Desensitization of endothelial nitric oxide synthase by receptor agonists

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Stimulation of endothelial cells with receptor agonists leads to an activation of endothelial nitric oxide synthase (eNOS) that only lasts for a short duration. A more prolonged effect, however, is observed in response to non-receptor agonists, such as Ca^{2+} ionophores or thapsigargin (TG). To investigate the molecular mechanisms underlying the rapid deactivation of eNOS after stimulation with receptor agonists, we measured the time courses of eNOS activation and intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to bradykinin (BK) and ATP. Incubation of porcine aortic endothelial cells with BK (1 μM) in the presence of 3 mM extracellular Ca^{2+} increased $[\text{Ca}^{2+}]_i$ from 110 to 350 nM and enhanced the rate of L-[³H]citrulline formation from 0.1 to 5 fmol/min. In the absence of extracellular Ca^{2+} , the BK-induced increase in $[\text{Ca}^{2+}]_i$ was only marginal (from 30 to 110 nM) and not sufficient to activate eNOS. When Ca^{2+} (final concentration 3 mM) was added 10 min after BK, $[\text{Ca}^{2+}]_i$ increased to 330 nM within 3 min, but interestingly, formation of

L-[³H]citrulline was not detectable. A similar phenomenon was observed with ATP, but not with Ca^{2+} ionophores or TG. This indicates that stimulation of endothelial cells with receptor agonists leads to desensitization of eNOS, which renders the enzyme insensitive to activation by subsequent increases in $[\text{Ca}^{2+}]_i$. However, when ATP was added to BK-pretreated cells or, conversely, BK to ATP-pretreated cells, activation of eNOS was comparable with that of untreated cells, suggesting that BK and ATP affect different pools of eNOS. The desensitization of eNOS was reversible, since removal of ATP or BK from the incubation buffer restored the response to the respective agonist within 20 min. In addition to the transient Ca^{2+} signal, desensitization of eNOS may represent a further mechanism by which endothelial cells rapidly terminate receptor-dependent NO formation.

Key words: ATP, bradykinin, Ca^{2+} .

INTRODUCTION

Endothelial nitric oxide synthase (eNOS) is activated in endothelial cells by a variety of stimuli, including several receptor agonists and fluid shear stress (see [1] for a review). Although shear stress-induced NO production appears to occur in a Ca^{2+} -independent manner, an increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is the essential trigger for activation of eNOS by receptor ligands. The signalling cascade for Ca^{2+} -dependent eNOS activation is initiated by ligand-induced activation of phospholipase C, which results in the formation of inositol (1,4,5)-trisphosphate, and the release of Ca^{2+} from inositol (1,4,5)-trisphosphate-sensitive intracellular Ca^{2+} stores. In the course of store depletion, store-operated Ca^{2+} channels are activated, resulting in a Ca^{2+} influx, referred to as capacitative Ca^{2+} entry (see [2,3] for a review). Both, release and influx of Ca^{2+} lead to a transient increase in $[\text{Ca}^{2+}]_i$, which promotes the binding of calmodulin (CaM) to eNOS leading to enzyme activation [4].

In addition to being controlled by Ca^{2+} -dependent CaM binding, ligand-induced eNOS activation is also influenced by phosphorylation [5,6] and by direct interactions with cellular proteins. One such protein is caveolin-1, which promotes the association of eNOS to caveolae [7–9]. The interaction with caveolin renders the enzyme inactive, apparently due to a functional competition with Ca^{2+} /CaM [10–13]. Ligand-induced increase of $[\text{Ca}^{2+}]_i$ promotes a reversible Ca^{2+} /CaM-mediated dissociation of the inhibitory eNOS–caveolin complex [14,15], resulting in transient activation of eNOS. In addition to its interaction with caveolin, eNOS also forms inhibitory complexes with the bradykinin (BK) B2, the angiotensin II AT1 and endothelin-1 ETB receptor, from which the enzyme is released in

a Ca^{2+} - and ligand-dependent manner on receptor activation [16,17]. Since all major components of the NO signalling cascade, including G-protein-coupled receptors, store-operated Ca^{2+} channels, eNOS and CaM are enriched in caveolae (see [18] for a review), these specialized cell surface domains may play a key role in regulating agonist-induced NO formation.

