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RESEARCH COMMUNICATION

Increases in endothelial cyclic AMP levels amplify agonist-induced formation of endothelium-derived relaxing factor (EDRF)

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The interaction between intracellular cyclic AMP and agonist-induced endothelium-derived relaxing factor (EDRF) (NO) formation was investigated in pig aortic endothelial cells. Three potent stimulators of adenylate cyclase, namely forskolin, adenosine and isoprenaline, amplified bradykinin- and ATP-induced biosynthesis and release of EDRF. None of the substances by itself affected basal EDRF formation. The effects of forskolin, adenosine and isoprenaline corresponded to an enhanced agonist-induced rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), were mimicked by the membrane-permeable cyclic AMP analogue dibutyryl cyclic AMP and were antagonized by the protein kinase inhibitor *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8). Our data suggest that cyclic AMP-dependent phosphorylation modulates Ca^{2+}-signalling and thus the function of endothelial cells. This mechanism may be of particular physiological importance, since it allows a joint regulation of endothelial functions by tissues factors such as bradykinin, which directly affects $[Ca^{2+}]_i$ and agonists which affect intracellular cyclic AMP levels.

INTRODUCTION

During the last few years the vascular endothelium has experienced an enormous increase in interest. It is now well established that endothelial cells serve many more functions than simply to serve as a passive barrier between interstitium and blood flow. For example, endothelial cells are known to regulate vascular tone by the formation and release of endothelium-derived relaxing factor (EDRF; for a review, see [1]), production of prostacyclin and the release of vasoconstricting factors such as endothelin. Most of these functions are known to be due to an increase in endothelial free Ca^{2+} concentrations ($[Ca^{2+}]_i$), which is regulated by the release of stored Ca^{2+} by inositol 1,4,5-trisphosphate [2,3] and stimulation of inositol 1,3,4,5-tetrakisphosphate- [6] and/or G-protein- [7,8] regulated Ca^{2+} entry through receptor-operated channels [4] (for a review, see [5]).

Although endothelial cells possess a large number of different adenylate cyclase-linked receptors, e.g. β_2-adrenoceptors [9] and adenosine A_2 receptors [10] (for a review, see [11]), the functional role of endothelial cyclic AMP is still obscure. Lückhoff *et al.* [12] reported that an increase in cyclic AMP attenuated ATP-induced Ca^{2+} increases in bovine aortic endothelial cells, while Brock *et al.* [13] and Buchan & Martin [14] have found an elevation of ATP-, bradykinin- and thrombin-induced plateau phases of $[Ca^{2+}]_i$ by cyclic AMP in the same tissue. On the other hand, Kuhn and co-workers [15] reported that neither cyclic GMP nor cyclic AMP modulated EDRF release from bovine aortic endothelial cells. However, Mackie *et al.* [16] presented evidence that cyclic AMP-dependent protein kinase phosphorylates a wide number of substrates in endothelial cells, suggesting a functional role for cyclic AMP. In agreement with this notion there is evidence for an involvement of endothelial cyclic AMP in the regulation of vascular tone (for reviews, see [11,17]). Adenosine, which is known to be involved in an 'autoregulation' of coronary blood flow via smooth muscle A_2 receptors, is thought to modulate EDRF biosynthesis (for a review, see [11]). Since we have previously reported that endothelial cyclic AMP does not regulate basal $[Ca^{2+}]_i$ and/or EDRF formation directly [14,18], we decided to investigate whether endothelial cyclic AMP modulates bradykinin-induced changes in $[Ca^{2+}]_i$ and EDRF formation in cultured porcine aortic endothelial cells.

MATERIALS AND METHODS

Materials

A23187, BSA, bradykinin, collagenase, EGTA, fura-2/AM, Hepes, IBMX (3-isobutyl-1-methylxanthine), isoprenaline, trypsin and trypsin inhibitor type II-S from soybean were purchased from Sigma, Munich, Germany. *N*-[2-Methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8) and ionomycin were obtained from Calbiochem, Frankfurt, Germany. Okadaic acid was purchased from Moana Bioproducts of Hawaii, Honolulu, Hawaii. All other chemicals were obtained from Merck, Darmstadt, Germany, and Dulbecco's minimum essential medium was from Flow Laboratories, Meckenheim, Germany. Fetal-calf serum, amino acids and vitamins were obtained from Boehringer Mannheim, and Opti-MEM was purchased from GIBCO/BRL, Eggenstein, Germany. Petri dishes and plates were purchased from Greiner, Kremsmünster, Austria.

