Cytosolic calcium concentration in resting and stimulated endothelium of excised intact rat aorta

Yuri M. Usachev, Sergey M. Marchenko and Stewart O. Sage*

The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

1. Optical fibres were used to excite and record fluorescence from the lumenal face of rat aorta or tail artery loaded with fura-2.

2. Acetylcholine (ACh) evoked an endothelium-dependent rise in the fura-2 340/380 nm excitation ratio in both vessels. High $[K^+]$ or phenylephrine evoked an endothelium-independent rise in ratio in tail artery but failed to increase the ratio in aorta. These observations indicate that fura-2 fluorescence and therefore cytosolic calcium concentration ($[Ca^{2+}]_i$) may be selectively recorded from the endothelium of intact rat aorta.

3. In aortic endothelium, resting $[Ca^{2+}]_i$ was 95 ± 8 nM ($n = 44$). ACh evoked a monophasic rise in $[Ca^{2+}]_i$ which was temporally coincident with a membrane hyperpolarization.

4. ATP in most (22/35) preparations evoked a rise in $[Ca^{2+}]_i$ which declined towards resting and was followed by a secondary rise. The biphasic $[Ca^{2+}]_i$ responses were accompanied by biphasic electrical responses of initial hyperpolarization followed by depolarization above the resting potential and subsequent restoration towards rest. In the presence of high $[K^+]$ or the K^+ ionophore valinomycin, ATP did not evoke changes in membrane potential and only monophasic rises in $[Ca^{2+}]_i$ were observed. In some (7/35) preparations, ATP evoked oscillations in $[Ca^{2+}]_i$, with membrane potential oscillating in antiphase.

5. These data suggest interplay between $[Ca^{2+}]_i$ and membrane potential in the generation of agonist-evoked responses in native endothelium *in situ*. The observed oscillations in $[Ca^{2+}]_i$ imply spatio-temporal synchronization of Ca^{2+} signalling in large groups of endothelial cells in intact rat aorta.

The elevation of cytosolic calcium concentration ($[Ca^{2+}]_i$) in endothelial cells influences endothelial barrier properties (Rotrosen & Gallin, 1986) and the release of prostacyclin (Hallam, Pearson & Needham, 1988) and nitric oxide (Moncada, Palmer & Higgs, 1991). Endothelial $[Ca^{2+}]_i$ has principally been studied in cultured cells (for review see Jacob, Sage & Rink, 1990). However, endothelial cells undergo significant changes in culture (see discussion in Marchenko & Sage, 1993), reducing the value of these results. Use of freshly isolated endothelial cells (e.g. Busse, Fichtner, Lückhoff & Kohlhardt, 1988) negates some shortcomings, although there is risk of mechanical or enzymatic damage. Furthermore, isolated endothelial cells, like those in culture, may not behave as they do physiologically since they lack contact with each other and with vascular smooth muscle.

Recently, measurements of $[Ca^{2+}]_i$ in single acetylcholine-stimulated endothelial cells in isolated rat aorta have been reported (Carter & Ogden, 1994). This study used furaptra, an indicator which has a low affinity for Ca^{2+} (K_d, 44 μM) and detects rises in $[Ca^{2+}]_i > 1$ μM. Resting $[Ca^{2+}]_i$, the prolonged effects of agonists, and moreover the spatial organization of agonist-evoked changes in $[Ca^{2+}]_i$ within native endothelium, are unknown.

We now describe a method using optical fibres for the selective measurement of $[Ca^{2+}]_i$ in the endothelium of excised intact rat aorta loaded with fura-2. This has allowed the determination of resting $[Ca^{2+}]_i$ and the effects of acetylcholine (ACh) and ATP. We show that in some respects Ca^{2+} signals in the endothelium of intact aorta differ from those observed in previous studies and are closely correlated with membrane potential changes.

METHODS

Preparation of vessels

Samples of intact rat aorta were prepared essentially as previously described (Marchenko & Sage, 1993). Briefly, 3- to 5-week-old rats were anaesthetized with diethylether and killed by cervical dislocation. The thoracic aorta was dissected out, cut into rings of 3–5 mm and stored in modified Krebs solution supplemented with

* To whom correspondence should be addressed.

gentamicin (50 μg ml^{-1}). Rings were used over 2 days, during which no changes in response were observed. Rat tail artery was similarly prepared.

Fluorescence measurement

Aortic (or tail artery) rings were loaded with fura-2 by incubation with 10 μM fura-2 AM and 0·02% pluronic F-127 in modified Krebs solution for 1 h at room temperature. Rings were then cut open and pinned lumen face up to the black rubber bottom of a 100 μl chamber continuously perfused with modified Krebs solution at 1–2 ml min^{-1}.

Fluorescence measurements used a Cairn spectrofluorimeter (Cairn Research, Sittingbourne, Kent, UK) modified for use with optical fibres (Thomas & Schwiening, 1992). Excitation light from a xenon lamp was filtered to provide wavelengths of 340, 360 and 380 nm using a rotating filter wheel and projected onto the vessel via a liquid light guide tapered to a final diameter of 8 mm. Fluorescence was collected with an optical fibre 0·4 mm in diameter, positioned using a manipulator 0·2–0·4 mm and almost vertically above the lumenal surface of the vessel. Fluorescence was estimated to be collected from a circular area 0·8–1·2 mm in diameter (about 250–560 cells). The collecting fibre was coupled through a 500 nm filter to a photomultiplier (EMI) run at about −900 V. The signal was then amplified and digitized by the Cairn spectrofluorimeter system connected to a computer. Signals at each excitation wavelength were sampled at 32 Hz and averaged to give an effective rate of 1 Hz.

