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Characteristics of adrenaline-driven receptor-mediated signals in human microvessel-derived endothelial cells

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Abstract:
Adrenaline (0.001–1,000 μM) strongly stimulated adenosine-3',5'cyclic monophosphate (cAMP) generation in cultured human microvascular-derived endothelial cells (HMEC-1). Isoprenaline mimicked the action of adrenaline, whereas noradrenaline appeared to be decisively less potent. Experiments carried out with an array of compounds acting selectively on different types/subtypes of adrenergic receptors revealed that the adrenaline cAMP effect in HMEC-1 cells did not possess either an α_1 or α_2 component. However, the effect may have been mediated through a receptor that did not fit β_1-, β_2-, or β_3-receptor classification. Supporting this assertion, various double and triple β-subtype selective drug combinations maximally inhibited the adrenaline effect by 50–60%, whereas the non-selective antagonist propranolol totally prevented the hormone-evoked cAMP effect. Based on results utilizing the phosphodiesterase (PDE)-isoform nonselective inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the PDE-4-selective inhibitor rolipram, the adrenaline-driven cAMP signal appeared to be regulated by PDE-4. In addition, the present study demonstrated that phenylephrine, a presumed selective α_1-adrenoceptor agonist, was capable of stimulating cAMP generation in HMEC-1 cells in a prazosin-insensitive and propranolol-sensitive manner. This result indicated that in at least this cell model system, phenylephrine may act nonspecifically. Microvessel-derived endothelial cells such as HMEC-1 exhibit functional differences when compared with macrovessel-derived endothelial cells (e.g. HUVEC sensitivity to adrenaline). Accordingly, these cell cultures represent a useful model system to study the biological effects of endogenous catecholamines, including adrenaline, as well as potential therapeutics targeting adrenergic receptors.

Key words:
adrenaline, cyclic AMP, rolipram, PDE-4, phenylephrine, β-adrenoceptor subtype, HMEC-1, human endothelial cell

Abbreviations: cAMP – adenosine-3',5'cyclic monophosphate, CGP-20712 – (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]-amino]ethoxy]-benzamide, ECs – endothelial cells, HMEC-1 – human microvascular endothelial cells, HUVEC – human umbilical vein endothelial cells, IBMX – 3-isobutyl-1-methylxanthine, ICI 118,551 – (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol, ISO – isoprenaline, PBS – phosphate buffered saline, PDE – phosphodiesterase, PRA – prazosin, PRO – propranolol, SR 59230A – 3-(2-ethylphenoxy)-1-{[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino}-(2S)-2-propanol

Introduction

Since its discovery and isolation more than a centenary ago by independent efforts of three scientists (Napoleon N. Cybulski, Jokichi Takamine, and John J. Abel), adrenaline/epinephrine was the subject of intense investigation and rapidly received the status of a vital hormone, colloquially named the "stress hormone" (for its essential role in the short-term stress reactions). In response to specific stimuli, e.g. low blood glucose, exercise, or stress, adrenaline is synthesized in the adrenal medulla and rapidly secreted into the bloodstream where it circulates throughout the whole body and reaches various target cells, with the endothelium being the first to be contacted. Then, the hormone binds to specific-adrenergic receptors localized on target cells, where it activates specific intracellular signaling system(s) and evokes/affects an array of vital processes, including cardiac function, blood vessels dynamics, and energy metabolism [9, 10, 29].

The endothelium represents the inner cellular lining of blood vessels and is composed of endothelial cells (ECs) displaying phenotypic and functional heterogeneity that is dependent on species, organ, vascular bed (e.g. micro- and macrovessels), as well as the different populations of receptors present on their cell membranes [1, 3, 4, 10, 22, 23, 29]. Among the various receptors present are adrenaline-sensitive adrenergic receptors including a prominent population of β-adrenoceptors that are positively coupled to the adenylyl cyclase/adenosine-3',5'cyclic monophosphate (cAMP) signaling system [10, 14, 15, 25].