Cell culture

Porcine aortic endothelial cells were cultured by the method described previously [7,19]. Briefly, endothelial cells were isolated by a 20 min enzymic digestion as described by Sturek *et al.* [20] [2 mg of BSA/ml, 200 units of collagenase/ml, 1 mg of trypsin inhibitor type II-S/ml, including non-essential amino acids and vitamins, in Dulbecco's minimum essential medium (without serum)], centrifuged for 5 min at 500 *g*, resuspended in Opti-MEM containing 3 % (v/v) fetal-calf serum and antibiotics, and seeded in plastic Petri dishes (diam. 95 mm). Confluent cells were

Abbreviations used: EDRF, endothelium-derived relaxing factor; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; L-NNA, N^{ω}-nitro-L-arginine.

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split by a ratio of 1:3 and subcultured for up to two passages. Most of the experiments were performed with cells from the first passage (about 10 days after isolation).

Ca^{2+} measurement

$[Ca^{2+}]_i$ was monitored by the method described previously [18]. Briefly, endothelial cells were harvested, centrifuged and incubated in culture medium including 2 μmol of fura-2/AM per litre. After 45 min incubation at 37 °C, cells were washed twice with Hepes buffer (mmol/litre: NaCl, 145; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1; Hepes acid, 10; adjusted with NaOH to pH 7.4) and resuspended in a final concentration of 1.25×10^6 cells/ml in Hepes-buffered solution containing 1 mmol of IBMX/litre. After an equilibration period of 15 min, fura-2 fluorescence was monitored by using the ratio-fluorescence-spectroscopy technique and $[Ca^{2+}]_i$ was calculated by using the equation given by Grynkiewicz *et al.* [21].

EDRF (= NO) measurements

Biosynthesis of EDRF (= NO). This was monitored by measuring endothelial cyclic GMP levels, which serve as biological markers for EDRF formation [19,22,23]. Since cyclic GMP levels were not measurable under any conditions in the absence of the phosphodiesterase inhibitor IBMX, all cyclic GMP experiments were performed in the presence of 1 mmol of IBMX/litre.

Release of NO. This was determined spectrophotometrically by the NO-mediated conversion of oxyhaemoglobin into methaemoglobin as described previously [23] in the absence of IBMX. All experiments were performed at 37 °C in Hepes buffer.

Statistical analysis

All experiments were performed in triplicate with at least three different batches of endothelial cells. EC_{50} values were extrapolated from individual concentration–response curves and are expressed as geometric means with 95% confidence limits calculated as S.E.M. × Student's *t* values. Unless otherwise indicated, the results are expressed as mean values ± S.E.M. The statistical significances were calculated by one- or two-way analysis of variance using Scheffe's *F*-test.

RESULTS AND DISCUSSION

As described previously [18], endothelial cells respond to bradykinin with a rapid increase in $[Ca^{2+}]_i$ from 142 ± 2.9 to 682 ± 6.3 nmol/l ($n = 48$), followed by a sustained plateau phase at 321 ± 5.2 nmol/l (Fig. 1). Addition of 10 μmol of forskolin/l during the bradykinin-induced plateau phase further increased $[Ca^{2+}]_i$ from 317 ± 10.2 to 507 ± 7.7 nmol/l, followed by a high plateau phase at 432 ± 7.9 nmol/l (Fig. 1; $n = 15$). Similarly, addition of 250 μmol of adenosine/l ($n = 12$) and 30 μmol of isoprenaline/l ($n = 8$) increased the bradykinin-induced plateau phase from 307 ± 4.7 to 354 ± 5.4 and 349 ± 7.3 nmol/l ($n = 12$) respectively. In these experiments forskolin, adenosine, as well as isoprenaline, increased endothelial cyclic AMP from 3.2 ± 0.07 to 15.4 ± 0.12, 10.6 ± 0.06 and 9.7 ± 0.09 respectively. Consistent with their effects on bradykinin, 10 μmol of forskolin/l, 250 μmol of adenosine/l or 30 μmol of isoprenaline/l also increased the effect of ATP on endothelial $[Ca^{2+}]_i$ from 283 ± 14.2 to 398 ± 7.6, 324 ± 3.2 and 327 ± 4.8 nmol/l respectively ($n = 9$). In all experiments described above, cells were preincubated for 5 min with 1 mmol of IBMX/l, which diminished the degradation of cyclic AMP, but did not alter the agonist-induced response in $[Ca^{2+}]_i$ alone [18]. In the absence of IBMX, i.e. under more physiological conditions, forskolin (10 μmol/l) as well as adenosine (250 μmol/l) also increased the bradykinin-induced (100 nmol/l) plateau from 303 ± 11.3 to 417 ± 4.3 (forskolin; $n = 8$) and 346 ± 8.2 (adenosine; $n = 9$) nmol of $[Ca^{2+}]_i$/l.

