The Mechanism of Injury-Induced Intracellular Calcium Concentration Oscillations in the Endothelium of Excised Rat Aorta

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Key Words

Rat aorta · Endothelial injury · Calcium oscillations · Gap junction blockers · Inositol-1,4,5-trisphosphate receptors · Fura-2

Abstract

Endothelial injury is the primary event that leads to a variety of severe vascular disorders. Mechanical injury elicits a Ca^{2+} response in the endothelium of excised rat aorta, which comprises an initial Ca^{2+} release from inositol-1,4,5-trisphosphate (InsP_3)-sensitive stores followed by a long-lasting decay phase due to Ca^{2+} entry through uncoupled connexons. The Ca^{2+} signal may also adopt an oscillatory pattern, the molecular underpinnings of which are unclear. In the light of the role played by Ca^{2+} spiking in tissue regeneration, this study aimed to unveil the mechanisms underlying injury-induced Ca^{2+} oscillations. The latter reversibly ceased upon removal of extracellular Ca^{2+} or addition of the gap junction blockers heptanol, 18 α,β -glycyrrhetic acid, La^{3+} and Ni^{2+} , but were insensitive to BTP-2 and SKF 96365. The spiking response was abolished by inhibiting the Ca^{2+} entry mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). The InsP_3 -producing agonist ATP resumed Ca^{2+} oscillations in silent cells, while the phospholipase C inhibitor U73122 suppressed them. Injury-induced Ca^{2+} transients were prevented by the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) blockers thapsigargin and

cyclopiazonic acid, while they were unaffected by suramin and genistein. These data show for the first time that the coordinated interplay between NCX-mediated Ca^{2+} entry and InsP_3 -dependent Ca^{2+} release contributes to injury-induced intracellular Ca^{2+} concentration oscillations.

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Introduction

The vascular endothelium does not merely serve as a passive barrier between circulating blood and underlying tissue, but forms a multifunctional signal-transducing interface that regulates vascular tone, coagulation and fibrinolysis, vascular inflammatory reactions and angiogenesis [1–3]. Significant wounding of endothelial cells (ECs) may dampen their inhibitory action on smooth muscle cells (SMCs) proliferation and platelet aggregation, thus causing a shift to conditions prone to coagulation, vasoconstriction and inflammation [1, 3]. Indeed, endothelial damage is regarded as the early event that leads to the onset of severe vascular disorders, such as ischemic disease, atherosclerosis and aneurysm [3]. After disruption of the intimal layer, ECs play a key role in the regeneration process. Proliferation and inward migration of ECs from the wound edge reseal the inner vessel wall at a rate that depends on the animal model and the ex-

perimental conditions [4]. The signal transduction pathway controlling ECs response to injury is still poorly understood. It has long been known that endothelial proliferation and migration are tightly regulated by an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [2]. Consistent with a role in wound healing, endothelial scraping induces an elevation in $[\text{Ca}^{2+}]_i$ in nearby survivors, which has been linked to the subsequent covering of the lesioned area [5–8]. In particular, a dramatic delay in the coverage of denuded area occurs when extracellular Ca^{2+} is removed from the bath [8–10]. Indeed, Ca^{2+} influx may induce the transcription of two immediate early genes, such as c-fos and c-jun, one of which (c-fos) stimulates ECs migration from the wound edge [7].

We have recently harnessed the excised rat aorta to analyze injury-induced Ca^{2+} signals in ECs adjacent to the lesion site [5]. Measuring $[\text{Ca}^{2+}]_i$ in intact endothelium represents a step forward in understanding the mechanisms underlying Ca^{2+} dynamics in vascular endothelium, as the latter undergoes remarkable alteration in the expression of cell surface receptors and ion channels under culture conditions [9]. Scraping 1–2 rows of cells with a microelectrode tip caused a rapid peak, mainly due to Ca^{2+} release from inositol-1,4,5-trisphosphate-sensitive receptors (InsP_3Rs), which was followed by a long-lasting decay phase mediated by Ca^{2+} influx through the plasma membrane [5]. InsP_3 synthesis was due to the stimulation of phospholipase C (PLC)-linked purinergic receptors, including the $\text{P}_{2\text{Y}1}$, $\text{P}_{2\text{Y}2}$ and $\text{P}_{2\text{Y}12}$ subtypes, that bound ATP released from ruptured cells [5]. The subsequent Ca^{2+} entry was insensitive to the inhibition of conventional plasma membrane channels, i.e. store-operated channels, receptor-operated channels, which comprise both second messenger-operated channels and ionotropic channels, such as $\text{P}_{2\text{X}}$ receptors, and voltage-activated Ca^{2+} channels, but was suppressed by gap junction blockers [5]. Each gap junction was formed by the docking of two hemichannels (connexons), which were each formed by six connexin (Cx) protein subunits and embedded within one of the two juxtaposed membranes [10]. Three Cx isoforms were expressed by rat aortic endothelium, namely Cx 37, 40, and 43 [11]. The aforementioned results led to the hypothesis that endothelium scraping uncouples Cx hemichannels of ECs freed from their severed neighbors: the hemichannels do not fully close and enable the long-lasting Ca^{2+} influx [5, 6]. Notably, a minor percentage of activated cells displayed oscillations in $[\text{Ca}^{2+}]_i$ that overlapped the prolonged decay phase [5]. This feature gains particular relevance when considering that Ca^{2+} oscillations may stimulate the ge-

netic program that leads to cell proliferation and migration [12–15]. Unveiling the molecular underpinnings of injury-induced Ca^{2+} oscillations in a physiological model, such as the intact endothelium of rat aorta, might lead to novel therapies aiming at restoring endothelial integrity.