Although the signalling cascade involved in receptor-induced eNOS activation is fairly well understood, the mechanisms by which the enzyme activation is terminated are less clear. Although the decrease in $[\text{Ca}^{2+}]_i$ may be the main trigger for eNOS deactivation, translocation of eNOS to intracellular compartments [14,19], rapid re-formation of an inhibitory eNOS–receptor complex [17] or disruption of the caveolae-associated signalling cascade by endogenously formed NO [20] have been suggested as additional mechanisms. To elucidate the molecular basis of eNOS deactivation, we measured the time-dependent effects of Ca^{2+} -mobilizing agonists on $[\text{Ca}^{2+}]_i$ and eNOS activity in cultured porcine aortic endothelial cells.

EXPERIMENTAL

Materials

Cell culture media, antibiotics and foetal calf serum were purchased from PAA Laboratories GmbH (Linz, Austria). L-[2,3,4,5-³H]arginine hydrochloride (40–80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.) via Humos Diagnostika (Salzburg, Austria). The fluorescent Ca^{2+} indicator fura 2 acetoxymethyl ester (fura 2/AM) was obtained from Molecular Probes (Europe BV, Leiden, The Netherlands). All other chemicals were from Sigma (Vienna, Austria).

Abbreviations used: BK, bradykinin; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; CaM, calmodulin; eNOS, endothelial nitric oxide synthase; fura 2/AM, fura 2 acetoxymethyl ester; TG, thapsigargin.

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Cell culture

Porcine aortic endothelial cells were isolated as described previously [21] and incubated in 5 % CO₂ atmosphere at 37 °C with Dulbecco's modified Eagle's medium containing 10 % heat-inactivated foetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1.25 µg/ml amphotericin B. After 3–5 days, primary cells were subcultured in Petri dishes or multi-well plates and used in experiments. Since repeated subculturing of endothelial cells reduced the response to receptor agonists, all experiments were performed with cells of the first passage.

Determination of intracellular Ca²⁺ levels

[Ca²⁺]_i was determined using the Ca²⁺ indicator fura 2 as described previously [22]. Briefly, endothelial cells from a single Petri dish were harvested, suspended in 5 ml of culture medium and incubated with 2 µM of fura 2/AM at 37 °C. After 45 min, cells were washed, resuspended in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 3 mM CaCl₂ (incubation buffer) or 0.1 mM EGTA (nominal Ca²⁺-free incubation buffer), transferred into a thermostated cuvette, and fluorescence was measured at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. After equilibration (5–10 min), experiments were started by the addition of agonist. [Ca²⁺]_i was calculated using the ratio *R* of fluorescence intensities at 340 and 380 nm and the fluorescence intensity *S*₂ at 380 nm according to the formula of Grynkiewicz et al. [23]:

$$[Ca^{2+}]_i = K_D[(R - R_{min}) / (R_{max} - R)](S_{f2} / S_{b2})$$

where *K_D* is the dissociation constant of the fura 2/Ca²⁺ complex, *R_{min}* the minimum fluorescence ratio, *R_{max}* the maximum fluorescence ratio, *S_{f2}* the fluorescence intensity at 380 nm of the free dye and *S_{b2}* the fluorescence intensity at 380 nm of the Ca²⁺-bound dye. With the exception of *K_D* (which was assumed to be 224 nM [23]), all calibration factors were determined at the end of each experiment by the addition of 0.1 % (v/v) Triton X-100 in the presence of 3 mM CaCl₂ (to obtain *R_{max}* and *S_{b2}*) and subsequent addition of excess (20 mM) EGTA (to obtain *R_{min}* and *S_{f2}*).

