Cyclic AMP enhances agonist-induced Ca\(^{2+}\) entry into endothelial cells by activation of potassium channels and membrane hyperpolarization

Wolfgang F. GRAIER,* Walter R. KUKOVETZ and Klaus GROSCHNER
Institut für Pharmakologie und Toxikologie, Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

The mechanism underlying cyclic AMP (cAMP)-mediated amplification of agonist-induced Ca\(^{2+}\) responses in endothelial cells was investigated in pig endothelial cells. Forskolin, adenosine and isoprenaline, as well as the membrane-permeant cAMP analogue dibutyl cAMP, enhanced bradykinin-induced rises in intracellular free Ca\(^{2+}\) as well as bradykinin-induced Mn\(^{2+}\) entry. These agents were also found to hyperpolarize endothelial cells without increasing intracellular Ca\(^{2+}\) by itself, i.e. in the absence of bradykinin. Both amplification of bradykinin effects and the hyperpolarizing action was blocked by the protein kinase inhibitor H-8. The involvement of K\(^{+}\) channels in the hyperpolarizing effects of forskolin was consequently studied in perforated outside-out vesicles. Two different types of K\(^{+}\) channels were recorded, one of which had a large conductance (170 pS) and was activated by forskolin. We suggest that stimulation of endothelial adenylate cyclase results in activation of large-conductance K\(^{+}\) channels and consequently in membrane hyperpolarization, which in turn enhances bradykinin-induced entry of Ca\(^{2+}\) by increasing its electrochemical gradient.

INTRODUCTION

The physiological role of endothelial Ca\(^{2+}\) has been extensively investigated during recent years (for review see [1]), whereas the role of cyclic nucleotides in regulation of endothelial functions is still obscure. It is meanwhile well established that vascular endothelial cells express a large number of adenylate cyclase-linked receptors [2,3]; however, there are some divergent hypotheses about the physiological role of endothelial cyclic AMP (cAMP). Lückhoff et al. [4] provided evidence that increases in cAMP attenuate ATP-induced Ca\(^{2+}\) increases in bovine aortic endothelial cells, and Brock et al. [5] and Buchan and Martin [6] have found amplification of ATP, bradykinin and thrombin effects in this tissue. We have recently reported that elevation of endothelial cAMP alone does not affect intracellular free Ca\(^{2+}\) or endothelium-derived relaxing factor (= NO) formation [7], whereas in the presence of agonists such as bradykinin cAMP amplifies agonist-induced NO formation in endothelial cells [8] as well as in neuronal cell lines [9]. cAMP-mediated amplification of agonist-stimulated NO formation may be of particular importance for regulation of vascular tone in vivo, since it enables joint regulation of NO formation by Ca\(^{2+}\)-increasing autacoids, such as ATP and bradykinin, and substances which increase endothelial cAMP levels, such as adenosine.

The mechanism underlying cAMP-mediated amplification of agonist-induced NO formation certainly involves activation of protein kinases and amplification of agonist-induced Ca\(^{2+}\) mobilization [8]. Here we present evidence for cAMP-mediated hyperpolarization of endothelial cells which involves activation of K\(^{+}\) channels. cAMP-mediated hyperpolarization enhances the driving force for Ca\(^{2+}\) entry and may thus represent the basis for amplification of endothelial NO synthesis.

MATERIALS AND METHODS

Materials

Fura-2/AM was obtained from LAMBDA Fluoreszenz-technology, Graz, Austria, and bis-(1,3-dibutylbarbituric acid)-pentamethine oxonol [DiBAC\(_4\)(5)] was from Molecular Probes, Eugene, OR, U.S.A. Tissue-culture media and materials were from GIBCO/BRL, Egggenstein, Germany, and fetal-calf serum, amino acids, vitamins and monoclonal smooth-muscle \(\alpha\)-actin antibodies were obtained from Boehringer, Mannheim, Germany. BSA, ADP, ATP, bradykinin, collagenase, EGTA, Heps, isoprenaline, trypsin and soybean trypsin-inhibitor type II-S were purchased from Sigma, Munich, Germany. Okadaic acid was obtained from Moana Bioproducts of Hawaii, Honolulu, HI, U.S.A., and H-8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride) and ionomycin were purchased from Calbiochem, Frankfurt, Germany.

