Exposure of endothelial cells to cyclic strain induces elevations of cytosolic Ca^{2+} concentration through mobilization of intracellular and extracellular pools

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We have previously reported that exposure of endothelial cells to cyclic strain elicited a rapid but transient generation of inositol 1,4,5-trisphosphate (IP_3), which reached a peak 10 s after the initiation of cyclic deformation. To address the effect of cyclic strain on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and its temporal relationship to IP_3 generation, confluent bovine aortic endothelial cells were grown on flexible membranes, loaded with aequorin and the membranes placed in a custom-designed flow-through chamber. The chamber was housed inside a photomultiplier tube, and vacuum was utilized to deform the membranes. Our results indicate that the initiation of 10% average strain induced a rapid increase in $[Ca^{2+}]_i$ which contained two distinct components: a large initial peak 12 s after the initiation of stretch which closely followed the IP_3 peak, and a subsequent lower but sustained phase. Pretreatment with 5 μM $GdCl_3$ for 10 min or nominally Ca^{2+}-free medium (CFM) for 3 min reduced the magnitude of the initial rise and abolished the sustained phase. Repetitive 10% average strain at a frequency of 60 cycles/min also elicited a single IP_3 peak at 10 s. However, there was also a large initial $[Ca^{2+}]_i$ peak followed by multiple smaller transient $[Ca^{2+}]_i$ elevations. Preincubation with 5 μM $GdCl_3$ or CFM diminished the initial $[Ca^{2+}]_i$ transient and markedly inhibited the late-phase component. Preincubation with 25 μM 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ) attenuated the initial $[Ca^{2+}]_i$ transient. Cyclic-strain-mediated IP_3 formation in confluent endothelial cells at 10 s, however, was not modified by pretreatment with 25 μM BHQ, 500 μM $NiCl_2$, 10 nM charybdotoxin, 5 μM $GdCl_3$ or CFM. We conclude that in endothelial cells exposed to cyclic strain, Ca^{2+} enters the cytosol from intracellular and extracellular pools but IP_3 formation is not dependent on Ca^{2+} entry via the plasma membrane.

INTRODUCTION

Endothelial cells (ECs) *in vivo* are continuously subjected to physical forces induced by the rhythmic contraction of the heart. These include shear stress from the flowing blood and wall deformation and stress, in three dimensions during systole and diastole [1]. In an effort to characterize the response of vascular cells to the physical forces exerted by the circulation, our laboratory has utilized a device that can apply different regimens of cyclic strain on attached monolayers of cultured vascular ECs [2–5]. This strain may be analogous to the pulsatile distension of the vessel wall. We have previously demonstrated that exposure of ECs *in vitro* to cyclic strain elicits a number of responses, including alterations in growth [5–7], morphology [8–10] and the secretory capacity of prostacyclin [11], tissue plasminogen activator [12], endothelin [13] and NO [14,15]. However, the mechanism by which ECs sense and react to physical forces remains unknown and is the subject of intense investigation. Lansman et al. [16] have described a single stretch-activated non-selective cation channel in porcine aortic ECs and have suggested that these channels could transduce the effects of mechanical forces of the circulation on vascular cells. Although other investigators have previously been unable to demonstrate the existence of these channels in other cell types [17] or detect their presence in cultured ECs derived from bovine pulmonary artery or rabbit aorta [18], recent reports have indicated that mechanical perturbation of human umbilical vein or bovine aortic ECs leads to alterations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [19–21].

Our laboratory has previously demonstrated activation of the adenylate cyclase pathway in vascular cells exposed to repetitive deformation [22,23]. We have also recently reported that either the initiation of strain or an acute change in cyclic strain frequency induced in ECs a transient generation of inositol 1,4,5-trisphosphate (IP_3) and its metabolites, inositol bisphosphate and monophosphate, the sustained production of diacylglycerol [24,25] and the translocation of protein kinase C [26]. Because in a great variety of cells the production of IP_3 elicits the release of Ca^{2+} from the endoplasmic reticulum, our studies suggested that $[Ca^{2+}]_i$ would also be altered in our system and could act as a potential transducer of the response of ECs to cyclic strain.

In view of the controversy about the existence of plasma-membrane stretch-activated Ca^{2+} channels in ECs and our data documenting the potential activation of the IP_3-release channel by cyclic strain, we sought to document the effect of dynamic strain on $[Ca^{2+}]_i$ and to characterize the relationship between $[Ca^{2+}]_i$ and IP_3 formation in ECs subjected to mechanical

Abbreviations used: EC, endothelial cell; $[Ca^{2+}]_i$, intracellular Ca^{2+}; IP_3, inositol 1,4,5-trisphosphate; BHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone; CFM, Ca^{2+}-free medium; DMEM, Dulbecco's modified Eagle's medium.

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deformation. To measure $[Ca^{2+}]_i$ in ECs subjected to cyclic strain, the photoprotein aequorin was used as an indicator of $[Ca^{2+}]_i$ and the strain apparatus was modified to permit the application of cyclic strain with real-time measurement of $[Ca^{2+}]_i$.

The results of the present study demonstrate that exposure of ECs to cyclic strain elicits a biphasic rise in $[Ca^{2+}]_i$ but only a transient single rise in IP_3 formation. The similar kinetics of the early rise in $[Ca^{2+}]_i$ and the monophasic rise in IP_3 and the attenuation of the initial $[Ca^{2+}]_i$ peak with 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ) suggest that mobilization of Ca^{2+} from intracellular stores is an important component of the initial large increase in $[Ca^{2+}]_i$ in ECs subjected to cyclic strain. In contrast, the late sustained rise in $[Ca^{2+}]_i$ appears to be predominantly supported by Ca^{2+} influx from the extracellular fluid, since this phase could be abolished by $GdCl_3$ or nominally Ca^{2+}-free medium (CFM). These results raise the possibility that the growth and secretory responses of ECs to cyclic strain *in vivo* may be dependent on internal as well as external pools of Ca^{2+}.

MATERIALS AND METHODS

EC culture

ECs were harvested as previously described [5–7,27], by mechanical scraping of the intima of calf thoracic aorta. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% heat-inactivated calf serum, 5 μg/ml deoxycytidine, 5 μg/ml thymidine, 1% penicillin/streptomycin and Fungizone, and grown to confluence at 37 °C in a humidified 5% CO_2 incubator. Cells from passages 2–10 were used. ECs were identified by their typical morphology, positive uptake of di-I-acetylated low-density lipoprotein and positive staining for Factor VIII antigen.

In vitro application of stress to cultured cells

We utilized the Flexercell strain unit (Flexcell Corp., McKeesport, PA, U.S.A.), which we have previously described and characterized [2–5]. Cells are grown on culture plates with flexible silastic bottoms coated with collagen type I (Flex I plate; Flexcell) which can be deformed to a known percentage elongation by applying a precise vacuum level. Release of the vacuum returns the plate bottoms to their original conformation. Vacuum is regulated by solenoid valves that are controlled by a computer program. Thus the magnitude, duration and frequency of the applied force can be varied. Force analysis of the strain that was exerted on the flex plate (attained by various vacuum levels) was determined mathematically, by finite element analysis [2–4], and empirically by measuring the distance between (radial strain), or the diameter (axial strain) of, concentric circles marked on the membrane. The strain is biaxial, but very little change is observed in the axial direction, and the force on the attached cells is predominantly radial.