Determination of eNOS activity

NOS activity in intact cells was determined by monitoring the conversion of incorporated L-[³H]arginine into L-[³H]citrulline as described previously [24]. Briefly, endothelial cells grown in 6-well plastic plates were washed and equilibrated for 10 min at 37 °C in incubation buffer or in nominal Ca²⁺-free incubation buffer (see above). Reactions were started by the addition of L-[³H]arginine (approx. 10⁶ d.p.m.; final concentration approx. 8 nM) and the agonist. After the indicated period of time (usually 3 min), cells were washed with chilled nominal Ca²⁺-free incubation buffer followed by the addition of 1 ml of HCl (10 mM). For determining eNOS activity in store-depleted cells, cells were preincubated for 10 min in nominal Ca²⁺-free incubation buffer containing 1 µM BK, 1 mM ATP or 0.1 µM TG. Reactions were started by addition of L-[³H]arginine and 3 mM CaCl₂, and terminated after 3 min as described above. Subsequent to the lysis of cells (approx. 1 h after addition of HCl), an aliquot was removed for determining the incorporated radioactivity. To the remaining sample, 200 mM sodium acetate buffer, pH 13.0, containing 10 mM L-citrulline was added (final pH approx. 5.0), and L-[³H]citrulline was separated from L-[³H]arginine by cation-exchange chromatography as described previously [24].

Data and statistical analysis

Data are presented as means ± S.E.M. for the indicated number of individual experiments. Statistical comparison was obtained using ANOVA followed by Scheffé's post-hoc test. Differences among means were considered significant at *P* < 0.05.

RESULTS

To determine the time-dependent activation of eNOS by receptor agonists, endothelial cells were stimulated with 1 µM BK or 1 mM ATP in the presence of extracellular Ca²⁺ (Figures 1A and

Figure 1 Time course of eNOS activation and intracellular Ca²⁺ levels in response to receptor agonists

(A, B) Endothelial cells equilibrated in incubation buffer containing 3 mM CaCl₂ (control), 3 mM CaCl₂ and 0.1 mM *N*^G-nitro-L-arginine (L-NNA), or 0.1 mM EGTA (Ca²⁺-free) were stimulated with 1 µM BK or 1 mM ATP in the presence of L-[³H]arginine. At the time points indicated, cells were lysed and the rate of L-[³H]citrulline formation was determined as described in the Experimental section. (C, D) Endothelial cells loaded with fura 2 were equilibrated in incubation buffer containing 3 mM CaCl₂ (control) or 0.1 mM EGTA (Ca²⁺-free) and then stimulated with 1 µM BK or 1 mM ATP. The ratio of fluorescence intensities at 340 and 380 nm was determined, and [Ca²⁺]_i calculated as described in the Experimental section. (E, F) Endothelial cells loaded with fura 2 were equilibrated in incubation buffer containing 0.1 mM EGTA and then stimulated with 1 µM BK or 1 mM ATP. After approx. 10 min, capacitative Ca²⁺ entry was induced by addition of CaCl₂ (3 mM). The ratio of fluorescence intensities at 340 and 380 nm was determined, and [Ca²⁺]_i calculated as described in the Experimental section. In (A) and (B), data are means ± S.E.M. (*n* = 4), and in panels (C–F), original traces each representative for 6–8 experiments were shown.

Figure 2 Intracellular Ca^{2+} levels and eNOS activity in store-depleted (A) and native (B) endothelial cells

(A) Endothelial cells were preincubated for 10 min in nominal Ca^{2+} -free incubation buffer containing 1 μM BK, 1 mM ATP or 0.1 μM TG, and then 3 mM CaCl_2 (determining $[\text{Ca}^{2+}]_i$) or L-[^3H]arginine and 3 mM CaCl_2 (determining eNOS activity) were added. After a further 3 min incubation, either the ratio of fluorescence intensities at 340 and 380 nm was determined or the conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined as described in the Experimental section. Data are means $\pm$ S.E.M. for 4 (eNOS activity) or 6–8 ($[\text{Ca}^{2+}]_i$) experiments. * $P < 0.01$ compared with values obtained in the absence of Ca^{2+} . (B) Endothelial cells equilibrated in incubation buffer containing 3 mM CaCl_2 were stimulated with 1 μM BK, 1 mM ATP or 0.1 μM TG. For determining $[\text{Ca}^{2+}]_i$, the ratio of fluorescence intensities at 340 and 380 nm was determined before and after addition of the agonists (maximal response). For determining the eNOS activity, stimulation of the cells was performed in the presence of L-[^3H]arginine, and conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined 3 min after addition of the agonists. Data are means $\pm$ S.E.M. for 4 (eNOS activity) or 6–8 ($[\text{Ca}^{2+}]_i$) experiments. * $P < 0.01$ compared with values obtained in the absence of the agonists.