In this study, we investigated the mechanisms underlying intracellular Ca^{2+} oscillations in native rat aortic endothelium loaded with the Ca^{2+} -sensitive dye, fura-2. As conventional plasmalemmal Ca^{2+} -permeable channels did not drive injury-induced Ca^{2+} oscillations, we searched for the involvement of alternative signaling pathways. More specifically, we probed whether injury-induced Ca^{2+} oscillations could be contributed by the interplay between Ca^{2+} release from InsP_3Rs and Ca^{2+} entry driven by the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX).

Methods

Dissection of the Aorta

Wistar rats aged 2–3 months were sacrificed with an overdose of diethyl ether. The thoracic and abdominal aorta were dissected out and perfused with physiological salt solution (PSS). The vessel was cleaned of the surrounding connective tissue, cut in ~5-mm-long rings, stored in PSS at room temperature (22–24°C) and used within 5 h. The animal handling was under the control of the veterinary surgeon of the University of Pavia and the research was approved by the Ethical Committee of the University of Pavia.

Solutions

PSS had the following composition (mM): 150 NaCl, 6 KCl, 1.5 CaCl_2 , 1 MgCl_2 , 10 glucose and 10 Hepes. In Ca^{2+} -free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Both solutions were titrated to pH 7.4 with NaOH. Na^+ -free solution was obtained by replacing extracellular Na^+ with an equimolar amount of N-methyl-D-glucamine and titrated with HCl.

$[\text{Ca}^{2+}]_i$ Measurements

The technique used to evaluate changes in $[\text{Ca}^{2+}]_i$ in intact endothelium has previously been described [5]. The aortic ring was opened and loaded with 16 μmol fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. In situ ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss $\times 63$ Achromplan objective (water immersion, 2.0 mm working distance, 0.9 numerical aperture). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. The exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, Calif., USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot online the fluorescence from 10 to 15 rectangular ‘regions of interest’ (ROI) enclosing one single cell. $[\text{Ca}^{2+}]_i$ was monitored by measuring, for each region of interest, the ratio of the mean fluorescence emitted at 510 nm

when exciting alternatively at 340 and 380 nm (shortly termed 'ratio'). An increase in $[Ca^{2+}]_i$ caused an increase in the ratio. Ratio measurements were performed and plotted online every 3–5 s. Every recording was conducted for no less than 15 min. The propagation of injury-induced Ca^{2+} oscillations from the first to the following rows was investigated by performing time-lapse recordings at 1 image/3 s by using the same equipment described above mounted on a different microscope, i.e. an upright Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan). Images were stored on the hard disk and converted offline to 340/380 ratio images by ImageJ software (National Institutes of Health, USA, <http://rsbweb.nih.gov/ij/>). The experiments were performed at room temperature (21–23°C).

Mechanical Disruption of ECs

As shown [5], aortic endothelium was injured under microscopic control by means of a glass microelectrode with a tip of about 30- μ m diameter, driven by an XYZ hydraulic micromanipulator (Narishige, Japan). The electrodes were fabricated using a Kopf 700D vertical pipette puller (David Kopf Instruments, USA). Images of fura-2-loaded ECs, together with numbered ROI, were taken before the lesion, in order to identify the cells facing the injury site. Preliminary experiments revealed that, when the endothelial monolayer was removed after the loading procedure, the underlying SMCs did not emit any detectable fluorescent signal. The microelectrode was first positioned almost parallel and very near to the endothelium surface. Then it was moved downward, along the z-axis, until the electrode tip gently touched the endothelium, and moved horizontally across the visual field to scrape 1–3 consecutive rows of ECs. This procedure allowed monitoring of $[Ca^{2+}]_i$ at the same time as microelectrode injury. Ethidium bromide, a fluorescent molecule unable to cross an intact plasma membrane and therefore indicative of damaged/dead cells, was used to check the viability of ECs near to the injury [5].

Data Analysis

For each protocol, data were collected from at least 3 rats; only cells facing the injured area were analyzed. Mean values are presented together with standard error of the mean and the whole number of tested cells 'n'. Statistical significance ($p < 0.05$) was evaluated by the Student t test for unpaired observations. Unless differently stated, tracings shown in the figures are single ROI recordings.

Chemicals

Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). All other chemicals were purchased from Sigma.

Results

Injury-Induced Intracellular Ca^{2+} Oscillations in the Excised Endothelium of Rat Aorta

Scraping the lumen of aortic rings with the tip of a glass microelectrode caused a rapid Ca^{2+} peak followed by a long-lasting decline towards the baseline in 163 out of 222 (73.4%) ECs adjacent to the lesion site [5]. The re-

