Stealth ryanodine-sensitive Ca^{2+} release contributes to activity of capacitative Ca^{2+} entry and nitric oxide synthase in bovine endothelial cells

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1. The involvement of ryanodine-sensitive Ca^{2+} release (RsCR) in bradykinin (Bk)-induced Ca^{2+} release, capacitative Ca^{2+} entry (CCE) and nitric oxide synthase (NOS) activation was assessed in freshly isolated bovine coronary artery endothelial cells.

2. Using deconvolution microscopy fura-2 was found throughout the whole cytosol, while the cell membrane impermeable dye FFP-18 was exclusively in the cell membrane. Thus, perinuclear ($[Ca^{2+}]_{pn}$) and subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sp}$) were monitored using fura-2 and FFP-18.

3. Inhibition of Na^+–Ca^{2+} exchange by lowering extracellular Na^+ concentration augmented the Bk-induced $[Ca^{2+}]_{pn}$ signal in Ca^{2+}-free solution. This effect was abolished when RsCR was prevented with 25 μmol l^{-1} ryanodine, while inhibition of RsCR had no effect on Bk-induced increase in $[Ca^{2+}]_{pn}$ without inhibition of Na^+–Ca^{2+} exchange.

4. Initiating RsCR by 200 nmol l^{-1} ryanodine increased $[Ca^{2+}]_{sp}$, while $[Ca^{2+}]_{pn}$ remained constant. However, when Na^+–Ca^{2+} exchange was prevented, ryanodine was also able to elevate $[Ca^{2+}]_{pn}$.

5. Blockage of RsCR diminished Ca^{2+} extrusion in response to stimulation with Bk in normal Na^+-containing solution.

6. Inhibition of RsCR blunted Bk-activated CCE, while inhibition of Na^+–Ca^{2+} exchange during stimulation enhanced CCE.

7. Although direct activation of RsCR failed to activate NOS, inhibition of RsCR diminished the effect of ATP and Bk on NOS, while the effect of thapsigargin remained unchanged.

8. These data suggest that during stimulation subplasmalemmal RsCR occurs, which contributes to the activities of CCE and NOS. Thus, the function of the subplasmalemmal Ca^{2+} control unit must be extended as a regulator for CCE and NOS.

In endothelial cells an increase in intracellular free Ca^{2+} concentration is an important mediator in the formation of vasoactive compounds, such as nitric oxide and endothelin (for review see Graier *et al.* 1994*b*). Agonists activate phospholipase C which synthesizes inositol-1,4,5 trisphosphate ($InsP_3$) and evokes Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores (Freay *et al.* 1989). Depletion of Ca^{2+} stores triggers activation of capacitative Ca^{2+} entry (CCE; Putney, 1986; Putney, 1990; Schilling *et al.* 1992), possibly mediated via tyrosine kinase (Fleming *et al.* 1996) and/or cytochrome P450 epoxygenase-derived metabolites (Graier *et al.* 1995; Hoebel *et al.* 1997).

Besides $InsP_3$-mediated Ca^{2+} release and CCE, the involvement of a Ca^{2+}-induced Ca^{2+} release mechanism (ryanodine-sensitive Ca^{2+} release; RsCR) has been proposed to contribute to endothelial Ca^{2+} signalling. Although binding sites for ryanodine have been demonstrated on the endoplasmic reticulum membrane in endothelial cells (Lesh *et al.* 1993; Graier *et al.* 1998), our knowledge of the importance of RsCR for Ca^{2+} signalling and endothelial cell function is limited. While there is evidence that RsCR occurs during agonist stimulation (Mozhayeva, 1996), direct stimulation of RsCR by ryanodine failed to evoke Ca^{2+} transients; instead ryanodine slowly increased the intracellular Ca^{2+}

concentration (Wang *et al.* 1995). A similar slow release of Ca^{2+} was described for caffeine, a non-specific activator of Ca^{2+}-induced Ca^{2+} release, in rabbit aortic endothelial cells (Wang *et al.* 1995). However, caffeine evoked rapid Ca^{2+} transients in human (Ziegelstein *et al.* 1994) and porcine endothelial cells (Graier *et al.* 1994*a*). In contrast to these reports indicating an involvement of RsCR in endothelial Ca^{2+} transients, several authors have failed to find its contribution to CCE (Graier *et al.* 1994*a*; Ziegelstein *et al.* 1994). Recently, we described the generation of localized Ca^{2+} gradients in the subplasmalemmal area and suggested RsCR may contribute to subplasmalemmal Ca^{2+} elevation (Graier *et al.* 1998). Although there is increasing evidence that localized Ca^{2+} signalling is important for the generation of vasoactive compounds, such as prostacyclin and nitric oxide (Lückhoff *et al.* 1988), very little is known about the contribution of RsCR to agonist-induced stimulation of nitric oxide synthase (NOS).

In the present study we used endothelial cells freshly isolated from bovine left circumflex coronary arteries to investigate the involvement of RsCR in agonist-induced intracellular Ca^{2+} release, CCE, and activation of NOS. Our data indicate that, during stimulation with agonists, RsCR occurs mainly in the subplasmalemmal area. Hence, although RsCR may not stimulate CCE *per se*, it contributes to activity of CCE and stimulation of NOS.

METHODS

Materials

Cell culture chemicals were purchased from Life Technologies, Vienna, Austria and fetal calf serum (FCS, premium quality) was from PAA Laboratories, Linz, Austria. Petri dishes were obtained from Corning, Vienna, Austria. Fura-2 AM and fura dextran potassium salt (MW 70 000) were from Molecular Probes, Leiden, The Netherlands. FFP-18 and ryanodine were purchased from Calbiochem, Vienna, Austria. Hepes acid was from Amresco, Vienna, Austria and buffer salts were purchased from Merck, Vienna, Austria. All other materials were from Sigma, Vienna, Austria. The human umbilical vein endothelial cell line EA.hy 926 was a generous gift from Dr Cora-Jean S. Edgell, Pathology Department, University of North Carolina, NC, USA (Edgell *et al.* 1983).

Cell isolation and culture

Endothelial cells were isolated from bovine left circumflex coronary arteries as described previously (Graier *et al.* 1992; Graier *et al.* 1995). Cattle were killed by a shot in the head in the local slaughterhouse. After they were exsanguinated, the hearts were extracted and put on ice for transport to the laboratory. For cell isolation, vessels were incubated at 37 °C in Dulbecco's minimum essential medium (DMEM) containing 200 U ml^{-1} collagenase (type II), trypsin inhibitor (soybean type I; 1 mg ml^{-1}), minimum essential medium (MEM) dilutions (v/v) of essential amino acids (2%), MEM non-essential amino acids (1%) and MEM (1%) vitamins (Life Technologies, Vienna, Austria). After 15 min incubation, parts of the incubation buffer were aspirated for cell culture followed by an additional 20 min for experiments with freshly isolated cells. Endothelial cells were cultured up to passage 2 in Opti-MEM containing 2% (v/v) fetal calf serum. Cell culture purity was tested by typical cobblestone morphology and the lack of smooth muscle α-actin. Cells from the EA.hy 926 cell line (passage 63 and higher) were grown in DMEM containing 10% fetal calf serum, 4·5 mg l^{-1} D-glucose and a 1% (w/v) hypoxanthine–aminopterine–thymidine mixture (HAT).