Cell culture

Pig aortic endothelial cells were isolated by the method described by Sturek et al. [10]. Briefly, fresh pig aortae were washed and incubated with Dulbecco’s Minimum Essential Medium, including 2 mg/ml BSA, 200 units/ml collagenase, 1 mg/ml soybean trypsin inhibitor type II-S, non-essential amino acids and vitamins. After 20 min, cells were centrifuged for 5 min at 500 g, resuspended in Opti-Minimum Essential Medium containing 3 % fetal-calf serum and antibiotics, and seeded in plastic dishes. Purity was indicated by the typical cobblestone morphology, as well as by immunofluorescence detection of contaminating smooth-muscle cells (\(\alpha\)-actin), which yielded over 99 % purity.

Measurements of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\))

[Ca\(^{2+}\)]\(_i\) was measured by the fura-2 technique, by the method described previously [7]. Experiments were performed with cultured cells about 10 days after isolation. Briefly, endothelial cells were harvested by incubation with 0.05 % trypsin and 0.02 % EDTA for 2 min, centrifuged and loaded over 45 min at 37 °C with fura-2 in Dulbecco’s Minimum Essential Medium including 2 \(\mu\)M fura-2/AM. After incubation, cells were washed twice with Heps buffer (145 mM NaCl, 5 mM KCl, 2.5 mM)

Abbreviations used: cAMP, cyclic AMP; [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\) concentration; H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride.

* To whom reprint requests and correspondence should be addressed.
CaCl₂, 1 mM MgCl₂, 10 mM Hepes acid, adjusted with NaOH to pH 7.4) and resuspended in a final concentration of 1.25 × 10⁴ cells/ml in Hepes buffer. After an equilibration period of 15 min, fura-2 fluorescence was monitored by the ratio technique (excitation at 340 and 380 nm, emission at 500 nm) and intracellular Ca²⁺ was calculated by the equation of Grynkiewicz et al. [11].

Simultaneous measurement of [Ca²⁺], and membrane potential
Endothelial cells were loaded with fura-2/AM as described above. Before starting the experiments, 375 nM DiBAC₄(5) was added (5 μl from a stock solution in dimethyl sulphoxide). Membrane potential was measured at 590 nm excitation and 616 nm emission, and [Ca²⁺], was simultaneously measured at 340 nm excitation and 500 nm emission with a dual-wavelength spectrofluorimeter (RF 5000; Shimadzu, Kyoto, Japan). Wave-lengths were changed every 8 s, and, due to the technical limitations of the fluorimeter, which admits only two pairs of excitation/emission wavelengths, intracellular free Ca²⁺ is simply shown as changes in the Ca²⁺-sensitive wavelength (340/500 nm). Membrane potential (mV), which is shown as changes in membrane-sensitive wavelength (590/616 nm), was also calculated in accordance with Rink et al. [12].

Patch-clamp recording
Freshly isolated endothelial cells were plated on glass coverslips, kept in primary culture and used for experimentation within 5 days after isolation. Single-channel currents were recorded as previously described [13], by using both the standard patch-clamp technique [14] as well as the amphoterin B-perforated patch technique [15]. For voltage clamp and current amplification, a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany) was used. Current records were stored on video tape with a VR10 digital data recorder (Instrutech Corp., Mineola, U.S.A.). Records were played back, filtered at 1 kHz (-3 dB) with a custom-made 4-pole Bessel filter and digitized at 5 kHz. For display of long current records, data were filtered at 100 Hz and digitized at 330 Hz. Data analysis was performed with pClamp 5.5 as well as custom-made software. Amplitudes of single-channel currents were derived from amplitude histograms, which were also used to calculate mean number of open channels during specified time intervals as a measure of channel activity.

