Synchronized repetitive spikes in cytoplasmic calcium in confluent monolayers of human umbilical vein endothelial cells

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Synchronized repetitive spikes in cytoplasmic free calcium concentration, $[Ca^{2+}]_i$, are evoked by histamine in confluent monolayers of human endothelial cells. The repetitive spikes, which are apparently dependent upon the establishment of cell coupling, are also induced by caffeine, indicating that they may be due to an oscillatory release of Ca^{2+} from the endoplasmic reticulum, and may not involve oscillations in inositol phosphates. It is suggested that sunchronized repetitive spikes in $[Ca^{2+}]_i$ might lead to oscillatory release of endothelial-derived substances such as prostacyclin, nitric oxide and endothelin, which have potent effects on the vascular system.

Cytoplasmic calcium; Ca^{2+} oscillation; Fura-2; Human endothelial cell

1. INTRODUCTION

Cytoplasmic free calcium concentration, $[Ca^{2+}]_i$, plays a critical role in endothelial cell function. A variety of humoral agents such as histamine, thrombin, bradykinin and ATP produce a rise in $[Ca^{2+}]_i$, which is then the trigger for release of prostacyclin [1], a potent vasodilator and inhibitor of platelet aggregation [2]. Endothelial cells also release other vasoactive substances such as nitric oxide [3] and endothelin [4], which modulate the activity of the underlying smooth muscle layers.

The regulation of $[Ca^{2+}]_i$ in human umbilical vein endothelial cells (HUVECS) has been studied by numerous groups. In cell populations, histamine has been shown to induce a rapid increase in inositol phosphates [5–8], which gives rise to an elevation of $[Ca^{2+}]_i$ [8–10]. The elevation of $[Ca^{2+}]_i$ has been reported to consist of two components; an early transient peak which is due to mobilization of Ca^{2+} from intracellular stores, and a sustained phase of elevated $[Ca^{2+}]_i$ which is due to stimulated Ca^{2+} entry [9–11]. In contrast, single cell studies have shown that low doses of histamine produce repetitive spikes in $[Ca^{2+}]_i$ which increase in frequency with increasing doses until they fuse to form an elevated plateau [12]. Oscillations in $[Ca^{2+}]_i$ in single cells have since been reported to occur in many other cell types [13,14]. However, since HUVECS form a monolayer configuration in the intact blood vessel it was important to determine whether spikes become synchronized, resulting in uniform fluctuations in Ca^{2+} over the whole monolayer. Digital imaging has indicated no clear evidence of synchronization in these cells, i.e. spikes occur independently of those in neighbouring cells [12,15]. However, oscillatory fluctuations in $[Ca^{2+}]_i$ have been observed in confluent monolayers of bovine pulmonary artery endothelial cells, which is suggestive of synchronization of the $[Ca^{2+}]_i$ response in thousands of cells [16]. In order to observe synchronization, the cells needed to be seeded thinly and grown to confluence, suggesting a requirement for cell communication to be established. We report here that synchronized repetitive spikes in $[Ca^{2+}]_i$ occur in response to histamine in HUVECS that are grown to confluency from an low initial seeding density. Furthermore, repetitive spikes in $[Ca^{2+}]_i$ appear to result from an oscillatory release of Ca^{2+} from the endoplasmic reticulum.

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2. MATERIALS AND METHODS

2.1. *Materials*

Human umbilical cords were obtained from the Rosie Maternity Hospital, Cambridge (UK). Thrombin (from human plasma), histamine dihydrochloride, caffeine, heparin (sodium salt), endothelial cell growth supplement, and collagenase (type II) were obtained from Sigma. Fura-2/AM was from Calbiochem.

2.2. *Cell culture*

Endothelial cells were isolated from segments of human umbilical-cord veins as previously described [8] with the following modifications. Endothelial cell growth supplement (20 μg/ml) was included in the growth medium and all tissue-culture plates and coverslips were gelatine-coated. Cells were passaged using trypsin/versene, seeded onto the coverslips at approximately 30% confluent density, and

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grown over a period of between 3–6 days to form a confluent monolayer. Only first passage cells were used for $[Ca^{2+}]_i$ measurements.

2.3. *Measurement of* $[Ca^{2+}]_i$

Coverslips containing fura-2-loaded monolayers of HUVECS were mounted into a coverslip-holding device which was inserted into a cuvette, maintained at 37°C and containing a physiological salt solution (composition in mM: NaCl 150, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1, Hepes 10, glucose 10, pH 7.4) and a magnetic stirrer bar. Fluorescence was monitored using a Perkin Elmer LS-5B fluorescence spectrophotometer, and $[Ca^{2+}]_i$ determined as described elsewhere [1]. The dimensions of the light beam were 0.6 × 1.0 cm, and thus, the fluorescence signal was collected from about 10 000 cells.