Experimental protocol

ECs were grown to confluence on 25 mm-well Flex I plates and maintained in specific media for each assay as described below. For $[Ca^{2+}]_i$ and IP_3 measurements, the membranes were deformed at 60 cycles/min (i.e. 0.5 s elongation alternating with 0.5 s relaxation) with 150 mmHg vacuum for zero (stationary control), one or up to 500 cycles at room temperature. This vacuum deformation results in 10% average strain on the membrane, which is translated to the attached cells. The unit was programmed to stop the vacuum deformation of the membrane bottoms at predetermined time points. For IP_3 and $[Ca^{2+}]_i$ measurements, we used Krebs–Ringer bicarbonate buffer, pH 7.2, containing 120 mM NaCl, 25 mM $NaHCO_3$, 3.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM sodium biphosphate, 5.6 mM dextrose, 1.25 mM $CaCl_2$ and 2 mg of BSA/ml. $CaCl_2$ was omitted in nominally CFM. Neither DMEM/F-12 nor Krebs–Ringer bicarbonate contained ATP, an agonist known to trigger receptor-mediated IP_3 activation [28,29].

To provide a comparison for the magnitude and character of the cyclic-strain-induced responses, the effect on $[Ca^{2+}]_i$ of the addition of 1 μM bradykinin to stationary ECs was also determined.

Aequorin loading and $[Ca^{2+}]_i$ measurements

Confluent ECs in six-well Flex plates were placed in serum-free DMEM/F12 for 48 h before the experiment. On the day of the experiment, cultured ECs were loaded with the Ca^{2+}-sensitive probe aequorin (Friday Harbor Photoproteins, Friday Harbor, WA, U.S.A.), and measurements were performed as described in detail elsewhere [30,31]. In brief, ECs were washed twice in CFM and then successively incubated in three solutions: (1) 120 mM potassium glutamate, 20 mM TES, 10 mM EGTA, 5 mM sodium ATP and 2 mM $MgCl_2$ at pH 7.1 for 20 min at 4 °C, to effect permeabilization; (2) 120 mM potassium glutamate, 20 mM TES, 0.1 mM EGTA, 5 mM sodium ATP, 2 mM $MgCl_2$ and 200 μg/ml aequorin at pH 7.1 for 30 min at 4 °C; (3) Krebs–Ringer bicarbonate Ca^{2+}-free buffer for 1 h. The extracellular Ca^{2+} concentration was restored to 1.25 mM incrementally over a 60 min period. More than 95% of ECs remained attached to the plates during incubation in the CFM and were viable at the end of the loading procedure, as assessed by Trypan Blue exclusion. The morphology of the ECs also remained unchanged. Aequorin-loaded ECs cultured on flexible membranes were clamped into a flow-through chamber and inserted into the photometer (Figure 1).

ECs were subjected to the strain protocols described above. In some experiments, ECs were pretreated with 500 μM $NiCl_2$ (an inhibitor of Ca^{2+} influx), 5–10 μM $GdCl_3$ (a non-selective cationic blocker of activated Ca^{2+} channels [31,32]), 25 μM BHQ (a depleter of the IP_3-sensitive Ca^{2+} pool [33]) or 10 nM charybdotoxin [a peptide toxin derived from the venom of the scorpion *Leiurus quinquestriatus* known to be a non-selective K^+ channel blocker at the applied dose (10 nM)] for 10 min or nominally CFM for 3 min. The aequorin-loaded ECs were subjected to only short durations of experimentation in order to minimize any potential long-term effects of aequorin on EC physiology. Emitted light from cells on the entire membrane was collected by ellipsoidal mirrors surrounding the flexible membrane and reflected on to a photomultiplier tube. Photon counts were accrued, filtered and amplified via a discriminator-analyser unit (Thorn–EMI) and averaged every second using a photon-counting board (Thorn–EMI) based in a 386 PC. In contrast with the Ca^{2+} dyes (fura 2, indo 1), which measure Ca^{2+} by monitoring a change in the fluorescence ratio at 340/380 nm, aequorin measures Ca^{2+} by monitoring the emission of blue–green light (470 nm) secondary to the peroxidation of the coelenterazine lumophore tightly bound to the apoprotein [34]. Thus the $[Ca^{2+}]_i$ measurement is not influenced by changes in light scattering induced by membrane deformation or attenuated by the obstruction of the light path by the presence of the semi-opaque membranes.

One limitation of the use of aequorin is that the light output is difficult to standardize. The amount of light emitted is dependent not only on the aequorin used but the loading conditions and the subsequent amplification by the photomultiplier tubes. Hence the experimental design was such that a