$[Ca^{2+}]_i$ was calculated using the fura-2 340/380 nm fluorescence ratio (F_{340}/F_{380}) assuming a K_d of 224 nM (Grynkiewicz, Poenie & Tsien, 1985) and calibrated by application of ionomycin (5–10 μM) in the presence of 2·5 mM Ca^{2+} then 5 mM EGTA. All signals were corrected for autofluorescence determined at the end of experiments by exposing the tissue to 50 μM digitonin and 0·5 mM Mn^{2+}. Autofluorescence was 40–70% of total signal.

Fluorescence microscopy was performed using a fluorescence microscope (Olympus BH-2) with excitation between 300 and 400 nm and collection over 500 nm with a ×20 fluor/phase objective.

Electrophysiology

Membrane potential was measured using the perforated patch technique as described previously (Marchenko & Sage, 1993), but nystatin (100 μg ml^{-1}) was used in the pipette filling solution. Pipettes had a resistance < 5 MΩ. All recordings were from endothelium located under the collecting optical fibre. The membrane potential was sampled by the Cairn system at the same rate as the optical signal.

Solutions and reagents

Modified Krebs solution had the following composition (mM): 118·3 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·2 NaH_2PO_4, 1·2 $MgCl_2$, 1 $CaCl_2$, 11 glucose, 0·02 Phenol Red; pH 7·4 at 20 °C, and was gassed with 95% O_2 and 5% CO_2. In high K^+ solutions, there was equimolar replacement of NaCl by KCl. In Ca^{2+}-free solutions, $MgCl_2$ was increased to 2·2 mM and 0·2 mM EGTA was added. Patch pipettes were filled with a solution containing (mM): 140 KCl, 10 Hepes; pH adjusted with NaOH to 7·3 at room temperature.

Values are given throughout as means ± S.E.M. of the number of observations indicated. All experiments were conducted at room temperature.

RESULTS

The fluorescence signal from fura-2-loaded intact vessels

Endothelial cells were clearly visible under the fluorescence microscope on the lumenal surface of fura-2-loaded rat aorta (Fig. 1*A*). No signs of fluorescence arising from underlying smooth muscle were seen. Only a weak fluorescence, somewhat fibrous in appearance, was seen in control, unloaded vessels (not shown).

In rat aorta, elevation of $[K^+]$ was either without effect on F_{340}/F_{380} or evoked a small fall (Fig. 1*B*). Stimulation of smooth muscle with the α_1-adrenergic agonist, phenylephrine (PE, 2 μM) was without effect on F_{340}/F_{380}, whereas stimulation of the endothelium in the same preparation with ACh (2 μM) evoked a rise in the ratio (Fig. 1*B*, $n = 9$). After removal of the endothelium by rubbing with filter paper, the fluorescence signal fell to near the autofluorescence level and addition of ACh had no effect on F_{340}/F_{380} (not shown). Increasing the concentration of fura-2 AM to 20 μM and the loading period to 2 h did not affect agonist-evoked responses (not shown).

In contrast to aorta, in tail artery both K^+ (30 mM) and phenylephrine (2 μM) elevated F_{340}/F_{380} (Fig. 1*C*, $n = 2$). ACh (2 μM) evoked a similar response in tail artery as in aorta (Fig. 1*C*). Removal of endothelium from tail artery reduced the fluorescent signal to about half. ACh was without effect on the denuded tail artery, whereas K^+ and phenylephrine still increased F_{340}/F_{380} (Fig. 1*D*).

Effects of agonists on $[Ca^{2+}]$ in rat aortic endothelium

In unstimulated aortic endothelium, $[Ca^{2+}]_i$ was 95 ± 8 nM ($n = 44$). ACh (2 μM) evoked a rapid rise in $[Ca^{2+}]_i$ to 413 ± 43 nM ($n = 23$), which was followed by a slow decline (Fig. 2*A*). After washout of ACh, $[Ca^{2+}]_i$ fell rapidly to the resting level. In the absence of external Ca^{2+}, ACh evoked a similar initial rise in $[Ca^{2+}]_i$, but this then declined rapidly towards the basal level or lower. Readdition of external Ca^{2+} in the continued presence of ACh resulted in a response similar to that observed under control (Ca^{2+} containing) conditions (Fig. 2*A*). Muscarine (10 μM) mimicked the effect of ACh ($n = 2$; not shown) whilst atropine (1 μM) abolished the effect of ACh on $[Ca^{2+}]_i$ ($n = 3$; not shown).

ATP (100 μM) evoked an elevation in $[Ca^{2+}]_i$ to 766 ± 91 nM ($n = 35$). The time course of the ATP-evoked response differed significantly from that evoked by ACh in most (22/35) aortic preparations. After the initial peak, $[Ca^{2+}]_i$ fell to 145 ± 15 nM ($n = 22$) and then underwent a secondary rise, peaking at 314 ± 31 nM ($n = 22$) before slowly declining again (Fig. 2*B*). Washout of ATP resulted in $[Ca^{2+}]_i$ returning to the resting level. Only the first phase of the ATP response was evoked in the absence of external Ca^{2+}, but a full biphasic response was seen if external Ca^{2+} was reintroduced (Fig. 2*B*).