maining 59 cells (26.6%) in the first row also displayed periodic Ca^{2+} oscillations which overlapped the decay phase and were initiated 203.2 ± 29.2 s ($n = 59$) after the initial Ca^{2+} spike (fig. 1B). The number of spikes following the initial Ca^{2+} peak ranged from 3 to 16 and averaged 7.02 ± 0.44 ($n = 59$) during our 15-min recordings. In the longest experiment performed, however, the oscillatory response was observed for as long as 50 min (see, for instance, the middle trace in fig. 6B). Oscillation amplitude (fig. 2A) and frequency (fig. 2B) remained constant after ignition of the train in each single cell. Indeed, there was no significant difference ($p > 0.05$) in either the amplitude of consecutive Ca^{2+} transients (fig. 2A) or the interspike interval (fig. 2B) throughout the Ca^{2+} burst. The height of each Ca^{2+} spike was lower than the initial raise in $[Ca^{2+}]_i$, but such a difference was not significant ($p = 0.36$; amplitude of the initial Ca^{2+} peak vs. that of the first Ca^{2+} oscillation). The oscillations in $[Ca^{2+}]_i$ elicited by mechanical scraping at the wound edge were never synchronous, either when the cells were located at the same side (fig. 1D) or at the opposite sides of the wound (fig. 1E). Repetitive Ca^{2+} spikes were detected also in ECs further back from the wound edge, as shown by the Ca^{2+} activity of cell 5 (cyan trace) in figure 1B. The kinetics of the Ca^{2+} signal recorded from cells 1 and 5 clearly show that Ca^{2+} oscillations arising at the lesion site and in the innermost tissue were not synchronized. Moreover, figure 1B displays that endothelial injury could induce Ca^{2+} oscillations both at the lesion edge and in the innermost tissue while evoking only subthreshold Ca^{2+} signals in intermediate cells (online suppl. video 1; for all suppl. material, see www.karger.com/doi/10.1159/000329618). Accordingly, while the initial Ca^{2+} peak at cell 1 was propagated to the adjoining cell 2 [fig. 1A (b, c); fig. 1C (black and red traces, respectively)] and 3 [fig. 1A (c, d); fig. 1C (red and green traces, respectively)], the subsequent Ca^{2+} oscillation was followed by small Ca^{2+} rise in cell 2 [fig. 1A (g); fig. 1C (asterisk on the red trace)] and in cell 3 [fig. 1A (h); fig. 1C (arrow on the green trace)] during the period under observation. Although cells from the 2nd to the 4th row did not exhibit Ca^{2+} oscillations, cell 5 (cyan trace in fig. 1C) started to oscillate at 243 s and these oscillations were associated with subthreshold Ca^{2+} signals in the adjacent cell 4 [fig. 1A (i); fig. 1B (blue trace); fig. 1C (enlargement)]. Synchronous Ca^{2+} spikes in adjacent ECs on the same side of lesion [cell 1, 6 and 7 in fig. 1A (j)]. The heterogeneous Ca^{2+} activity recorded both at the wound edge and in the innermost tissue might be ascribed to nonuniform dye loading or fura-2 compartmentalization in intracellular organelles. Therefore, rat aortic rings

Fig. 1. Injury-induced intracellular Ca^{2+} oscillations in the endothelium of excised rat aorta. **A** Fluorescence images of the surface region of a rat aortic ring at sequential time points before and after mechanical denudation displayed in pseudocolors (color scale: 0–255) as the ratio of fura-2 fluorescence intensity at 340/380 nm. The two white lines encompass the area scratched by the micro-electrode. The times before and after the lesion are indicated at the top of each image. The bar length is 40 μm . **B** Mean fluorescence ratios of the cells 1–5 labelled in **A**. Cell borders have been

manually outlined to delineate the position of individual cells. **C** Expanded section of the traces depicted in **B** to highlight the lack of synchrony between Ca^{2+} oscillations at the wound edge (black trace) and those arising in the innermost tissue (cyan trace). **D** Asynchronous Ca^{2+} spikes in adjacent ECs on the same side of the lesion (cells 1, 6 and 7 in **A**). **E** Asynchronous Ca^{2+} oscillations arising in two cells located on the opposite sides of the wound. In this and the following figures, the mechanical damage carried out at the time is marked by the black arrow.

Fig. 2. Spike amplitude and frequency are constant during the oscillatory response to injury. The amplitude of the Ca^{2+} transients (**A**) and the interspike interval (**B**) did not undergo significant changes after the onset of the oscillations. The frequency was

evaluated by measuring the peak-to-peak interval between two successive Ca^{2+} pulses. In both **A** and **B**, each bar is the mean $\pm$ SE of 7–58 cells recorded from at least three different rat aortic rings.

were challenged with a high dose (100 μM) of ATP after the injury. In three separate experiments, this stimulation produced an increase in $[\text{Ca}^{2+}]_i$ in all the cells within the visual field (not shown), which suggests that the fluorophore was adequately loaded within all the cells and thus able to detect a Ca^{2+} spike should it have occurred. The mechanism leading to the Ca^{2+} transients in cells away from the lesion was not investigated further and is the object of current investigation. Overall, these findings indicate that asynchronous Ca^{2+} oscillations may arise in a fraction of ECs adjacent to the lesion on both sides of the wound and that they are not transmitted in a 1:1 ratio to following cells. Nevertheless, Ca^{2+} oscillations may also occur at remote sites from the wound edge.