Ca^{2+} measurement

Intracellular free Ca^{2+} concentration was determined in single endothelial cells using microfluorometry (Graier *et al.* 1995). As described previously (Graier *et al.* 1998), different Ca^{2+}-sensitive dyes were used to monitor perinuclear free Ca^{2+} concentration ($[Ca^{2+}]_{pn}$; fura-2) and the subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sp}$; FFP-18). For fura-2 loading, the cells were incubated in the dark with 2 μmol l^{-1} fura-2 AM in DMEM. After 45 min at room temperature, cells were centrifuged, washed twice and resuspended in Hepes-buffered solution containing (mmol l^{-1}): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2·5 $CaCl_2$, 10 Hepes acid, pH = 7·4 (Hepes-buffered solution; HBS). The cell membrane impermeable dye FFP-18 was loaded using the laser-generated stress wave loading technique (Graier *et al.* 1998). Briefly, approximately 106 cells were suspended in 350 μl Phenol Red-free MEM containing 2 μmol l^{-1} FFP-18 and transferred into a well of a microtitre plate. After the well had been sealed with 1·25 mm aluminum foil, laser-induced stress waves were generated by 3 to 5 shots of a Nd:YAG laser (70 mJ) to the foil, focused 0·5 cm behind the foil. After an equilibration period of 10 to 30 min in the dark, cells were centrifuged and resuspended in HBS. For Ca^{2+} measurements Ca^{2+}-free HBS was used containing (mmol l^{-1}): either 145 NaCl, 5 KCl, 1 $MgCl_2$, 0·01 EGTA, 10 Hepes-acid, pH = 7·4 (i.e. control buffer) or 19 NaCl, 5 KCl, 1 $MgCl_2$, 126 choline chloride, 0·01 EGTA, 10 Hepes acid, pH = 7·4 (i.e. low Na^+ solution). For measurement of Ca^{2+} extrusion, cells were resuspended in Ca^{2+}-free HBS containing 1 μmol l^{-1} fura dextran potassium salt (MW 70 000).

Data acquisition

Single cell $[Ca^{2+}]_{pn}$ was recorded in a microfluorometer, which excited cells with alternating 360 and 380 nm light, while emission was detected at 510 nm using a modified photon counting technique (Sturek *et al.* 1991; Graier *et al.* 1995). Counts were converted to an analog signal by an optical processor and the fluorescence intensity for each excitation and/or emission wavelength was acquired by a personal computer running AxoBASIC® 1·0 (Axon Instruments, Foster City, CA, USA). For measurements of $[Ca^{2+}]_{sp}$, fluorescence of FFP-18 at 335–365 nm excitation and 500 nm emission was monitored in suspended cells every 0·2 s in a spectrofluorometer (Hitachi F-4500). Extrusion of Ca^{2+} was measured with fura dextran by monitoring extracellular Ca^{2+} concentration at 340 and 364 nm excitation and 500 nm emission.

Data analysis

Due to the reported difficulties in the calibration techniques for intracellular Ca^{2+} concentration (Sturek *et al.* 1991; Graier *et al.* 1995; Wang *et al.* 1995), Ca^{2+} concentrations are expressed in ratio units (ratio F_{360}/F_{380} for fura-2; ratio F_{335}/F_{365} for FFP-18 and ratio F_{340}/F_{364} for fura dextran).

Digital confocal microscopy

Images were obtained with a scientific-grade CCD camera using conventional lamp illumination. Out-of-focus fluorescence was calculated with a computer algorithm using the three-dimensional point spread function for each microscope objective (Waters & Brown, 1996). The instrument setup included a liquid-cooled CCD camera (Quantix, Photometrics, Munich, Germany) on an inverted

microscope (Nikon Eclipse TE300, Nikon, Vienna, Austria) with z-stage motor controlled by the Ludl-Z-stage control box (Ludl, Inc., Fairfield Imaging, Turnbridge Wells, UK) in association with Image Pro 3·0 software (Media Cybernetics, Fairfield Imagining, Turnbridge Wells, UK). The camera head was cooled to −40 °C and the gain level was 2. Images were collected with a CFI Plan Fluor × 100 oil immersion objective (NA 1·3; 0·068 μm pixel^{-1}, Nikon, Vienna, Austria) and the distance between images in the z scan was 0·3 μm. Images were collected with Volume Scan® (VayTek, Inc., Fairfield Imaging, Turnbridge Wells, UK) and out-of-focus fluorescence was calculated with Micro Tome® deconvolution software (VayTek, Inc., Fairfield Imaging, Turnbridge Wells, UK) using the advanced constrained iterative deconvolution algorithm (5 iterations). These deconvolution procedures removed out-of-focus fluorescence, thus producing 'digital confocal' images.

Three-dimensional reconstruction of data

The deconvolved images were transformed into 8-bit images to conduct three-dimensional reconstruction with the volume rendering program VoxBlast®, which utilizes a back propagation-weighted average algorithm (VayTek, Inc., Fairfield Imaging, Turnbridge Wells, UK).

Measurements of NOS activity

Activity of NOS was determined by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described previously (Graier *et al.* 1996). Briefly, cells were cultured in 6-well plastic dishes. Experiments were performed in HBS and the extracellular Na^+ concentration ($[Na^+]_o$) indicated. After equilibrating the cells in HBS containing the solvent (0·5% DMSO) or ryanodine for 5 min, [^{3}H]-L-arginine (106 c.p.m. dish^{-1}) was added together with the compound to be tested. After 15 min, incubation buffer was aspirated and 1 ml chilled 0·01 mol l^{-1} HCl was added. After 1 h at 4 °C, a 100 μl aliquot was saved (i.e. incorporated radioactivity) and to the remaining 900 μl, 100 μl of 200 mmol l^{-1} sodium acetate buffer containing 10 mmol l^{-1} L-citrulline (pH = 13·0) was added. From this mixture, 900 μl were placed on a Dowex AG50W-X8 and [^{3}H]-L-citrulline was purified by washing with 1 ml water. After considering the column recovery for each column and a dilution correction, activity of NOS was calculated as the percentage of [^{3}H]-L-citrulline radioactivity within the total incorporated radioactivity. In the presence of 100 μmol l^{-1} L-GN-nitroarginine, which reduced basal [^{3}H]-L-citrulline formation by about 40–50%, no increase in the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline with agonists was found. The L-GN-nitroarginine resistant formation of [^{3}H]-L-citrulline under each condition was subtracted to demonstrate NOS-dependent formation of [^{3}H]-L-citrulline.

