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Spiking of intracellular calcium ion concentration in single cultured pig aortic endothelial cells stimulated with ATP or bradykinin

Thomas D. CARTER,*‡ Richard G. BOGLE† and Tone BJAALAND†

*Division of Neurophysiology and Neuropharmacology, N.I.M.R., The Ridgeway, Mill Hill, London NW7 1AA, and †Section of Vascular Biology, MRC Clinical Research Centre, Watford Road, Harrow HA1 3UJ, U.K.

Single pig aortic endothelial cells in culture loaded with the Ca^{2+}-sensitive fluorescent dye Indo-1 were stimulated with ATP (0.1–100 μM) or bradykinin (0.1–5.0 nM). Spiking or oscillations of $[Ca^{2+}]_i$ were seen in approx. 50% of cells stimulated with either agonist. Non-spiking or transient responses in which $[Ca^{2+}]_i$ returned to pre-stimulation levels rapidly (120–250 s), or sustained responses in which $[Ca^{2+}]_i$ remained elevated for many minutes, were seen in a further 20% of cells in each case, stimulated with either agonist. There was a marked variation between individual cells in the latency, magnitude, frequency and overall pattern of oscillations induced by ATP and bradykinin, although the patterns of response to bradykinin were less variable. In cells where repetitive spikes were seen, a relation between concentration of ATP and the latency of the response and the frequency of spiking was evident. Effects of removal of extracellular Ca^{2+}, elevation of extracellular K^+ concentration (35 or 70 mM) or exposure to phorbol 12,13-dibutyrate or 1,2-dioctanoyl-*sn*-glycerol were tested on the spiking Ca^{2+} responses. Each of these procedures reversibly slowed or prevented Ca^{2+} spiking evoked by ATP or bradykinin. In contrast, the inactive phorbol ester 4α-phorbol didecanoate had no effect on Ca^{2+} spiking evoked by these hormones. Our results thus indicate that the responses of single cells to ATP or bradykinin exhibit marked heterogeneity, and suggest that secretory events driven by extracellular Ca^{2+} may be regulated by repetitive spikes or oscillations of Ca^{2+}.

INTRODUCTION

In non-excitable cells, elevations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) are a central event linking hormone–receptor binding to alterations in cell activity. Populations of endothelial cells respond to a wide variety of hormones, including ATP, bradykinin, histamine and thrombin, typically with a biphasic change in $[Ca^{2+}]_i$ [1–3] and the subsequent production of vasoactive factors such as endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2). The initial phase is a transient elevation of $[Ca^{2+}]_i$, and is provided by release from an intracellular Ca^{2+} store. This appears to be the major, if not the exclusive, mediator of thrombin- or ATP-induced PGI_2 release [3,4]. The second component of the agonist response is a sustained elevation of Ca^{2+}. This phase is entirely dependent on the presence of external Ca^{2+} ions, increases in a stepwise fashion after cumulative additions of hormone [5], and is decreased by elevating extracellular $[K^+]$ [6–9] or by activation of protein kinase C (PKC) [10]. The similarities between the time course and pharmacology of this response and those of hormone-evoked EDRF release have led to the suggestion that this phase is involved in the regulation of the maintained release of this potent vasoactive mediator [11].

Many non-excitable cells display repetitive spikes and/or oscillations of Ca^{2+} in response to stimulation [12,13]. Recently, such behaviour has been reported in single human umbilical-vein endothelial cells stimulated with histamine [14] or ATP [5]. Similarly to the oscillations observed in single hepatocytes [15–17], the frequency of histamine-stimulated Ca^{2+} spiking was related to the concentration of agonist applied, suggesting a frequency-coding mechanism for hormone responses.

Oscillatory behaviour has occasionally been observed in populations of endothelial cells [18–20], suggesting synchronization of single-cell responses. However, asynchronous oscillations would not be detected in population of cells.

In the present experiments, we have examined the responses to Ca^{2+} of single isolated pig aortic endothelial cells stimulated with ATP and bradykinin. The results suggest that secretory events driven by extracellular Ca^{2+} (e.g. EDRF release) may be regulated by repetitive spikes or oscillations of $[Ca^{2+}]_i$.

METHODS

Pig aortic endothelial cells were isolated as previously described [1], seeded directly on to 40 mm-diameter no. 1 coverslips and used 1–2 days later at sub-confluent density. At this time more than 95% of cells took up acetylated low-density lipoprotein (Di-Ac-LDL), but none were positive for anti-(α smooth-muscle actin) antibody. Cells were loaded with the Ca^{2+}-sensitive fluorescent indicator Indo-1 by incubating the cells in Dulbecco's Modified Eagle's Medium, supplemented with 20% (v/v) serum and containing 7 μM indo-1/AM, for 60 min at 37 °C. Cells were then transferred to a Hepes-buffered physiological saline (PSS: 145 mM-NaCl, 5.6 mM-KCl, 1 mM-$MgSO_4$, 1 mM-$CaCl_2$, 10 mM-Hepes, 10 mM-glucose, pH 7.4) at room temperature. Indo-1 fluorescence was measured by epifluorescence microscopy with an inverted microscope (Nikon Diaphot) equipped with an oil-immersion ×40 1.4 NA Neofluora objective. Excitation at 360 nm (±5 nm) was from a xenon arc lamp. Cells were maintained at 32 °C and superfused at 1.5 ml/min with PSS via a two-way tap, which allowed rapid changes of the superfusate to be effected. Emitted light was directed through a rectangular iris

Abbreviations used: EDRF, endothelium-derived relaxing factor; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PKC, protein kinase C; PGI_2, prostaglandin I_2 (prostacyclin); PSS, Hepes-buffered physiological saline; PDBu, phorbol 12,13-dibutyrate; 4αPDD, 4α-phorbol didecanoate.

