}, CPA did so each time it was applied.

4. Discussion

This study provided direct evidence in endothelial cells of bovine pulmonary artery origin for VIP-mediated elevation of cytosolic concentration of Ca^{2+}. Ca^{2+} transients associated with application of VIP in Ca^{2+}-free bath medium showed that it is capable of mobilizing internally stored Ca^{2+}. The spread of Ca^{2+} from the periphery to the center of the cell appears to be consistent with the hypothesis that Ca^{2+} stores are closely associated with the plasma membrane.

Studies using fluorescent dyes such as fura-2, which directly monitor the concentration of Ca^{2+} in the cytoplasm, confirmed that agents, such as CPA, ryanodine and bradykinin, that interfere with mobilization of Ca^{2+} from the intracellular Ca^{2+} store, promoted Ca^{2+} entry, although the time course and pattern of Ca^{2+} release differed among these agents. In Ca^{2+}-free medium, receptor agonists, such as bradykinin, which release Ca^{2+} from the

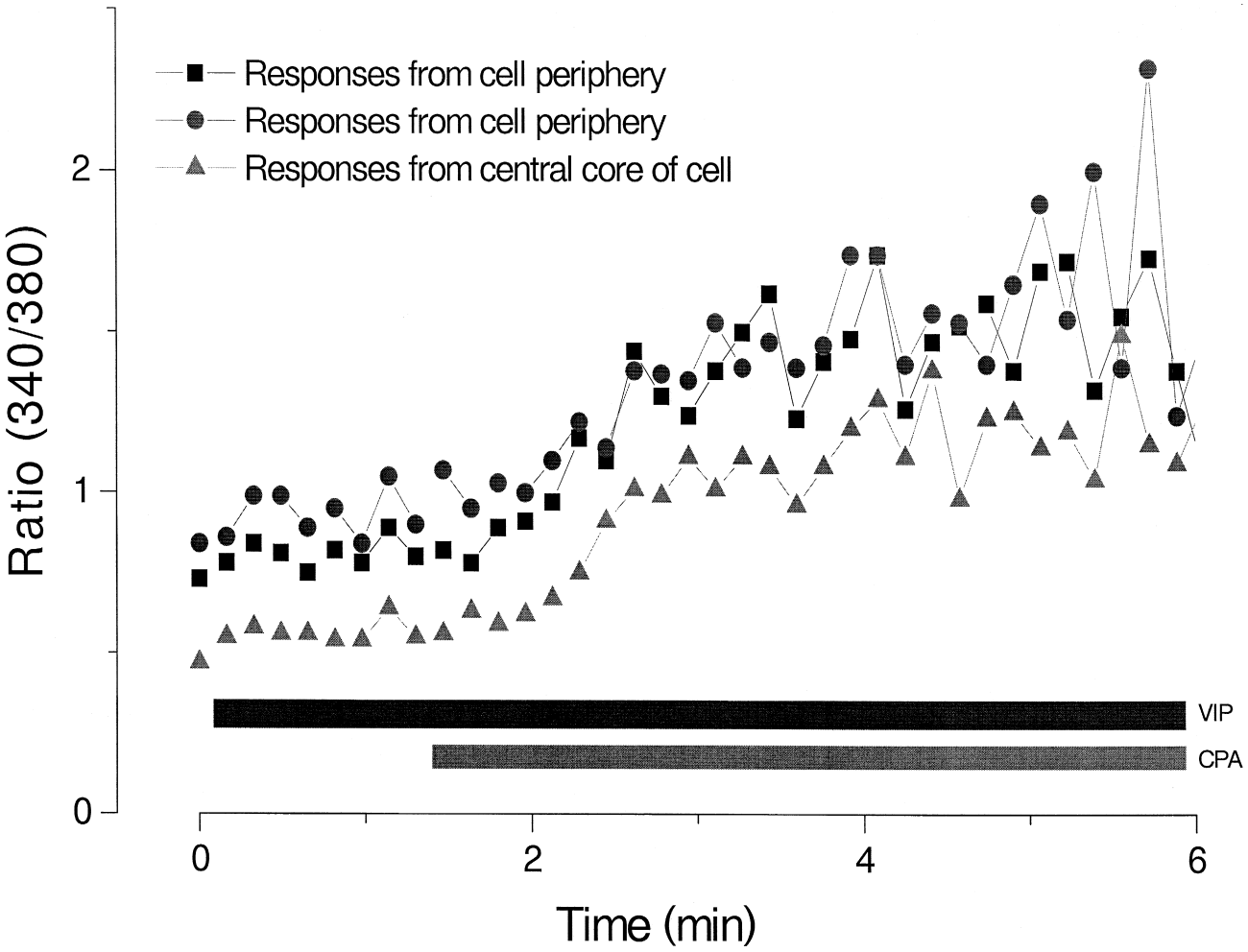

Fig. 5. This shows a typical example of the 75% of the cells which did not response to VIP (1 μM) but responded to CPA (10 μM) in nominally-Ca^{2+}-free HEPES buffer. Ca^{2+} levels as deduced by the ratio values were higher around the periphery of the cell compared to the central regions of the cell.