Statistics

All data points represent the mean ± S.E.M. Data evaluation was performed by analysis of variance (ANOVA) and statistical significance was estimated by Scheffé's *post hoc* test. The level of significance was defined as $P < 0·05$.

RESULTS

Contribution of RsCR to $[Ca^{2+}]_{pn}$ elevation

In the absence of extracellular Ca^{2+}, lowering $[Na^+]_o$ from 145 to 19 mmol l^{-1} increased the Bk-induced elevation of $[Ca^{2+}]_{pn}$ by 112%, while resting $[Ca^{2+}]_{pn}$ remained unchanged (Fig. 1). Under low $[Na^+]_o$ conditions, the mobilization of $[Ca^{2+}]_{pn}$ following the addition of 2·5 mmol l^{-1} extracellular Ca^{2+} (a marker for CCE) was augmented by 55% in cells pre-stimulated by Bk (Fig. 1). Accordingly, Bk-induced Mn^{2+} quench was increased by 23% when cells were stimulated with 100 nmol l^{-1} Bk under low $[Na^+]_o$ conditions (data not shown). Similar findings were obtained in the EA.hy926 cell line using 10 μmol l^{-1} histamine (data not shown).

To investigate the involvement of RsCR in the Bk-induced Ca^{2+} signalling, Bk-initiated Ca^{2+} release and CCE in the absence and presence of 25 μmol l^{-1} ryanodine at normal (i.e. 145 mmol l^{-1}) $[Na^+]_o$ were compared. There was no difference in the increase in $[Ca^{2+}]_{pn}$ to 100 nmol l^{-1} Bk in the Ca^{2+}-free HBS in the absence and presence of ryanodine, while CCE activity was reduced by 60% in the presence of

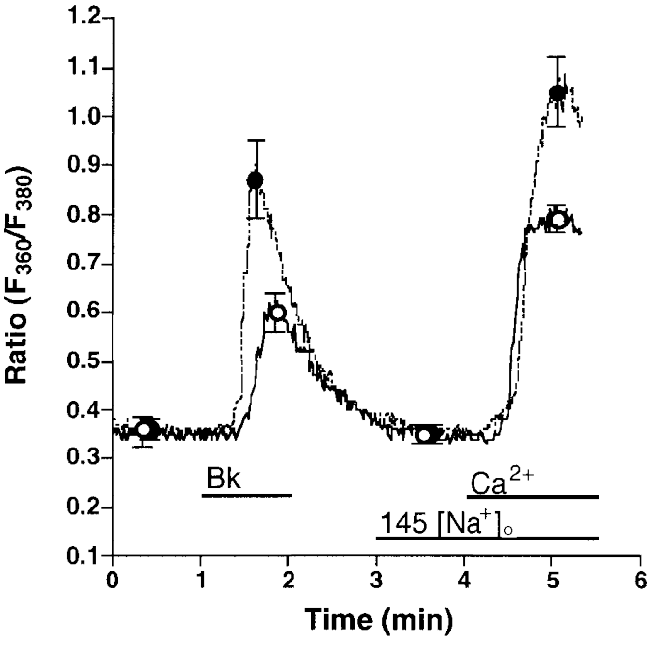

Figure 1. Effect of low extracellular $[Na^+]_o$ on agonist-induced changes of $[Ca^{2+}]_{pn}$ in endothelial cells freshly isolated from bovine left circumflex arteries

Freshly isolated endothelial cells were loaded with fura-2 to measure $[Ca^{2+}]_{pn}$. In Ca^{2+}-free HBS containing 145 (145 $[Na^+]_o$, ○, continuous line) or 19 (19 $[Na^+]_o$, ●, dashed line) mmol l^{-1} Na^+, cells were stimulated for 1 min with 100 nmol l^{-1} Bk followed by an addition of 2·5 mmol l^{-1} Ca^{2+} as indicated. In experiments using 19 mmol l^{-1} Na^+-containing solution, $[Na^+]_o$ was changed to 145 mmol l^{-1} at time 3 min. Points represent means ± S.E.M. ($n = 22$).

ryanodine (Fig. 2*A*). Similar results were obtained in Mn^{2+} quench experiments. Thus, in the presence of ryanodine Bk-induced Mn^{2+} quench was reduced by 78% (7·3 ± 2·2 to 1·6 ± 0·2% decrease of initial fluorescence at 360 nm excitation min^{-1}; $n = 4$, $P < 0·05$). In contrast, addition of ryanodine to Bk-prestimulated cells immediately prior to the addition of Ca^{2+} had no effect on CCE activity (data not shown).

When Na^+–Ca^{2+} exchange was prevented by lowering $[Na^+]_o$ to 19 mmol l^{-1}, 25 μmol l^{-1} ryanodine diminished the effect of Bk on $[Ca^{2+}]_{pn}$ in Ca^{2+}-free solution by 51% (Fig. 2*B*). Moreover, CCE stimulated by Bk was diminished by 77% when ryanodine was present (Fig. 2*B*).

Figure 3 summarizes the data obtained in the above protocols in terms of Bk-induced intracellular Ca^{2+} release (Fig. 3*A*) and CCE (Fig. 3*B*).

Measurement of RsCR in endothelial cells

Endothelial RsCR was further investigated using 200 nmol l^{-1} ryanodine in Ca^{2+}-free HBS. As shown in Fig. 4, there was no detectable increase in $[Ca^{2+}]_{pn}$ to 200 nmol l^{-1} ryanodine in 145 mmol l^{-1} Na^+-containing buffer. In contrast, under low $[Na^+]_o$ conditions 200 nmol l^{-1} ryanodine evoked a small, but significant, increase in $[Ca^{2+}]_{pn}$ (Fig. 4). In cells stimulated with 200 nmol l^{-1} ryanodine in 19 mmol l^{-1} $[Na^+]_o$, elevation of $[Ca^{2+}]_{pn}$ following the external addition of 2·5 mmol l^{-1} Ca^{2+} was not significantly different from that observed using 145 mmol l^{-1} Na^+-containing solution. This increase in $[Ca^{2+}]_{pn}$ was also similar to that measured in resting cells (data not shown). Furthermore, 200 nmol l^{-1} ryanodine failed to initiate Mn^{2+} entry in either 19 or 145 mmol l^{-1} Na^+-containing solution (data not shown).