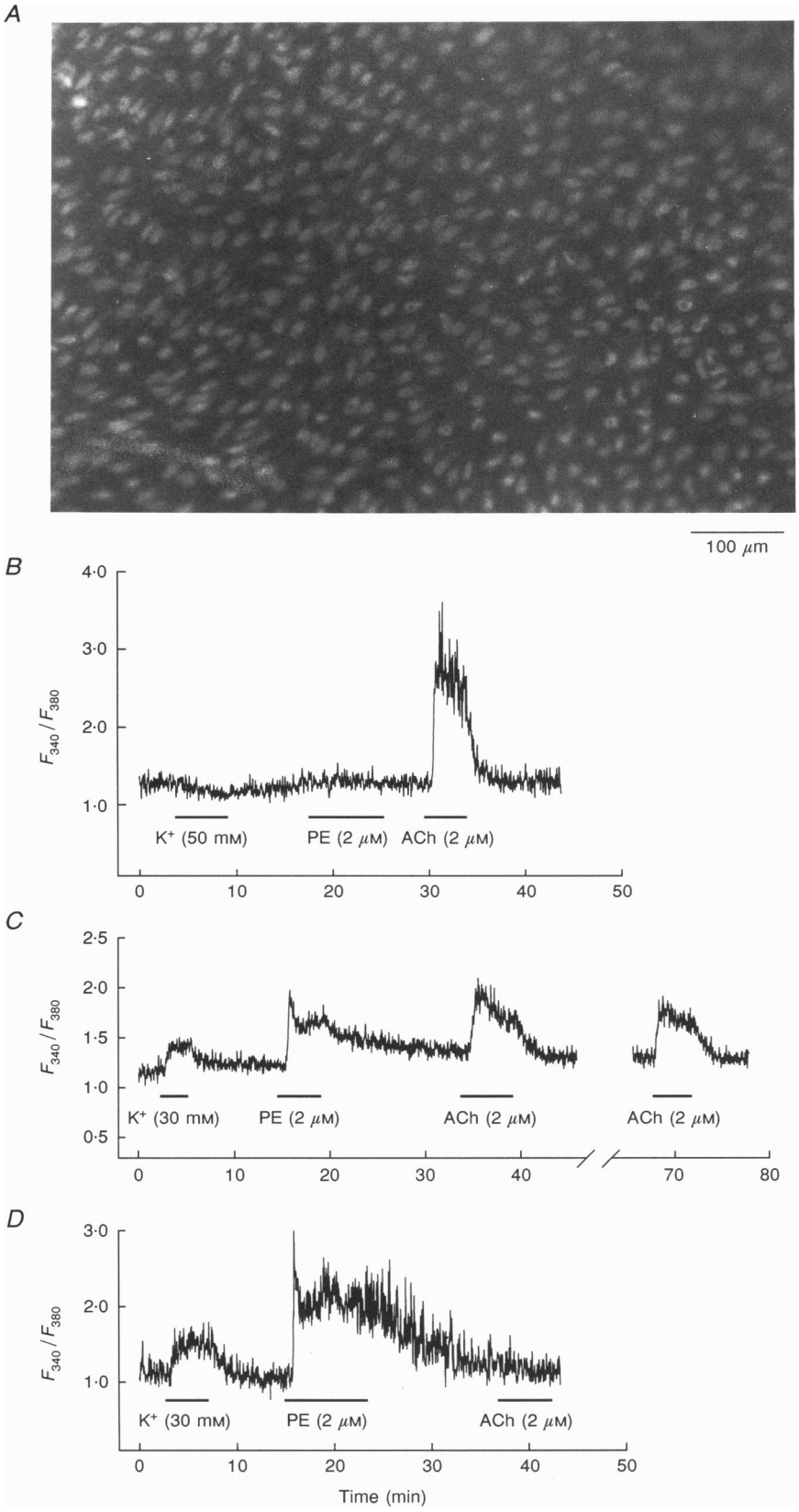

Figure 1. Fluorescence from fura-2-loaded rat aorta and tail artery

A, fluorescence micrograph of lumenal face of fura-2-loaded rat aorta. Excitation, 300–400 nm; emission, > 500 nm. *B–D*, fura-2 fluorescence ratio (F_{340}/F_{380}) recorded from the lumenal face of rat aorta (*B*), rat tail artery (*C*) or rat tail artery after removal of endothelium (*D*). Duration of application of high [K^+], phenylephrine (PE) or ACh in perfusate at the shown concentrations is indicated by the horizontal bars.

Mn^{2+} permeates many entry Ca^{2+} pathways and may be used as a probe for divalent cation entry, being detected from quenching of the fura-2 signal excited at the isoemissive (Ca^{2+}-insensitive) wavelength of 360 nm (Sage, Merritt, Hallam & Rink, 1989). Mn^{2+} quench experiments revealed that the later stages of the ATP-evoked rise in $[Ca^{2+}]_i$, including the secondary rise, are accompanied by divalent cation entry (Fig. 2*C*). Mn^{2+} entry commenced shortly after the initial rise in $[Ca^{2+}]_i$ and was then detectable throughout the period of ATP application. A similar Mn^{2+} entry was evoked by ACh (not shown).

In some cases, ATP evoked either a monophasic response like that evoked by ACh (6/35 preparations; not shown) or oscillations in $[Ca^{2+}]_i$ (7/35 preparations; Fig. 3*D*). Basal $[Ca^{2+}]_i$ was usually higher (190 ± 23 nM, $n = 7$) in preparations showing oscillations compared with those which did not.