endoplasmic reticulum via elevation of IP_3 level caused Ca^{2+} transients that peaked rapidly (within 30 s). CPA, on the other hand, affected Ca^{2+} levels more slowly (> 30 s) whereas ryanodine caused small and very slow elevation of intracellular Ca^{2+} (minutes). Functional ryanodine- or caffeine-sensitive Ca^{2+} stores have also been reported in endothelial cells from the rat aorta, human aorta and human umbilical vein (Ziegelstein et al., 1994; Corda et al., 1995; Jino et al., 1992). VIP elicited a response that was similar in time course to bradykinin, suggesting a rapid coupling of surface membrane receptors and the intracellular signalling system with the release of internally-stored Ca^{2+}, which can activate numerous physiological responses such as activation of nitric oxide synthase and the release of vasoactive compounds.

Restoring Ca^{2+} in the presence of agonists (other then VIP), and in the presence of endoplasmic reticulum Ca^{2+} pump inhibitors (CPA and thapsigargin), caused a rapid increase in intracellular Ca^{2+} concentration, with little difference in the rate of rise between the different modes of Ca^{2+} release. Addition of external Ca^{2+} in the presence of VIP did not always produce cytosolic Ca^{2+} elevation, suggesting that Ca^{2+} entry did not usually follow the initial Ca^{2+} release. When Ca^{2+} entry occurred following Ca^{2+} release by bradykinin or ryanodine, it was reversed by SK and F 96365, a nonselective cation channel blocker (Merritt et al., 1990; Inazu et al., 1995; Low et al., 1996). It is also interesting to note that emptying of the internal Ca^{2+} stores by VIP did not trigger capacitative Ca^{2+} entry as predicted by Putney's model of Ca^{2+} entry (Putney, 1986). It is possible that VIP does not sufficiently empty the Ca^{2+} stores to induce Ca^{2+} entrance for refilling. This is consistent with the observations that subsequent stimulation by bradykinin caused a second transient.

We had previously reported that in the endothelial cells, VIP did not enhance cAMP accumulation, even in the presence of a phosphodiesterase inhibitor, whereas experiments conducted in parallel using isoproterenol raised cAMP significantly above basal levels (Pasyk et al., 1996). In this study, we have gathered evidence for VIP ability to release internally-stored Ca^{2+}, consistent with the results from rat astrocytes where IP_3 levels were increased above control values after treating the astrocytes with VIP (Fatatis et al., 1994). In addition, it was also reported that exposure to the cAMP analog, 8-bromo-cAMP, did not cause elevation of cytosolic concentration of Ca^{2+} (Fatatis et al., 1994).

It is unclear why VIP only had an effect in 1 of 4 endothelial cells examined in single cell studies or in cell suspension studies. A similar proportion of cells responding to VIP was also reported by Fatatis et al. (1994) in type I astrocytes from rat cerebral cortex, who attributed the small percentage to cellular heterogeneity. In the human umbilical vein, it was reported that VIP-immunoreactive cells comprise only 12% of a total of over 5,000 cells examined (Cai et al., 1993). Our in-progress studies with angiotensin II also show that only a subpopulation of cells responds to angiotensin II (A.M. Low and L. Sormaz, unpublished observations), whereas, all cells respond to CPA or 4-chloro-ethyl phenol, another compound which releases Ca^{2+} from the endoplasmic reticulum (Low et al., 1997). Uncoupling of surface receptors from intracellular signal transduction pathways in cultured cells could contribute to the low percentage of responding cells especially when we have previously obtained high VIP binding sites in these cells (Pasyk et al., 1992), or, perhaps, different subtypes of VIP receptors which exhibit high and low affinity for the peptide are present and these may use different signalling systems.

In summary, our studies provided direct evidence for the first time for the Ca^{2+}-mobilizing capability of VIP on endothelial cells, a compound which has been thought to mediate its physiological response via cAMP accumulation. The observation that Ca^{2+} elevation in single cells by VIP and bradykinin in the periphery was greater than that in the central regions of the cells suggests proximity of the Ca^{2+} stores to the plasma membrane and that there stores were activated first.

Acknowledgements

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