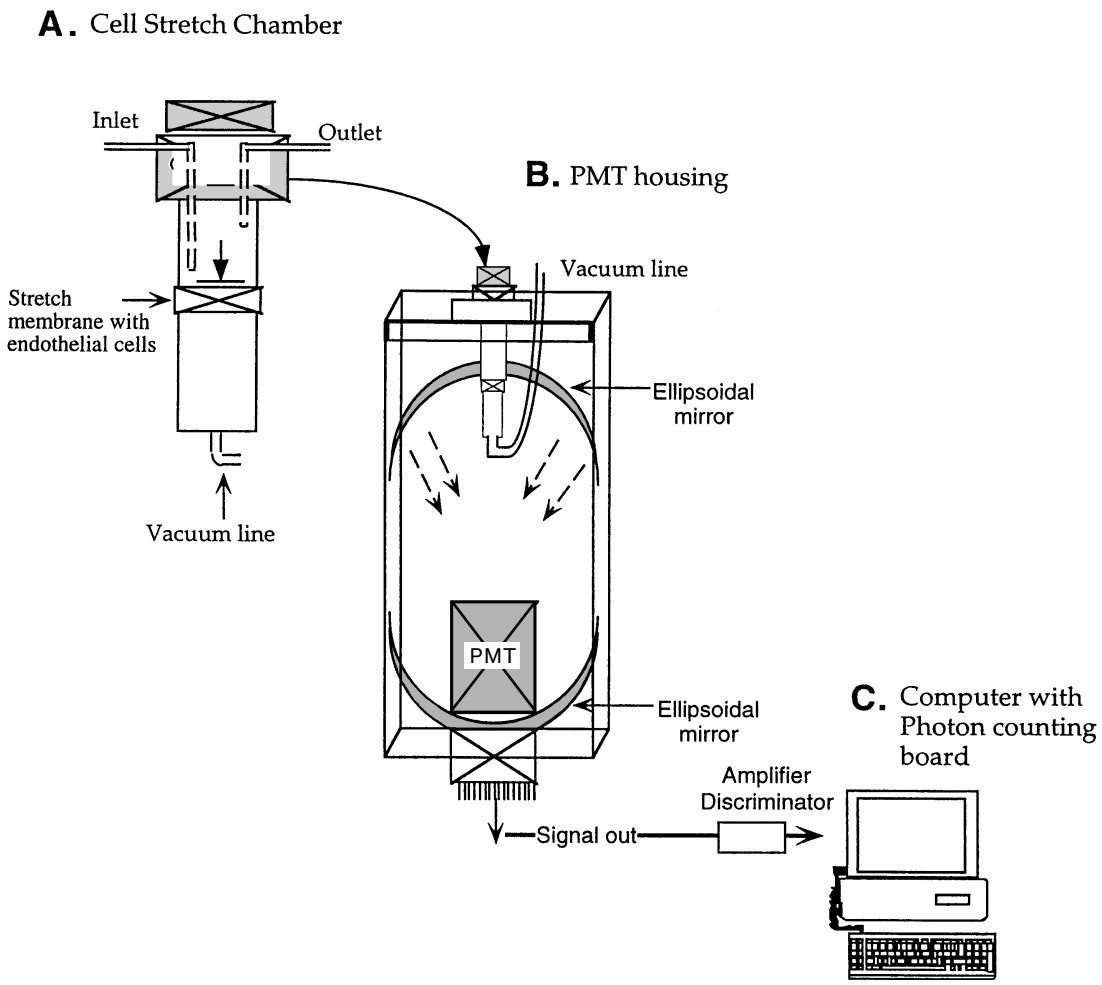

Figure 1 Schematic of the stress unit used to measure $[Ca^{2+}]_i$ in bovine aortic EC monolayers subjected to cyclic strain

ECs were cultured on deformable membrane bottoms and loaded with aequorin on the day of the experiment. The membranes were then placed on the custom-designed flow-through chamber with a vacuum line (**A**) inside the photomultiplier tube (PMT) housing (**B**). When a precise vacuum level was applied to the system, the culture plate bottoms were deformed to a known percentage of elongation, which was translated to the cultured cells. The light emitted from aequorin-loaded cells was collected by ellipsoidal mirrors surrounding the stretch membrane and reflected on to the photomultiplier tube through an amplifier/discriminator and averaged every second via a photon-counting board in a 386-based PC (**C**).

series of experiments was performed on the same cells and the experiments repeated at least four times with different cell lines and passage levels.

One theoretical concern is whether intracellular aequorin can be released during cyclic strain and produce a large luminescence signal. Since it is difficult to measure aequorin release directly, we performed preliminary experiments in which a radioactive label of similar size to aequorin ([^{14}C-*methyl*]cytochrome *c*; 13 kDa; New England Nuclear, Boston, MA, U.S.A.) was loaded into ECs using the same loading protocol as above, then subjected to zero, one or 500 cycles of strain. There was no detectable trichloroacetate-precipitable radioactivity in the medium (75.6 ± 4.1, 76.7 ± 7.0, 77.0 ± 6.4 c.p.m. respectively; means $\pm$ S.D.) and no significant difference in the amount of trichloroacetate-precipitable radioactivity in the cell lysates ($19\,500 \pm 2500$, $20\,860 \pm 2070$, $20\,330 \pm 2030$ c.p.m. respectively), indicating no release of protein with the different cycles of strain.

Another concern is the possibility that aequorin may become depleted to some extent during the experiments and this may affect the reproducibility of the measurements as well as the ability of the system to accurately signal genuine sustained increases in Ca^{2+}. However, additional experiments in which ECs were exposed to the Ca^{2+} ionophore A23187 (100 nM) for 75 min demonstrated the ability to elicit a maximum light output with Triton X-100 lysis (results not shown). Thus aequorin is not expended and subsequent stimulation does not result in a diminished light output.

RIA for IP_3

IP_3 measurements were performed as previously described [25]. Confluent ECs in six-well Flex I plates were placed in serum-free DMEM/F12 medium for 48 h before the experiment. On the day of the experiment, culture medium was replaced with Krebs–Ringer bicarbonate (with or without Ca^{2+}) and cells were subjected to the cyclic strain protocol described above. The cyclic regimen was computer-terminated at the predetermined cycle, and the response stopped by buffer aspiration and the immediate addition of 1 ml of cold 15% trichloroacetic acid. Plates were chilled (0 °C; 30 min), and the trichloroacetate-soluble extract was removed. Extracts were washed three times with ethyl ether, adjusted to pH 7.4 with $NaHCO_3$, and subjected to RIA for IP_3

utilizing a commercially available kit (TRK 1000; Amersham, Arlington Heights, IL, U.S.A.). Our previous report demonstrated that cyclic-strain-mediated IP_3 production reached a peak after a single deformation of the membrane [25]. For technical reasons, however, the earliest time point at which we could be confident in halting the IP_3 reaction was 10 s after the cessation of the vacuum deformation. Therefore this delay was timed equally for each experiment.

Reagents

$NiCl_2$ was obtained from Sigma, St. Louis, MO, U.S.A., $GdCl_3$ from Aldrich Chemical and BHQ from EGA Chemie (Steinheim, Germany). Charybdotoxin was a gift from Dr. Howard Lippton (Louisiana State University, New Orleans, LA, U.S.A.).

Statistical analysis

All experiments were replicated at least four times. $[Ca^{2+}]_i$ (light output) is expressed in units of luminescence (photons/s) and standardized to the initial baseline level of aequorin luminescence, i.e. before stimulation. The absolute values can vary with each experiment since it is dependent on the specific aequorin used, the loading conditions and the efficiency of the photomultiplier tube. IP_3 mass levels were expressed as pmol/10^6 cells (means $\pm$ S.D.). Results were analysed by two-way analysis of variance, Bonferroni-Dunn *post hoc* testing (SuperANOVA, Abacus, Berkeley, CA, U.S.A.). $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

In the presence of 1.25 mM extracellular Ca^{2+}, the initiation of sustained 10% average strain elicited a biphasic increase in EC $[Ca^{2+}]_i$. This response was characterized by two distinct phases: a rapid initial rise in $[Ca^{2+}]_i$, which reached a peak at about 12 s after the initiation of cyclic strain and closely followed in time the peak of IP_3 formation [25], and a submaximal but sustained phase (Figure 2, left, baseline to peak ratio of four experiments was 7.1 ± 3.3). Because of the similar temporal-dependence of the early $[Ca^{2+}]_i$ and IP_3 transients, a major component of the initial large increase in $[Ca^{2+}]_i$ in ECs subjected to cyclic strain appeared to be Ca^{2+} mobilized from intracellular stores. This hypothesis was confirmed by manoeuvres that reduced the contribution of Ca^{2+} influx to the measured rise in $[Ca^{2+}]_i$. Incubation in nominally CFM or a 10 min pretreatment with 5 or 10 μM $GdCl_3$, a Ca^{2+}-channel antagonist that reduces Ca^{2+} entry from the extracellular medium, resulted in preservation of the early $[Ca^{2+}]_i$ transient induced by strain, although the observed rise in $[Ca^{2+}]_i$ was smaller than that obtained in the presence of 1.25 mM extracellular Ca^{2+}. However, the most striking change observed in the $[Ca^{2+}]_i$ response after either pretreatment was the marked attenuation of the sustained rise in $[Ca^{2+}]_i$ induced by stretch (Figure 2, right; baseline to peak ratio of four experiments was 3.3 ± 1.1).

