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Stewart O. Sage
University of Miami

David J. Adams
University of Miami, djadams@uow.edu.au

Cornelis Van Breemen
University of Miami

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Abstract

Bradykinin-evoked rises in [Ca2+](i) were measured in fura-2-loaded bovine pulmonary artery endothelial cell monolayers by dual wavelength excitation fluorimetry. In monolayers seeded thinly and grown to confluence, bradykinin, in the presence of external Ca2+, evoked a rise in [Ca2+](i) composed of an initial peak and subsequent oscillating plateau. In the absence of external Ca2+, bradykinin evoked a rise in [Ca2+](i) which then returned to the basal value without oscillating. In monolayers seeded near confluent density, the bradykinin-evoked peak in [Ca2+](i) was followed by a steady plateau which showed no oscillation. The addition of the phorbol ester, phorbol 12,13-dibutyrate, to a monolayer during bradykinin-evoked oscillations abolished the oscillations and lowered [Ca2+](i) partway back toward the basal level. The addition of the protein kinase C inhibitor, H7, did not abolish oscillatory activity, although the frequency of oscillation was reduced. These results indicate that synchronized oscillatory activity can occur in endothelial cell monolayers. It is suggested that these oscillations are dependent on intercellular coupling developed when the cells are grown to confluence and that the mechanism responsible for generating oscillations in [Ca2+](i) requires extracellular Ca2+ and involves protein kinase C.

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Synchronized Oscillations in Cytoplasmic Free Calcium Concentration in Confluent Bradykinin-stimulated Bovine Pulmonary Artery Endothelial Cell Monolayers*

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Stewart O. Sage‡, David J. Adams, and Cornelis van Breemen

From the Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

Bradykinin-evoked rises in $[Ca^{2+}]_i$ were measured in fura-2-loaded bovine pulmonary artery endothelial cell monolayers by dual wavelength excitation fluorimetry. In monolayers seeded thinly and grown to confluence, bradykinin, in the presence of external Ca^{2+}, evoked a rise in $[Ca^{2+}]_i$ composed of an initial peak and subsequent oscillating plateau. In the absence of external Ca^{2+}, bradykinin evoked a rise in $[Ca^{2+}]_i$ which then returned to the basal value without oscillating. In monolayers seeded near confluent density, the bradykinin-evoked peak in $[Ca^{2+}]_i$ was followed by a steady plateau which showed no oscillation. The addition of the phorbol ester, phorbol 12,13-dibutyrate, to a monolayer during bradykinin-evoked oscillations abolished the oscillations and lowered $[Ca^{2+}]_i$ partway back toward the basal level. The addition of the protein kinase C inhibitor, H7, did not abolish oscillatory activity, although the frequency of oscillation was reduced. These results indicate that synchronized oscillatory activity can occur in endothelial cell monolayers. It is suggested that these oscillations are dependent on intercellular coupling developed when the cells are grown to confluence and that the mechanism responsible for generating oscillations in $[Ca^{2+}]_i$ requires extracellular Ca^{2+} and involves protein kinase C.

Vascular endothelial cells modulate the activity of adjacent smooth muscle cells and of blood platelets in response to a number of stimuli by the release of the vasoactive mediators PGI_2[1] (1), EDRF (2), which is believed to be nitric oxide (3, 4), and endothelin (5). A variety of endothelium-dependent vasoactive agents, including histamine, bradykinin, thrombin, ATP, and platelet-activating factor, has been shown to elevate $[Ca^{2+}]_i$ in endothelial cells loaded with fluorescent Ca^{2+} indicator dyes (6–10). The elevation in $[Ca^{2+}]_i$ appears to be the major, if not exclusive, mediator of thrombin- and ATP-evoked PGI_2 production (8)[2] and is also believed to mediate, at least in part, EDRF release (12–14).