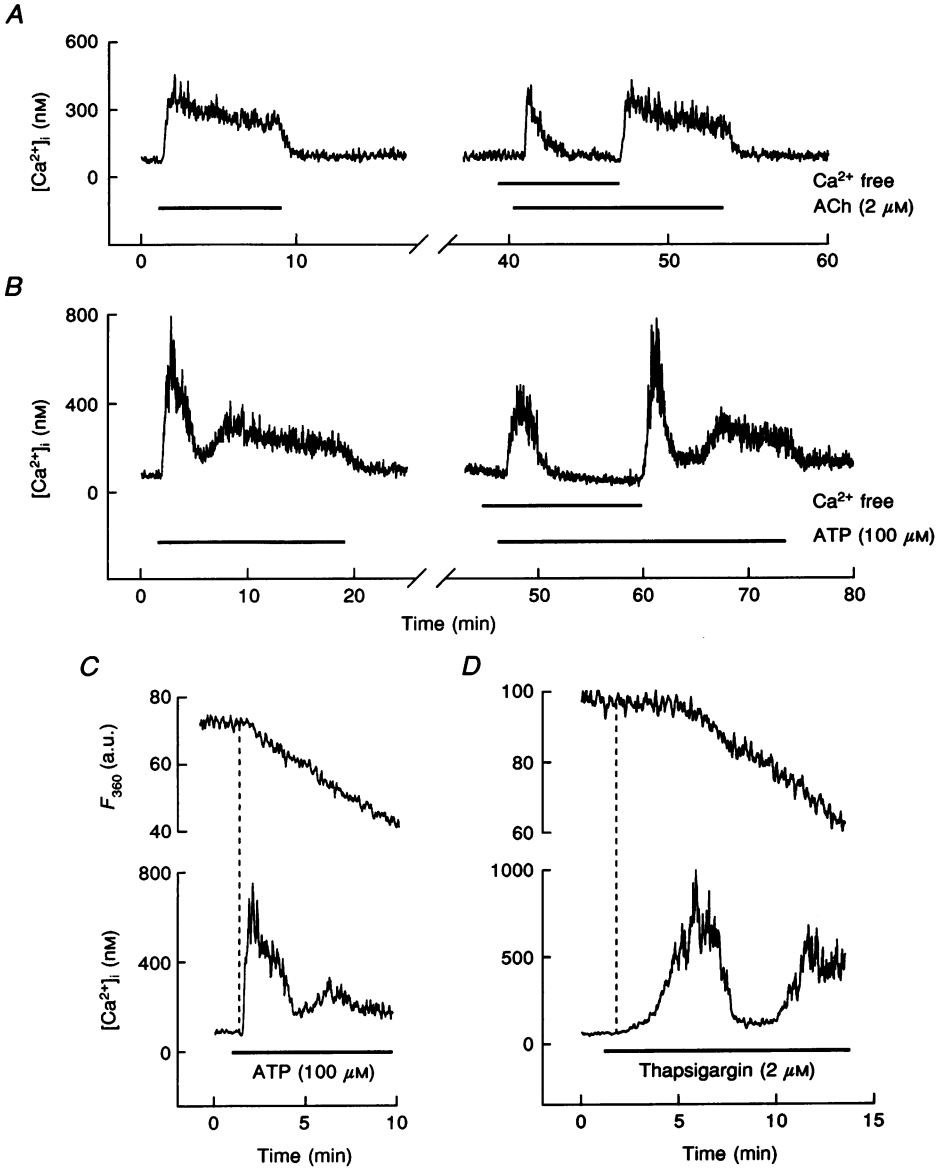

Figure 2. Dependence of ACh-, ATP- and thapsigargin-evoked $[Ca^{2+}]_i$ signals in endothelium of intact rat aorta on external Ca^{2+}

A and *B*, effects of ACh (2 μM; *A*) or ATP (100 μM; *B*) on $[Ca^{2+}]_i$ in control (1 mM Ca^{2+}) or Ca^{2+}-free (0·2 mM EGTA) modified Krebs solution. Duration of agonist application and external Ca^{2+} absence are indicated by the horizontal bars. *C* and *D*, effects of ATP (100 μM; *C*) or thapsigargin (2 μM; *D*) on $[Ca^{2+}]_i$ and fura-2 fluorescence excited at 360 nm (F_{360}) in the presence of 50 μM Mn^{2+}. F_{360} is in arbitrary units (a.u.); a fall indicates Mn^{2+} entry.

ADP (100 μM), like ATP, evoked a prolonged elevation in $[Ca^{2+}]_i$ ($n = 3$), whilst AMP (100 μM) or adenosine (100 μM) were without effect in the same preparations (not shown).

The endothelial responses evoked by ACh and ATP were concentration dependent. With ACh, the concentrations for detectable and maximal effects on $[Ca^{2+}]_i$ were 1–5 nM and 1 μM (EC_{50}, 0·2 μM). With ATP, these concentrations were 1 μM and 100 μM (EC_{50}, 35 μM).

Thapsigargin (2 μM), a specific inhibitor of an endomembrane Ca^{2+}-ATPase (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990) usually (7/9 preparations) evoked a biphasic rise in $[Ca^{2+}]_i$ (Fig. 2*D*). The initial phase was independent of external Ca^{2+} (not shown), whilst later stages were accompanied by divalent cation entry, detectable using Mn^{2+} quench (Fig. 2*D*). Application of ACh or ATP after thapsigargin failed to increase $[Ca^{2+}]_i$ ($n = 4$; not shown).