‡ To whom correspondence should be addressed.

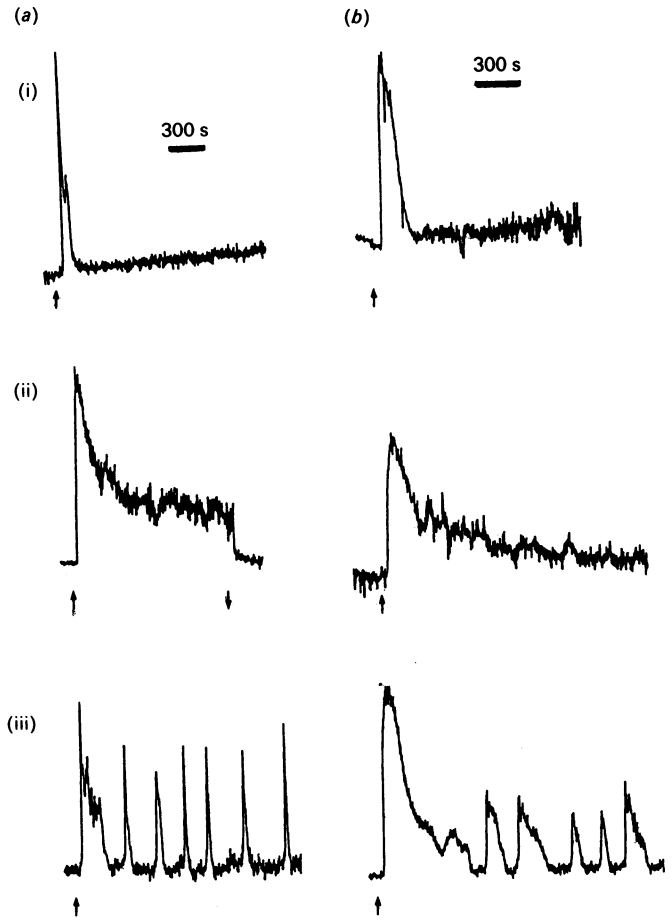

Fig. 1. Patterns of response to ATP (1 μM; *a*) or bradykinin (0.5 nM; *b*) obtained in single pig aortic endothelial cells

Arrows indicate time of addition or removal (downward arrow) of agonist.

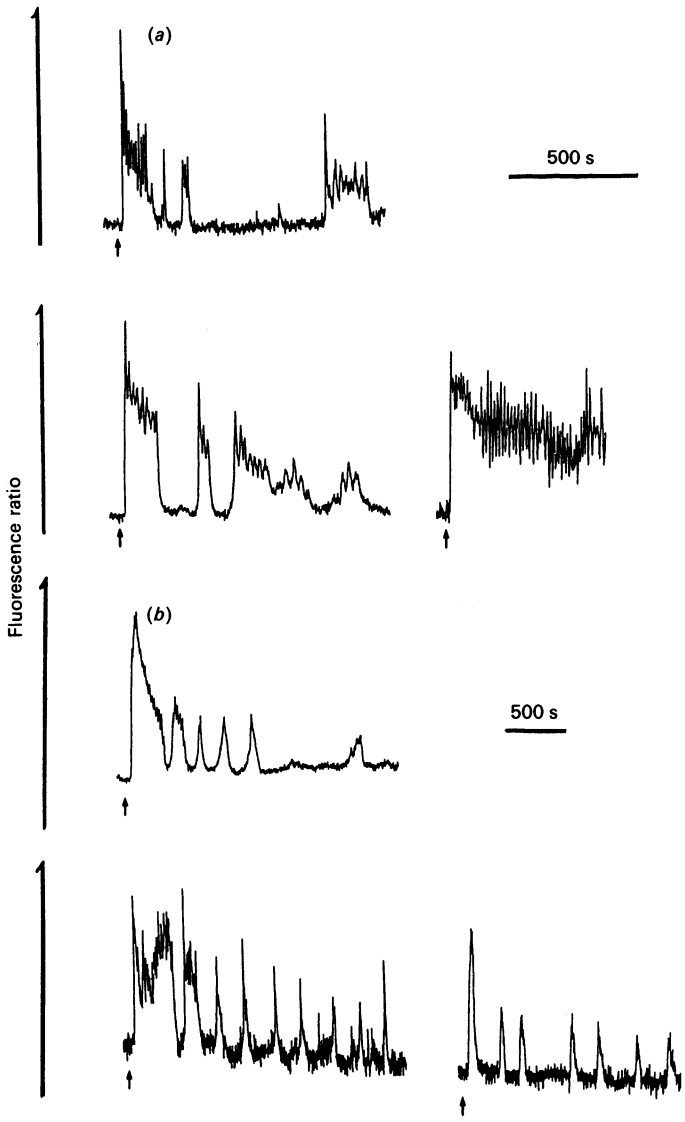

Fig. 2. Patterns of Ca^{2+} oscillatory/spiking responses induced by ATP and bradykinin

(*a*) Examples of the different oscillatory responses to 1 μM-ATP. (*b*) Examples of the Ca^{2+} spiking responses to 1 nM-bradykinin.

which limited the light collected to the cell or group of confluent cells under observation. The emitted beam was split by a mirror and the intensities were centred on 400 ± 20 and 480 ± 20 nm, measured by photon-counting photomultipliers interfaced by a pulse counter with a PC AT computer. Data acquisition and processing were controlled by software.