Agonist-evoked rises in $[Ca^{2+}]_i$ in endothelial cell monolayers or suspensions have been reported to consist of an initial peak followed by a sustained plateau. The removal of extracellular Ca^{2+}, or the blockade of Ca^{2+} influx using extracellular Ni^{2+}, leaves the initial peak unaffected or a little reduced in magnitude while the plateau is abolished under these conditions (6–9, 15).[2] These observations indicate that the initial peak is largely due to the release of Ca^{2+} from intracellular stores while the subsequent plateau is maintained by Ca^{2+} influx from the extracellular medium. Recently, histamine has been reported to evoke asynchronous repetitive spikes in $[Ca^{2+}]_i$ in single fura-2-loaded human umbilical vein endothelial cells (16). These spikes merged to give an elevated plateau in $[Ca^{2+}]_i$ at high agonist concentrations. Agonist-evoked oscillations in $[Ca^{2+}]_i$ have also been reported in single rat hepatocytes (17, 18) and parotid acinar cells (19).

Here we report for the first time synchronized oscillatory activity about a plateau in $[Ca^{2+}]_i$ evoked by bradykinin in monolayers of fura-2-loaded cultured bovine pulmonary artery endothelial cells grown to confluence on quartz coverslips. These responses are compared with those evoked in monolayers seeded near confluent density. The possible role of protein kinase C in generating oscillations in $[Ca^{2+}]_i$ was investigated.

MATERIALS AND METHODS

Fura-2/AM and H7 were from Calbiochem. Bradykinin, ATP-Na_2, PDBu, EGTA, and digitonin were from Sigma.

Endothelial cells used in this study were from a continuous cell culture line obtained from calf pulmonary artery without proteolytic digestion and cultured in Ryan Red medium as described previously (20, 21). Eleventh or twelfth passage cells were harvested from flasks using a rubber policeman and seeded onto quartz coverslips at approximately one-seventh of the original (confluent) density. These cells grew to form a confluent monolayer after 3 days of incubation. In some cases, cells were seeded at near confluent density and used the following day.

Following incubation, coverslips were washed in a physiological salt solution (PSS) containing 135 mM NaCl, 5 mM KCl, 0.75 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4, at 37 °C with NaOH. Cells were then loaded with fura-2 by incubation at 37 °C for 30 min in PSS to which 1 μM Fura-2/AM had been added. Coverslips were then placed in fresh PSS and incubated at 37 °C for at least a further 15 min before use. This procedure yielded cells which were evenly loaded with fura-2 as assessed by epifluorescence microscopy (Zeiss × 100 Neofluor oil immersion objective fitted to a Nikon Diophot microscope, excitation wavelength 360 nm). Longer loading periods and higher ester concentrations were avoided as cytoplasmic localization of the dye, similar to that reported by Steinberg *et al.* (22), was found to occur.

Fura-2 fluorescence was measured in a dual-excitation wavelength Spex Fluorolog 1681 spectrometer (Spex Industries, Inc., Edison, NJ). Coverslips were placed against one wall of square polystyrene cuvettes containing PSS or a similar medium from which the $CaCl_2$

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‡ To whom correspondence should be addressed. Present address: The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge, CB2 3EG, United Kingdom.

[1] The abbreviations used are: PGI_2, prostaglandin I_2 (prostacyclin); EDRF, endothelium-derived relaxing factor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PDBu, phorbol 12,13-dibutyrate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $Ins(1,4,5)P_3$, inositol 1,4,5-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PSS, physiological salt solution.

[2] Carter, T. D., Hallam, T. J., Cusack, N. J., and Pearson, J. D. (1989) *Br. J. Pharmacol.*, in press.

had been omitted. Cuvettes were continuously stirred using a magnetic stir bar. Excitation was at 340 and 380 nm, and emission was collected in front-face mode at 505 nm. Fluorescence records were corrected by subtraction of autofluorescence measured prior to dye loading and then processed using the following relation.