Dependence of agonist-evoked changes in $[Ca^{2+}]_i$ on membrane potential

Since ACh- and ATP-evoked changes in endothelial $[Ca^{2+}]_i$ were dependent in part on Ca^{2+} entry across the plasma membrane, the responses might be significantly influenced by or dependent on the membrane potential. To explore the correlation between agonist-evoked changes in $[Ca^{2+}]_i$ and

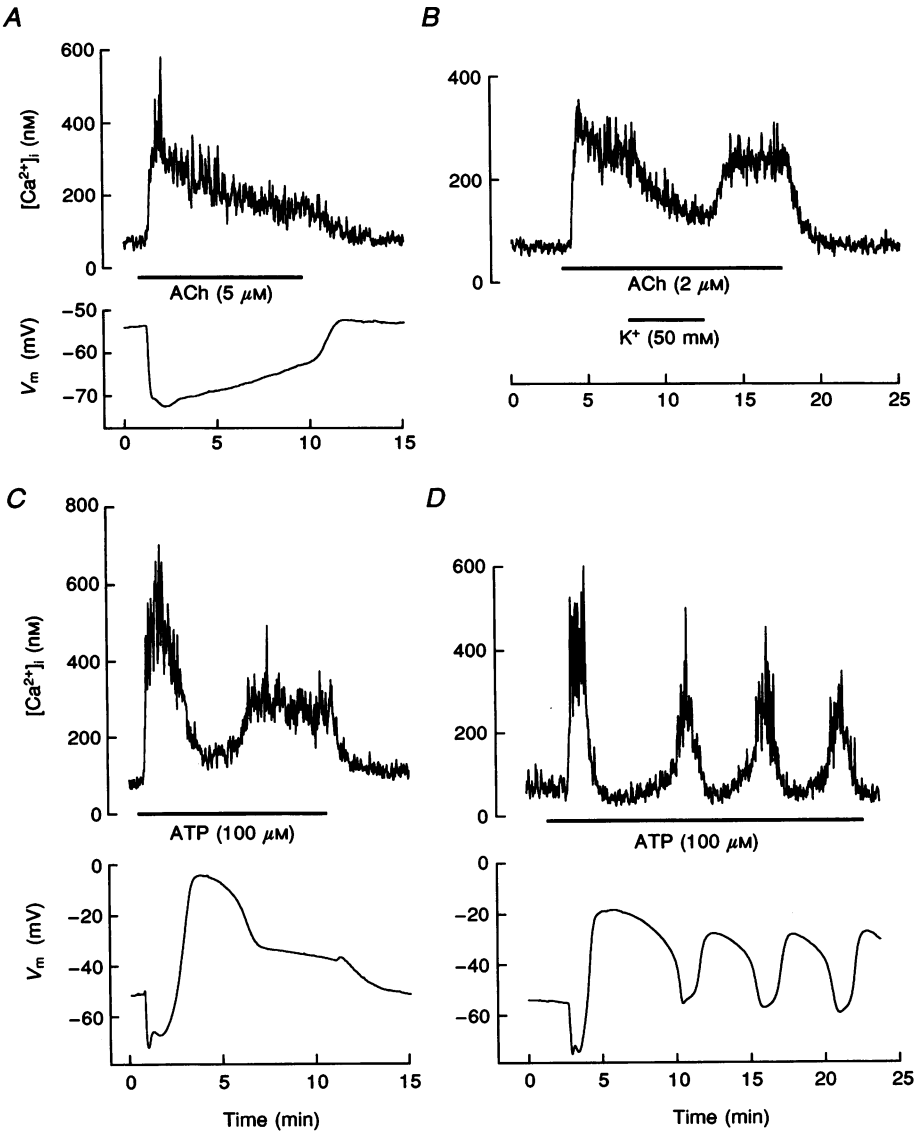

Figure 3. Relationships between membrane potential and $[Ca^{2+}]_i$ in endothelium of intact rat aorta

A, *C* and *D*, simultaneous recordings of $[Ca^{2+}]_i$ and membrane potential (V_m) during application of ACh (5 μM; *A*) or ATP (100 μM; *C* and *D*). *B*, effect of high $[K^+]$ on the $[Ca^{2+}]_i$ response evoked by ACh (2 μM). Agonist and high $[K^+]$ applications are indicated by the horizontal bars.

the complex agonist-evoked changes in membrane potential we have reported previously (Marchenko & Sage, 1993; Marchenko & Sage, 1994*a*), simultaneous recording of both variables was undertaken.

Resting, fura-2-loaded endothelium had a membrane potential of -52 ± 1 mV ($n = 8$), similar to unloaded endothelium in intact rat aorta (Marchenko & Sage, 1993). ACh (5 μM) evoked a rise in $[Ca^{2+}]_i$ accompanied by a membrane hyperpolarization to -71 ± 1 mV ($n = 3$). This was followed by a slowly developing depolarization and gradual decline in $[Ca^{2+}]_i$ (Fig. 3*A*). Depolarizing the membrane using 50 mM K^+ during ACh stimulation decreased $[Ca^{2+}]_i$ (Fig. 3*B*).