Ca^{2+} Entry Sustains Injury-Induced Intracellular Ca^{2+} Oscillations: The Role of Gap Junction Channels/Hemichannels

The contribution of Ca^{2+} entry to the spiking signal was assessed by removing Ca^{2+} from the bathing solution. As shown in figure 3A, such a maneuver reversibly inhibited injury-elicited $[\text{Ca}^{2+}]_i$ oscillations in 61 out of 61 cells adjacent to the lesion site. These data indicate that Ca^{2+} entry during the decay phase maintains the repetitive Ca^{2+} waves occurring in a minor fraction of the cells next to the lesion site. Consistent with these data, endothelial scraping induced an evident influx of Mn^{2+} , which is a marker for Ca^{2+} inflow and quenches fura-2 fluorescence

at 360 nm when it enters into the cytosol through Ca^{2+} -permeable channels (online suppl. fig. 1A). The entry of Mn^{2+} and the consequent quenching of intracellular fluorescence were strongly reduced by the gap junction blocker, heptanol (4 mM) (online suppl. fig. 1B). Injury-induced Ca^{2+} influx in the excised endothelium of rat aorta is not sensitive to the blockage of either store-dependent or store-independent Ca^{2+} channels [5]. Accordingly, neither BTP-2 (50 μM) (fig. 3B), a powerful store-operated channel inhibitor, nor SKF 96365 (20 μM) (fig. 3C), which interferes with both store-operated channels and receptor-operated channels, impaired the spiking response to mechanical damage. Therefore, we probed the effect of two well-known gap junction blockers, heptanol and 18 α,β -glycyrrhetic acid [6, 16, 17]. As illustrated in figure 4A, heptanol (4 mM) reversibly abolished the intracellular Ca^{2+} oscillations occurring in 24 out of 24 cells at the lesion margin. A similar effect was exerted by 18 α,β -glycyrrhetic acid (25 μM) in 20 out of 20 cells (fig. 4B). These data were corroborated by testing La^{3+} and Ni^{2+} , two divalent cations that may also interfere with gap junction gating [6, 16]. We found that La^{3+} (2.5 μM) inhibited the oscillatory response to injury in 18 out of 24 cells (fig. 4C). Similarly, Ni^{2+} (10 μM) halted the repetitive Ca^{2+} spikes in 8 out of 12 cells (fig. 4D). Collectively, these findings strongly suggest that gap junction channels/hemichannels sustain injury-induced oscillations in $[\text{Ca}^{2+}]_i$.

Fig. 3. Ca²⁺ inflow through nonconventional membrane pathways sustains injury-elicited Ca²⁺ oscillations. **A** Withdrawing extracellular Ca²⁺ (0Ca²⁺) during an established response immediately interrupted the Ca²⁺ spikes and caused a decline in the baseline, which represents the long-lasting recovery of the [Ca²⁺]_i to the resting levels measured before the initial Ca²⁺ peak (not shown). Therefore, the drop in the baseline concurs with the findings reported elsewhere [7]. The oscillatory behavior resumed upon readmission of external Ca²⁺. Conversely, neither BTP-2 (50 μM; **B**) nor SKF 96365 (20 μM; **C**) modified the oscillatory response to injury in 15 out of 15 cells and in 19 out of 19 cells, respectively.

The Reverse Mode of NCX Is Recruited in Oscillating Cells

The aforementioned experiments have shown that the periodic fluctuations in [Ca²⁺]_i, which may overlap the decay phase of its injury-elicited increase, require the opening of gap junction channels/hemichannels. Therefore, the same membrane pathway is operating in both spiking and nonspiking cells. The question now arises as to the nature of the additional mechanism conferring an oscillatory behavior to the decay phase. Besides Ca²⁺, gap junction channels/hemichannels are permeable to Na⁺, which is driven into the cell by the large electrochemical gradient existing across the plasma membrane [18]. It is now firmly established that an elevation in submembranous Na⁺ levels may turn NCX into the reverse mode (3 Na⁺ out:1 Ca²⁺ in) and cause Ca²⁺ entry across the plasma membrane [19]. Notably, NCX-mediated Ca²⁺ inflow has recently been reported in the intact endothelium of excised rat aorta challenged with acetylcholine [20]. In a

first set of experiments, we found that blocking NCX activity by replacing extracellular Na⁺ with an equimolar amount of N-methyl-D-glucamine [21], or KB-R 7943 (8 μM) (a selective blocker of the reverse mode) [20, 22], did not affect the prolonged decay phase (fig. 5A, B). More specifically, Na⁺ removal and KB-R 7943 addition were performed in 140 and 165 nonspiking cells, respectively. Conversely, both treatments exerted a profound inhibitory effect on injury-induced Ca²⁺ oscillations (fig. 5C, D). In more detail, removal of extracellular Na⁺ abrogated the intracellular Ca²⁺ waves in 57 out of 57 cells, and KB-R 7943 was successfully tested in 58 out of 58 cells. The blockade of the Ca²⁺ waves was partially reversible in both cases (fig. 5C, D). Both maneuvers did not affect the decay phase upon which the Ca²⁺ oscillations were superimposed (fig. 5C, D), which is consistent with the findings depicted in figure 5A and B. These data outline for the first time the role served by the reverse mode of NCX in wound-induced oscillations in [Ca²⁺]_i.

Fig. 4. Gap junction blockers abolished the spiking response to mechanical damage. The widely used gap junction blockers, heptanol (4 mM; **A**) and 18 α,β -glycyrrhetic acid (18 α,β Gly) (25 μ M; **B**), abolished the repetitive increases in $[Ca^{2+}]_i$ triggered by injury and return the $[Ca^{2+}]_i$ towards basal levels. Note that Ca^{2+} oscillations were recovered on heptanol washout, whereas the ef-

fect of 18 α,β Gly was irreversible. Nevertheless, the occurrence of a Ca^{2+} response to ATP (20 μ M) indicated that the cells were still viable in the presence of 18 α,β Gly. Similarly, La^{3+} (2.5 μ M; **C**) and Ni^{2+} (10 μ M; **D**) abolished injury-induced Ca^{2+} oscillations. The effect of both drugs was not reversible (not shown).