1B, open circles). After addition of the agonists, the rate of L-[^3H]citrulline formation increased sharply to reach a peak of approx. 5 fmol/min after 1 min and thereafter decreased to a 20 times lower value (approx. 0.25 fmol/min) at 5 min. No effect of BK or ATP was observed in cells preincubated for 30 min with 100 μM N^G -nitro-L-arginine (filled circles). To investigate whether the apparent deactivation of eNOS observed after approx. 3 min correlated with a decrease in $[\text{Ca}^{2+}]_i$, we measured the time course of the intracellular Ca^{2+} response with the fluorescence indicator fura 2. As shown in Figures 1(C) and 1(D) (solid lines), addition of BK or ATP evoked a sharp increase in $[\text{Ca}^{2+}]_i$, which reached a peak after approx. 1 min and then declined to basal values within 4 min. The time course of $[\text{Ca}^{2+}]_i$ roughly correlated with that of eNOS activity, suggesting that the deactivation of eNOS may be mainly due to a decrease in $[\text{Ca}^{2+}]_i$.

Since it is known that stimulation of endothelial cells with receptor agonists results in both release of Ca^{2+} from intracellular stores and Ca^{2+} influx, experiments were also performed in nominal Ca^{2+} -free buffer to determine the contribution of Ca^{2+} release in regulation of $[\text{Ca}^{2+}]_i$ and eNOS activity. As shown in Figures 1(C) and 1(D) (dotted lines), omission of Ca^{2+} from the incubation buffer decreased the basal intracellular Ca^{2+} levels and significantly reduced the increases in $[\text{Ca}^{2+}]_i$ induced by the agonists. Moreover, stimulation of the cells with the agonists in the absence of extracellular Ca^{2+} did not evoke an increase in L-

Figure 3 Effect of successive addition of Ca^{2+} -mobilizing agonists on intracellular Ca^{2+} levels and eNOS activity

Endothelial cells were preincubated for 10 min in nominal Ca^{2+} -free incubation buffer containing 1 μM BK (A) or 1 mM ATP (B), and then 3 mM CaCl_2 (determining $[\text{Ca}^{2+}]_i$) or L-[^3H]arginine and 3 mM CaCl_2 (determining eNOS activity) and, where indicated, TG (0.1 mM), BK (1 mM) or ATP (1 mM) were added. After a further 3 min incubation, either the ratio of fluorescence intensities at 340 and 380 nm was determined or the conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined as described in the Experimental section. Data are means $\pm$ S.E.M. for 4 (eNOS activity) or 6–8 ($[\text{Ca}^{2+}]_i$) experiments. * $P < 0.01$ compared with values obtained in the presence of BK and Ca^{2+} alone (A) or ATP and Ca^{2+} alone (B). Endothelial cells equilibrated in incubation buffer containing 3 mM CaCl_2 were stimulated with 1 μM BK (C) or 1 mM ATP (D) in the presence of L-[^3H]arginine. After 10 min, the second agonist was added and then cells were incubated for a further 3 min. At the time points indicated, cells were lysed and the rate of L-[^3H]citrulline formation was determined as described in the Experimental section. Data are means $\pm$ S.E.M. for 4 (eNOS activity) or 6–8 ($[\text{Ca}^{2+}]_i$) experiments.