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

K_d for the fura-2·Ca^{2+} complex was taken to be 224 nM (23). R is the experimentally determined 505-nm fluorescence ratio at the two excitation wavelengths. R_{max} is the maximal fluorescence ratio determined after addition of 50 μM digitonin to permeabilize the cells in the presence of 1 mM Ca^{2+}, and R_{min} is the minimal ratio determined after the subsequent addition of 30 mM EGTA and 30 mM Tris base. S_{f2}/S_{b2} is the ratio of fluorescence values at 380-nm excitation in Ca^{2+}-free and -replete media, respectively. The area of illumination was 0.3 × 10 mm. The confluent density of the cells, determined using a Coulter Counter (model ZF) after trypsin/EDTA dispersal, was approximately 4.0 × 10^5 cm^{-2}. Thus the fluorescence signal was collected from about 12,000 cells.

RESULTS AND DISCUSSION

Fig. 1 shows that in the presence of 1 mM external Ca^{2+}, 10 μM bradykinin elevated $[Ca^{2+}]_i$ from a basal value of 60 ± 3 nM to a peak of 335 ± 23 nM (S.E., n = 23). $[Ca^{2+}]_i$ then declined to a plateau above the basal level. In monolayers which had been seeded thinly and grown to confluence over several days, the bradykinin-evoked plateau showed oscillations in $[Ca^{2+}]_i$ (Fig. 1*a*). These oscillations were uniform excursions through approximately 100 nM in $[Ca^{2+}]_i$ with a period, in this example, of 78.5 ± 0.5 s (S.E., 8 oscillations). Oscillations continued for at least 10 min. Similar oscillations were evoked in 11 preparations by bradykinin at concentrations between 100 nM and 10 μM. The mean frequency of oscillation did not correlate significantly with agonist concentration (r = −0.35, 0.5 > p > 0.1) but was significantly correlated to a mean plateau $[Ca^{2+}]_i$ (r = −0.82, 0.01 > p > 0.001). The termination of stirring after agonist addition did not affect oscillatory activity, indicating that no movement artifacts were involved. In 12 monolayers which had been grown to confluence as described above, bradykinin-evoked oscillations in $[Ca^{2+}]_i$ did not persist but were damped over varying times, eventually reaching a steady plateau (Fig. 1*b*). In monolayers which were seeded near confluent density and used the following day, the bradykinin-evoked plateau in $[Ca^{2+}]_i$ showed no oscillation (Fig. 1*c*). Similar nonoscillatory responses were evoked in preconfluent monolayers (data not shown). The same type of response (undamped, damped, or nonoscillating) was observed in all monolayers from a given batch of coverslips which had been prepared at the same time.

Fig. 2 shows the response evoked by 10 μM bradykinin in the absence of external Ca^{2+}. When 1 mM Ca^{2+} was subsequently added, $[Ca^{2+}]_i$ rose to a value typical of the plateau of responses evoked in the presence of external Ca^{2+} and began to oscillate.

These results indicate that a field of cells is capable of generating synchronized oscillations in $[Ca^{2+}]_i$ in response to an agonist. These oscillations appear to be dependent on Ca^{2+} influx, since oscillatory activity was not observed in the absence of external Ca^{2+}. For oscillatory activity to be observed in a monolayer, there must be some form of intercellular communication to synchronize events in individual cells. It appears that this communication is established between cells of monolayers which have been seeded thinly and grown to confluence but is absent from cells which are used shortly after seeding near confluent density. Asynchronous oscillations at the single cell level, as reported to be evoked by histamine in human umbilical vein endothelial cells (16), would be expected to be obscured and appear as a constant plateau in $[Ca^{2+}]_i$ in a signal from a large number of cells. The requirement for establishment of intercellular communication in order to synchronize activity between cells may explain the failure of other authors to observe oscillations in $[Ca^{2+}]_i$ in endothelial monolayers in response to bradykinin