Injury-Induced Intracellular Ca^{2+} Transients Depend on Ca^{2+} Release from $InsP_3$ Rs

The contribution of $InsP_3$ -dependent Ca^{2+} release to repetitive Ca^{2+} spikes was investigated by inhibiting PLC activity with U73122 (10 μ M). U73122 abrogated the oscillatory response to injury in 30 cells (fig. 6A), but it did not hamper the long-lasting decay phase [7]. Moreover, Ca^{2+} oscillations resumed in 27 out of 27 cells exposed to a low dose (20 μ M) of the $InsP_3$ -releasing agonist ATP [23] (fig. 6B, upper and lower traces). Notably, this treatment did not significantly alter the frequency of ongoing oscillations in 30 out of 30 cells from the same damaged areas (fig. 6B, middle trace). Conversely, ryanodine (20 μ M), which blocks ryanodine receptors at $>10\text{-}\mu$ M con-

centrations [24], did not exert any detectable effect on injury-induced Ca^{2+} transients (fig. 6C). This finding is consistent with the lack of functional ryanodine receptors in the endothelium of excised rat aorta [6]. Taken together, these data demonstrate that the PLC/ $InsP_3$ -signaling cascade contributes with the reverse mode of NCX to injury-induced oscillations in $[Ca^{2+}]_i$. This feature was further investigated by interfering with endoplasmic reticulum recharging during the ongoing variations in $[Ca^{2+}]_i$ induced by endothelial scraping. SERCA (sarcoplasmic-endoplasmic reticulum calcium ATPase) blockade was achieved by exposing the cells to two structurally different drugs, cyclopiazonic acid (10 μ M) and thapsigargin (5 μ M). As depicted in figure 7, thapsigargin

Fig. 5. The reverse mode of NCX sustains the rhythmical changes in $[Ca^{2+}]_i$ induced by injury. **A** Preventing NCX activity by removing extracellular Na^+ ($0Na^+$) did not significantly affect the prolonged decay phase observed in nonoscillating cells. **B** KB-R 7943 ($8 \mu M$), which selectively inhibits the reverse-mode of NCX, did

not impair the long-lasting decline in $[Ca^{2+}]_i$ which followed the initial Ca^{2+} peak. Conversely, perfusing the rings either with an extracellular solution devoid of Na^+ ($0Na^+$; **C**) or with KB-R 7943 ($8 \mu M$; **D**) abolished the oscillatory signal, although neither maneuver altered the underlying decay phase.

($n = 42$) and cyclopiazonic acid ($n = 55$) rapidly inhibited injury-induced Ca^{2+} spikes in all tested cells, despite the presence of Ca^{2+} in the extracellular solution. Both drugs induced an initial elevation in $[Ca^{2+}]_i$, due to Ca^{2+} leakage from the endoplasmic reticulum, which contains most of the $InsP_3$ -sensitive Ca^{2+} reservoir. These results demonstrate that refilling of the $InsP_3$ -sensitive Ca^{2+} pool by SERCA is required to maintain injury-induced Ca^{2+} oscillations.

P_{2Y} Receptors and Tyrosine-Kinase Receptors Do Not Contribute to Injury-Induced Ca^{2+} Oscillations

We have previously shown that the initial Ca^{2+} response to mechanical damage is elicited by ATP (or its

derivatives) binding to P_{2Y} receptors, which in turn leads to PLC activation and $InsP_3$ -dependent Ca^{2+} release [5]. In order to assess whether purinergic receptors stimulate PLC to synthesize $InsP_3$ also during the following $[Ca^{2+}]_i$ oscillations, we probed the effect of suramin ($300 \mu M$), a broad-spectrum blocker of P_{2Y} receptors which dampens the initial Ca^{2+} response to injury [5]. Suramin did not affect the ongoing Ca^{2+} transients in 16 out of 16 cells (fig. 8A). As tissue injury may also liberate a number of growth factors in the extracellular space, we assessed the involvement of tyrosine-kinase receptors (TKRs) on the spiking response to injury. Figure 8B shows that genistein ($100 \mu M$), a widely employed TKR blocker, did not impair injury-elicited Ca^{2+} oscillations in 20 out of 20 cells. Both

Fig. 6. The role of the PLC/InsP₃ pathway in injury-induced Ca²⁺ oscillations. **A** Blockade of PLC with U73122 (10 μM) inhibited the periodic changes in [Ca²⁺]_i following the initial Ca²⁺ peak without interfering with the underlying decline to resting levels, as shown elsewhere [8]. **B** Addition of a low dose of the InsP₃-mobilizing agonist, ATP (20 μM), resumed Ca²⁺ oscillations in nonspiking cells (upper and lower traces), whereas it did not modify the interspike interval of ongoing transients (middle trace). Note that, at the concentration employed here, ATP did not stimulate Ca²⁺ entry in the endothelium of excised rat aorta [23]. **C** Ryanodine (20 μM), a selective ryanodine receptor inhibitor at this dose, did not cause any detectable change in the spiking response to injury.

Fig. 7. Inhibition of endoplasmic reticulum reuptake abrogates injury-elicited [Ca²⁺]_i oscillations. Thapsigargin (5 μM; **A**) and cyclopiazonic acid (CPA) (10 μM; **B**) irreversibly hindered the spiking response to mechanical damage.

the addition of suramin and genistein caused a decrease in fura-2 fluorescence, which did not affect the ability of the recording system to detect the increases in [Ca²⁺]_i (fig. 8B). Overall, these experiments indicate that PLC activity driving injury-induced Ca²⁺ oscillations is not stimulated by either P_{2Y} receptors or TKRs.