To test whether the stimulatory effects of forskolin and isoprenaline on agonist-mediated changes in $[Ca^{2+}]_i$ are indeed mediated by cyclic AMP, the effect of the membrane-permeable cyclic AMP analogue dibutyryl cyclic AMP was studied. Similarly to forskolin, adenosine and isoprenaline, 100 μmol of dibutyryl cyclic AMP increased the bradykinin-induced plateau phase from 312 ± 11.3 to 489 ± 13.7 nmol/l within 120 s ($n = 11$). Forskolin, adenosine, isoprenaline and dibutyryl cyclic AMP alone did not alter endothelial $[Ca^{2+}]_i$ significantly ([18]; results not shown). These data clearly indicate that amplification of agonist-induced Ca^{2+} responses are due to increases in endothelial cyclic AMP. This idea was further confirmed by our findings that the EC_{50} values for the effects of forskolin and isoprenaline on endothelial cyclic AMP [forskolin, 1.2 (0.6–2.5) × 10^{-5} mol/l; isoprenaline, 9.5 (7.5–12.1) × 10^{-7} mol/l] are essentially the same as for the amplification of the bradykinin-induced Ca^{2+} response [forskolin, 2.0 (0.9–4.3) × 10^{-5} mol/l; isoprenaline, 10.4 (6.7–16.1) × 10^{-7} mol/l].

Our data indicate that an increase in endothelial cyclic AMP promotes mechanisms which are either directly or indirectly involved in the stimulatory mechanisms of bradykinin- and ATP-induced Ca^{2+} mobilization. Buchan & Martin [14], who described similar effects in bovine aortic endothelial cells, suggested that cyclic AMP may enhance agonist-induced Ca^{2+} mobilization. Furthermore, these authors exclude an inhibitory effect of cyclic AMP on protein kinase C, which would prevent the inhibition of phospholipase C by this enzyme. A slight augmentation of ATP-induced Ca^{2+} changes in bovine aortic endothelial cells has also been reported by Brock *et al.* [13]. By contrast, Lückhoff *et al.* [12] reported inhibition of ATP-induced changes in $[Ca^{2+}]_i$ by cyclic AMP in bovine endothelial cells. To date the reason for these divergent results is obscure. However, one might speculate about tissue specificities in the regulation of $[Ca^{2+}]_i$, particularly since Lückhoff *et al.* reported that the observed changes in $[Ca^{2+}]_i$ mainly depended on activation of phospholipase C [12], while in our cells the agonist-induced plateau phase is definitely due to phospholipase C-independent mechanisms [3,7,8].

Since it is well known that endothelial $[Ca^{2+}]_i$ regulates the

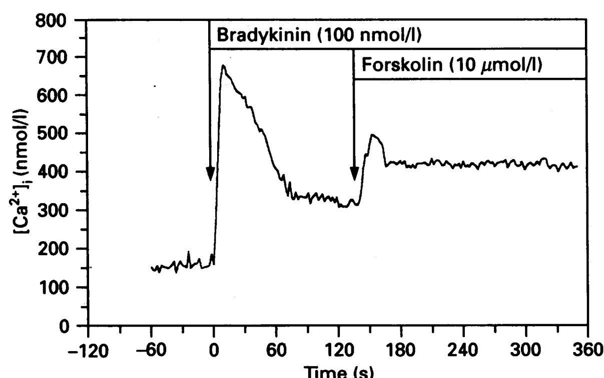

Fig. 1. Original tracing of the effect of forskolin on the bradykinin-induced plateau phase in endothelial $[Ca^{2+}]_i$

Cells were loaded with fura-2/AM (2 μmol/l) at 37 °C for 45 min, centrifuged, washed twice and resuspended in Hepes-buffered solution. During an equilibration period of 15 min, cells were preincubated with IBMX (1 mmol/l). Bradykinin (100 nmol/l) and forskolin (10 μmol/l) were added at the times indicated. $[Ca^{2+}]_i$ is expressed as nmol of free endothelial Ca^{2+}/l, which was calculated as described in the Materials and methods section.