A comparative study carried out on human microvessel- and macrovessel-derived ECs revealed that adrenaline is a powerful stimulator of cAMP generation in the former cells (human microvascular endothelial cells – HMEC-1), and comparatively weakly affects the nucleotide formation in the latter cells (human umbilical vein endothelial cells – HUVEC) [20, 30]. In addition, previous findings have shown the opposite cAMP response in HUVEC and HMEC-1 cells to the adenosine A_2-type receptor agonist NECA and EC type-specific sensitivity to hypoxic conditions (HMEC-1 > HUVEC) [30]. Thus, these findings illustrate functional differences between human micro- and macrovessel-derived ECs and suggest that adrenaline may primarily and specifically influence microvessel-derived ECs. This work is a continuation of our earlier studies on cAMP-directed effects in different types of ECs, and focuses on a more detailed pharmacological characterization of adrenaline-driven receptor-mediated cAMP signaling in microvessel-derived ECs using HMEC-1 cells as a model system.

Materials and Methods

Chemicals

The substances used were the following: MCDB 131 medium, fetal bovine serum, penicillin-streptomycin solution (5,000 units/ml penicillin and 5,000 μg/ml streptomycin sulphate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, CA, USA). Epinephrine bitartare (adrenaline), 3-isobutyl-1-methylxanthine (IBMX), rolipram, isoprenaline hydrochloride, norepinephrine bitartare, phenylephrine hydrochloride, methoxamine hydrochloride, propranolol hydrochloride, and prazosin hydrochloride were purchased from Sigma (St. Louis, MO, USA), (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide (CGP 20712) dihydrochloride, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)-amino]-2-butanol (ICI 118,551) hydrochloride, 3-(2-ethylphenoxy)-1-{[(1S)-1,2,3,4-tetrahydronaphth-1-yl]-amino}-(2S)-2-propanol (SR 59230A) hydrochloride were purchased from Tocris (Ellisville, MO, USA). Radioactive compounds including 2,8-[^{3}H]adenine (specific activity 24.40 Ci/mmol) was from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA) and [^{14}C]cyclic AMP (specific activity 56 mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA).

Cell culture

HMEC-1 were kindly provided by Dr. F. Candal from the Center for Disease Control and Prevention (Atlanta, GA, USA). The cells were used between passages 32–41 and cultured in 25 cm^3 flasks in MCDB 131 medium supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone and penicillin-streptomycin solution, in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were harvested every third day in a trypsin-EDTA (0.25% trypsin, 1 mM EDTA) solution.

Assay of cAMP formation

Cells used in these experiments were seeded in 12-well plates at a density of 250,000 cells/well in 500 µl of culture medium and cultured overnight. The next day, the culture medium was removed, fresh serum-free culture medium was added, and cells were incubated in the presence of [^{3}H]adenine for 2 h at 37°C. After this incubation, the medium was removed, cells were rinsed two times with pre-warmed PBS and serum-free culture medium was added. Subsequently, cells were preincubated for 20 min at 37°C in the presence of IBMX (0.1 mM). After this preincubation period, cells were exposed to appropriate agonists for a further 15 min. Antagonists were applied 15 min before agonist treatment. The reaction was stopped by adding 500 µl of ice-cold 10% trichloroacetic acid. The resulting mixture was then transferred to test tubes, centrifuged, and the supernatant fraction was examined for cAMP generated. The formation of [^{3}H]cAMP in [^{3}H]adenine-prelabeled cells was assayed according to Shimizu et al. [26] with some modifications [30]. The formed [^{3}H]cAMP was isolated by sequential Dowex-alumina column chromatography according to Salomon et al. [24]. The results were individually corrected for percent recovery that was normalized with the aid of [^{14}C]cAMP added to each column system prior to the nucleotide extraction. The accumulation of cAMP during a 15 min stimulation period was assessed as a percentage of the conversion of [^{3}H]adenine to [^{3}H]cAMP.

Data analysis

All data are expressed as the mean ± SEM values. For statistical evaluation of the results, an analysis of variance (ANOVA) was used followed by a *post-hoc* Student-Newman-Keuls test.