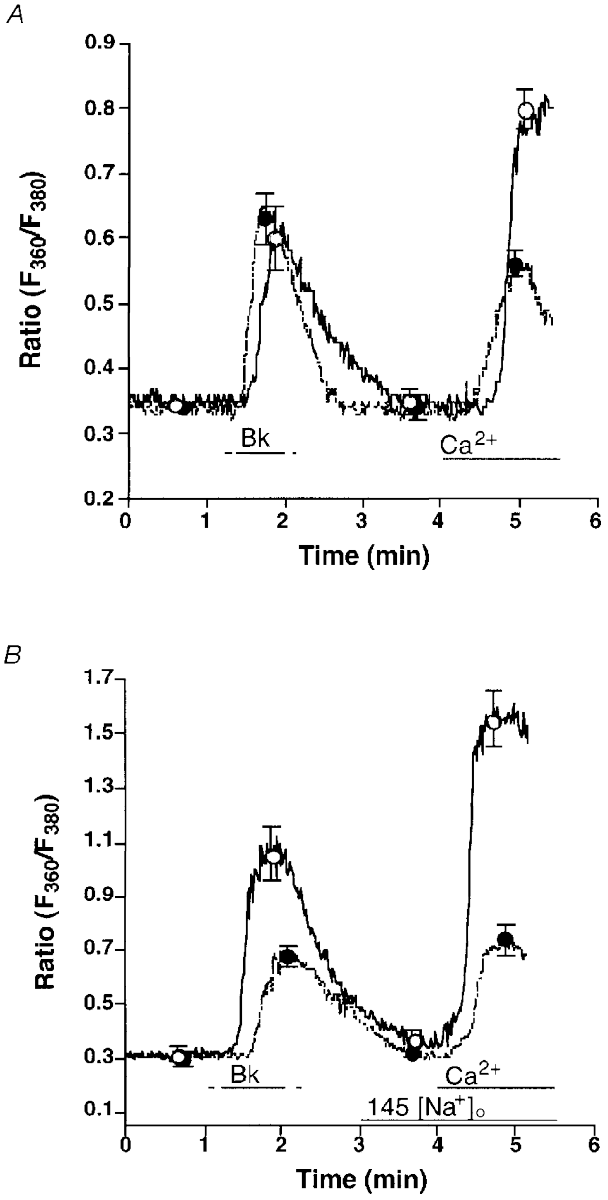

Figure 2. Effect of 25 μmol l^{-1} ryanodine on Bk-induced changes of $[Ca^{2+}]_{pn}$ in single freshly isolated bovine left circumflex endothelial cells in 145 (panel *A*) and 19 (panel *B*) mmol l^{-1} Na^+-containing solution

Freshly isolated endothelial cells were loaded with fura-2 to measure $[Ca^{2+}]_{pn}$. Panel *A*, in Ca^{2+}-free HBS containing 145 mmol l^{-1} Na^+ without (control, ○, continuous line) or with 25 μmol l^{-1} ryanodine (●, dashed line) endothelial cells were stimulated with 100 nmol l^{-1} Bk followed by the addition of 2·5 mmol l^{-1} Ca^{2+} as indicated. Panel *B*, in Ca^{2+}-free HBS containing 19 mmol l^{-1} Na^+ without (control, ○, continuous line) or with 25 μmol l^{-1} ryanodine (●, dashed line) cells were stimulated with 100 nmol l^{-1} Bk followed by the addition of 2·5 mmol l^{-1} Ca^{2+}. As indicated, $[Na^+]_o$ was increased to 145 mmol l^{-1}. Points represent means ± S.E.M. (panel *A*, $n = 36$; panel *B*, $n = 32$).

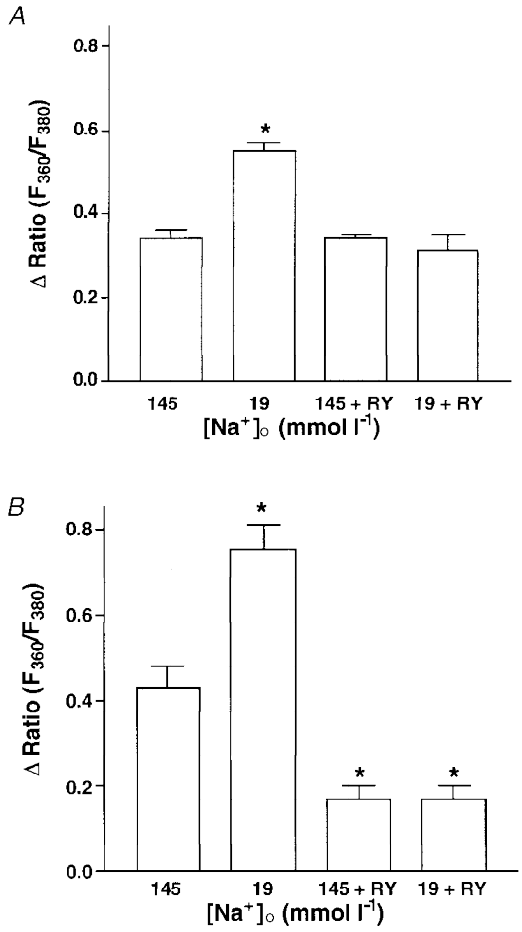

Figure 3

Summary of the results on the effect of Bk on intracellular Ca^{2+} release (*A*) and CCE (*B*) in normal and low Na^+-containing solution in the absence and presence of ryanodine.

To verify whether, in 145 mmol l^{-1} Na^+-containing solution, ryanodine induces an elevation of $[Ca^{2+}]_{sp}$ which is insulated from $[Ca^{2+}]_{pn}$, experiments were conducted using cells that were loaded with FFP-18. This is a fura-2 analogue which selectively monitors $[Ca^{2+}]_{sp}$ (Etter *et al.* 1996; Graier *et al.* 1998). As shown in Fig. 5, dye distribution of FFP-18 markedly differed from that of fura-2. Both dyes were loaded in freshly isolated bovine left circumflex coronary artery endothelial cells (panels *A* and *B*) or EA.hy 926 cells (panels *C* and *D*) using the laser-pulse wave loading

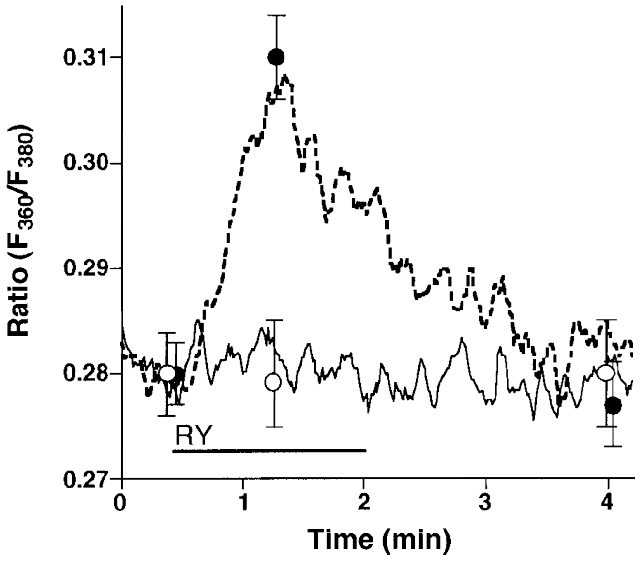

Figure 4. Prevention of Na^+–Ca^{2+} exchange by lowering $[Na^+]_o$ unmasked ryanodine-induced Ca^{2+} release