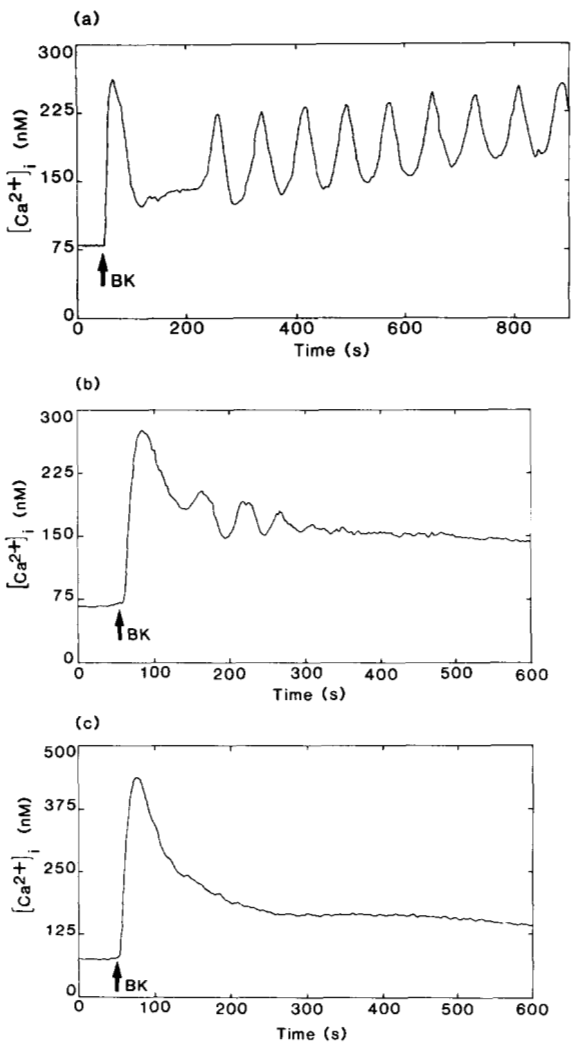

FIG. 1. **Responses evoked by bradykinin in fura-2-loaded pulmonary artery endothelial cell monolayers.** 10 μM bradykinin (*BK*) was added to monolayers (*a*, *b*) seeded thinly and grown to confluence over 3 days and (*c*) seeded near confluent density and used the following day. External $[Ca^{2+}]$ was 1 mM in each case.

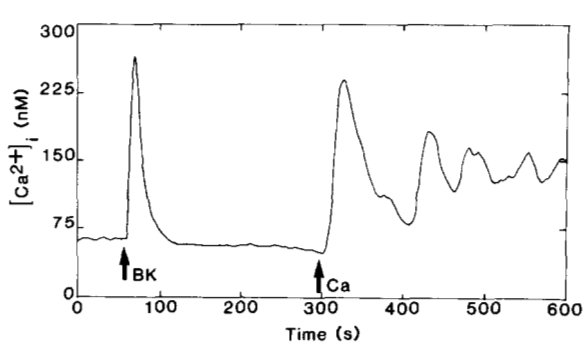

FIG. 2. **Bradykinin-evoked response in the absence of external Ca^{2+}.** 10 μM bradykinin (*BK*) was added to an endothelial cell monolayer which had been placed in medium without added Ca^{2+} approximately 1 min prior to the start of the experiment. 1 mM $CaCl_2$ was subsequently added as indicated.

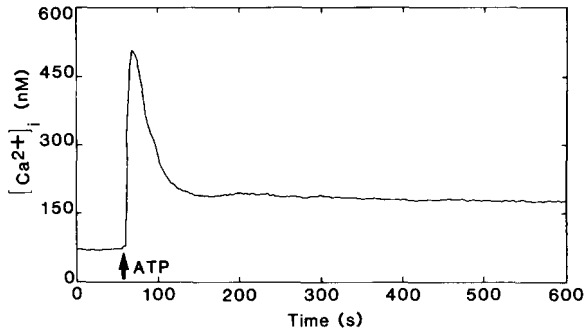

FIG. 3. **ATP-evoked rises in $[Ca^{2+}]_i$ do not oscillate.** 100 μM ATP was added to an endothelial cell monolayer which had been grown to confluence. External $[Ca^{2+}]$ was 1 mM.

(24), histamine (6, 15), and other agonists (8, 10).