Discussion

Oscillations in [Ca²⁺]_i have long been known to engage the transcriptional program leading to cell proliferation and resistance to apoptosis and may drive cell motility [21, 25]. Therefore, we endeavored to elucidate the signaling

Fig. 8. Suramin and genistein do not affect the spiking response to injury. Suramin (300 μM ; **A**) and genistein (100 μM ; **B**) did not affect the repetitive Ca^{2+} spikes triggered by endothelial damage in rat aortic rings.

cascade shaping the repetitive Ca^{2+} spikes that may occur at the lesion site of a physiological model, such as the excised rat aorta. We found that Ca^{2+} oscillations superimposed on the decay phase that follows the initial Ca^{2+} peak reversibly cease in the absence of extracellular Ca^{2+} and in the presence of the gap junction blockers, La^{3+} , Ni^{2+} , heptanol and 18 α,β -glycyrrhetic acid. These findings strongly suggest that the same membrane pathway is involved in both the monotonic decline of the $[\text{Ca}^{2+}]_i$ towards the baseline and the overlapping Ca^{2+} spikes. The question then arose as to the nature of the additional mechanism conferring the oscillatory behavior to the decay phase. We showed that the coordinated interplay between NCX-mediated Ca^{2+} entry and InsP_3 -dependent Ca^{2+} release contributes to injury-induced Ca^{2+} oscillations. The following pieces of evidence support this hypothesis. First, gap junction channels/hemichannels serve as nonselective cation conduits and they may thus allow Na^+ flux into the cells surrounding the wound edge [18]. Second, our study showed that blocking NCX activity by replacing extracellular Na^+ with an equimolar amount of N-methyl-D-glucamine, or selective inhibition of its reverse mode with KB-R 7943, did not affect the decay phase in nonspiking cells. These data indicate that NCX does not mediate Ca^{2+} inflow in the latter. Nevertheless, both maneuvers reversibly abrogated the oscillations in intracellular Ca^{2+} levels overlapping the long recovery of $[\text{Ca}^{2+}]_i$ towards the baseline. These observations suggest that, in oscillating cells, Ca^{2+} entry is mediated by NCX, which is switched in the backward mode by gap junction channel/

hemichannel-mediated Na^+ entry. Third, PLC inhibition with U73122 abrogated the repetitive Ca^{2+} transients, while the InsP_3 -releasing agonist, ATP, resumed Ca^{2+} oscillations in nonspiking cells. Fourth, depletion of the InsP_3 -sensitive Ca^{2+} pool with either cyclopiazonic acid or thapsigargin hindered injury-elicited Ca^{2+} oscillations. Fifth, both the amplitude of Ca^{2+} spikes and the interspike interval were constant during the observation period. These features are typical of regenerative Ca^{2+} release from InsP_3Rs and have already been described in other cell types [12, 25]. Therefore, we suggest that open gap junction channels/hemichannels elevate $[\text{Ca}^{2+}]_i$ and this is responsible for the decaying phase with or without oscillations. Repetitive Ca^{2+} spikes arise in cells where NCX-mediated Ca^{2+} entry and PLC-dependent InsP_3 -induced Ca^{2+} release have been detected. Therefore, such mechanisms are likely to contribute to the onset of the Ca^{2+} waves following the initial Ca^{2+} peak, although additional pathways may not be ruled out. Consistently, the pharmacological experiments described in figure 5A, B and in Berra-Romani et al. [5] demonstrate that NCX does not mediate any detectable Ca^{2+} inflow and PLC is inactive after the initial Ca^{2+} peak in nonspiking cells. PLC stimulation might depend on the transmitters, mainly ATP and its derivatives, and the growth factors, which are liberated by the injury in the extracellular space [6]. However, neither suramin, an antagonist of P_{2Y} receptors, nor genistein, a well-known blocker of TKRs, affected the spiking response to endothelial scraping. Therefore, PLC activity detected in oscillating cells after the initial Ca^{2+} peak is

not driven by purinoreceptors or TKRs. Consistent with this, a recent study reported that PLC δ , a PLC isoform that is stimulated independently on membrane receptors by an increase in $[Ca^{2+}]_i$ (0.1–10 μM), is activated in injured endothelium [26]. As either P_{2Y} receptors or TKRs are apparently not involved and do not drive the spiking signal at the lesion site, an alternative stimulus for PLC activation must be conceived. The Ca^{2+} -dependent δ -isotype might be a suitable candidate to supply the $InsP_3$ necessary to elicit Ca^{2+} release from the endoplasmic reticulum during the oscillatory signal and might be activated in those cells experiencing higher Ca^{2+} concentrations due to the opening of gap junction channels/hemichannels (either directly or indirectly via NCX). The positive feedback between NCX-mediated Ca^{2+} inflow, PLC (possibly PLC δ), and $InsP_3$ might provide a mechanism for regenerative Ca^{2+} signals to occur, and drive only a minor percentage of cells in and out of oscillations. If and how the reverse mode of NCX and PLC activation are arranged and whether additional pathways are recruited by injury in oscillating cells remains to be elucidated. Cell-to-cell heterogeneity is a hallmark of $[Ca^{2+}]_i$ oscillations occurring in ECs studied in *in situ* preparations [27, 28], including rat aortic rings [29]. The molecular underpinnings of the variability in the spatiotemporal organization of Ca^{2+} signals in native ECs is still under debate, although the heterogeneous topography of ECs at the luminal surface of the vessel wall, the influence of neighboring cells within the vascular wall (e.g. adjacent ECs and smooth muscle cells), and the different density or distribution of the signaling components might play a role [27–32].