Table 1. Effects of forskolin (Forsk.), adenosine (Ado) and isoprenaline (Iso) on agonist-induced increases in endothelial cyclic GMP levels

Confluent monolayers of cultured endothelial cells were preincubated for 15 min with IBMX (1 mmol/l) in Hepes buffer without or with L-NNA (100 μmol/l). After 13 min, forskolin (10 μmol/l), adenosine (250 μmol/l) or isoprenaline (30 μmol/l) was added. After 2 min, bradykinin, ATP, A23187 or sodium nitroprusside (SNP) was added and the reaction was stopped after 4 min by removal of the incubation buffer and addition of 2.0 ml of HCl (0.01 mol/l). Intracellular cyclic GMP levels were measured by radioimmunoassay as described in the Materials and methods section. Data are expressed as mean values ± S.E.M. ($n = 12$). *$P < 0.01$ versus the respective value without forskolin, adenosine or isoprenaline.

	Cyclic GMP level (pmol/10^6 cells)					
		Bradykinin				
Addition	Control	(30 nmol/l)	(1 μmol/l)	ATP (10 μmol/l)	A23187 (1 μmol/l)	SNP (1 mmol/l)
None	1.9±0.10	4.6±0.14	8.4±0.22	6.9±0.08	27.1±0.31	39.4±1.27
Forsk.	1.9±0.14	7.3±0.17*	12.7±0.28*	10.9±0.11*	27.6±0.53	40.4±1.67
Ado	1.8±0.09	6.2±0.21*	10.1±0.14*	8.7±0.06*	27.4±0.33	39.2±1.66
Iso	1.8±0.07	5.9±0.13*	10.3±0.31*	9.0±0.21*	26.8±0.47	39.7±1.53
L-NNA	1.6±0.09	1.7±0.17	1.5±0.21	1.7±0.19	1.5±0.12	38.6±1.44

Table 2. Effects of bradykinin, ATP, A23187 and sodium nitroprusside on forskolin-, adenosine- and isoprenaline-induced increases in endothelial cyclic AMP

Cultured endothelial cells were preincubated for 15 min with IBMX (1 mmol/l) in Hepes buffer. After 13 min, bradykinin, ATP, A23187 or SNP were added. Incubation was started by addition of forskolin (10 μmol/l) or isoprenaline (30 μmol/l) and stopped after 4 min by removal of the incubation buffer and addition of 2.0 ml of HCl (0.01 mol/l). Intracellular cyclic AMP levels were measured by radioimmunoassay [18]. Results are expressed as means ± S.E.M. ($n = 9$). *$P < 0.01$ versus the respective value without forskolin, adenosine or isoprenaline. No statistical significance was found between the effects of forskolin, adenosine and isoprenaline in the absence or in the presence of bradykinin, ATP, A23187 or SNP. Abbreviations are the same as those used in Table 1.

	Cyclic AMP level (pmol/10^6 cells)				
Addition	Control	Bradykinin (1 μmol/l)	ATP (10 μmol/l)	A23187 (1 μmol/l)	SNP (1 mmol/l)
None	1.8±0.13	1.9±0.22	1.7±0.08	1.5±0.06	1.9±0.17
Forsk.	14.6±0.40*	13.5±0.36*	13.2±0.31*	14.3±0.27*	14.7±0.44*
Ado	8.3±0.36*	7.9±0.42*	8.1±0.22*	7.7±0.26*	8.2±0.37*
Iso	7.2±0.25*	7.5±0.38*	7.6±0.38*	7.9±0.18*	7.4±0.47*