3. RESULTS

The effect of histamine on $[Ca^{2+}]_i$ was examined in confluent monolayers of HUVECS. When cells were seeded onto coverslips at confluent density and $[Ca^{2+}]_i$ measured the following day, histamine (100 μM) evoked a rise in $[Ca^{2+}]_i$ which consisted of an initial peak and a sustained plateau phase (Fig. 1A) as has been observed previously [9,10]. However, when cells were seeded at low density (i.e. approx. 30% of confluence) and grown over 3–6 days to form a confluent monolayer, histamine (100 μM) evoked repetitive spikes in $[Ca^{2+}]_i$ (Fig. 1B). Individual spikes consisted of a slow early rise, followed by a rapid increase, and then an equally rapid decrease in $[Ca^{2+}]_i$. Levels of $[Ca^{2+}]_i$ returned to basal after each spike. At the concentration of histamine used, there was no detectable period of non-activity between individual spikes. The amplitude of the spikes varied somewhat between batches of cells, between coverslips on the same batch, and between spikes on the one coverslip. Some spikes maintained their amplitude over a 30 min period whereas others dampened, with the amplitude becoming progressively smaller, and often were followed by periods of increased noise. Also, in some preparations, no spiking was observed and we do not know as yet what determines whether a batch of cells will spike or not. Although the spike amplitude varied, the frequency of spiking in response to 100 μM histamine remained remarkably constant and averaged 0.37 ± 0.02 min^{-1} ($n = 6$).

Thrombin (0.3 units/ml), another agonist which mobilizes Ca^{2+} in endothelial cells by stimulating inositide hydrolysis, did not produce $[Ca^{2+}]_i$ spikes whereas, on the same coverslip, repetitive spikes were produced by histamine (100 μM; Fig. 2). In addition, on 3 separate coverslips, a series of 3–4 small spontaneous oscillations in $[Ca^{2+}]_i$ which peaked at 50–100 nM above baseline, were observed.

Repetitive spikes in $[Ca^{2+}]_i$ were not seen when histamine was added in the absence of external Ca^{2+} suggesting a requirement for extracellular Ca^{2+}. To determine whether spikes involve Ca^{2+} release from internal stores, the effect of caffeine was examined. Addition of caffeine (6 mM) to some cell preparations produced repetitive spikes of $[Ca^{2+}]_i$ (Fig. 3A) which were

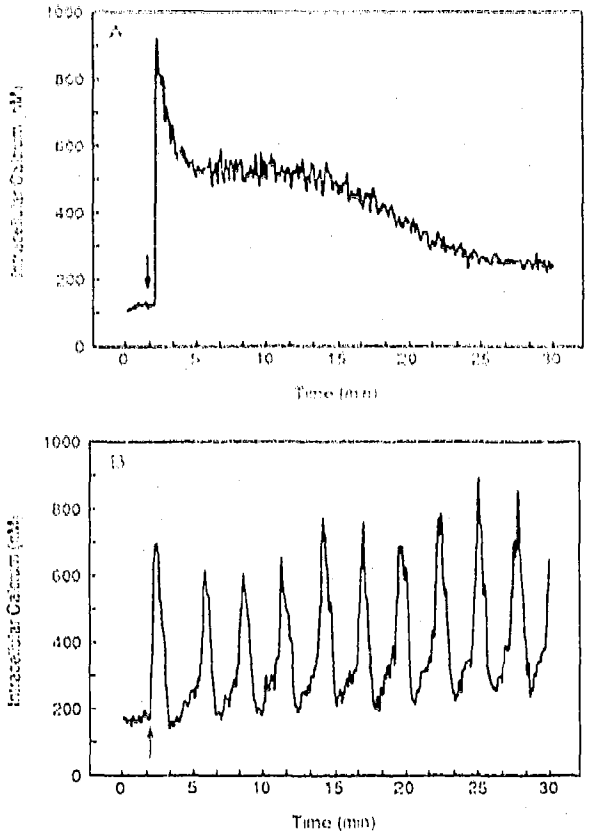

Fig. 1. Effect of histamine (100 μM) on $[Ca^{2+}]_i$ in confluent monolayers of human umbilical vein endothelial cells. The upper trace (A) shows the response in a monolayer in which cells were seeded at confluent density the previous day, and the lower trace (B) shows the response in cells seeded at low density and grown to confluence over 4 days. External Ca^{2+} was 1 mM.