[^3H]citrulline formation (Figures 1A and 1B, triangles), demonstrating that Ca^{2+} influx is essential for eNOS activation. To substantiate these findings, we used a methodology-based approach, which allowed temporal separation of Ca^{2+} influx from Ca^{2+} release. Endothelial Ca^{2+} stores were first depleted by pretreatment of the cells with the agonists in Ca^{2+} -free buffer and, after $[\text{Ca}^{2+}]_i$ had declined back to basal levels, Ca^{2+} influx was induced by subsequent addition of extracellular CaCl_2 . As shown in Figures 1(E) and 1(F), this procedure resulted in a small and transient increase in $[\text{Ca}^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores, and a rapid and sustained increase in $[\text{Ca}^{2+}]_i$ when extracellular Ca^{2+} was added. The intracellular Ca^{2+} levels reached 3 min after addition of CaCl_2 to BK- and ATP-pretreated cells were 330 and 354 nM respectively (Figure 2A), and thus virtually identical with the peak values of $[\text{Ca}^{2+}]_i$ obtained after stimulation of native cells with BK (350 nM) and

Figure 4 Recovery of eNOS activity after enzyme desensitization

Endothelial cells were preincubated for 10 min in nominal Ca^{2+} -free incubation buffer containing 1 μM BK (A) or 1 mM ATP (D), and then 3 mM CaCl_2 (determining $[\text{Ca}^{2+}]_i$) or L-[^3H]arginine and 3 mM CaCl_2 (determining eNOS activity) were added. After a further 3 min incubation, either the ratio of fluorescence intensities at 340 and 380 nm was determined or the conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined as described in the Experimental section. Endothelial cells were preincubated for 10 min in nominal Ca^{2+} -free incubation buffer containing 1 μM BK (B) or 1 mM ATP (E). After two washing steps, cells were incubated for 20 min in nominal Ca^{2+} -free incubation buffer and then re-stimulated with the agonist in the presence of 3 mM CaCl_2 (determining $[\text{Ca}^{2+}]_i$) or L-[^3H]arginine and 3 mM CaCl_2 (determining eNOS activity). After a further 3 min incubation, either the ratio of fluorescence intensities at 340 and 380 nm was determined or the conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined as described in the Experimental section. Endothelial cells equilibrated in incubation buffer containing 3 mM CaCl_2 were stimulated with 1 μM BK (C) or 1 mM ATP (F). For determining $[\text{Ca}^{2+}]_i$, the maximal ratio of fluorescence intensities at 340 and 380 nm was obtained after addition of the agonists. For determining the eNOS activity, stimulation of the cells was performed in the presence of L-[^3H]arginine, and conversion of incorporated L-[^3H]arginine into L-[^3H]citrulline determined 3 min after addition of the agonist. Data are means $\pm$ S.E.M. for 4 (eNOS activity) or 6–8 ($[\text{Ca}^{2+}]_i$) experiments.

ATP (387 nM) in the presence of extracellular Ca^{2+} (Figure 2B). Despite this increase in $[\text{Ca}^{2+}]_i$, no activation of eNOS was detectable on the induction of Ca^{2+} entry in BK- or ATP-pretreated cells (Figure 2A), whereas the conversion of L-[^3H]arginine into L-[^3H]citrulline was increased from 3 to 18 and 16%, respectively by the addition of BK or ATP to non-pretreated cells (Figure 2B). Interestingly, the ineffectiveness of Ca^{2+} entry on eNOS activation was not observed when TG was used as the Ca^{2+} -mobilizing compound, suggesting that pre-incubation of endothelial cells with receptor agonists but not with TG leads to a desensitization of eNOS, apparent as insensitivity of the enzyme to subsequent activation by increases in $[\text{Ca}^{2+}]_i$.

To determine whether desensitized eNOS is activated by a more pronounced increase in $[\text{Ca}^{2+}]_i$, endothelial cells were pretreated with BK or ATP, and Ca^{2+} entry was induced by the addition of CaCl_2 in the presence of TG. As shown in Figures 3(A) (BK pretreatment) and 3(B) (ATP pretreatment), the resulting increase in $[\text{Ca}^{2+}]_i$ was approx. two times higher (approx. 700 nM) than that obtained after addition of CaCl_2 alone