Single endothelial cells were loaded with fura-2 to measure $[Ca^{2+}]_{pn}$. As indicated, cells were stimulated with 200 nmol l^{-1} ryanodine (RY) in Ca^{2+}-free HBS containing 145 (RY, ○, continuous line) or 19 mmol l^{-1} Na^+ (RY, ●, dashed line). Each point represents the mean ± S.E.M. ($n = 24$).

technique (Graier *et al.* 1998). FFP-18 fluorescence was mainly found at the edge of the cells (i.e. plasma membrane; Fig. 5, panels *A* and *C*). By contrast, fura-2 was distributed throughout the cytosol (Fig. 5, panels *B* and *D*). In FFP-18-loaded cells 200 nmol l^{-1} ryanodine induced an elevation in $[Ca^{2+}]_{sp}$ in Ca^{2+}-free solution containing 145 mmol l^{-1} Na^+ (Fig. 6). In the presence of 2·5 mmol l^{-1} extracellular Ca^{2+}, ryanodine (200 nmol l^{-1}) yielded a similar elevation in $[Ca^{2+}]_{sp}$ to that observed in Ca^{2+}-free HBS (data not shown).

Next we tested whether in the presence of 145 mmol l^{-1} Na^+, RsCR is linked to Ca^{2+} extrusion. Here, extrusion of Ca^{2+} was monitored in cells stimulated with Bk in Ca^{2+}-free HBS. Inhibition of RsCR with 25 μmol l^{-1} ryanodine diminished Ca^{2+} extrusion in response to a stimulation with 100 nmol l^{-1} Bk by 66 % (Fig. 7).

Correlation of RsCR with activity of CCE

The data shown above indicate that RsCR contributes to activity of CCE (Fig. 3*B*). Next, the (cumulative) amplitude

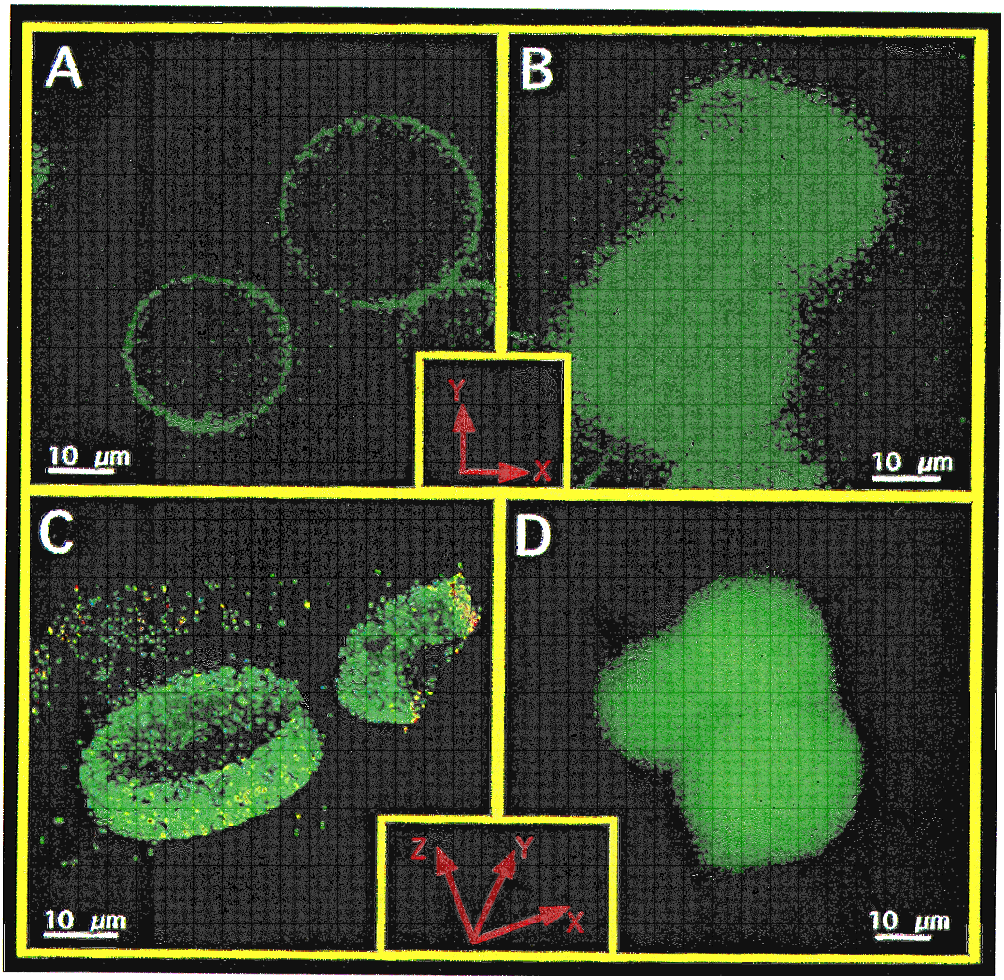

Figure 5. Distribution of FFP-18 and fura-2 in the bovine left circumflex coronary artery endothelial cells and in EA.hy 926 cells

Freshly isolated bovine left circumflex coronary artery endothelial cells (panels *A* and *B*) and cultured EA.hy 926 (panels *C* and *D*) were loaded with FFP-18 (panels *A* and *C*) or fura-2 free acid (panels *B* and *D*) using laser-stress wave loading technique (Graier *et al.* 1998), equilibrated for 10 min at room temperature, washed three times in HBS and placed in a glass-bottomed chamber. Fluorescence images were collected using a × 100 objective (NA 1·3) with an excitation of 340 ± 5 nm and emission of 510 ± 10 nm for both dyes (5 to 10 s exposure time). Panels *A* and *B*: 2-D image of a slice in the middle depth of freshly isolated bovine endothelial cells. Panels *C* and *D*: 3-D reconstructed image of EA.hy 926 cells. Images were collected throughout the whole cell with 0·3 μm interslice distance, and out-of-focus fluorescence was removed using the advanced constrained iterative algorithm (MicroTome, Vaytek, Turnbridge Wells, UK). 3-D reconstruction was performed with VoxBlast (Vaytek, Turnbridge Wells, UK). In the case of FFP-18 (panel *C*) just 10 slices at the cell equator are shown, while panel *D* (fura-2) shows the whole cell. Identical distribution of fura-2 was found when fura-2 was loaded conventionally using 2 μmol l^{-1} fura-2 AM over 30 min.