As with $[Ca^{2+}]_i$, the membrane potential response evoked by ATP (Fig. 3*C*) was more complicated than that evoked by ACh. The initial large ATP-evoked rise in $[Ca^{2+}]_i$ was accompanied by membrane hyperpolarization to -67 ± 1 mV ($n = 8$), followed by a brief plateau and then rapid depolarization to -17 ± 4 mV ($n = 8$). The depolarization was temporally coincident with the fall in $[Ca^{2+}]_i$ from the

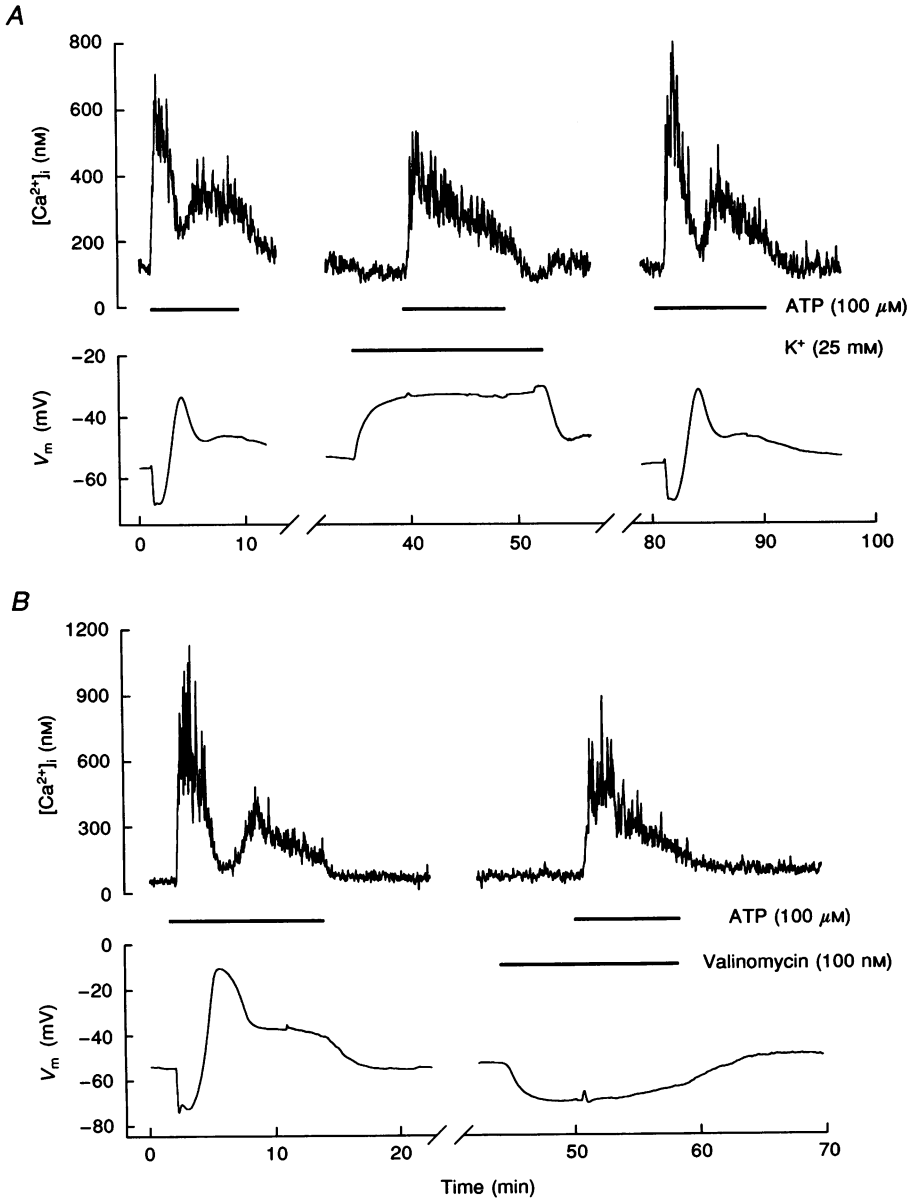

Figure 4. Effects of altering membrane potential on ATP-evoked $[Ca^{2+}]_i$ responses

A, simultaneous recordings of $[Ca^{2+}]_i$ and membrane potential during ATP (100 μM) application under control conditions and with elevated $[K^+]$. *B*, similar recordings of ATP responses under control conditions and in the presence of valinomycin (100 nM).

initial peak, but was maximal slightly earlier (by 34 ± 8 s, $n = 8$) than the minimum value reached by the $[Ca^{2+}]_i$ prior to the secondary rise (Fig. 3*C*). Following the depolarization peak, the membrane hyperpolarized again, reaching a value somewhat higher than the resting potential and continuing to slowly decline. This period of slow decline in membrane potential temporally corresponded to the slow decline in $[Ca^{2+}]_i$ after the secondary peak. Washout of ATP resulted in $[Ca^{2+}]_i$ and membrane potential returning to resting values (Fig. 3*C*).

In preparations in which ATP evoked oscillations in $[Ca^{2+}]_i$, membrane potential also oscillated in temporal correlation, with $[Ca^{2+}]_i$ decreasing as the membrane depolarized and increasing as it hyperpolarized (Fig. 3*D*).