Repetitive deformation of ECs at 60 cycles/min (i.e. one cycle = 1 s) with an average strain of 10% elicited a large initial peak in $[Ca^{2+}]_i$ followed by multiple elevations of lesser magnitude. $[Ca^{2+}]_i$ remained above baseline levels throughout the stretch protocol (Figure 3A). Preincubation with 5 or 10 μM $GdCl_3$ for 10 min or nominally CFM for 3 min also resulted in a diminished initial rise in $[Ca^{2+}]_i$ and marked inhibition of the sustained component (Figure 3B). This pattern was also observed in experiments in which strain at a frequency of 100 cycles/min was exerted on ECs (results not shown).

To determine the role of Ca^{2+} mobilization from internal stores as a source for the initial elevation of $[Ca^{2+}]_i$ after the initiation of cyclic strain, ECs were preincubated with BHQ. Figure 4(A) confirms that bovine aortic ECs exposed to 1 μM bradykinin had a large early $[Ca^{2+}]_i$ transient followed by a sustained level (baseline to peak ratio was 5.5 ± 1.3; $n = 5$). As expected incubation with 25 μM BHQ markedly attenuated this early spike (Figure 4B; baseline to peak ratio was 1.2 ± 0.2; $n = 3$). Figure 5(A) shows that the addition of 1 μM bradykinin to ECs that were undergoing repetitive deformation did not result in any further increases in $[Ca^{2+}]_i$ (baseline to peak ratio was 3.2 ± 2.0, $n = 4$). The multiple small spikes seen in Figure 3(A) are also present but less evident in Figure 5(A) because of the expanded time scale. Preincubation with 25 μM BHQ blunted the initial $[Ca^{2+}]_i$ spike but did not seem to affect the sustained

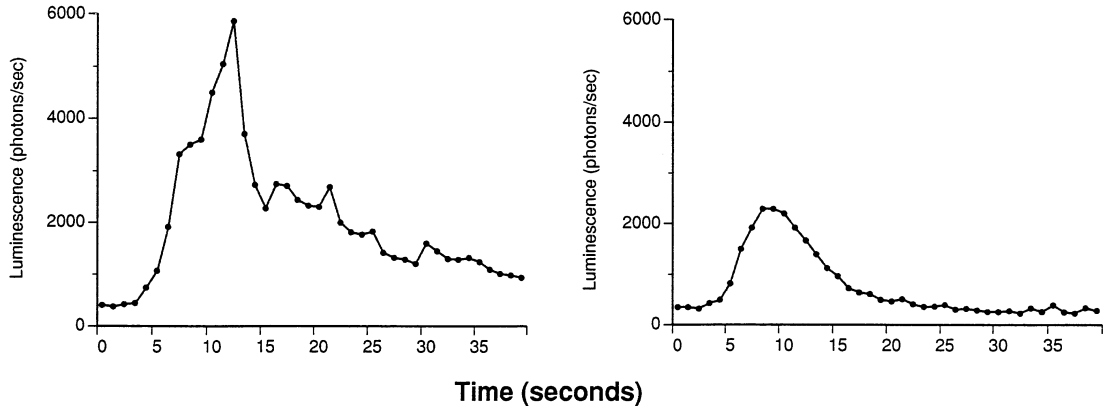

Figure 2 Effect of 10% average strain on $[Ca^{2+}]_i$ in confluent ECs

Left, in the presence of 1.25 mM extracellular Ca^{2+}, the initiation of 10% average strain induced a biphasic increase in $[Ca^{2+}]_i$. A representative trace is shown which depicts the large initial transient elevation which reached a peak at about 12 s after the initiation of strain and a lower, more sustained component. Right, the effect of 5 or 10 μM $GdCl_3$ on $[Ca^{2+}]_i$ in ECs subjected to the same strain protocol is shown. Pretreatment with $GdCl_3$ for 10 min diminished the magnitude of the initial rise and abolished the sustained phase. Incubation in nominal CFM for 3 min resulted in a similar monophasic response to strain (results not shown). $[Ca^{2+}]_i$ in aequorin-loaded bovine aortic ECs is expressed as luminescence in photons/s. The traces are from a representative experiment. The experiments were repeated at least four times with similar results.

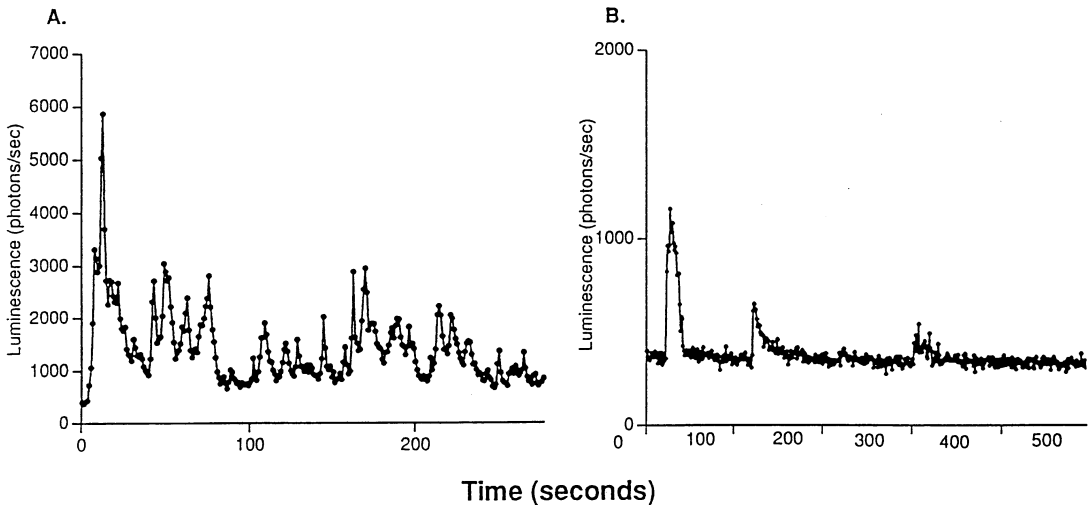

Figure 3 Effect of repetitive deformation of confluent bovine aortic ECs on $[Ca^{2+}]_i$

(**A**) Repetitive deformation at 60 cycles/min with a maximum strain of 24% elicited a large initial peak in $[Ca^{2+}]_i$ followed by multiple elevations of lesser magnitude. Note that $[Ca^{2+}]_i$ remained above baseline levels throughout the strain protocol. (**B**) Preincubation with 5 or 10 μM $GdCl_3$ for 10 min or nominal CFM significantly reduced the magnitude of the early transient and abrogated the sustained phase response. $[Ca^{2+}]_i$ is expressed as luminescence in photons/s. The traces are from a representative experiment. The experiments were repeated at least four times with similar results.

rise in $[Ca^{2+}]_i$ induced by repetitive stretch (Figure 5B; baseline to peak ratio was 1.2 ± 0.1; $n = 4$).