Background fluorescence and the photomultiplier dark counts from unloaded cells and coverslip were subtracted from values for the indo-1-loaded cells. All traces are expressed as the 400 nm/480 nm ratios of the corrected fluorescence. Indo-1 fluorescence was found to be evenly distributed throughout the cells, with no apparent sequestration of dye in intracellular organelles under the loading conditions used.

For experiments in high external $[K^+]$, the solutions used were: for 35 mM external K^+, 145 mM-sodium gluconate, 23.25 mM-KCl, 11.75 mM-potassium gluconate, 10 mM-Hepes, 1 mM-$MgSO_4$, 1 mM-$CaCl_2$, 10 mM-glucose, pH 7.4; and for 70 mM external K^+, 145 mM-sodium gluconate, 11.13 mM-KCl, 58.87 mM-potassium gluconate, 10 mM-Hepes, 1 mM-$MgSO_4$, 1 mM-$CaCl_2$, 10 mM-glucose, pH 7.4. Combinations of sodium gluconate, potassium gluconate and KCl used in these solutions allowed the $[K^+]/[Cl^-]$ product to be kept constant, thus decreasing potential changes in cell volume.

Statistical analysis was performed by one-way analysis of variance. Significant differences between the means of individual groups were revealed by *t* test using the standard error calculated from the within-groups mean square.

Materials

Phorbol 12,13-dibutyrate (PDBu), 4α-phorbol didecanoate (4αPDD), 1,2-dioctanoyl-*sn*-glycerol and bradykinin were from Sigma. ATP was from BDH. Indo-1/AM was obtained from Calbiochem.

RESULTS

ATP- and bradykinin-stimulated changes in $[Ca^{2+}]_i$ in single pig aortic endothelial cells

Single pig aortic endothelial cells in Ca^{2+}-containing PSS responded to superfusion with 0.1–100 μM-ATP (66 cells) or 0.1–10.0 nM-bradykinin (73 cells) with an initial rapid elevation of $[Ca^+]_i$, which reached a peak within 10 s. Three patterns of response were seen, as illustrated in Fig. 1. Transient responses, in which the initial peak Ca^{2+} elevation declined to resting levels

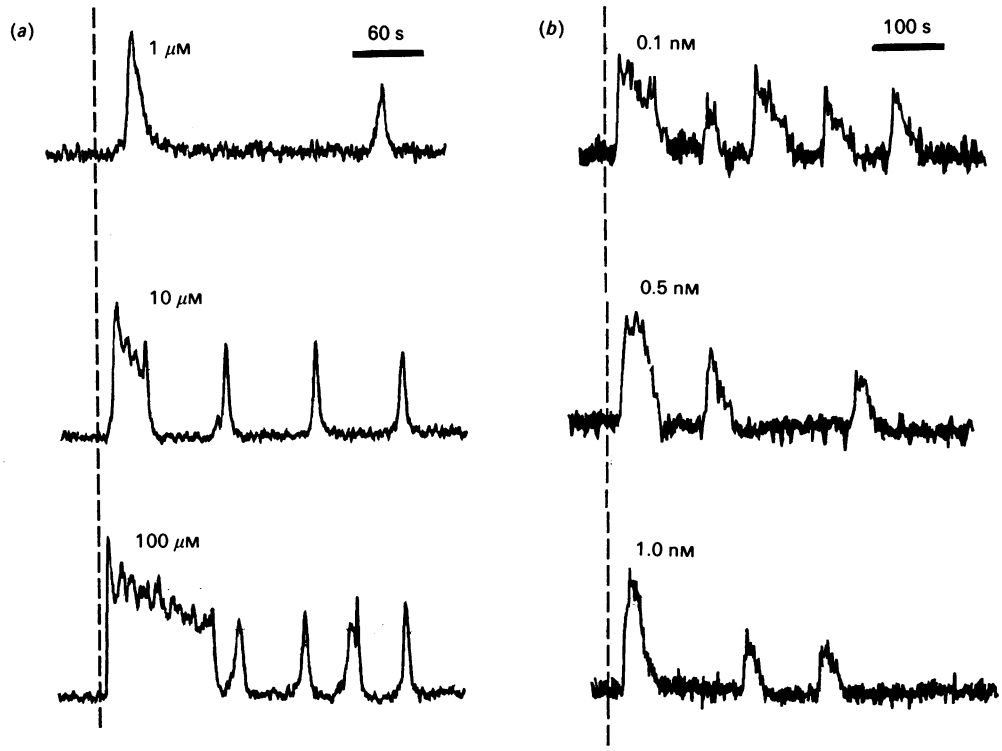

Fig. 3. Examples of the effect of agonist concentration on latency and spike frequency, with (*a*) ATP (1, 10 and 100 μM) and (*b*) bradykinin (0.1, 0.5 and 1 nM)

Dashed line indicates the time of application of each agonist concentration.

Table 1. Effect of bradykinin (0.1–1.0 nM) on latency of response and initial change in indo-1 fluorescence

Values show the latency of response (s) and change in initial fluorescence ratio (arbitrary units) after sequential exposure of individual cells (1–5) to increasing concentrations of bradykinin (BK). Cells were exposed to the agonist for 300–350 s, with a 300–600 s wash-out period between exposures.