Mechanical damage does not only induce Ca^{2+} oscillations in ECs adjacent to the lesion site. Indeed, the scraping procedure turns the endothelium of excised rat aorta from a quiescent state to an active mode, as even cells located further away from the lesion edge could display both subthreshold (i.e. nonspiking) and suprathreshold (i.e. spiking) Ca^{2+} events. These injury-dependent Ca^{2+} signals were not synchronized with the Ca^{2+} oscillations occurring at the wound site and future analysis will reveal whether they are intimately correlated, i.e. whether Ca^{2+} oscillations in the first row of cells drive the Ca^{2+} activity occurring in the innermost tissue, and why some cells spike and others do not. These features are different from the results obtained in cultured endothelial monolayers, where the Ca^{2+} waves elicited by injury are transmitted in a 1:1 ratio and in a nonregenerative fashion to cells further back from the wound edge [6]. As extracellular transmitters are unlikely to underlie injury-induced Ca^{2+} oscillations in rat aortic rings, variability in intercellular com-

munication might account for the random onset of Ca^{2+} spikes at remote sites from the lesion margin. The lack of synchrony between oscillating cells at the first row and those spiking in the innermost tissue might depend on a failure in the intercellular propagation of Ca^{2+} -releasing second messengers (e.g. $InsP_3$ or Ca^{2+} itself). Moreover, SMCs within the vascular wall may regulate endothelial Ca^{2+} activity through the so-called myoendothelial junctions, which allow for the diffusion of either second-messengers or electrotonic currents able to impact on endothelial Ca^{2+} signaling [30–32]. The possibility that SMCs influence the propagation of the Ca^{2+} waves emanating from the lesion edge to more remote cells by sensitizing or depressing EC excitability in the innermost tissue cannot presently be ruled out. Understanding how injury-induced Ca^{2+} oscillations spread away from the wound edge was, however, beyond the scope of our study.

The appearance of intracellular Ca^{2+} oscillations at the edge of a discrete wounded area might signal the activation of a number of prosurvival pathways within vascular endothelium. It has already been reported that a monotonic Ca^{2+} signal stimulates ECs facing the lesion site to replicate and migrate in order to close the wound [6]. The molecular decoders which translate the Ca^{2+} signal occurring at the lesion margin into a biological response capable of repairing the wound are still unclear. Ca^{2+} oscillations have been shown to selectively recruit a number of Ca^{2+} -dependent transcription factors, such as NFAT and NF- κB , which may activate the genetic programme that leads to cell division, differentiation, and migration [12–15]. The interspike interval in the injured endothelium of excised rat aorta is about 100 s (fig. 2B), which is particularly suited to enable Ca^{2+} spikes to activate both NFAT and NF- κB [14]. More specifically, NFATc1 drives cell cycle progression by inducing the cell cycle-related genes cyclin D1, cyclin D2, retinoblastoma protein, and c-Myc, which permit the passage of the G₁/S checkpoint [21]. Furthermore, repetitive Ca^{2+} spikes promote the transcription of the immediate early genes, such as c-Fos and c-Jun, which stimulate the G₀/G₁ transition and induce cell migration [33]. In this context, gene expression induced by Ca^{2+} oscillations may occur hours after the onset of the spikes, which is consistent with their long-term impact on the healing process [13]. Finally, Ca^{2+} oscillations may directly drive cell migration by rearranging the actin cytoskeleton and disassembling focal adhesions [6]. It is, therefore, conceivable that the recurrent Ca^{2+} waves occurring at the lesion site might play a key role in promoting endothelial regeneration by stimulating EC growth and motility.

In conclusion, this study described the molecular underpinnings of the oscillatory response to injury in the endothelium of excised rat aorta. We demonstrated for the first time that the coordinated interaction between NCX-mediated Ca^{2+} entry and InsP_3 -dependent Ca^{2+} release shapes the oscillatory response to mechanical damage. Na^+ entry responsible for reversing NCX activity mode in ECs facing the lesion site is provided by gap junction channels/hemichannels. Injury-induced oscillations in $[\text{Ca}^{2+}]_i$ might play a key role in healing damaged endothelium, by stimulating ECs adjacent to the lesion to prolifer-

ate and migrate in order to cover the denuded membrane basement. Unraveling the signal transduction pathways engaged by mechanical injury is essential to design novel therapeutic treatment aimed at restoring endothelial integrity and limiting cardiovascular diseases [10].