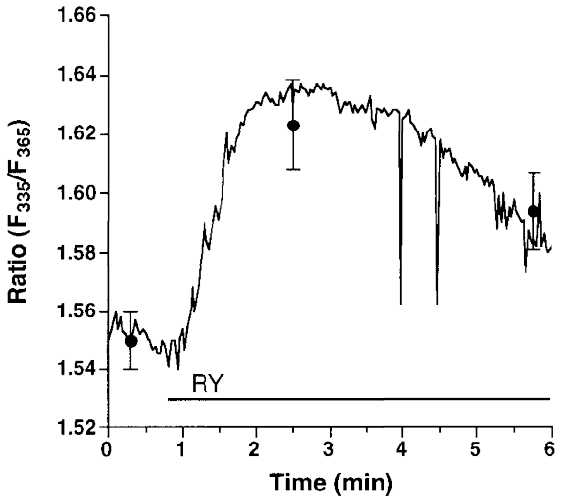

Figure 6. Ryanodine induced subplasmalemmal Ca^{2+} increase in bovine coronary endothelial cells monitored by FFP-18

Endothelial cells freshly isolated from bovine circumflex coronary artery were loaded with FFP-18 using laser-generated stress waves to monitor changes in $[Ca^{2+}]_{sp}$. After 10 min equilibration time at room temperature, cells were washed twice, resuspended in Ca^{2+}-free HBS and placed into a stirred cuvette. Changes in $[Ca^{2+}]_{sp}$ were monitored at 335 and 365 nm excitation and 500 nm emission. Each point represents the mean ± S.E.M. ($n = 14$).

of Bk-induced Ca^{2+} transients after (repetitive) stimulation taken as an index of Ca^{2+} store depletion, was correlated with the magnitude of CCE. Under control conditions (i.e. 145 mmol l^{-1} $[Na^+]_o$), activity of CCE increased stepwise with the number of repeats of the Bk stimulation (open circles; Fig. 8). In contrast, in low Na^+ solution, CCE was already maximal after the first stimulation with Bk and did not increase further after repetitive stimulation with Bk (filled circles; Fig. 8). Inhibition of RsCR with 25 μmol l^{-1} ryanodine blunted CCE under normal and low $[Na^+]_o$ conditions and prevented increases in CCE, induced by repetitive stimulation with Bk (squares; Fig. 8).

Importance of RsCR for NOS activity

To test whether RsCR itself stimulates NOS, cultured endothelial cells from bovine left circumflex coronary artery (passage 2) were stimulated for 15 min with 20, 200 and 500 nmol l^{-1} ryanodine in the presence of 2·5 mmol l^{-1} Ca^{2+} under normal and low $[Na^+]_o$ conditions. Ryanodine, in all concentrations tested, failed to stimulate NOS (Fig. 9*A*). However, inhibition of RsCR with 5 μmol l^{-1} ryanodine diminished NOS activation by Bk and ATP (Fig. 9*B*). The inhibitory effect of ryanodine on autacoid-induced NOS activation was more pronounced at lower agonist concentrations (i.e. 10 μmol l^{-1} ATP, 10 nmol l^{-1} Bk), and was less effective for the highest concentrations tested (i.e. 100 μmol l^{-1} ATP, 100 nmol l^{-1} Bk). In contrast, thapsigargin-induced activation of NOS was not affected by inhibition of RsCR (Fig. 9*B*). Similar findings were obtained in the human umbilical vein endothelial cell-derived cell line EA.hy 926 using 10 and 100 μmol l^{-1} ADP and 10 and 100 μmol l^{-1} histamine (data not shown).

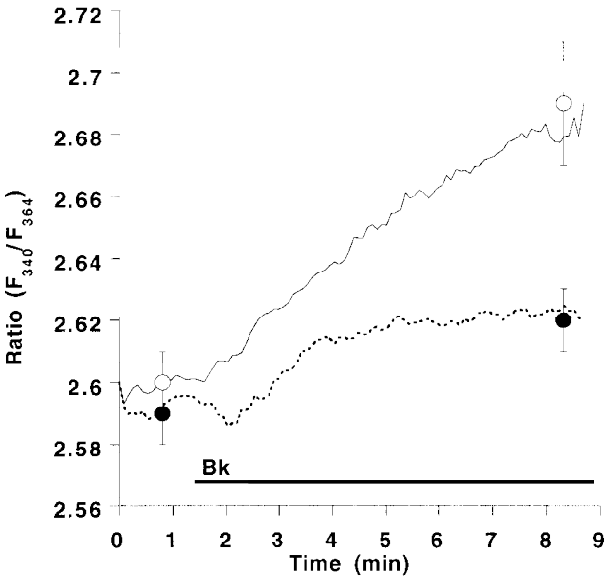

Figure 7. Inhibition of ryanodine-sensitive Ca^{2+} release attenuates Ca^{2+} extrusion in response to bradykinin

Suspended cultured endothelial cells (passage 2; 7×10^7 cells ml^{-1}) were stimulated with 100 nmol l^{-1} Bk in the absence (control, ○, continuous line) or presence (●, dashed line) of 25 μmol l^{-1} ryanodine in Ca^{2+}-free HBS containing 1 μmol l^{-1} fura-2 dextran. The Ca^{2+} extrusion was monitored at 340 and 364 nm excitation and 500 nm emission. Each point represents the mean ± S.E.M. ($n = 6$).

DISCUSSION

Role of Na^+–Ca^{2+} exchange and RsCR in intracellular Ca^{2+} signalling

Impairment of Na^+–Ca^{2+} exchange by lowering $[Na^+]_o$ increased the Bk-induced elevation in $[Ca^{2+}]_{pn}$. This suggests that during Bk stimulation a large amount of the Ca^{2+} released is extruded via the Na^+–Ca^{2+} exchange in endothelial cells. This is in agreement with the proposed involvement of Na^+–Ca^{2+} exchange in endothelial cell Ca^{2+} handling during agonist stimulation (Sage *et al.* 1991).

In contrast to the prevention of Na^+–Ca^{2+} exchange activity, inhibition of RsCR by 25 μmol l^{-1} ryanodine did not affect Bk-induced mobilization of $[Ca^{2+}]_{pn}$ in 145 mmol l^{-1} Na^+-containing buffer. Hence, under these conditions RsCR does not contribute to Bk-induced elevation of $[Ca^{2+}]_{pn}$. However, under low $[Na^+]_o$ conditions inhibition of RsCR attenuated the ability of Bk to increase $[Ca^{2+}]_{pn}$, suggesting that under these conditions RsCR contributes to the observed elevation of $[Ca^{2+}]_{pn}$.