To investigate the dependence of $[Ca^{2+}]_i$ responses on membrane potential changes, we attempted to clamp the membrane potential. Since it is not possible to clamp the membrane potential in thousands of electrically coupled cells using traditional methods, we used the indirect approach of applying high $[K^+]$ or the K^+ ionophore, valinomycin. Application of 25 mM K^+ depolarized the membrane to −28 ± 1 mV ($n = 4$). This new potential was rather stable and almost unaffected by stimulation with ATP (100 μM). Under these conditions, the ATP-evoked rise in $[Ca^{2+}]_i$ was somewhat reduced in magnitude and lacked the secondary rise (Fig. 4*A*, $n = 4$). A typical, biphasic rise in $[Ca^{2+}]_i$ was evoked by ATP after $[K^+]$ was returned to the control value. Valinomycin (100 nM) hyperpolarized the membrane and the membrane potential was little affected by subsequent application of ATP (100 μM). The ATP-evoked rise in $[Ca^{2+}]_i$, like that with high $[K^+]$, was monophasic (Fig. 4*D*, $n = 2$). Removal of valinomycin from the perfusate did not restore a normal ATP-evoked $[Ca^{2+}]_i$ response, presumably because the ionophore was poorly washed out (not shown).

DISCUSSION

In fura-2-loaded rat aorta, ACh evoked a rise in F_{340}/F_{380} whereas high $[K^+]$ or the α_1-adrenoreceptor agonist, phenylephrine, had little or no effect. The action of ACh was abolished by removal of the endothelium. Stimulation of α_1-adrenoreceptors and depolarization with high $[K^+]$ both increase $[Ca^{2+}]_i$ in smooth muscle cells of rat aorta (McDonald, Pelzer, Trautwein & Pelzer, 1994). These data therefore indicate that fura-2 fluorescence was being recorded only from the aortic endothelium. Similarly, endothelial cells but not smooth muscle cells were visible in fura-2-loaded aorta under the fluorescence microscope. Smooth muscle cells isolated from rat aorta have been loaded with fura-2, and $[Ca^{2+}]_i$ successfully recorded in them (e.g. Batille, Godinich, La Pointe, Munoz, Carone & Mehring, 1991). Therefore poor loading with fura-2 seems unlikely to be the cause of the lack of signal from the smooth muscle in intact rat aorta. In fura-2-loaded rat tail artery, ACh, high $[K^+]$ and phenylephrine all increased F_{340}/F_{380}, indicating that fluorescence from endothelial and smooth muscle cells contributed to the recorded signal. Rat aorta has morphology typical of elastic arteries (see Rhodin, 1980). Its media contains significant amounts of extracellular material, elastin and collagen fibres which form a meshwork embedding the smooth muscle cells. Rat tail artery is of muscular type, with less extracellular material. It may be that the extracellular material in the elastic artery (aorta) absorbs or reflects excitation and/or emitted light, allowing selective recording from the endothelium of this vessel type. This method thus provides a relatively simple approach for the selective study of $[Ca^{2+}]_i$ in endothelium *in situ*. Selective measurement of $[Ca^{2+}]_i$ in endothelium of rat arterioles has been reported (Falcone, Kuo & Meininger, 1993), but this required microperfusion to selectively load the endothelium using fura-2 AM and the use of imaging technology.

In resting aortic endothelium, $[Ca^{2+}]_i$ was 95 ± 8 nM, similar to values reported for cultured endothelial cells but almost twice that estimated for endothelium in intact rat cremaster skeletal muscle arterioles (Falcone *et al.* 1993). In aortic endothelium, ACh and ATP evoked rapid rises in $[Ca^{2+}]_i$, the first component of which was largely independent of external Ca^{2+}, indicating release from intracellular stores. Later stages of the responses were affected by removal of external Ca^{2+}, indicating agonist-evoked Ca^{2+} entry which was confirmed using the Mn^{2+} quench technique.

The ACh-evoked rise in $[Ca^{2+}]_i$ was mimicked by muscarine and was inhibited by atropine, indicating activation of muscarinic cholinergic receptors. The effect of ATP on $[Ca^{2+}]_i$ was mimicked by ADP but not AMP or adenosine, which suggests activation of P_2-purinergic receptors.

Changes in $[Ca^{2+}]_i$ evoked by ACh (and a minority evoked by ATP) were simple monophasic responses, similar to those reported to be evoked by other agonists in cultured endothelial cells (e.g. Gosink & Forsberg, 1993). The ACh-evoked rise in $[Ca^{2+}]_i$ in aortic endothelium was similar in form to that reported in endothelium of intact rat arterioles, although the latter responses were only a third of the amplitude of those in aortic endothelium (Falcone *et al.* 1993). In a majority of aortic preparations, ATP evoked more complex changes in $[Ca^{2+}]_i$, with an initial large peak being followed by a fall towards basal levels and then a secondary, better sustained rise. In some preparations, the decline in $[Ca^{2+}]_i$ after the first peak reached values below the initial resting level ($n = 5$). These results contrast with the monophasic ATP-evoked $[Ca^{2+}]_i$ rises observed in cultured endothelium (e.g. Gosink & Forsberg, 1993).

Our previous work has shown that the initial agonist-evoked hyperpolarization is likely to be due to a rise in $[Ca^{2+}]_i$ acting on a Ca^{2+}-activated K^+ conductance (Marchenko & Sage, 1993, 1994*a*, *b*). Simultaneous recording of $[Ca^{2+}]_i$ and membrane potential showed that the agonist-evoked hyperpolarization of the endothelium was indeed always accompanied by a rise in $[Ca^{2+}]_i$ sufficient to activate the Ca^{2+}-activated K^+ channels that have been identified in the endothelium of intact rat aorta (Marchenko & Sage, 1994*b*).