Results and Discussion

Role of PDE-4 type phosphodiesterase in cAMP inactivation in human microvessel-derived endothelial cells

Our earlier experiments carried out on HMEC-1 cells incubated with or without the phosphodiesterase (PDE) inhibitor IBMX showed that under specified routine experimental conditions, the basal activity contributing to cAMP generation in this cell type is rather low [20]. However, the addition of forskolin (a direct activator of adenylyl cyclase), an adenosine A_2-agonist, adrenaline, or isoprenaline to the incubation mixture led to significant increases in cAMP production. Notably, the effects of these compounds were highly pronounced in the presence of IBMX, as is observed in Figure 1 and as previously reported [20, 30]. IBMX is a widely used PDE inhibitor, which effectively blocks PDE-dependent hydrolysis of cAMP when applied at high concentrations. Mammalian PDEs are encoded by many isoenzymes, which based on primary amino acid sequence, overall domain structure, and catalytic or regulatory characteristics are classified into 11 distinct families. Of these family members, eight hydrolyze cAMP selectively (PDE-4, -7, -8), the remaining hydrolyze cAMP in addition to guanosine-3',5' cyclic monophosphate to varying degrees (PDE-1, -2, -3, -10, -11) [2, 17]. The understanding of which particular PDE subtype may be involved in cAMP inactivation in a given biological model system is important in light of the cell-specificity of PDE isoenzymes and PDE subtype-dependent subcellular compartmentalization of cyclic nucleotide signaling [7, 28]. Since it has been suggested that the PDE-4 enzyme is in microvascular endothelial cells [21, 31], our next experiments utilized a PDE-4-selective inhibitor, rolipram [17, 21, 27]. In rolipram (100 µM) pretreated HMEC-1 cells, the cAMP-elevating effects of adrenaline were comparable to those seen in the presence of IBMX, which is a PDE multi-isoform inhibitor (Fig. 2). Thus, the obtained results indicated that in human microvascular-derived ECs (such as HMEC-1) cAMP

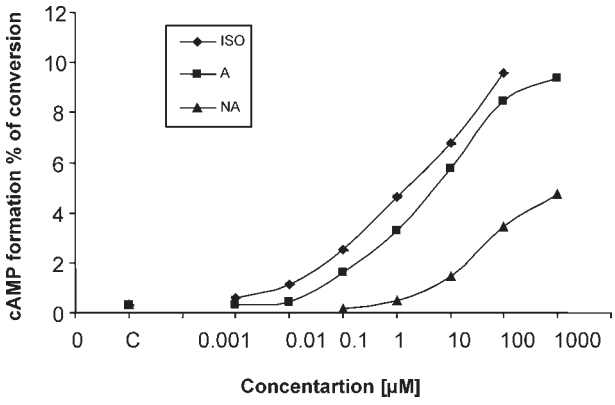

Fig. 1. Concentration-dependent effects of noradrenaline (NA), adrenaline (A), and isoprenaline (ISO) on cAMP synthesis in HMEC-1 cells. Data represent the means of 5–25 experiments; C – control values

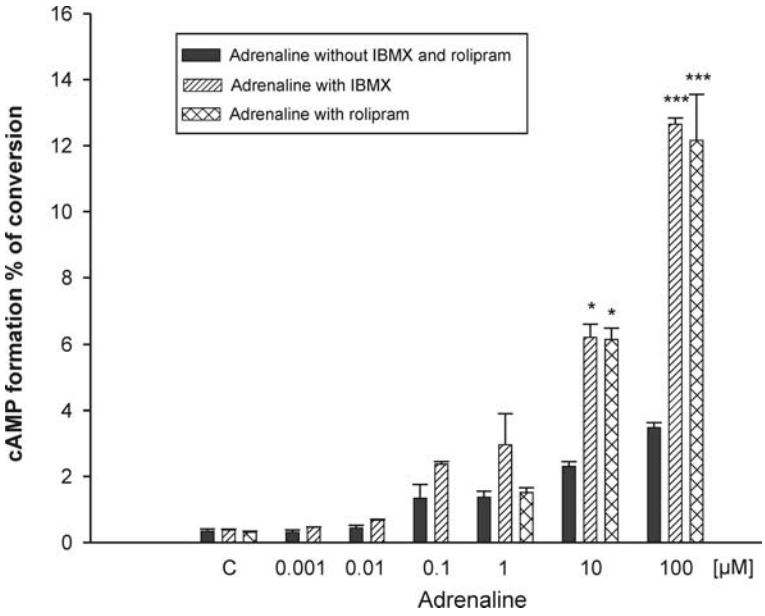

Fig. 2. Effects of adrenaline on cAMP synthesis in HMEC-1 cells in the absence and presence of the PDE inhibitor IBMX (100 μM) and PDE-4 inhibitor rolipram (100 μM). Bars represent the means ± SEM of 5–9 experiments. C – control values. * $p < 0.05$, *** $p < 0.001$ *vs.* adrenaline without inhibitors of PDE