Our results are in agreement with recent reports that confirmed that the sustained component of $[Ca^{2+}]_i$ in ECs after pretreatment with bradykinin was completely dependent on influx of extracellular Ca^{2+}, as it was abolished on removal of external Ca^{2+} or by pretreatment with inhibitors of Ca^{2+} influx, such as Ni^{2+} and La^{3+} [35–40]. It is also widely known that cells kept in CFM (i.e. in the presence of EGTA) will relatively rapidly become depleted of their intracellular Ca^{2+} stores [41]. In the studies presented here, the experiments were performed in nominally CFM (without added EGTA) to which cells were exposed for 3 min. On the basis of preliminary experiments, we had determined that cells had to be maintained in CFM for at least 15 min to significantly attenuate a bradykinin-induced elevation in intracellular Ca^{2+}. Thus we believe that, although there may be a small component of attenuation of the Ca^{2+} transient from depletion of intracellular Ca^{2+} stores, the main cause for attenuation of the Ca^{2+} transient is inhibition of Ca^{2+} influx and that this is also responsible for the disappearance of any sustained component in the elevation of intracellular Ca^{2+}. These data are consistent with data reported by others using bradykinin in ECs [35,42].

We have previously reported that the initiation of cyclic strain stimulated a transient monophasic accumulation of immunoreactive IP_3 in ECs. Peak levels occurred 10 s after the initiation of strain and returned to control values 25 s later. IP_3 levels remained low up to 110 s after the initiation of strain [25]. Subsequent experiments have confirmed the monophasic nature of the rise in IP_3 with strain protocols extending up to 500 s (O. R. Rosales, C. M. Isales, P. Q. Barrett, C. Brophy and B. E. Sumpio, unpublished work). To examine the effect of extracellular Ca^{2+} on IP_3 levels, ECs were pretreated with Ca^{2+}-influx antagonists and subsequently subjected to cyclic strain (Table 1). In pilot experiments, these agents had no effect on IP_3 production in the absence of strain. $NiCl_2$ (500 μM) for 10 min induced a small, but non-significant reduction in IP_3 levels measured at 10 s after the initiation of cyclic strain. Pretreatment with 5–10 μM $GdCl_3$ for 10 min failed to reduce the IP_3 response to cyclic strain. Similarly, incubation in nominally CFM for 3 min had no effect on IP_3 production in control (stationary) ECs and ECs subjected to cyclic strain. Although ECs are known to lack voltage-dependent Ca^{2+} channels [42], perturbations that affect membrane potential should change the driving force for Ca^{2+} entry and thus alter influx through stretch- or receptor-activated Ca^{2+} channels. Incubation with charybdotoxin for 10 min, which would be expected to reduce the driving force for Ca^{2+} entry, also failed to significantly modify the production of IP_3 elicited by cyclic strain.

Table 2 demonstrates the lack of dependence of IP_3 production by ECs on external Ca^{2+}. In the presence of Ca^{2+} in the culture medium (1.25 mM), there was a twofold increase in total IP_3 production by ECs on stimulation with 100 nM A23187 or 1 μM bradykinin. However, in CFM, IP_3 increased on exposure of ECs to bradykinin but not to A23187. Depletion of IP_3-sensitive Ca^{2+} pools (by either agonist addition or thapsigargin) stimulates Ca^{2+} influx through the plasma membrane [43]. Thus it is likely that initiation of stretch, with the subsequent increase in IP_3 and mobilization of Ca^{2+} from intracellular stores, leads to some capacitative Ca^{2+} influx. This would not appear to be a major component, however, because in the experiments presented, the Ca^{2+}-influx component is inhibited by $GdCl_3$ (which is not thought to affect capacitative Ca^{2+} entry).

Taken together, these data strongly suggest that IP_3 production elicited by cyclic strain is not dependent on Ca^{2+} entry via plasma-membrane pathways. Moreover, the lack of effect of 25 μM BHQ on IP_3 formation in response to mechanical deformation (Table 1) further suggests that in ECs the activation of phosphoinositide-specific phospholipase C is not modulated by $[Ca^{2+}]_i$.

A number of investigators have demonstrated that lanthanides, including gadolinium, are potent Ca^{2+}-channel [31,44,45] and Na^+/Ca^{2+} exchange-blocking agents. Although the availability of a more specific blocker of stretch-activated channels would have been greatly desirable for this present study, $GdCl_3$ has been generally utilized [46]. Of principal interest to this study is

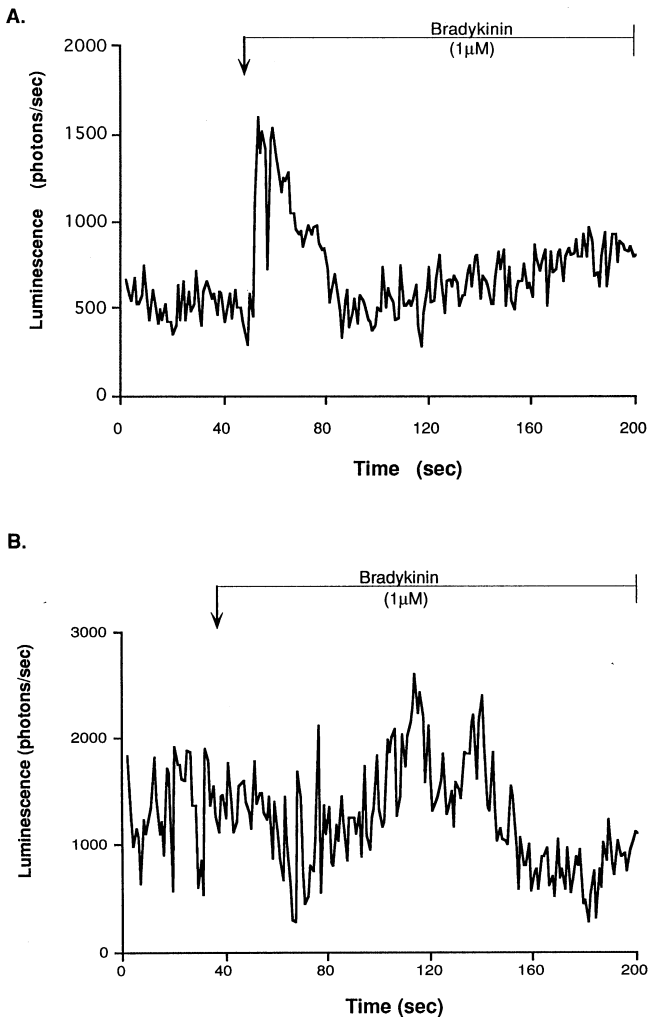

Figure 4 Effect of (A) bradykinin and (B) bradykinin + BHQ on $[Ca^{2+}]_i$ in bovine aortic ECs

(**A**) Bovine aortic ECs were loaded with aequorin and then incubated with 1 μM bradykinin. There was a large early $[Ca^{2+}]_i$ transient followed by a sustained level. (**B**) Incubation with 25 μM BHQ abolished this early spike. The traces are from a representative experiment. The experiments were repeated at least four times with similar results.