(approx. 350 nM), and accompanied by a marked conversion of L-[^3H]arginine into L-[^3H]citrulline (approx. 35%). Then we investigated whether a similar effect is observed when store-depleted endothelial cells are stimulated with CaCl_2 and a receptor agonist. The results showed that addition of ATP to BK-pretreated cells (Figure 3A) or addition of BK to ATP-pretreated cells (Figure 3B) did not enhance the CaCl_2 -induced increase in $[\text{Ca}^{2+}]_i$, but completely restored eNOS activity. This stimulatory effect was only observed when the second agonist was different from the first, i.e. addition of BK and CaCl_2 to BK-pretreated cells, or addition of ATP and CaCl_2 to ATP-pretreated cells did not increase eNOS activity. Similar results were obtained in the continuous presence of 3 mM CaCl_2 . Addition of ATP to endothelial cells stimulated with BK (Figure 3C), or conversely, addition of BK to ATP-stimulated cells (Figure 3D) led to a pronounced increase in L-[^3H]citrulline formation.

To test for a reversibility of eNOS desensitization, endothelial cells pretreated with BK were washed, incubated for 20 min in Ca^{2+} -free buffer, and then stimulated with BK in the presence of extracellular Ca^{2+} . In contrast with its ineffectiveness in unwashed endothelial cells (Figure 4A), addition of BK and Ca^{2+} to washed endothelial cells (Figure 4B) led to a pronounced conversion of L-[^3H]arginine into L-[^3H]citrulline (approx. 17%), a response that was almost similar to that obtained in non-pretreated endothelial cells (Figure 4C). Similar results were obtained with ATP (Figures 4D–4F), demonstrating that eNOS completely recovers from BK- or ATP-induced desensitization within 20 min after removal of the agonist.

DISCUSSION

In the present study, we investigated the mechanism underlying deactivation of eNOS in response to stimulation of endothelial cells by receptor agonists. We observed that the time course of eNOS activity roughly correlated with the transient intracellular Ca^{2+} signal evoked by the agonists, suggesting that the decrease in $[\text{Ca}^{2+}]_i$ may be the key trigger for eNOS deactivation. In contrast with our results obtained with porcine aortic endothelial cells, it has been reported that the Ca^{2+} response elicited by BK in human umbilical vein endothelial cells was significantly shortened as compared with subsequent NO production [25]. The sustained activation of eNOS was attributed to an intracellular alkalization mediated by BK-induced Na^+/H^+ exchange. Since we observed a very similar time course of Ca^{2+} response and eNOS activity, NO formation appears to be regulated by $[\text{Ca}^{2+}]_i$, and not by intracellular pH in porcine aortic endothelial cells.

In addition to this crucial role of $[\text{Ca}^{2+}]_i$, our experiments revealed an additional mechanism of eNOS regulation that became evident after a more prolonged incubation of the cells with BK or ATP. This mechanism involved desensitization of eNOS, apparent as decreased sensitivity of the enzyme to increases in $[\text{Ca}^{2+}]_i$. The effect on eNOS occurred after one single challenge with either BK or ATP and was not accompanied by a diminished $[\text{Ca}^{2+}]_i$ response, ruling out the involvement of receptor desensitization. As BK did not affect ATP-induced eNOS activation, and vice versa, each agonist appears to stimulate and desensitize a distinct pool of eNOS.

The occurrence of distinct eNOS pools was suggested previously by Venema and co-workers [16,17], who demonstrated that eNOS forms inhibitory complexes with BK B2, the angiotensin II AT1 and endothelin-1 ETB receptors, from which the enzyme is released in a Ca^{2+} - and ligand-dependent manner on receptor activation. Since eNOS may not bind to more than one receptor at a time, these authors proposed that each type of