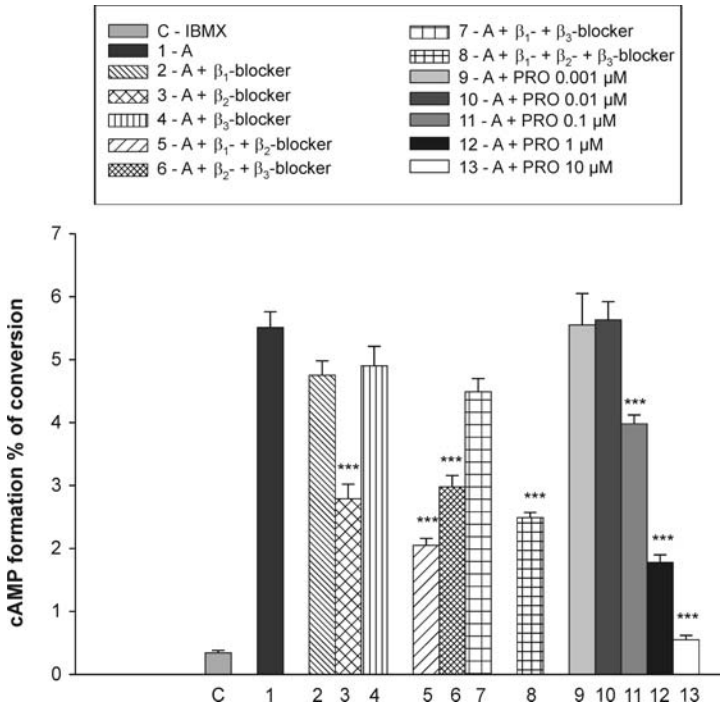

Fig. 3. Effects of the following β-adrenergic receptor antagonists: β_1-blocker CGP 20712, β_2-blocker ICI 118,551, β_3-blocker SR 59230A (each at 10 μM), and propranolol (PRO, 0.001–10 μM), on adrenaline (A, 100 μM)-evoked stimulation of cAMP accumulation in HMEC-1 cells. Bars represent the means ± SEM of 5–23 experiments. C – control value. *** $p < 0.001$ *vs.* adrenaline

was chiefly, if not exclusively, inactivated by the actions of the PDE-4 enzyme subfamily.

Role of β-adrenergic receptor subtype mediating the cAMP effect of adrenaline

Based on reports from the literature and our own data [14, 20, 25, 30], the action of adrenaline on cAMP generation in different types of ECs, including HMEC-1 cells, is mediated through β-adrenergic receptors, with two of the following facts being consistently observed: (1) the β-adrenoceptor antagonist propranolol (PRO) prevented the effect of adrenaline, and (2) the β-adrenoceptor agonist isoprenaline mimicked the action of adrenaline (Fig. 1, 3). However, the β-adrenergic receptor is comprised of at least three subtypes (β_1,