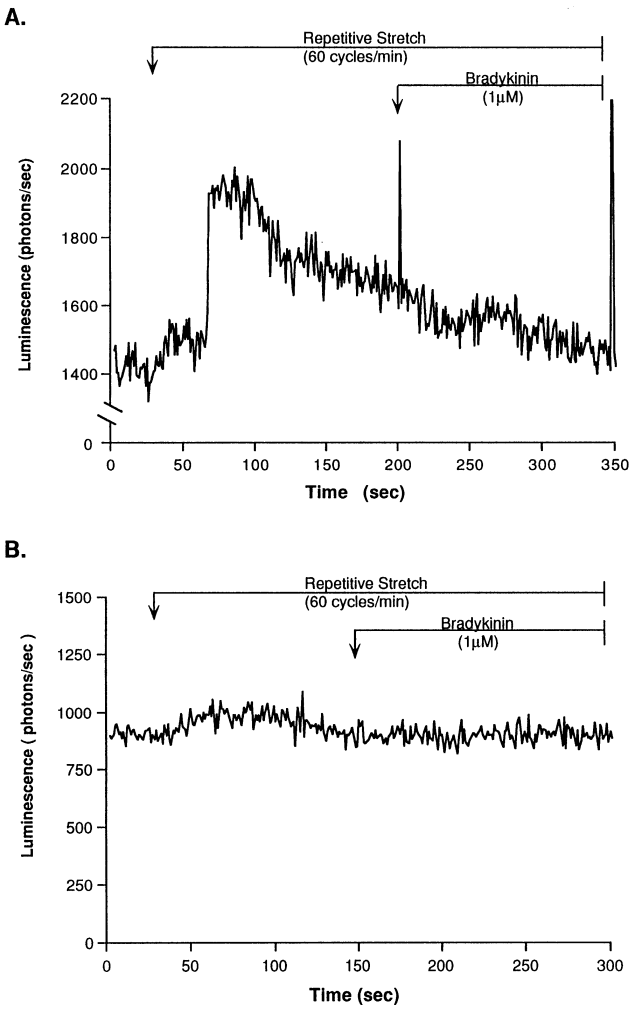

Figure 5 Effect of bradykinin on $[Ca^{2+}]_i$ in ECs undergoing repetitive deformation

(**A**) 1 μM bradykinin was added to ECs that were undergoing repetitive deformation at 60 cycles/min. There was no further increase in $[Ca^{2+}]_i$. Note that the spike seen with the addition of bradykinin is a 'marking' artifact. (**B**) Preincubation with 25 μM BHQ abolished the initial $[Ca^{2+}]_i$ spike induced by repetitive stretch. The traces are from a representative experiment. The experiments were repeated at least four times with similar results.

the dose-dependent block of stretch-activated channels by $GdCl_3$ using patch-clamp techniques in *Xenopus* oocytes [46]. In our experiments, 5 or 10 μM $GdCl_3$ effectively diminished intracellular Ca^{2+} levels, probably by blocking Ca^{2+} entry, without evidence of cell toxicity. Since the cell membrane is relatively impermeable to lanthanides [47], it is unlikely that the changes observed in $[Ca^{2+}]_i$ in ECs pretreated with $GdCl_3$ were due to a direct inhibitory effect on the mobilization of Ca^{2+} from internal stores. Furthermore additional studies indicate that both Ni^{2+} and Gd^{3+} can induce transient elevations in intracellular Ca^{2+}. However, neither Ni^{2+} nor Gd^{3+} were toxic to the aequorin since the maxima elicited by Triton X-100 lysis were similar to those cells not exposed to Ni^{2+} or Gd^{3+} (results not shown). Although $GdCl_3$ proved to be a potent Ca^{2+}-influx blocker, our data only provide indirect support for the presence of stretch-activated channels in bovine aortic ECs.

Although Ca^{2+} influx appears to regulate the sustained phase, the precise nature of the initial transient elevation of $[Ca^{2+}]_i$ remains controversial. Using human umbilical vein and bovine aortic ECs respectively, Hallam et al. [48] and Luckhoff et al. [49] reported that removal of external Ca^{2+} had no effect on the magnitude of the initial agonist-induced transient elevation, whereas other studies [35,42] demonstrated a significant reduction in the magnitude of the initial-phase peak in the absence of extracellular Ca^{2+}. Additional studies using fura 2 confirmed that the magnitude of the initial agonist (bradykinin)-induced elevation of $[Ca^{2+}]_i$ was reduced in Ca^{2+}-free conditions [50]. Our studies suggest that internal mobilization of Ca^{2+} from the IP_3-sensitive pool and from the extracellular space participate in the early rise that follows the initiation of cyclic strain in view of the fact that the magnitude of the initial peak was diminished by manoeuvres that limited Ca^{2+} influx, such as removal of extracellular Ca^{2+} and pretreatment with $GdCl_3$. Furthermore addition of BHQ, a depleter of intracellular Ca^{2+} stores, blunted this initial peak. It is important to note, however, that BHQ may,

Table 1 Effect of initiation of cyclic strain on IP_3 mass

Confluent bovine aortic ECs ($n = 4$) were subjected to 10% average strain at 60 cycles/min (0.5 s elongation alternating with 0.5 s relaxation), and IP_3 mass was determined by RIA. See the Materials and methods section for details. Previous time-course experiments demonstrated that IP_3 mass peaked 10 s after the initiation of cyclic strain [25]. Cyclic-strain-mediated IP_3 generation at 10 s was not altered by CFM and the inhibitors of Ca^{2+} influx, $NiCl_2$ and $GdCl_3$. Charybdotoxin, a non-selective K^+-channel blocker, and BHQ, a depleter of the IP_3-sensitive Ca^{2+} pool, also failed to modify the IP_3 response to mechanical deformation. Results are means ± S.D.