In spite of the existence of ryanodine receptors in endothelial cells (Lesh *et al.* 1993; Graier *et al.* 1998), direct stimulation of RsCR by 200 nmol l^{-1} ryanodine failed to elevate $[Ca^{2+}]_{pn}$ under control conditions. These findings are consistent with the report of Wang *et al.* (1995) who observed that ryanodine did not induce Ca^{2+} transients. Our findings that inhibition of Na^+–Ca^{2+} exchange unmasked ryanodine-induced Ca^{2+} release suggest that activation of RsCR occurs in the subplasmalemmal compartment and that the Ca^{2+} released by RsCR is extruded by the Na^+–Ca^{2+} exchange without increasing $[Ca^{2+}]_{pn}$. This hypothesis is further supported by results obtained with FFP-18. In contrast to fura-2, which is found in the whole cytosol, FFP-18 has been shown to accumulate only in the plasma membrane and the nuclear membrane in smooth muscle cells when introduced into the cell from a patch pipette (Etter *et al.* 1996). In human neutrophils loaded for 1 to 3 h with the membrane permeable ester FFP-18 AM, FFP-18 was found mainly in the plasma membrane (Davies & Hallett, 1996). In agreement with these findings, when

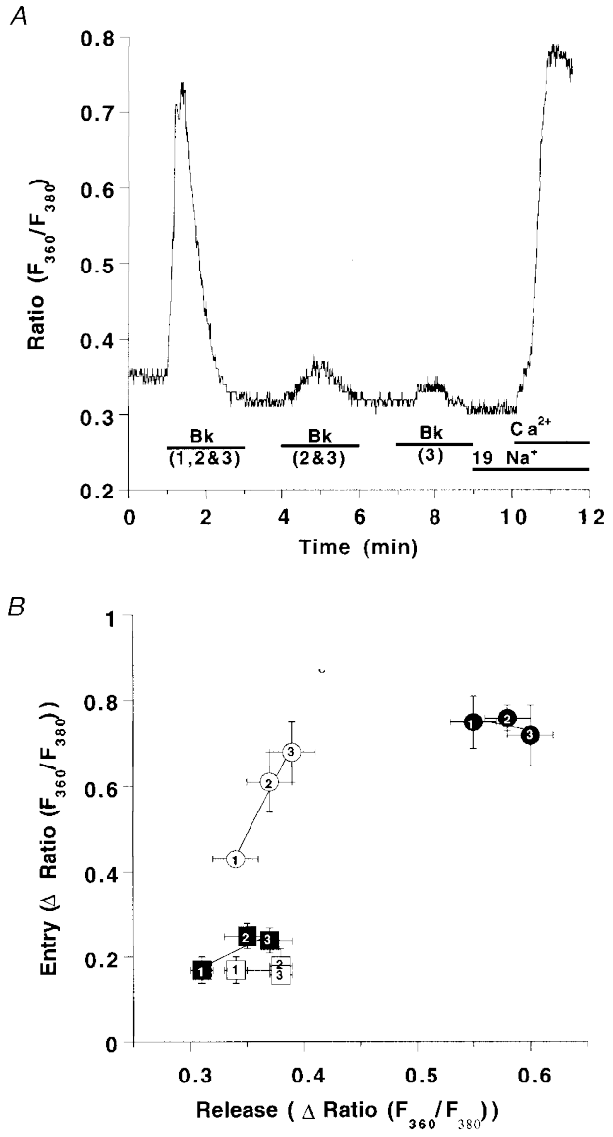

Figure 8. Correlation of Ca^{2+} release and CCE on repetitive stimulation with Bk in normal and low $[Na^+]$ conditions in the absence and presence of ryanodine

Panel *A* shows the experimental protocol: fura-2-loaded endothelial cells freshly isolated from bovine circumflex arteries were stimulated with 100 nmol l^{-1} Bk for 2 min either at time point 1 min (1), at time points 1 and 4 min (2) or at time points 1, 4 and 7 min (3). At time 9 min $[Na^+]_o$ was set to 19 mmol l^{-1} and 2·5 mmol l^{-1} Ca^{2+} was added at time 10 min. Panel *B*, elevation of $[Ca^{2+}]_{pn}$ to Bk at time 1 min (1), or the total sum of the increases of $[Ca^{2+}]_{pn}$ upon two (2: 1st peak plus 2nd peak) or three (3: 1st peak plus 2nd peak plus 3rd peak) stimulations is shown on the *x*-axis. The increase in $[Ca^{2+}]_{pn}$ to the addition of Ca^{2+} is shown on the *y*-axis. Bk stimulation was performed in Ca^{2+}-free HBS containing 145 mmol l^{-1} Na^+ (○), 19 mmol l^{-1} Na^+ (●), 145 mmol l^{-1} Na^+ and 25 μmol l^{-1} ryanodine (□) and 19 mmol l^{-1} Na^+ plus 25 μmol l^{-1} ryanodine (■). Each point represents the mean ± S.E.M. ($n = 12$).

cells were loaded with FFP-18 using a laser-generated stress wave loading technique (Graier *et al.* 1998) FFP-18 was found mainly in the plasma membrane. Using FFP-18, ryanodine-induced subplasmalemmal Ca^{2+} release could be monitored even under control conditions (i.e. 145 mmol l^{-1} $[Na^+]_o$). Hence, in 145 mmol l^{-1} Na^+-containing solution, inhibition of RsCR blunted the amount of Ca^{2+} extruded during stimulation with Bk. These data suggest that during stimulation of endothelial cells RsCR increases $[Ca^{2+}]_{sp}$ by vectorial Ca^{2+} release towards plasmalemmal Na^+–Ca^{2+} exchange proteins without increasing $[Ca^{2+}]_{pn}$. A similar functional organization of the plasmalemmal Na^+–Ca^{2+} exchange with sarcoplasmic reticulum Ca^{2+} release was described in smooth muscle cells (Moore *et al.* 1993), a cell type where subplasmalemmal Ca^{2+} handling is also insulated from $[Ca^{2+}]_{pn}$ (van Breemen *et al.* 1986; Sturek *et al.* 1992; Fay, 1995; Nelson *et al.* 1995; Yamaguchi *et al.* 1995).

Role of RsCR for CCE activity

The activity of CCE has been shown to increase progressively with enhanced Ca^{2+} store depletion (Hofer *et al.* 1998). Recently, we have shown that inhibition of Na^+–Ca^{2+} exchange during autacoid stimulation enhanced depletion of Ca^{2+} stores due to Ca^{2+}-induced Ca^{2+} release initiated by Ca^{2+} normally extruded by Na^+–Ca^{2+} exchange (Graier *et al.* 1998). In agreement with these reports CCE was increased when cells were stimulated under low $[Na^+]_o$ conditions in our experiments, a phenomenon possibly linked to the pronounced depletion of intracellular Ca^{2+} stores by RsCR when Na^+–Ca^{2+} exchange activity was prevented. Consistent with this explanation, inhibition of RsCR diminished CCE under normal and low $[Na^+]_o$ conditions. Moreover, ryanodine uncoupled the correlation of intracellular Ca^{2+} release and activity of CCE. These data may indicate that RsCR contributes to the depletion of Ca^{2+} stores and hence activation of CCE. Although this explanation seems attractive, the contribution of caffeine- and/or ryanodine-sensitive compartments of the endoplasmic reticulum in CCE activation in endothelial cells was excluded very recently (Sasajima *et al.* 1997). Hence, several authors have failed to measure CCE in response to activation of RsCR by either caffeine (Graier *et al.* 1994; Ziegelstein *et al.* 1994; Wang *et al.* 1995) or ryanodine (Wang *et al.* 1995). Consistent with these reports, direct stimulation of RsCR by 200 nmol l^{-1} ryanodine did not evoke CCE in this study. In agreement with these findings, we have previously reported that ryanodine failed to activate microsomal cytochrome P450 epoxygenase (Hoebel *et al.* 1997), an enzyme thought to be involved in the