		Latency (s)			Change in initial indo-1 fluorescence		
Cell	[BK]...	0.1 nM	0.5 nM	1 nM	0.1 nM	0.5 nM	1.0 nM
1		17.4	15.6	19.1	0.26	0.29	0.36
2		10.8	12.4	16.2	0.47	0.46	0.50
3		13.3	7.4	10.2	0.61	0.57	0.68
4		11.2	6.4	5.9	0.56	0.54	0.51
5		8.6	8.2	9.4	0.42	0.36	0.32
Mean ± S.E.M.		12.3 ± 1.5	10.0 ± 1.7	12.2 ± 2.4	0.46 ± 0.1	0.44 ± 0.1	0.47 ± 0.1

within 120–250 s (Fig. 1, i), were seen in 12 cells with ATP and in 17 with bradykinin. In 17 cells stimulated with ATP and 15 cells with bradykinin, $[Ca^{2+}]_i$ declined to an intermediate level which was maintained as long as the agonist was present (Fig. 1, ii). In 37 cells stimulated with ATP and 41 cells stimulated with bradykinin, spikes or oscillations of $[Ca^{+}]_i$ were seen (Fig. 1, iii). Variations in the latency (time between application of agonist and the initial Ca^{2+} response) and magnitude of the initial Ca^{2+} responses were found between different pig aortic endothelial cells in response to a single dose of either ATP or bradykinin. Similarly, the frequency and overall pattern of Ca^{2+} responses to a single dose of ATP also varied between different cells, although the overall pattern of responses to bradykinin was less variable (Figs. 1 and 2).

To determine if a relationship existed between the concentration of agonist and the magnitude of the initial peak elevation of Ca^{2+}, the latency of the response or the frequency of Ca^{2+} spiking/oscillations, cells were exposed for 300–350 s to graded doses of ATP (0.1–100 μM; 7 cells) or bradykinin (0.1–1.0 nM; 5 cells), with a wash-out interval of 5–10 min between exposures. Initial experiments demonstrated that this wash-out period was sufficient to allow for full recovery of responses to repeated exposure to 100 μM-ATP (results not shown). The experiments were carried out in the presence of 1 mM extracellular Ca^{2+}. Although no consistent relationship between the concentration of either ATP or bradykinin and the magnitude of the initial rise in $[Ca^{+}]_i$ (Fig. 3; Tables 1 and 2) was found within any individual cell, both the latency of the response and the frequency of Ca^{2+} spikes induced by ATP (7 cells), but not by bradykinin (5 cells), showed a dose-relationship. In addition, cells exhibited marked

Table 2. Effect of ATP (1–100 μM) on latency of response and initial change in indo-1 fluorescence

Values show the latency of response (s) and change in initial fluorescence ratio (arbitrary units) after sequential exposure of individual cells (1–7) to increasing concentrations of ATP. Cells were exposed to the agonist for 300–350 s, with a 300–600 s wash-out period between exposures. *$P < 0.01$ ATP 1 μM versus 10 μM; †$P < 0.01$ ATP 10 μM versus 100 μM.

		Latency (s)			Initial change in indo-1 fluorescence		
Cell	[ATP]...	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
1		10.4	5.2	3.1	0.29	0.35	0.40
2		8.2	5.9	4.2	0.24	0.28	0.27
3		10.8	6.7	3.9	0.43	0.37	0.36
4		6.7	6.2	4.8	0.26	0.31	0.30
5		7.9	5.2	3.4	0.39	0.31	0.25
6		9.6	6.4	4.2	0.18	0.17	0.19
7		7.2	5.8	3.8	0.36	0.36	0.32
Mean ± S.E.M.		8.7 ± 0.6	5.9 ± 0.2*	3.9 ± 0.2†	0.31 ± 0.03	0.31 ± 0.02	0.30 ± 0.03