β_2, β_3), all being positively coupled to adenylyl cyclase and all showing similar sensitivity to isoprenaline and PRO. Therefore, in order to determine the receptor subtype mediating the effects of adrenaline, the three following established β-receptor subtype-specific antagonists were used: CGP 20712 (β_1), ICI 118,551 (β_2), and SR 59230A (β_3) [13, 19]. Each antagonist was used at a relatively high concentration (10 μM) so as to elicit a clear response. When used separately, only the β_2-selective agent significantly reduced (by 49%) the action of adrenaline (100 μM) (Fig. 3). In addition, all possible double-antagonist combinations ($\beta_1 + \beta_2$, $\beta_2 + \beta_3$, $\beta_1 + \beta_3$) produced an inhibition of the adrenaline-evoked effect. However, the observed actions were variable with the most pronounced and statistically significant effects noted for combinations of $\beta_1 + \beta_2$ (inhibition by 63%) and $\beta_2 + \beta_3$ (inhibition by 46%) antagonists, whereas the least effective was a combination of $\beta_1 + \beta_3$ (non-significant inhibition by 19%) antagonists. Interestingly, simultaneous pretreatment of HMEC-1 cells with three drugs ($\beta_1 + \beta_2 + \beta_3$) led to inhibition of the adrenaline effect by only 55%. This effect was roughly similar to that produced by a β_2-selective blocker or a drug combination containing a β_2-selective agent (Fig. 3). It should be stressed that parallel experiments utilizing the non-selective β-adrenoceptor antagonist PRO at a similar dose (10 μM) as the other β-blockers completely inhibited the adrenaline-evoked cAMP effect (Fig. 3). In our earlier experiments, lower concentrations (< 10 μM) of PRO were also highly effective, with usually a 1 μM dose producing complete antagonism (unpublished results). These findings do not provide a straightforward conclusion concerning the role of a particular β-receptor subtype in the adrenaline-evoked cAMP response in HMEC-1 cells. Nevertheless, the following possibilities should be considered: (1) β_2-adrenergic receptors mediate only a portion of the adrenaline-induced cAMP effect, (2) β-receptor subtype-selective drugs used at 10 μM concentration do not effectively block particular types of β-receptor in HMEC-1 cells, and (3) the β-receptor expressed in human microvessel-derived ECs does not fit the β_1-, β_2-, or β_3-classification profile. In other words, it is likely that HMEC-1 cells possess an atypical subtype of β-adrenergic receptor that is sensitive to PRO, partially sensitive to ICI 118,551, and insensitive to CGP 20712 and SR 59230. Interestingly, these findings in HMEC-1 cells do not appear to be unique. In the literature, there are many conflicting findings regarding β-adrenoceptor subtypes expressed in the heart, blood vessels, or specifically in the endothelium, with such terms as e.g. "putative β_4-adrenergic receptor" or "atypical β-adrenoceptor" being introduced and supported by some authors and neglected by others [6, 8, 11, 12, 18, 19]. Although the putative

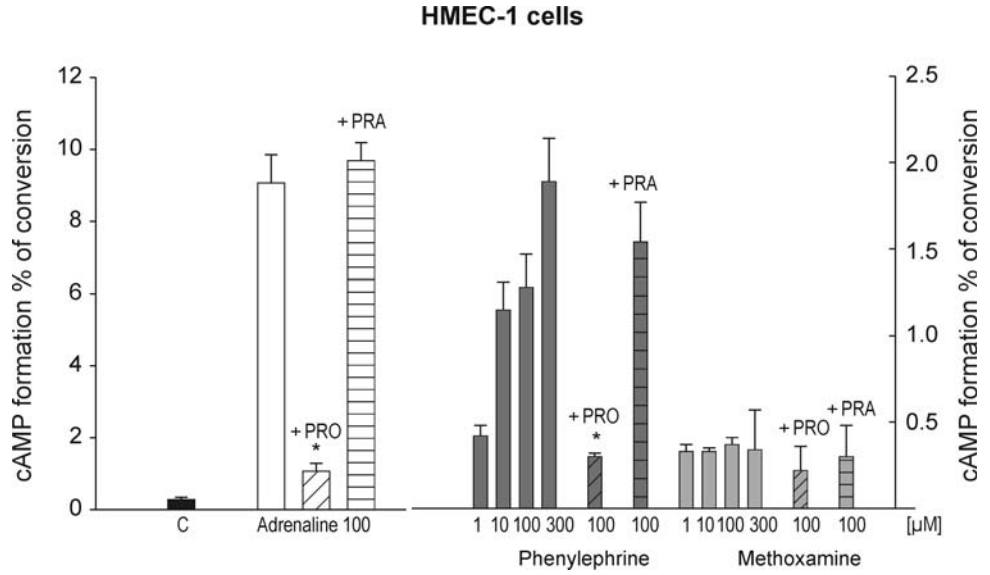

Fig. 4. Effects of phenylephrine (1–300 μM) and methoxamine (1–300 μM) on cAMP synthesis, and effects of the adrenergic receptor antagonists propranolol (PRO, 10 μM) and prazosin (PRA, 10 μM) on cAMP accumulation evoked by adrenaline, phenylephrine, and methoxamine (each at 100 μM) in HMEC-1 cells. Note that y-axis scales in the left and right figure are different. Bars represent the means ± SEM of 5–15 experiments, * $p < 0.05$ *vs.* adrenaline or phenylephrine

β_4-adrenoceptor appears to be a low-affinity form of the β_1-adrenoceptor [11], the cardiac β_3-adrenoceptor remains a matter of intensive debate [see 11], although the β_3-receptor has been cloned, molecularly characterized, and its presence in the endothelium has been demonstrated [5, 12, 16]. Moreover, such endothelial β_3-adrenoceptors may signal *via* a mechanism independent of adenylyl cyclase/cAMP, including nitric oxide generation [12, 16].