At present it is not clear what form the intercellular communication synchronizing the oscillations takes, but this could be electrical or chemical. Electrical coupling between endothelial cells has been demonstrated (25, 26). It has been reported that high K^+ depolarization does not affect histamine-evoked spikes in $[Ca^{2+}]_i$ in single endothelial cells (16). We find that high K^+ solutions largely abolish bradykinin-evoked Ca^{2+} influx[3] on which oscillations appear to rely; thus we are unable to comment at present on the possible role of electrical coupling in monolayers.

It is not as yet clear why in some preparations bradykinin-evoked oscillations were continuous while in others they were damped, but the consistency between the type of response observed within sets of coverslips seeded at the same time suggests differing degrees of establishment of intercellular communication. Damped responses may arise from more than one group of daughter cells which are not in communication and in which responses are initially entrained but then go out of phase.

Fig. 3 shows the response evoked by 100 μM ATP in the presence of 1 mM external Ca^{2+} in a fura-2-loaded monolayer grown to confluence. $[Ca^{2+}]_i$ was elevated to a peak of 449 ± 28 nM and then declined to a plateau at 207 ± 25 nM (S.E., n = 10). Oscillations were not observed in response to ATP (56 preparations). This suggests that there may be differences between the signal transduction processes underlying the responses evoked by ATP and bradykinin. A similar proposal has been made on the basis of kinetic differences between responses evoked by ADP and other agonists in human platelets (27). In view of this, the nature of purinergic responses in endothelial cells merits further investigation.

To test for the possible involvement of protein kinase C in generating oscillations in $[Ca^{2+}]_i$, the effects of the phorbol ester, PDBu, which activates protein kinase C (28), and H7, which is reported to be a relatively specific inhibitor of protein kinase C (29), were investigated. Fig. 4*a* shows the effect of adding 200 nM PDBu to a preparation showing uniform oscillations evoked by 10 μM bradykinin in the presence of 1 mM external Ca^{2+}. PDBu immediately abolished the oscillatory activity and lowered $[Ca^{2+}]_i$ partway back toward the basal value. In oscillating and nonoscillating monolayers, PDBu, even at very high concentrations (2 μM), never reduced $[Ca^{2+}]_i$ completely back to the basal value from the plateau (data not shown). Preincubation of monolayers with 20 μM H7 for up to 10 min did not abolish bradykinin-evoked oscillations in $[Ca^{2+}]_i$ (data not shown). However, the addition of H7 to a monolayer oscillating in response to bradykinin did

[3] S. O. Sage, D. J. Adams, and C. van Breeman, unpublished observations.

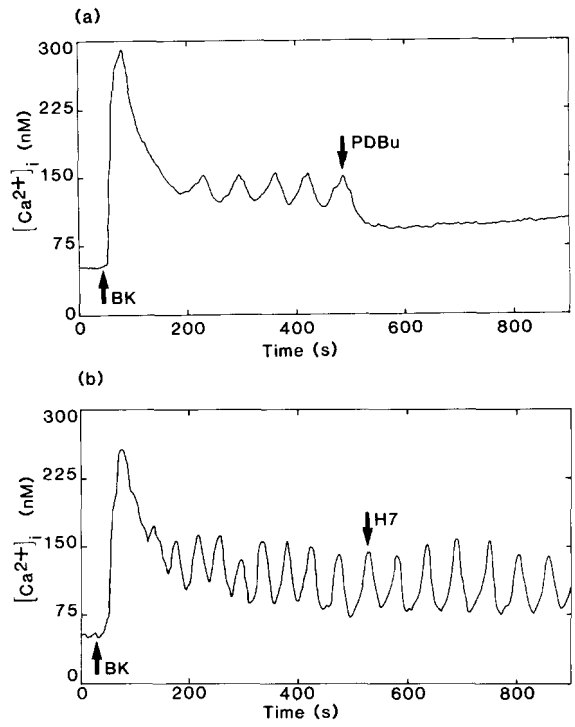

FIG. 4. **Effects of phorbol ester and protein kinase C blocker on bradykinin-evoked oscillations.** Oscillations in $[Ca^{2+}]_i$ were evoked by 10 μM bradykinin and then (*a*) 200 nM PDBu or (*b*) 20 μM H7 added as indicated. External $[Ca^{2+}]$ was 1 mM.