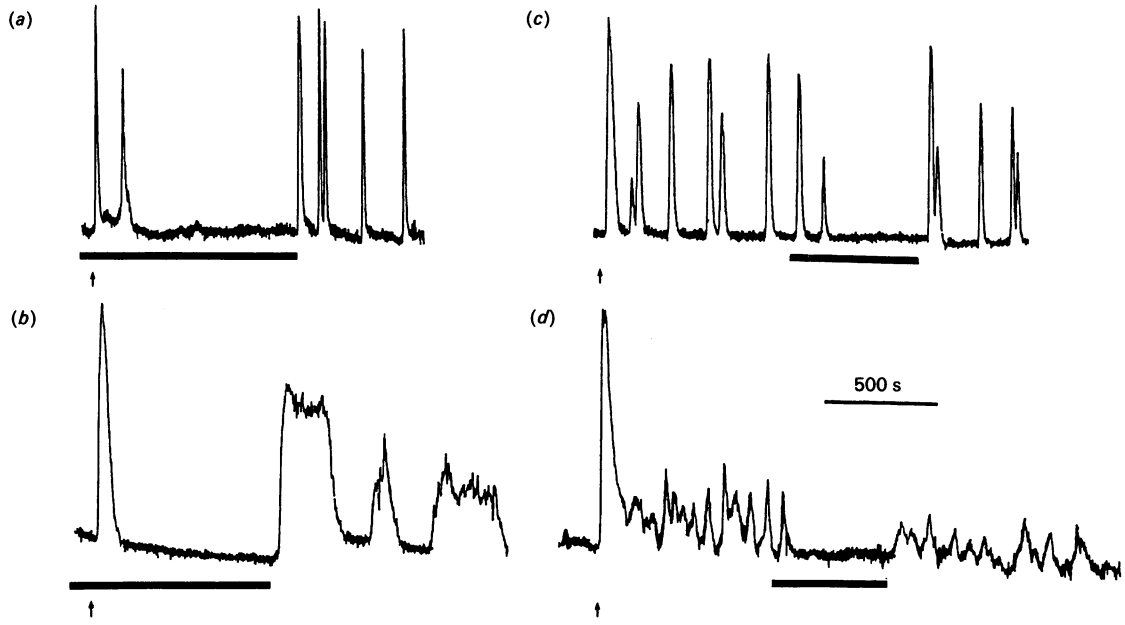

Fig. 4. Dependency of spiking on extracellular Ca^{2+} concentration

The Figure shows representative traces obtained after removal of extracellular Ca^{2+} before addition of 10 μM-ATP (*a*) or 1 nM-bradykinin (*c*), and removal of extracellular Ca^{2+} during Ca^{2+} spiking evoked by 10 μM-ATP (*b*) or 1 nM-bradykinin (*d*) in separate endothelial cells. Arrows indicate time of application of agonist; solid bar indicates absence of extracellular Ca^{2+}.

variations in their sensitivity to either ATP or bradykinin. Some cells responded to concentrations as low as 0.1 μM-ATP or 0.1 nM-bradykinin, whereas others required higher concentrations, e.g. 10 μM-ATP or 1 nM-bradykinin, before changes in $[Ca^{2+}]_i$ were observed (results not shown).

Effect of removal of extracellular Ca^{2+} before addition of ATP or bradykinin

Figs. 4(*a*) and 4(*b*) illustrate representative recordings of the response of single pig aortic endothelial cells to ATP (10 μM) or bradykinin (0.5 nM) in the absence of extracellular Ca^{2+} and in the presence of 200 μM-EGTA. The absence of extracellular Ca^{2+} did not affect resting fluorescence. Both hormones evoked an initial rapid elevation of Ca^{2+} that declined back to pre-stimulated levels over 90–120 s (5/8 cells with ATP and 6/6 cells with bradykinin). In 3/8 cells stimulated with ATP one to three spikes of Ca^{2+} were seen before the response ceased. When Ca^{2+} was restored to the superfusing buffer, cells exposed to ATP exhibited either oscillations of Ca^{2+} (5/8 cells) or a rapid rise in Ca^{2+} to an elevated level (Fig. 4*a*). In cells exposed to bradykinin, Ca^{2+} rose rapidly to an elevated level (4/6 cells) or showed complex oscillations (Fig. 4*b*).

Effect of removal of extracellular Ca^{2+} during Ca^{2+} oscillations induced by ATP or bradykinin

Oscillatory responses to ATP (10 μM) or bradykinin (1.0 nM) were established (in the presence of 1 mM extracellular Ca^{2+}) before manipulating the extracellular $[Ca^{2+}]$. After removal of extracellular Ca^{2+} (no added Ca^{2+}, 200 μM-EGTA) a variable number (one to three) of spikes of $[Ca^{2+}]_i$ were seen before the response ceased (ATP, 5 cells; bradykinin, 7 cells) (Figs. 4*c* and 4*d*); however, the magnitudes of the spikes were often decreased, particularly the second or subsequent spikes. Upon re-addition of extracellular Ca^{2+} (1 mM) to the perfusate, cells exposed to

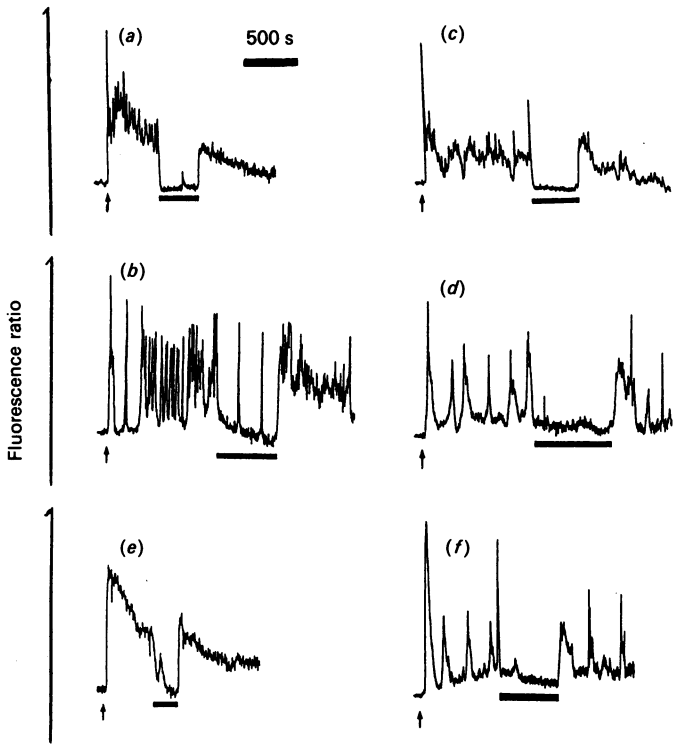

Fig. 5. Effect of elevated extracellular K^+ concentration during Ca^{2+} responses evoked by ATP or bradykinin in single pig aortic endothelial cells

The Figure shows representative traces of responses elicited by ATP (10 μM; *a–d*) or bradykinin (1 nM; *e*, *f*). During agonist application extracellular K^+ was elevated from 5 mM to 35 mM (*a*, *b*) or 70 mM (*c–f*) where indicated by the solid bar. Arrows show time of application of agonist.