biosynthesis of NO (= EDRF) [25,26,31], we investigated the effect of cyclic AMP-increasing substances on agonist-stimulated NO biosynthesis by measuring endothelial cyclic GMP levels [22–24]. As shown in Table 1, forskolin, adenosine and isoprenaline increased bradykinin- and ATP-induced NO biosynthesis, whereas the effects of the Ca^{2+} ionophore A23187 or sodium nitroprusside were not affected by these compounds (Table 1). These data suggest that forskolin, adenosine and isoprenaline affect receptor-mediated Ca^{2+} signalling by receptor agonists (i.e. bradykinin and ATP), while neither a direct stimulation of guanylate cyclase by sodium nitroprusside nor the activation of the NO synthase by the 'unphysiological' Ca^{2+} entry induced by the Ca^{2+} ionophore A23187 was affected by increasing cyclic AMP. The rises in cyclic AMP induced by forskolin, adenosine and isoprenaline were, on the other hand, not altered by bradykinin, ATP, A23187 or sodium nitroprusside (Table 2). Thus modulation of forskolin-, adenosine- or isoprenaline-stimulated adenylate cyclase can be excluded. To test whether agonist-induced increases in cyclic GMP levels are indeed due to stimulated NO synthase, we studied the effect of the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA). L-NNA at 100 μmol/l abolished the effects of bradykinin, ATP and A23187 on endothelial cyclic GMP levels, but did not affect those of sodium nitroprusside (Table 1).

In order to find out whether the effect of cyclic AMP is mediated by cyclic AMP-dependent phosphorylation, we used H-8, an inhibitor of protein kinase A [27], as a tool. As shown in Fig. 2, H-8 abolished the stimulatory effect of forskolin on bradykinin-induced NO formation in a concentration-dependent manner, while the effect of bradykinin alone remained un-

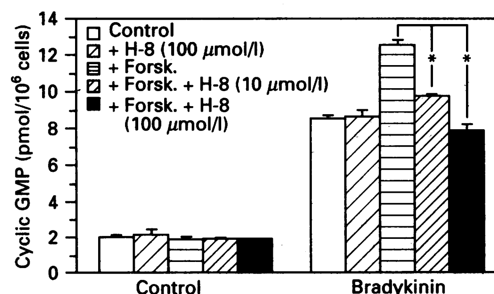

Fig. 2. The protein kinase A inhibitor H-8 antagonizes forskolin-mediated amplification of bradykinin-induced EDRF biosynthesis, measured as increases in endothelial cyclic GMP levels

Confluent cells were preincubated over a period of 15 min in Hepes-buffered solution containing IBMX (1 mmol/l) alone (Control), or including 100 μmol of H-8/l (H-8), 10 μmol of forskolin/l (Forsk.) or forskolin plus H-8 (10 or 100 μmol/l). Incubation was started by the addition of water (Control) or 1 μmol of bradykinin/l, and stopped after 4 min by aspirating the incubation solution and addition of 2.0 ml of HCl (0.01 mol/l). Results are means ± S.E.M. ($n = 9$). *$P < 0.01$ compared with the effect of forskolin in the absence of H-8.

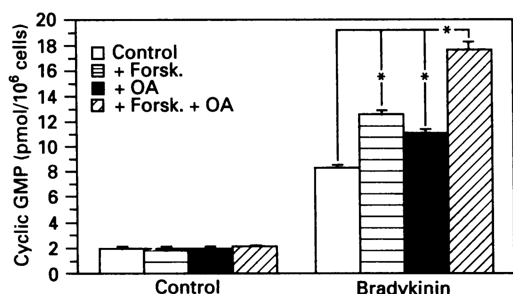

Fig. 3. Okadaic acid (OA) amplifies the effect of forskolin on bradykinin-induced EDRF biosynthesis

Endothelial cells were preincubated in Hepes-buffered solution containing IBMX (1 mmol/l) ± okadaic acid (0.7 μmol/l) at 37 °C. After 13 min, water or forskolin (10 μmol/l) was added, and, after a further 2 min, incubation was started by addition of the compound to be tested. The experiment was stopped after removal of incubation buffer and addition of 2.0 ml of HCl (0.01 mmol/l). Results are means ± S.E.M. ($n = 9$). $*P < 0.01$ compared with the effect under control conditions.

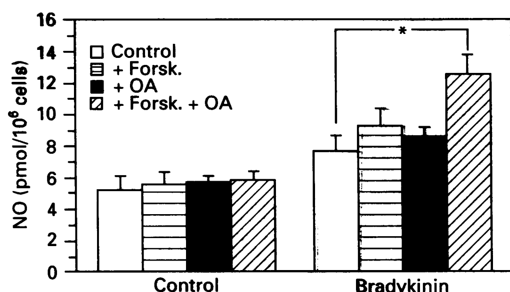

Fig. 4. Amplification of the bradykinin-induced NO release from endothelial cells, measured by NO-induced conversion of oxyhaemoglobin into methaemoglobin as described in the Materials and methods section