similar in form to those produced by histamine. Furthermore, addition of caffeine to a cell preparation in which histamine-induced spikes had dampened to basal levels, re-established the repetitive spikes (Fig. 3B). These results lead us to suggest that spikes of $[Ca^{2+}]_i$

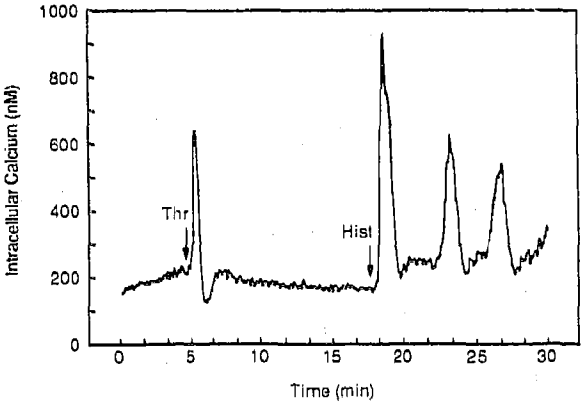

Fig. 2. Effect of thrombin (0.3 units/ml) and histamine (100 μM) on $[Ca^{2+}]_i$ in a confluent monolayer of endothelial cells.

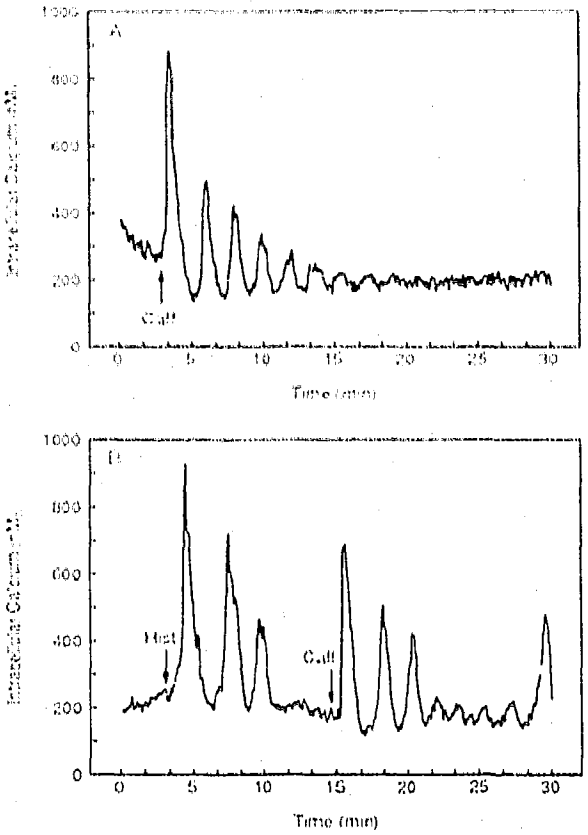

Fig. 3. Effects of caffeine (6 mM) on $[Ca^{2+}]_i$ in confluent monolayers of HUVECS. The upper trace (A) shows the ability of caffeine to induce repetitive spiking in $[Ca^{2+}]_i$. The lower trace (B) shows the ability of caffeine to establish spikes on a coverslip in which histamine (100 μM)-induced spikes had dampened to baseline.

result from an oscillatory release of Ca^{2+} from a caffeine-sensitive intracellular pool.

4. DISCUSSION

We have observed synchronized repetitive spikes in $[Ca^{2+}]_i$ in histamine-stimulated confluent monolayers of human endothelial cells. The spikes were observed only in cells seeded thinly and grown to confluence suggesting a requirement for the establishment of cell coupling. HUVECS have been shown to form efficient intercellular coupling, demonstrated by transfer of dye between cells in monolayers [17]. Thus, it is likely that in order to see synchronized activity, cells need to be grown under conditions which favour the formation of gap junctions [14]. We have found that inclusion of 'endothelial cell growth supplement' is clearly helpful in this regard. Also, allowing the cells to grow to confluence on the coverslip, and the use of a gelatine support on tissue culture surfaces, were found to be essential for the development of spiking behaviour.

Repetitive spikes were evoked by high concentrations of histamine, and closely resemble those reported to occur in individual HUVECS [12]. However, in single cells, the spikes increase in frequency with increasing levels of stimulation until they fuse to form an elevated plateau [12]. Imaging of $[Ca^{2+}]_i$ spikes in individual cells within a confluent monolayer has indicated no clear evidence of synchrony [12,15], although in one study, averaging of the Ca^{2+} response in eight neighbouring cells revealed a somewhat modest oscillatory pattern [15]. On the other hand, synchronized oscillations have recently been reported to occur in bovine pulmonary artery endothelial [16], rat aortic smooth muscle [18], and pancreatic acinar cell populations [19]. Since we have found that spiking behaviour occurs only in confluent cells, and is not seen in either pre- or post-confluent monolayers, synchronized spiking of individual cells within a monolayer might only be observed, in Ca^{2+}-imaging studies, during the short time period when the cells are at confluent density.