In conclusion, the difficulties of identifying subtypes of Gs protein/adenylyl cyclase-coupled β-adrenoceptors in HMEC-1 cells remain to be overcome.

α-Adrenergic receptors do not participate in the cAMP effects of adrenaline

Our earlier experiments excluded the role of α_2-adrenergic receptors in the cAMP effect of adrenaline because the selective α_2-antagonist yohimbine (10 μM) did not modify the hormone (100 μM)-evoked response of HMEC-1 cells [20, 30]. A similar situation was observed while using a selective α_1-type adrenergic receptor antagonist, prazosin (PRA, 10 μM). However, this compound slightly (up to 10%) and non-significantly, yet consistently, increased the effects of adrenaline. To resolve the problem of α_1-receptor mediated signals contributing to the overall cAMP effect of adrenaline, two selective α_1-adrenoceptor agonists, phenylephrine and methoxamine (both at concentrations of 1–300 μM), were used alone and in combination with PRA and PRO (each antagonist at 10 μM and agonists at 100 μM). These results are presented in Figure 4. Methoxamine appeared to be inactive at the concentration tested, whereas phenylephrine affected the cAMP generation in HMEC-1 cells by increasing nucleotide formation in a concentration-dependent manner. Its action was decisively weaker than that of adrenaline, and at the highest dose tested (300 μM) phenylephrine produced a net rise in the nucleotide level marked by a 1.61% increase in conversion ($p < 0.01$). In parallel experiments, 100 μM adrenaline produced increases of 8–11% conversion. The effect of phenylephrine (100 μM) was practically insensitive to PRA, but was completely prevented by the presence of PRO. In other experiments, neither phenylephrine (1 μM; at this concentration the drug by itself had no effect on cAMP generation; Fig. 4) nor methoxamine (100 μM) affected isoprenaline (ISO; 10 μM)-induced cAMP formation. The obtained results were the following: ISO, 7.43 ± 0.51(4); ISO + phenylephrine, 7.67 ± 0.19(4); ISO + methoxamine, 7.26 ± 0.25(4)% conversion. These results suggest the following: (1) methoxamine did not stimulate cAMP production, (2) the phenylephrine-evoked cAMP effect was PRO-sensitive and PRA-insensitive, and (3) the two α_1-agonists did not affect the β-agonist ISO-driven cAMP effect. These observations favor the conclusion that there was no α_1-adrenoceptor-dependent component contributing to the overall effect of adrenaline on cAMP production in HMEC-1 cells. The phenylephrine-evoked cAMP effect in the tested human microvessel-derived ECs indicates to us that this α_1-adrenoceptor agonist, which is widely used in cardiovascular research, exhibits a nonspecific interaction with adenylyl cyclase-coupled β-adrenergic receptors and should be used with caution.

In conclusion, there are four major findings of this study. First, adrenaline is a powerful stimulator of cAMP formation in human microvessel-derived ECs, i.e. HMEC-1, and the formed nucleotide is likely hydrolyzed by the PDE-4 isoenzyme. Second, although it is clear that the cAMP effect of adrenaline in HMEC-1 cells results from activation of β-adrenergic receptors, we were unable to precisely identify the receptor isoform involved in the observed phenomenon using selective β_1-, β_2-, β_3-adrenoceptor blockers. Third, there was no α_1- or α_2-dependent signal/component influencing the adrenaline-driven cAMP effect in HMEC-1 cells. Fourth, microvessel-derived ECs and HMEC-1 cells have emerged as important physiological targets in human blood vessels. Therefore, they may serve as a good model system with which to study the biological effects of endogenous catecholamines, including adrenaline, as well as potential therapeutics targeting adrenergic receptors.

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