Experiments were performed with cells preincubated in the absence or presence of forskolin (10 μmol/l), okadaic acid (OA; 0.7 μmol/l), or forskolin + okadaic acid. NO release was measured over a period of 10 min under control conditions and after stimulation with bradykinin (1 μmol/l). Results are means ± S.E.M. ($n = 6$). $*P < 0.05$ versus the effect of bradykinin under control conditions.

changed. The IC_{50} value of H-8 in our experiments [1.9 (0.6–5.8) × 10^{-6} mol/l] was essentially the same as described for cyclic AMP-dependent protein kinase (IC_{50} = 1.2 μmol/l; [27]). Thus inhibition of cyclic AMP-dependent protein kinase abolished the stimulatory effect of cyclic AMP on agonists-induced NO biosynthesis, suggesting an involvement of this enzyme in the amplification of agonist-induced NO biosynthesis.

This hypothesis was further confirmed by the observation that okadaic acid, an inhibitor of phosphatases [28], potentiated the effect of forskolin (Fig. 3). In our experiments okadaic acid at 0.7 μmol/l alone increased the effect of bradykinin from 8.4 ± 0.22 to 11.1 ± 0.26 pmol of cyclic GMP/10^6 cells, which may reflect a basis phosphatase activity in endothelial cells [28]. In the presence of okadaic acid at 0.7 μmol/l the effect of forskolin on bradykinin-induced NO formation was also markedly enhanced from 12.7 ± 0.28 to 17.69 ± 0.67 pmol of cyclic GMP/10^6 cells (Fig. 3). In our experiments the EC_{50} values for okadaic acid-induced stimulation of bradykinin-induced NO [23 (12–44) × 10^{-9} mol/l] formation perfectly correlates with the described IC_{50} values for inhibition of cyclic AMP-stimulated protein phosphatase-1 {(10–15) × 10^{-9} mol/l; [29]}. An evaluation of the combined effects of okadaic acid and forskolin by the method of Pöch [32] provided evidence for a synergistic interaction of these two compounds. Thus our data suggest that inhibition of cyclic AMP-regulated phosphatase by okadaic acid amplifies bradykinin-induced NO biosynthesis. This is in line with our hypothesis that cyclic AMP-stimulated protein phosphorylation is involved in the amplification of agonist-induced $[Ca^{2+}]_i$ and NO (= EDRF) responses.

The physiological significance of cyclic AMP-mediated enhancement of agonist-induced Ca^{2+} responses and EDRF formation was finally also tested by measuring NO release using NO-induced conversion of oxyhaemoglobin into methaemoglobin (Fig. 4). In contrast with basal NO release, which remained unaffected (5.2 ± 0.88 versus 5.5 ± 0.81 pmol of NO/min per 10^6 cells), forskolin at 10 μmol/l enhanced NO release in the presence of bradykinin from 7.7 ± 1.00 to 9.3 ± 0.84 pmol of NO/min per 10^6 cells. In agreement with the results shown in Fig. 3, okadaic acid at 0.7 μmol/l again amplified the effect of forskolin (Fig. 4). Similarly to forskolin, adenosine at 250 μmol/l also amplified the effect of bradykinin on NO release (7.0 ± 0.38 in the absence and 9.0 ± 0.67 in the presence of adenosine), while the basal release remained unchanged (5.1 ± 0.38 versus 5.2 ± 0.26). These data indicate that elevated endothelial cyclic AMP levels caused amplification of agonist-induced NO release, and thus enhanced endothelium-dependent vasodilation, while basal NO release remained unchanged. These findings are in agreement with reports demonstrating separate control mechanisms for basal and agonist-induced NO formation [33,34].

Since it is well known that vascular endothelial cells possess β_2-adrenoceptors [9] and adenosine A_2-receptors [10] (for reviews, see [11,30]), it can be expected that endothelial cyclic AMP plays an important role in EDRF-mediated vasodilation under physiological conditions. Recently, it has been suggested that adenosine, which is involved in the 'autoregulation' of coronary blood flow via interaction with smooth-muscle A_2-receptors, may also modulate vascular tone via an endothelium-dependent mechanism by interaction with endothelial A_2-receptors (for reviews, see [11,17]). Consequently, an enhanced NO release in the presence of adenosine was postulated. We now report that increases in endothelial cyclic AMP indeed amplify agonist-induced rises in endothelial $[Ca^{2+}]_i$, leading to an enhanced agonist-induced biosynthesis and release of NO. This mechanism may represent the physiological control of vascular tone via endothelial adenylate cyclase-coupled receptors.

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