Treatment	Peak IP_3 mass (pmol/10^6 cells)
Static	
Control	4.12 ± 1.33
1 μM bradykinin	8.26 ± 1.43
Cyclic strain	
Control	8.82 ± 1.90
500 μM $NiCl_2$	6.84 ± 1.84
5 μM $GdCl_3$	8.23 ± 1.72
10 μM $GdCl_3$	7.67 ± 2.35
25 μM BHQ	7.50 ± 1.62
10 nM charbydotoxin	8.56 ± 2.11
CFM	7.94 ± 1.96

Table 2 Effects of A23187 and bradykinin on IP_3 measurements in ECs

Total IP_3 (c.p.m./well) was measured by RIA in ECs in the presence or absence of Ca^{2+} in the medium and exposed to either 100 nM A23187, a calcium ionophore, or 1 μM bradykinin, an agonist known to increase inositol turnover and compared with non-stimulated cells (control). Results are means ± S.E.M. ($n = 4$). The results show that the Ca^{2+} ionophore increases inositol turnover by increasing Ca^{2+} influx. In contrast, bradykinin can increase inositol turnover in the presence or absence of extracellular Ca^{2+}, suggesting a receptor-coupled G-protein-mediated phospholipase C mechanism.

	Total IP_3		
	Control	+ A23187	+ Bradykinin
1.25 mM Ca^{2+} in medium	100.5 ± 9.9	173.0 ± 1.5	211.0 ± 2.5
CFM	69.3 ± 2.4	59.0 ± 2.5	200.4 ± 7.4

in a dose-dependent manner, also inhibit Ca^{2+} influx [51]. However, there is no known 'exclusive' depleter of intracellular Ca^{2+} stores. Even thapsigargin has been shown to stimulate Ca^{2+} influx at nanomolar concentrations and block voltage-activated Ca^{2+} channels at micromolar concentrations in bovine adrenal glomerulosa cells [52]. Despite these limitations, the results indicate that BHQ (as well as other measures that would lower $[Ca^{2+}]_i$ by decreasing extracellular Ca^{2+} influx) did not significantly alter IP_3 production in ECs subjected to cyclic strain, while blunting the initial $[Ca^{2+}]_i$ elevation. These data support the idea that IP_3 generation in ECs subjected to strain is independent of changes in $[Ca^{2+}]_i$ and unaffected by manoeuvres that decrease extracellular Ca^{2+} influx.

During the preparation of this manuscript, three different groups reported on the effect of mechanical perturbation on EC $[Ca^{2+}]_i$ [19–21]. Our observations are in general agreement with these findings and confirm the dual importance of Ca^{2+} entry and release from the IP_3-sensitive pool in $[Ca^{2+}]_i$ changes in response to mechanical deformation. These earlier studies, however, failed to address the response to repetitive strain and were performed at much shorter stretch intervals utilizing different protocols of mechanical deformation and experimental design.

It should be emphasized that the mechanical load experienced by cells on the flexible membrane is heterogeneous [3,15]. Therefore, since aequorin luminescence is obtained from cells from the entire membrane, we cannot determine whether this heterogeneity of strain influences the Ca^{2+} spiking profile (Figure 3A), although our results probably underestimate the magnitude of the rise in $[Ca^{2+}]_i$. Likewise, we cannot absolutely exclude the premise that there may be a regulatory influence by cells in the low- or no-strain region on cells growing in the high-strain periphery or that the agitation of the medium with repetitive deformation of the membrane could be responsible for the findings. These possibilities are unlikely since, in previous studies in which cells were grown exclusively in either the high-strain or low-strain region and exposed to cyclic strain, no enhancement of proliferation and expression of strain-responsive genes, such as tissue plasminogen activator or nitric oxide synthase, was detected in the high-strain region compared with cells grown on the entire membrane [7,12,15]. Likewise, the calculated shear ($< 10^{-7}$ N/cm^2) in our system is too low to account for these sustained responses [3,4], and Sigurdson and co-workers have demonstrated that up to 10^{-6} N/cm^2 shear could not elicit a rise in $[Ca^{2+}]_i$ [21].

The exact mechanism by which ECs sense and respond to physical forces is not known, although there are many indirect clues. For example, there are a number of different ways of activating phospholipase C including a G-protein-mediated mechanism [53–55] (both pertussis-toxin-sensitive and -insensitive), tyrosine phosphorylation [9,56] or through an increase in Ca^{2+} influx [57]. Our present studies suggest that stretch may be activating a G-protein-linked phospholipase C, although further experiments are needed to define this link. These results are in contrast with a recent report [58] that demonstrates that stretch in smooth-muscle cells resulted in inositol turnover and phospholipase C activation through an increase in Ca^{2+} influx through Gd^{3+}-sensitive ion channels. It is possible that these differences reflect intrinsic differences in the tissues studied. It is also possible that we did not inhibit Ca^{2+} influx sufficiently, using nominally CFM, Ni^{2+} or Gd^{3+}, which although they demonstrate a decrease in IP_3 levels (see Table 1), were not statistically significant.

The implications of the present *in vitro* studies for the situation *in vivo* is also not well defined. *In vivo* vessels are continuously exposed to cyclic stretch throughout a life time, albeit with varying magnitudes and frequencies of stretch. Our previous studies support the notion that these variances in rhythmic distension may have a higher priority than the absolute levels, i.e. varying the conditions of stretch will result in repeated stimulation of the cells in the vessel wall. For instance, we have demonstrated that, during chronic stretch of ECs for 24 h at 60 cycles/min, IP_3 production remains at baseline levels, but that immediately on switching to 100 cycles/min there is an increase in IP_3 production [25]. Likewise, chronic exposure to 100 cycles/min frequency followed by a decrease to 60 cycles/min will also result in IP_3 release [59].

In summary, the results of the present study show that EC $[Ca^{2+}]_i$ is modulated by cyclic strain and that the generation of IP_3 in ECs subjected to mechanical deformation is not impaired by measures that alter $[Ca^{2+}]_i$. Our studies demonstrate that the IP_3–Ca^{2+} pathway is activated in response to cyclic strain and that both phospholipase C and a $GdCl_3$-inhibitable Ca^{2+}-entry pathway may be transducers of the responses of ECs to cyclic strain *in vivo*.

This work was supported by grants to B.E.S. from the NIH (HL R29-40305, HL R01-47345), the American Heart Association (National) and the Department of Veterans

Affairs Merit Review. O.R.R. was a recipient of a research fellowship award from the American Heart Association, Connecticut Affiliate.