ATP resumed spiking (Fig. 4*d*). In cells exposed to bradykinin, 3/7 cells resumed spiking, whereas in 4/7 cells $[Ca^{2+}]_i$ rose rapidly to a maintained level.

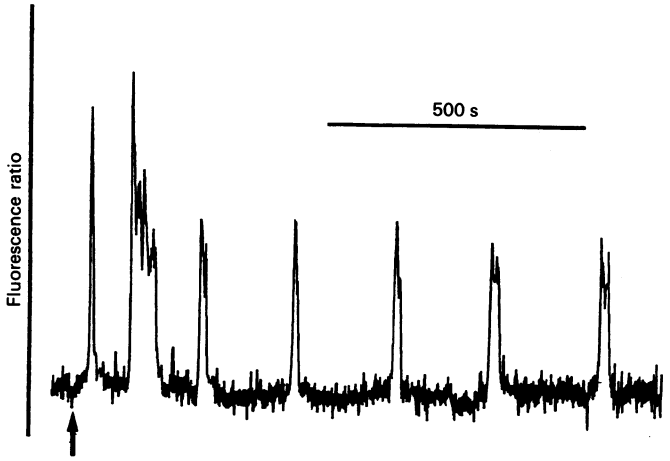

Fig. 7. Response of groups of confluent pig aortic endothelial cells to 100 μM-ATP

Arrow indicates time of application of agonist.

Effect of raising extracellular K^+ concentration on Ca^{2+} oscillations induced by ATP or bradykinin

Cells were exposed to ATP (10 μM) or bradykinin (1.0 nM) in buffer containing 5 mM-K^+ and 1 mM-Ca^{2+}. Once an oscillatory response was established, the extracellular $[K^+]$ was raised to either 35 mM or 70 mM. In each of three cells exposed to ATP, elevating extracellular K^+ to 35 mM decreased the frequency of Ca^{2+} spiking, whereas in each of another three cells tested 70 mM extracellular K^+ reversibly prevented spiking (Fig. 5). Application of 70 mM extracellular K^+ during spiking responses elicited by 1.0 nM-bradykinin also reversibly prevented spiking in each of three cells studied (Fig. 5).

Effect of PKC activators on Ca^{2+} oscillations induced by ATP or bradykinin

Oscillatory responses were established to 1 μM-ATP or 1.0 nM-bradykinin (in the presence of 1 mM extracellular Ca^{2+}) before

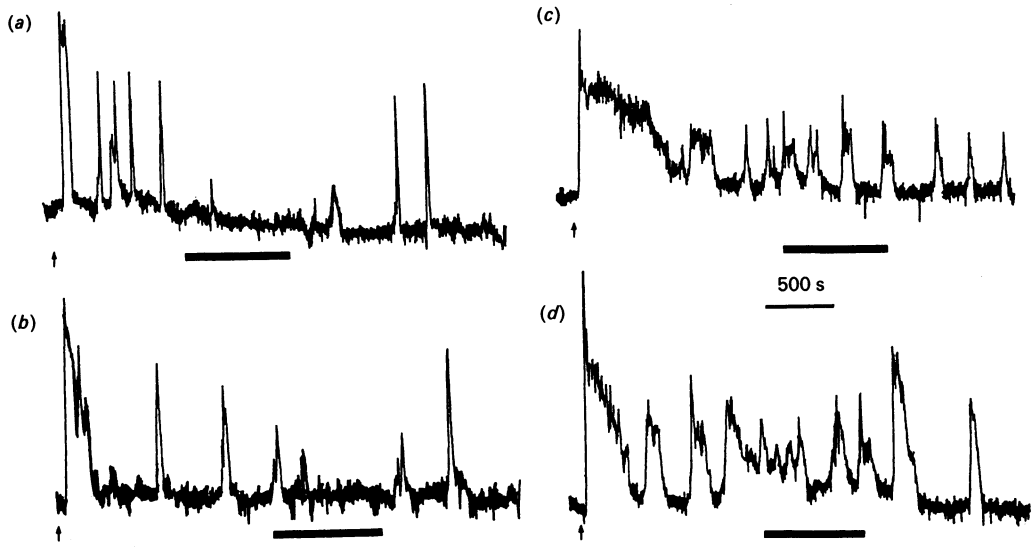

Fig. 6. Effects of PKC activation on agonist-induced Ca^{2+} spiking in single pig aortic endothelial cells

The Figure shows representative traces after application of ATP (1 μM; *a*, *c*) or bradykinin (1 nM; *b*, *d*). Solid bar indicates the period of application of (*a*, *b*) 20 nM-PDBu or (*c*, *d*) 4α-PDD. Arrows indicate time of application of agonist.

addition of either 20 nM-4αPDD or 20 nM-PDBu to the superfusate. The inactive phorbol ester 4αPDD had no significant effect on the response to either hormone in three cells (Fig. 6). However, after addition of PDBu, the Ca^{2+} oscillations induced by ATP (four cells) or bradykinin (three cells) were prevented (Fig. 6). After removal of PDBu, the oscillatory response recovered in 3/4 cells stimulated with ATP and 2/3 cells with bradykinin, after a lag of 120–250 s. Similar results were obtained with the synthetic diacylglycerol 5 μM-DiC_8 (results not shown).