Agonist-evoked electrical responses of rat aortic endothelium in many preparations were biphasic and included a large depolarization phase. Simultaneous recordings revealed that the depolarization attains values positive to the resting potential, yet $[Ca^{2+}]_i$ remains above its resting level. This depolarization thus cannot be explained by effects on Ca^{2+}-activated K^+ channels. We have shown previously that agonist-evoked depolarization depends absolutely on Ca^{2+} entry and can be reduced or abolished by increasing $[Ca^{2+}]_i$ buffering using BAPTA (Marchenko & Sage, 1993, 1994*a*). These data suggest that the rise in $[Ca^{2+}]_i$ is involved in the development of depolarization, but in contrast to the agonist-evoked hyperpolarization, it affects an as yet unknown membrane conductance.

Simultaneous recording of $[Ca^{2+}]_i$ and membrane potential revealed that biphasic $[Ca^{2+}]_i$ responses were only evoked by ATP in preparations which also showed biphasic electrical responses. The depolarization reached its maximum about 30 s before $[Ca^{2+}]_i$ reached its minimum. Depolarization of the endothelium with high $[K^+]$ strongly reduced $[Ca^{2+}]_i$ after its elevation by stimulation with ACh or ATP. These observations are compatible with the idea that the changes in membrane potential influence the Ca^{2+} entry-dependent component of the ATP-evoked Ca^{2+} signal. In particular, the decline in $[Ca^{2+}]_i$ after the initial ATP-evoked peak is probably a result of a reduced driving force for Ca^{2+} entry during the depolarization phase, whereas the secondary rise in $[Ca^{2+}]_i$ may be due to an increased driving force for Ca^{2+} entry as the membrane potential recovers towards the resting level. Support for these hypotheses comes from experiments where membrane potential changes were minimized. In high $[K^+]$ (Marchenko & Sage, 1993) or in the presence of the K^+ ionophore valinomycin, the endothelial membrane potential was close to the K^+ equilibrium potential and ATP-evoked electrical responses were essentially abolished. Under these conditions, ATP evoked only monophasic rises in $[Ca^{2+}]_i$, in contrast to the biphasic responses seen in controls.

In 20% of preparations, ATP evoked oscillations in $[Ca^{2+}]_i$. Since the fluorescent signal was collected from an area containing hundreds of cells, these results indicate that synchronized oscillations in $[Ca^{2+}]_i$ can occur in native vessel endothelium *in situ*, as previously reported for cultured endothelium (Sage, Adams & Van Breemen, 1989). These observations indicate that endothelial functions may be synchronized over large areas. Synchronized oscillations in $[Ca^{2+}]_i$ have also been observed in rabbit cardiac valve endothelium *in situ* (Laskey, Adams & Van Breemen, 1994). However, the oscillations in cardiac endothelium were only observed under unphysiological, K^+-free conditions, and synchrony was confined to small clusters of 6–8 cells. The mechanism of the more extensive synchronization of oscillations in $[Ca^{2+}]_i$ in rat aortic endothelium and the rate and extent of propagation of signals within the endothelium are unknown and require further work.

In this study, ACh did not evoke synchronized oscillations in $[Ca^{2+}]_i$ and the rises in $[Ca^{2+}]_i$ recorded from populations of aortic endothelial cells were always monophasic. We have previously reported that ACh-evoked electrical responses, although the same in different samples from one aorta, vary between aortas and that only some exhibit ACh-evoked oscillations in membrane potential (Marchenko & Sage, 1993). The reason for this variation between animals is not clear, but may explain the absence of oscillatory ACh-evoked responses here. It seems unlikely that the failure of ACh to evoke oscillations in this study relates to calcium buffering effects of fura-2 loading. ACh did not evoke oscillatory electrical responses in control, unloaded, vessel samples taken from the same animals (data not shown) and ATP was able to evoke oscillations. In furaptra-loaded endothelium of intact rat aorta, ACh has been reported to evoke repetitive spikes in single endothelial cells, but it is not clear if synchronized responses were occurring in other cells (Carter & Ogden, 1994). In the experiments reported here we cannot say whether ACh failed to evoke spiking in $[Ca^{2+}]_i$ or whether such responses failed to synchronize between sufficient numbers of cells to be detected since we have no single cell data. In rabbit cardiac endothelium, synchrony of ACh-evoked oscillations in $[Ca^{2+}]_i$ was limited to just a few cells (Laskey *et al.* 1994).

Simultaneous recordings indicated that aortic endothelial membrane potential oscillated in antiphase with oscillations in $[Ca^{2+}]_i$ evoked by ATP. Oscillations in $[Ca^{2+}]_i$ were not observed when the membrane potential was stabilized using high $[K^+]$. These observations suggest that the oscillations may result from interplay between $[Ca^{2+}]_i$ and membrane potential, with changes in $[Ca^{2+}]_i$ leading to changes in membrane potential which in turn alter $[Ca^{2+}]_i$ due to a change in driving force for Ca^{2+} entry. Support for a membrane-based oscillatory mechanism comes from the observations that both agonist-evoked oscillations in $[Ca^{2+}]_i$ in cultured endothelium (Sage *et al.* 1989; Laskey, Adams, Cannell & Van Breemen, 1992) and in membrane potential in native endothelium (Marchenko & Sage, 1993) are absolutely dependent on entry of extracellular Ca^{2+}.

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Author's present address

Y. M. Usachev: A. A. Bogomoletz Institute of Physiology, 4 Bogomoletz Street, Kiev 024, Ukraine.

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