receptor sequesters a small pool of total cellular eNOS. These results suggest that stimulation of the cells with a ligand may only, or at least preferentially, activate the eNOS pool associated with the respective receptor, but a distinct regulation of eNOS pools by receptor agonists has not been demonstrated so far. In the present study, we provide the first functional evidence for the occurrence of two distinct eNOS pools, which are separately and independently regulated by BK and ATP. Since Venema and co-workers [17] did not observe a direct interaction between eNOS and the ATP P2Y2 receptor, and endothelial cells also express P2Y4 and P2Y6 receptors [26], we first speculated that the ATP-sensitive eNOS pool identified in our study may be linked to one of these receptor subtypes. However, the rank order of potency of different nucleotides to induce L-citrulline formation exhibited the typical characteristics of a P2Y2 receptor (ATP = UTP > ADP = UDP $\gg$ 2-Me-S-ATP; results not shown). A similar profile has been reported for the endothelial $[Ca^{2+}]_i$ response to purinoceptor agonists [27], demonstrating the prominent role of the P2Y2 receptor in regulating endothelial Ca^{2+} signalling and eNOS activation by ATP. Therefore the reason for the discrepancy between our functional data, which suggest the existence of an ATP-sensitive eNOS pool and, thus, a direct interaction between the enzyme and the P2Y2 receptor, and the contradictory binding data of Venema et al. [17] remains unclear. However, based on the previous results with the BK B2 receptor, the binding studies were performed with a peptide corresponding to the intracellular domain 4 of the P2Y2 receptor and under rather stringent conditions (1 M NaCl). Thus, the possibility that the P2Y2 receptor binds to eNOS via another domain or is only loosely associated with the enzyme cannot be ruled out. Based on the results of Venema and co-workers [16,17], which showed that eNOS also interacts with the AT1 and ETB receptors, we probed the cells for eNOS pools sensitive to angiotensin II and endothelin-1. However, neither of these agonists produced a detectable increase in the conversion of L-arginine into L-citrulline (results not shown), suggesting that the respective receptors are not expressed in the cultured cells that were used for the present study.

In endothelial cells, eNOS is targeted to specific intracellular domains, including Golgi and caveolae. The localization of eNOS in caveolae is apparently a prerequisite for an effective enzyme activation, as HEK 293 cells transfected with a palmitoylation-deficient eNOS mutant (which does not target to caveolae) were found to release less NO on stimulation with agonists than those transfected with the wild-type enzyme [28]. Similarly, exposure of endothelial cells to oxidized or hypochlorite-modified low-density lipoprotein, which promotes a translocation of eNOS from caveolae, markedly reduced agonist-induced enzyme activation [29–31]. Since caveolae compartmentalize all major components involved in the control of intracellular Ca^{2+} homeostasis (see [18] for a review), the spatial separation of eNOS from these specialized Ca^{2+} -signalling microdomains apparently interrupts, or at least impairs the signalling cascade involved in eNOS activation. Interestingly, a reversible translocation of eNOS from the membrane to structures in the cytosol was also observed after treating endothelial cells with Ca^{2+} -mobilizing agonists, leading to the assumption that this mechanism may represent a means for desensitization and thus deactivation of eNOS after agonist stimulation [14,19]. Since previous studies (S. Wagner and K. Schmidt, unpublished work; [32,33]) failed to observe a translocation of eNOS to subcellular compartments in response to agonists, we suggest the following mechanism for eNOS desensitization: binding of an agonist to its receptor promotes dissociation of the respective eNOS–caveolin–receptor complex, and in the presence of extracellular Ca^{2+} , activation of the enzyme due to the increase in

$[Ca^{2+}]_i$ mediated by Ca^{2+} influx. Although the interaction of eNOS with caveolin is disrupted, the enzyme apparently remains associated with the plasma membrane, albeit spatially separated from the Ca^{2+} channels (which are localized in caveolae), and thus largely insensitive to increases in $[Ca^{2+}]_i$. Since stimulation of the cells with the agonist only activates and desensitizes the eNOS pool associated with the respective receptor, the cellular response to a second receptor agonist, which activates a distinct eNOS pool, remains unaltered.

In summary, our results provide functional evidence for the existence of two distinct eNOS pools, which are independently regulated by BK and ATP. Stimulation of the cells with either agonist leads to a desensitization of the respective eNOS pool, which renders the enzyme less sensitive to subsequent activation by increases in $[Ca^{2+}]_i$. We therefore suggest that, in addition to the transient Ca^{2+} signal, desensitization of eNOS might contribute to the termination of receptor-dependent NO formation in endothelial cells.

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