Effects of ATP on Ca^{2+} responses in confluent groups of pig aortic endothelial cells

Ca^{2+} responses of confluent groups of pig aortic endothelial cells (15–20 cells) were obtained during superfusion with 100 μM-ATP. Of twelve groups examined, two exhibited biphasic responses similar to those previously observed in monolayers of pig aortic endothelial cells [1]. Of the remaining confluent groups, 4/12 demonstrated spikes of $[Ca^{2+}]_i$ as shown in Fig. 7, and 6/12 showed complex patterns of sustained and oscillatory responses.

DISCUSSION

In populations of pig aortic endothelial cells, the Ca^{2+} response elicited by either ATP or bradykinin is biphasic, consisting of a transient elevation, owing to the release of Ca^{2+} from internal stores, and a sustained component, entirely dependent on the presence of extracellular Ca^{2+} [1]. Studies of single human umbilical-vein endothelial cells, however, have shown that qualitatively different responses are obtained, consisting of either repetitive spikes or oscillations of Ca^{2+} [14]. Oscillatory changes in K^+ currents have been observed recently in single pig aortic [8,21,22], rabbit aortic [23,24] and guinea-pig coronary-artery [25] endothelial cells after stimulation with ATP, bradykinin or acetylcholine, which have been interpreted as indicating underlying oscillations of $[Ca^{2+}]_i$. These studies do not, however, provide direct evidence for Ca^{2+} oscillations in these cells.

Our experiments demonstrate that, in contrast with the typical biphasic change in $[Ca^{2+}]_i$ detected in response to ATP or bradykinin in populations of pig aortic endothelial cells, the responses of single cells to these hormones exhibit marked heterogeneity. In approx. 50% of cells studied with either ATP or bradykinin, repetitive spikes or oscillations of $[Ca^{2+}]_i$ were seen. In the remaining cells studied, either transient (approx. 20% of cells) or sustained (approx. 20% of cells) responses were seen with either hormone. These results provide the first direct evidence of different patterns of Ca^{2+} responses in pig aortic endothelial cells.

There was a marked variability between cells in the latency, magnitude, frequency and overall pattern of responses to any given stimulation by ATP or bradykinin in cells exhibiting Ca^{2+} spikes/oscillations (e.g. Figs. 1–3; Tables 1 and 2), though the pattern of response elicited with bradykinin was less variable (Fig. 2). The reasons for such variation are unclear, but may reflect differences in the individual cell content of receptors, coupling proteins or enzymes, as well as other parameters, such as the size of the intracellular Ca^{2+} stores [17] and the effectiveness of the agonist to release stored intracellular Ca^{2+} [5].

Within individual cells there was no apparent relationship between the concentration of hormone applied and the magnitude of the initial elevation of $[Ca^{2+}]_i$, unlike responses elicited in populations of cells, which do show such a relationship [2,4]. These observations, coupled with the variability in the sensitivity of individual cells to either of these hormones, suggest that changes in the magnitude of responses observed in populations of cells results from the recruitment of less sensitive cells to the overall response, and not from a graded increase in the magnitude of the initial response.

In contrast with single human umbilical-vein endothelial cells stimulated with ATP [5], we observed that both the latency of the responses and the frequency of Ca^{2+} spikes were related to the concentration of ATP (Fig. 3, Table 2). The decreased latency of the initial Ca^{2+} transient may reflect more rapid generation of intracellular second messengers responsible for Ca^{2+} mobilization (e.g. $InsP_3$) as the ATP concentration is increased. Similar results have been reported in histamine-stimulated human umbilical-vein endothelial cells [14] and other cell types [15,17]. No such relationship between latency of response or frequency of spiking was found for bradykinin. The reason for this is unclear, but may reflect a more prolonged period of desensitization of the bradykinin response, unlike that of ATP, which recovers after a 5–10 min wash-out period.

An interesting feature of the spiking responses elicited by both ATP and bradykinin in these cells was the slower rise in $[Ca^{2+}]_i$ often seen at the beginning of a spike (Fig. 3). Such slow rises in Ca^{2+} before the up-stroke of the response have been observed in histamine-stimulated human umbilical-vein endothelial cells [14], but the function of this phase of the response is not known.

Effect of removal of extracellular Ca^{2+}

Both ATP and bradykinin induced a transient elevation of Ca^{2+} in single pig aortic endothelial cells in the absence of extracellular Ca^{2+}, but $[Ca^{2+}]_i$ returned more rapidly to pre-stimulated levels with ATP. Only ATP elicited more than one elevation of Ca^{2+} (up to three spikes) under these conditions, suggesting that exposure to ATP releases less of the stored Ca^{2+} during each spike than does bradykinin. Alternatively, these two hormones could differentially modulate Ca^{2+} sequestration or efflux. In contrast, when Ca^{2+} was removed during spiking, one to three further Ca^{2+} spikes were seen, although the magnitude of the second or subsequent spikes was often decreased. Similarly, in single histamine-stimulated human umbilical-vein endothelial cells, a decrease in the frequency and amplitude of spikes was observed [14]. The decrease in the magnitude of spikes in zero Ca^{2+} may reflect: (1) the incomplete discharge of the internal Ca^{2+} store during each spike, or (2) complete discharge, followed by partial sequestration of the released Ca^{2+} back into an internal store, or (3) a combination of these two processes.