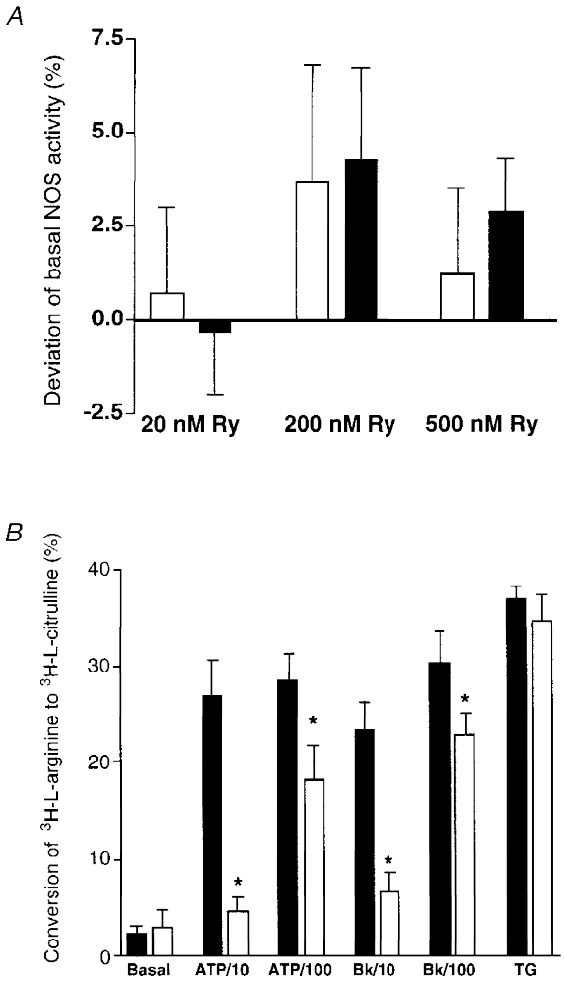

Figure 9. Effect of ryanodine-induced RsCR on NOS activity (*A*) and changes in autacoid-induced NOS activation by inhibition of RsCR with a high concentration of ryanodine (*B*)

Cultured endothelial cells from the bovine circumflex coronary artery (passage 2) were stimulated with 20, 200 and 500 nmol l^{-1} ryanodine (panel *A*), or with ATP (10 and 100 μmol l^{-1}), Bk (10 and 100 nmol l^{-1}) and 2 μmol l^{-1} thapsigargin (TG) in the absence or presence of 5 μmol l^{-1} ryanodine (panel *B*) for 15 min in HBS. Columns represent the percentage deviation from basal NOS activity (panel *A*) or the percentage of conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline (panel *B*). Each column represents the mean ± S.E.M. (*A*, $n = 4$; *B*, $n = 5$). * $P < 0{\cdot}05$ *vs.* absence of ryanodine.

regulation of CCE (Graier *et al.* 1995). Therefore, a direct involvement of RsCR in the activation of CCE seems unlikely.

In endothelial cells stimulated by agonists, the amount of Ca^{2+} entering the cell depends strictly on the driving force provided by membrane hyperpolarization due to activation of Ca^{2+}-activated K^+ channels (Colden-Stanfield *et al.* 1987; Busse *et al.* 1988). Since we have shown that RsCR preferably occurs in the subplasmalemmal region, one might expect that RsCR may activate Ca^{2+}-activated K^+ channels to promote membrane hyperpolarization and thus increase the driving force for Ca^{2+} influx. This is consistent with the finding that caffeine, an activator of RsCR, induced Ca^{2+}-sensitive K^+ current in freshly isolated rabbit aortic endothelial cells (Rusko *et al.* 1995). Hence, caffeine has been shown to elevate CCE in activated endothelial cells (Corda *et al.* 1995). Therefore, the reduction of CCE activity by inhibition of RsCR might be due to a reduction of agonist-induced membrane hyperpolarization. However, a more detailed investigation on the contribution of RsCR on CCE is necessary to determine the exact mechanism by which RsCR affects CCE activity.

Role of RsCR in autacoid-induced NOS activation

There is little known about the involvement of RsCR in the activation of NOS. In rat aortic rings inhibition of RsCR attenuated endothelium-dependent relaxation in response to caffeine (Hatano *et al.* 1995). In isolated rabbit arterial rings ryanodine induced nitric oxide-mediated relaxation (Hutcheson & Griffith, 1997), while in rat lung and pulmonary arteries ryanodine failed to induce nitric oxide release (Muramatsu *et al.* 1996). In agreement with the latter findings, ryanodine at a concentration where it activates RsCR (20, 200 and 500 nmol l^{-1}) failed to activate NOS in the present study. However, inhibition of RsCR diminished activation of NOS, evoked either by ADP, ATP, Bk or histamine. These findings suggest that the partial inhibition of CCE by ryanodine accounts for the reduced NOS activation to autacoids. Since the inhibitory properties of ryanodine were diminished with increased agonist concentration, one might speculate that RsCR is more important for regulation of NOS under submaximal and/or physiological stimulation. Under supramaximal stimulation the strong $InsP_3$-mediated Ca^{2+} release and/or the maximal CCE may compensate for the lack of RsCR.

Recently, a subplasmalemmal Ca^{2+} control unit (SCCU) was introduced to explain the control of subplasmalemmal Ca^{2+} gradients under submaximal stimulation (Graier *et al.* 1998). The data presented here extend the function of the SCCU as a regulator of activation of NOS, which is localized in cavaeoli of the plasma membrane (García-Cardena *et al.* 1996).

In conclusion, our data provide evidence that during autacoid stimulation RsCR occurs in endothelial cells. Ca^{2+} is vectorially released towards plasmalemmal Na^+–Ca^{2+} exchange proteins for rapid extrusion without any impact on $[Ca^{2+}]_{pn}$. However, RsCR elevates $[Ca^{2+}]_{sp}$ and contributes to the activity of CCE, while RsCR alone does not activate CCE. Finally, the subplasmalemmal Ca^{2+} release by RsCR is important for activation of NOS under submaximal stimulation. These data extend the function of the SCCU not only to control $[Ca^{2+}]_{sp}$ but to the regulation of NOS activity.

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