REFERENCES

1 Sumpio, B. E. (1993) Hemodynamic forces and vascular cell biology, RG Landes Publisher, Austin, TX
2 Banes, A. J., Link, G. W., Gilbert, J. W., Son Tay, R. T. and Monbureau, O. (1990) Am. Biotech. Lab., May, 13–22
3 Gilbert, J. A., Banes, A. J., Link, G. V. W. and Jones, G. J. (1989) in ANSYS Conference Proceedings, (Dietrich, D. E., ed.), pp. 13.2–13.7, Swanson Analysis System Company, Houston, PA
4 Gilbert, J. A., Banes, A. J., Link, G. W. and Jones, G. L. (1990) Exp. Tech. **1415**, 43–45
5 Sumpio, B. E., Banes, A. J., Levin, L. G. and Johnson, G. J. (1987) J. Vasc. Surg. **6**, 252–256
6 Iba, T., Maitz, S., Fubert, T., Rosales, O., Widmann, M. D., Spillane, B., Shin, T., Sonoda, T. and Sumpio, B. E. (1991) Circ. Shock **35**, 193–198
7 Li, G., Mills, I. and Sumpio, B. E. (1994) Endothelium **22**, 177–181
8 Yano, Y., Saito, Y., Narumiya, S. and Sumpio, B. (1996) Biochem. Biophys. Res. Commun. **224**, 508–515
9 Yano, Y., Geibel, J. and Sumpio, B. (1996) Am. J. Physiol. **271**, C635–C649
10 Sumpio, B. E., Banes, A. J., Buckley, M. and Johnson, G. J. (1988) J. Vasc. Surg. **7**, 130–138.
11 Sumpio, B. E. and Banes, A. J. (1988) Surgery **104**, 383–389
12 Iba, T. and Sumpio, B. E. (1992) Cell Transplant. **1**, 43–50
13 Sumpio, B. E. and Widmann, M. D. (1990) Surgery **108**, 277–282
14 Awolesi, M. A., Sessa, W. C. and Sumpio, B. E. (1994) Surgery **116**, 439–444
15 Awolesi, M. A., Sessa, W. C. and Sumpio, B. E. (1995) J. Clin. Invest. **96**, 1449–1454
16 Lansman, J. B., Hallam, T. J. and Rink, T. J. (1987) Nature (London) **325**, 811–813
17 Morris, C. and Horn, R. (1991) Science **251**, 1246–1249
18 Adams, D. J., Barakeh, J., Laskey, R. and Van Breemen, C. (1989) FASEB J. **3**, 2389–2400
19 Demer, L., Wortham, C., Dirksen, E. and Sanderson, M. (1993) Am. J. Physiol. **2**, H2094–H2102
20 Naruse, K. and Sokabe, M. (1993) Am. J. Physiol. **2**, C1037–1044
21 Sigurdson, W., Sachs, F. and Diamond, S. (1993) Am. J. Physiol. **264**, H1745–1752
22 Mills, I., Letsou, G., Rabban, J., Sumpio, B. and Gewirtz, H. (1990) Biochem. Biophys. Res. Commun. **171**, 143–147
23 Cohen, C. R., Mills, I., Du, W., Sumpio, B. E. (1997) Exp. Cell Res. **231**, 184–189
24 Evans, L., Frenkel, L., Brophy, C. M., Rosales, O. R., Sudhaker, S., Li, G., Du, W. and Sumpio, B. (1997) Am. J. Physiol. **41**, C650–C659
25 Rosales, O. R. and Sumpio, B. E. (1992) Am. J. Physiol. **262**, C956–C962
26 Rosales, O. R. and Sumpio, B. E. (1992) Surgery **112**, 459–466
27 Iba, T. and Sumpio, B. E. (1991) Microvasc. Res. **42**, 245–254
28 Mo, M., Eskin, S. and Schilling, W. (1991) Am. J. Physiol. **260**, H1698–H1707
29 Nollert, M. U., Eskin, S. G. and McIntire, L. V. (1990) Biochem. Biophys. Res. Commun. **170**, 281–287
30 Isales, C. M., Bollag, W. B., Kiernan, L. C. and Barrett, P. Q. (1989) Am. J. Physiol. **256**, C89–C95
31 Nathanson, M. H., Gautam, A., Bruck, R., Isales, C. M. and Boyer, J. L. (1992) Hepatology **15**, 107–116
32 Evans, C. H. (1989) Biochemistry of the Lanthanides, Plenum Publishing Corp., New York and London
33 Kass, G. E. N., Llopis, J., Chow, S. C., Duddy, S. K. and Orrenius, S. (1990) J. Biol. Chem. **265**, 17486–17492
34 Shimomura, O. and Johnson, F. (1979) in Detection and Measurement of Free Ca^{2+} in Cells, (Ashley, C. and Campbell, A., eds.), pp. 73–83, Elsevier, North Holland
35 Colden-Stanfield, M., Schilling, W., Ritchie, A., Eskin, S., Navarro, L. and Kunze, D. (1987) Circ. Res. **61**, 632–640
36 Hallam, T. J. and Pearson, J. D. (1986) FEBS Lett. **207**, 95–99
37 Hallam, T. J., Jacob, R. and Merritt, J. E. (1988) Biochem. J. **255**, 179–184
38 Rotrosen, D. and Gallin, J. I. (1986) J. Cell. Biol. **103**, 2379–2387
39 Johns, A., Lategan, T. W., Lodge, N. J. and Adams, D. J. (1987) Tissue Cell **19**, 733–745
40 Bussolino, F., Aglietta, M., Sanavio, F. and Camussi, G. (1985) J. Immunol. **135**, 2748–2753
41 Hoth, M. and Penner, R. (1992) Nature (London) **355**, 353–356
42 Schilling, W. P., Ritchie, A. K., Navarro, L. T. and Eskin, S. G. (1988) Am. J. Physiol. **255**, H219–H227
43 Jacob, R. (1990) J. Physiol. (London) **421**, 55–77
44 Biagi, B. and Enyeart, J. (1990) Am. J. Physiol. **259**, C515–520
45 Docherty, R. J. (1988) J. Physiol. (London) **398**, 33–47
46 Yang, X.-C. and Sachs, F. (1989) Science **243**, 1068–1071
47 Hansen, D. E., Craig, C. S. and Hongeghem, L. M. (1990) Circulation **81**, 1094–1105
48 Hallam, T. J., Pearson, J. D. and Needham, L. A. (1988) Biochem. J. **251**, 243–249
49 Luckhoff, A., Zeh, R. and Busse, R. (1988) Pflügers Arch. **412**, 654–658
50 Buchan, K. W. and Martin, W. (1991) Br. J. Pharmacol. **102**, 35–40
51 Mason, M., Garcia-Rodriguez, C. and Grinstein, S. (1991) J. Biol. Chem. **268**, 20856–20862
52 Rossier, M., Python, C., Burnay, M., Schlegel, W., Vallotton, M. and Capponi, A. (1993) Biochem. J. **296**, 309–312
53 Liedtke, C. (1994) Am. J. Physiol. **266**, C397–C405
54 Rhee, S. and Choi, K. (1992) J. Biol. Chem. **267**, 12393–12396
55 Sanchez, M. and Groberna, R. (1994) J. Cell Biochem. **55**, 173–181
56 Tkachuk, V. and Voyno, Y. (1991) Am. J. Physiol. **261**, 118–122
57 Watson, S., Poole, A. and Asselin, J. (1995) Mol. Pharmacol. **47**, 823–830
58 Matsumoto, H., Baron, C. and Coburn, R. (1995) Am. J. Physiol. **268**, C458–C465
59 Brophy, C., Mills, I., Rosales, O., Isales, C. and Sumpio, B. E. (1993) Biochem. Biophys. Res. Commun. **190**, 576–581

Received 13 December 1996/4 April 1997; accepted 18 February 1997