significantly increase the mean period of the oscillations. In the example shown, the period increased from 44 ± 2 s (S.E., nine oscillations) to 55 ± 2 s (S.E., six oscillations) (Student's t test, different means, $p < 0.001$). This result is typical of that observed in three preparations.

Possible models capable of generating oscillations in $[Ca^{2+}]_i$ have recently been reviewed by Jacob *et al.* (16). The basic process could involve oscillating levels of $Ins(1,4,5)P_3$, perhaps involving feedback by diacylglycerol via protein kinase C on receptors, G-proteins, or phospholipase C, as suggested by Woods *et al.* (18) in hepatocytes. Alternatively, Ca^{2+} feedback loops might generate oscillations in the presence of a constant level of $Ins(1,4,5)P_3$. Our results suggest a role for protein kinase C in generating oscillations since these were abolished by PDBu, which activates this enzyme (28). Following the application of PDBu, $[Ca^{2+}]_i$ fell to a new steady plateau but was not returned to the basal value as has been reported in platelets (30). Phorbol esters have been reported to inhibit agonist-evoked activation of phospholipase C in endothelial cells, so reducing the formation of $Ins(1,4,5,)P_3$ (31), which is believed to mediate discharge of the intracellular Ca^{2+} stores (32, 33). The inhibition of agonist-evoked EDRF and PGI_2 secretion by phorbol esters has also been demonstrated (34, 35). The finding that the protein kinase C blocker H7 slows bradykinin-evoked oscillations in $[Ca^{2+}]_i$ supports the idea that this enzyme is involved in the generation of oscillatory behavior, but an action of H7 at a site other than protein kinase C cannot at present be excluded.

The mechanism(s) by which agonists evoke Ca^{2+} influx in endothelial cells are still uncertain. Agonist-evoked currents, carried in part by Ca^{2+}, have been reported (26, 36, 37), and it has also been suggested that Ca^{2+} may enter via an indirect route dependent on discharge of the intracellular Ca^{2+} stores, rather than receptor occupation *per se* (15, 38). The failure of PDBu to completely inhibit the plateau phase of the brady-

kinin-evoked response suggests that some route of Ca^{2+} entry, presumably that conducted directly by plasma membrane channels, is insensitive to protein kinase C. Direct Ca^{2+} entry across the plasma membrane might contribute to the plateau in $[Ca^{2+}]_i$, while the oscillatory component of the signal could be derived from the intracellular stores. Oscillations would result if the stores discharged and then refilled due to cyclical feedback by protein kinase C on the generation of $Ins(1,4,5)P_3$. Cycles of protein kinase C activity could arise from negative feedback in the production of diacylglycerol. If such a dual route model for Ca^{2+} entry were correct, the prolonged activation of protein kinase C by PDBu would be expected to abolish oscillatory activity, as observed, while leaving some influx, and so a steady plateau, intact. Similarly, the partial inhibition of protein kinase C by H7 might be expected to produce the observed reduction in the frequency of oscillation by prolonging the time for sufficient protein kinase C activity to be produced to periodically terminate Ca^{2+} entry via the intracellular stores.

In summary, we have observed for the first time synchronized agonist-evoked oscillations in $[Ca^{2+}]_i$ in a monolayer of cells. The basis of these oscillations, the possible role of protein kinase C in their generation, and the relationship of these events to the oscillations reported in single cells (16–18) require further investigation. The possible physiological implications of synchronized oscillatory activity in endothelium, if translated into release of vasoactive mediators, could be of considerable importance in the control of vascular tone and peripheral resistance. A role for the endothelium in the generation of oscillations in active tension in hamster aorta has recently been proposed (11).

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