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References

- Michiels C: Endothelial cell functions. *J Cell Physiol* 2003;196:430–443.
- Munaron L: Intracellular calcium, endothelial cells and angiogenesis. *Recent Pat Anti-cancer Drug Discov* 2006;1:105–119.
- Tesfamariam B, DeFelice AF: Endothelial injury in the initiation and progression of vascular disorders. *Vascul Pharmacol* 2007;46:229–237.
- Van Belle E, Bauters C, Asahara T, Isner JM: Endothelial regrowth after arterial injury: from vascular repair to therapeutics. *Cardiovasc Res* 1998;38:54–68.
- Berra-Romani R, Raqeeb A, Avelino-Cruz JE, Moccia F, Oldani A, Speroni F, Taglietti V, Tanzi F: Ca^{2+} signaling in injured in situ endothelium of rat aorta. *Cell Calcium* 2008;44:298–309.
- Moccia F, Avelino-Cruz JE, Sánchez-Hernández Y, Tanzi F: Ca^{2+} signalling in damaged endothelium: do connexin hemichannels aid in filling the gap? *Curr Drug Ther* 2010;5:277–287.
- Tran PO, Hinman LE, Unger GE, Sammak PJ: A wound-induced $[\text{Ca}^{2+}]_i$ increase and its transcriptional activation of immediate early genes is important in the regulation of motility. *Exp Cell Res* 1999;246:319–326.
- Zhao Z, Walczysko P, Zhao M: Intracellular Ca^{2+} stores are essential for injury induced Ca^{2+} signaling and re-endothelialization. *J Cell Physiol* 2008;214:595–603.
- Nilius B, Droogmans G: Ion channels and their functional role in vascular endothelium. *Physiol Rev* 2001;81:1415–1459.
- Figueroa XF, Isakson BE, Duling BR: Connexins: gaps in our knowledge of vascular function. *Physiology* 2004;19:277–284.
- Gabriels JE, Paul DL: Connexin43 is highly localized to sites of disturbed flow in rat aortic endothelium but connexin37 and connexin40 are more uniformly distributed. *Circ Res* 1998;83:636–643.
- Berridge MJ: Inositol trisphosphate and calcium oscillations. *Biochem Soc Symp* 2007;74:1–7.
- Parekh AB: Decoding cytosolic Ca^{2+} oscillations. *Trends Biochem Sci* 2011;36:78–87.
- Lewis RS: Calcium oscillations in T-cells: mechanisms and consequences for gene expression. *Biochem Soc Trans* 2003;31:925–929.
- Berridge MJ: Calcium signalling and cell proliferation. *Bioessays* 1995;17:491–500.
- Schalper KA, Palacios-Prado N, Orellana JA, Sáez JC: Currently used methods for identification and characterization of hemichannels. *Cell Commun Adhes* 2008;15:207–218.
- Griffith TM: Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? *Br J Pharmacol* 2004;141:881–903.
- Ruiz-Meana M, Garcia-Dorado D, Hofstaetter B, Piper HM, Soler-Soler J: Propagation of cardiomyocyte hypercontracture by passage of Na^+ through gap junctions. *Circ Res* 1999;85:280–287.
- Poburko D, Fameli N, Kuo KH, van Breemen C: Ca^{2+} signaling in smooth muscle: TRPC6, NCX and LNats in nanodomains. *Channels (Austin)* 2008;2:10–12.
- Bondarenko A: Sodium-calcium exchanger contributes to membrane hyperpolarization of intact endothelial cells from rat aorta during acetylcholine stimulation. *Br J Pharmacol* 2004;143:9–18.
- Lipskaia L, Lompré AM: Alteration in temporal kinetics of Ca^{2+} signaling and control of growth and proliferation. *Biol Cell* 2004;96:55–68.
- Berra-Romani R, Raqeeb A, Guzman-Silva A, Torres-Jácome J, Tanzi F, Moccia F: Na^+ - Ca^{2+} exchanger contributes to Ca^{2+} extrusion in ATP-stimulated endothelium of intact rat aorta. *Biochem Biophys Res Commun* 2010;395:126–130.
- Berra-Romani R, Rinaldi C, Raqeeb A, Castelli L, Magistretti J, Taglietti V, Tanzi F: The duration and amplitude of the plateau phase of ATP- and ADP-evoked Ca^{2+} signals are modulated by ectonucleotidases in situ endothelial cells of rat aorta. *J Vasc Res* 2004;41:166–173.
- Carafoli E, Santella L, Branca D, Brini M: Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol* 2001;36:107–260.
- Berridge MJ: Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 2009;1793:933–940.
- Mi LY, Ettenson DS, Edelman ER: Phospholipase C-delta extends intercellular signalling range and responses to injury-released growth factors in non-excitable cells. *Cell Prolif* 2008;41:671–690.
- Duza T, Sarelius IH: Localized transient increases in endothelial cell Ca^{2+} in arterioles in situ: implications for coordination of vascular function. *Am J Physiol Heart Circ Physiol* 2004;286:H2322–H2331.
- Mumtaz S, Burdya G, Borisova L, Wray S, Burdya T: The mechanism of agonist induced Ca^{2+} signalling in intact endothelial cells studied confocally in situ arteries. *Cell Calcium* 2011;49:66–77.
- Huang TY, Chu TF, Chen HI, Jen CJ: Heterogeneity of $[\text{Ca}^{2+}]_i$ signaling in intact rat aortic endothelium. *FASEB J* 2000;14:797–804.
- Lambole M, Pittet P, Koenigsberger M, Sauer R, Bény JL, Meister JJ: Evidence for signaling via gap junctions from smooth muscle to endothelial cells in rat mesenteric arteries: possible implication of a second messenger. *Cell Calcium* 2005;37:311–320.
- Rahman A, Hughes A, Matchkov V, Nilsson H, Aalkjaer C: Antiphase oscillations of endothelium and smooth muscle $[\text{Ca}^{2+}]_i$ in vasomotion of rat mesenteric small arteries. *Cell Calcium* 2007;42:536–547.
- Sandow SL, Haddock RE, Hill CE, Chadha PS, Kerr PM, Welsh DG, Plane F: What's where and why at a vascular myoendothelial microdomain signalling complex. *Clin Exp Pharmacol Physiol* 2009;36:67–76.
- Roderick HL, Cook SJ: Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival. *Nat Rev Cancer* 2008;8:361–375.