Although extracellular Ca^{2+} is clearly needed to maintain the spiking, perhaps by repleting the Ca^{2+} store(s), the results suggest that the mechanism which triggers spiking does not require extracellular Ca^{2+}. The mechanism underlying Ca^{2+} spiking in these and other non-excitable cells is not known. Although several models have been proposed to explain this kind of behaviour [13], there is as yet no good evidence to distinguish between them.

Effect of raising extracellular $[K^+]$

There is little information about the mechanisms underlying Ca^{2+} entry into endothelial cells. Voltage-sensitive Ca^{2+} channels do not appear to play an important role [26,27], but several recent studies have shown that the magnitude of the Ca^{2+}-dependent phase of responses measured in populations of endothelial cells is decreased by depolarization [6–9,27,28], suggesting that membrane potential is a driving force for Ca^{2+} influx. Membrane hyperpolarizations induced by several vasoactive mediators, including ATP and bradykinin, have also been suggested as a possible mechanism for augmenting Ca^{2+} entry during the late phase of the response, and for the maintained synthesis and release of autacoids such as EDRF [8,21,29].

Indeed, there is evidence that hormone-evoked EDRF release is prevented under depolarizing conditions [8]. It was therefore important to examine whether responses were modified by changes in membrane potential. Treating cells with increasing extracellular concentrations of K^+ caused a decrease in the magnitude of the sustained phase of the Ca^{2+} response in cells exhibiting a biphasic rise in $[Ca^{2+}]_i$, whereas in spiking cells spike frequency was decreased or the response was prevented. Whether this effect of high K^+ results from a negation of the driving force for calcium down its electrochemical gradient, or some other effect on the underlying mechanism of spiking in these cells, is unclear. Interestingly, in human umbilical-vein endothelial cells, iso-osmotic K^+ solutions were found to have no effect on Ca^{2+} spiking elicited by histamine [14], suggesting that in these cells depolarization does not interfere with the underlying mechanism of spiking. What role membrane potential plays in regulating Ca^{2+} entry into human umbilical-vein endothelial cells is unclear, but evidence suggests that the state of the internal Ca^{2+} store may be of more importance in this process [30].

Effect of activators of PKC

Activators of PKC have variable effects on Ca^{2+} spiking in different cells. In mouse oocytes, phorbol 12-myristate 13-acetate produces Ca^{2+} oscillations [31], whereas in hepatocytes and pancreatic acinar cells Ca^{2+} spiking is slowed or blocked [32,33], and cholecystokinin-induced oscillations in parotid acinar cells are unaffected by activators of PKC [34]. Activation of PKC in populations of bovine pulmonary-artery endothelial cells has been reported to block oscillations of $[Ca^{2+}]_i$ [19] and to decrease the magnitude of the sustained component of the biphasic Ca^{2+} response in addition to inhibiting agonist-evoked EDRF release [10,35]. We investigated the effect of activation of PKC on the spiking responses in single pig aortic endothelial cells. Both the phorbol ester PDBu and DiC_8 reversibly blocked Ca^{2+} spiking in these cells, whereas the inactive analogue 4αPDD was without effect. The specificity for the PKC activators strongly suggests that these effects are mediated via PKC. It remains to be determined which intermediary step (agonist–receptor binding, G-protein function, phosphoinositide turnover, $InsP_3$ binding/function) in the signal-transduction cascade is the site for regulation by PKC and whether PKC exerts a functional regulatory role in hormone-evoked Ca^{2+} spiking.

Responses of confluent groups of pig aortic endothelial cells to ATP

In preliminary experiments, measurements from groups of confluent endothelial cells exhibited two patterns of response. Almost all (10/12) showed complex sustained/oscillatory changes in Ca^{2+} or Ca^{2+} spikes (cf. Fig. 7). In the remaining preparations a simple biphasic response, similar to that previously observed in populations of cells, was observed [1]. Oscillations or repetitive spikes of Ca^{2+} have recently been reported in populations of endothelial cells from bovine pulmonary artery, bovine atrium, human umbilical vein and rabbit aorta [18,20,36].

Oscillatory or spiking in endothelial-cell monolayers is suggestive of synchronization of Ca^{2+} responses over many cells. In these experiments, like those of Sage *et al.* [19] and Neylon & Irvine [20], the cells were seeded at low density and allowed to grow to confluency, suggesting a requirement for cell-to-cell communication to become established. The synchronized oscillatory/spiking activity in endothelium, if translated into the release of autacoids, may be of some importance in the regulation of vascular tone. Further experiments are required to characterize fully the importance of intercellular communication in the generation of synchronous Ca^{2+} response.

Conclusions

The widespread occurrence of dose-related Ca^{2+} spiking in non-excitable cells [12,13] has led to the suggestion that the frequency of transient elevations of intracellular Ca^{2+} may be an important mechanism for controlling cellular processes. This type of signalling system may allow a more precise regulation of cell function, particularly when the effect of Ca^{2+} is highly co-operative, as when its actions are mediated by calmodulin [16]. Although little is yet known of the precise mechanisms by which Ca^{2+} regulates the release of EDRF from endothelial cells, there is evidence that a Ca^{2+}–calmodulin-dependent enzyme is involved [23,37]. The dose-related changes in Ca^{2+} spiking observed with ATP, and the sensitivity of the spikes to procedures known to inhibit agonist-induced EDRF release, suggest that the sustained release of this potent vasodilator may be regulated by complex spiking or oscillations of Ca^{2+}. However, this can only be determined by parallel single-cell assays of Ca^{2+} and EDRF release.

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