Dampening of spike amplitude could be caused by different groups of cells spiking increasingly out of phase. This is supported by the finding that there was often increased noise after the spikes had dampened than there was before histamine addition, as would be expected from an averaged response of various loci spiking asynchronously. Interestingly, addition of caffeine could establish repetitive spikes which had been induced initially by histamine but had dampened to baseline, possibly indicating that caffeine brought the different groups of cells back into phase.

The spikes could occur by propagation of the $[Ca^{2+}]_i$ rise both within cells, and from cell to cell. In single HUVECS, the rise in $[Ca^{2+}]_i$ seen during an oscillation, begins in a discrete region of the cell, and then propagates throughout the cytoplasm in the form of a 'Ca^{2+} wave' [15]. These waves might propagate from cell to cell via gap junctions as has been observed in trains of hepatocytes [20]. Cell to cell propagation of the $[Ca^{2+}]_i$ signal has also been demonstrated in cultured astrocytes [21], vascular smooth muscle cells [22], and tracheal epithelium [23], and appears to be an important mechanism used to coordinate cellular activity.

The finding that caffeine also induces repetitive spikes in $[Ca^{2+}]_i$ leads us to suggest that repetitive spikes result from an oscillatory release of stored Ca^{2+} since, at least in skeletal muscle, caffeine is thought to lower the threshold for Ca^{2+} release from the sarcoplasmic reticulum [24]. However, this Ca^{2+} store must have access, either directly or indirectly, to the extracellular medium since the spikes observed here, and those observed in bovine endothelium [16], appear to be dependent on the presence of extracellular Ca^{2+}. The results obtained with caffeine also indicate that spikes can occur in the absence of receptor-stimulated inositol phosphate generation [25[, although it is possible that the Ca^{2+} released by caffeine might activate phospholipase C to generate IP_3 as in the model propos-

ed by Cobbold [26]. Repetitive spikes in $[Ca^{2+}]_i$ occurring in a synchronized cell monolayer should allow for the first time examination of whether there are changes in the concentrations of intracellular messengers such as inositol phosphates during an individual spike, although, as discussed here, we do not yet know whether these synchronized oscillations are generated by the same mechanism as those seen in single cells as low agonist concentrations [26].

Repetitive spikes in HUVECS bear some similarities to the oscillations observed in bovine endothelial cells [16] in that they are dependent on extracellular Ca^{2+} and the frequency does not appear to be dose-dependent. However, in bovine endothelial cells, the oscillations occur over a sustained plateau, whereas in HUVECS, $[Ca^{2+}]_i$ returns to basal after each spike. The oscillations also appear more sinusoidal in the bovine cells, in contrast to the 'spikes' observed in the present study. The different patterns of oscillations observed between bovine and human endothelial cells might result from different mechanisms of Ca^{2+} entry present in the two cell types. HUVECS, in which we observe spikes of $[Ca^{2+}]_i$ arising from baseline, appear to possess only one mechanism of Ca^{2+} entry, i.e. that which is controlled by the concentration of Ca^{2+} in the intracellular store [10,11]. However, it has been suggested that bovine endothelial cells might contain two mechanisms of Ca^{2+} entry which are stimulated by bradykinin, i.e. one which supplies Ca^{2+} for synchronized oscillations, and the other, a PKC-independent mechanism, which accounts for the sustained plateau of elevated Ca^{2+} [16].

A number of important implications have arisen from this study. Firstly, $[Ca^{2+}]_i$ responses consisting of an initial peak and a sustained plateau phase in cell monolayer preparations which were not given time to form gap junctions between cells may give an erroneous picture of how the cells would normally behave in vivo where they would be expected to have efficient intercellular coupling. Secondly, it is apparent that $[Ca^{2+}]_i$ is not controlled independently in individual cells within a monolayer but is strongly regulated by neighbouring cells. Thus, the finding that Ca^{2+} responses are heterogeneous between cells may in itself be an artifact of studying individual cells in isolation. Alternatively, it becomes plausible that some cells might have unique $[Ca^{2+}]_i$ responses, which enable them to act as trigger cells to induce Ca^{2+} waves which propagage throughout the cell monolayer. The third possibility is that synchronized spiking of $[Ca^{2+}]_i$ may lead to oscillatory release of vasoactive substances such as prostacyclin, nitric oxide and endothelin.

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