RESULTS AND DISCUSSION
Addition of bradykinin is known to elevate endothelial free Ca²⁺ in a biphasic manner. In pig aortic endothelial cells, initially a transient spike (from 143 ± 4.7 to 687 ± 14.0 nM) was induced, corresponding to a transient inositol 1,4,5-trisphosphate-mediated Ca²⁺ release. This spike was followed by a sustained plateau phase at 306 ± 13.2 nM, which reflects Ca²⁺ entry through non-voltage-operated ion channels. Simultaneously with changes in [Ca²⁺], bradykinin initially hyperpolarized endothelial cells from -34 ± 5.3 to -68 ± 7.3 mV, thereafter causing sustained hyperpolarization at -48 ± 6.6 mV (Figure 1a; n = 8). This bradykinin-induced membrane hyperpolarization has been demonstrated to result from stimulation of Ca²⁺-activated K⁺ channels [13,16,17]. We have previously reported that increases in cAMP levels enhanced the agonist-induced [Ca²⁺], plateau [8], and have also demonstrated that the endothelial membrane potential controls Ca²⁺ entry via the electrochemical gradient [13]. Consequently, we now investigated whether this amplifying effect of cAMP involves membrane hyperpolarization. As shown in Figure 1(a), forskolin enhanced bradykinin-induced membrane hyperpolarization from -48 ± 6.6 to -60 ± 4.6 mV (n = 8). Similar results were observed with adenosine (250 μM) and isoprenaline (30 μM), which also hyperpolarized endothelial cells in the presence of bradykinin from -44 ± 4.2 to -55 ± 3.7 mV (n = 6) and -51 ± 6.8 mV (n = 6) respectively. In order to test whether forskolin hyperpolarizes endothelial cells either by elevation of [Ca²⁺], and subsequent activation of Ca²⁺-dependent potassium channels, or by a Ca²⁺-independent hyperpolarizing mechanism, the effect of forskolin on [Ca²⁺], and membrane potential was studied in the absence of bradykinin (Figure 1b). Forskolin (10 μM) by itself hyperpolarized endothelial cells from -31 ± 2.1 to -45 ± 5.3 mV (n = 10), while leaving [Ca²⁺], unchanged (Figure 1b). Further stimulation with bradykinin resulted in the well-known response of [Ca²⁺], and the membrane potential, which was, however, markedly enhanced (-77 ± 7.1 mV; n = 10). Similar to the results obtained with forskolin, also adenosine (250 μM) and isoprenaline (30 μM) by themselves hyperpolarized endothelial cells (from -29 ± 2.1 to -42 ± 4.2 and -38 ± 3.9 mV respectively; n = 12). These results are in agreement with those reported by Mehrke and Daut [18] and Mehrke et al. [19], who described adenosine-induced membrane hyperpolarization in coronary endothelial cells. To confirm that the observed membrane hyperpolarization was indeed mediated by cAMP, we investigated the effect of the membrane-permeant analogue dibutyryl cAMP. Dibutyl cAMP (100 μM) mimicked

---

**Figure 1** Simultaneous recording of changes in [Ca²⁺], and membrane potential in cultured vascular endothelial cells

Cells were loaded with fura-2 and the membrane-sensitive dye DiBAC₄(5) was added before starting the experiment. Increases in [Ca²⁺], are expressed as changes in the Ca²⁺-sensitive wavelength of fura-2, and membrane potential, which is expressed as mV, was calculated as described in the Materials and methods section. (a) Effect of forskolin (10 μM) on bradykinin-induced increases in [Ca²⁺], and membrane potential. Cells were stimulated with bradykinin (100 nM) at zero time, and forskolin was added as indicated. (b) effect of forskolin (10 μM) in the absence of bradykinin, followed by further addition of bradykinin (100 nM).
the effect of forskolin and hyperpolarized vascular endothelial cells from $-36 \pm 7.3$ to $-62 \pm 4.6$ mV ($n = 9$), indicating that rises in cAMP levels are actually involved in forskolin-induced membrane hyperpolarization.

Since we have recently provided evidence for the involvement of protein kinase A in cAMP-mediated amplification of agonist-induced endothelium-derived relaxing factor (NO) formation [8], it is tempting to speculate that the hyperpolarizing effect of forskolin is also based on activation of protein kinase A. To test this hypothesis, we preincubated endothelial cells with H-8, an inhibitor of protein kinase A. As shown in Figure 2, H-8 inhibited the hyperpolarizing effect of forskolin. Under control conditions forskolin hyperpolarized endothelial cells from $-30 \pm 2.6$ to $-54 \pm 3.8$ mV ($n = 3$), whereas in the presence of H-8 (10 μM) the effect of forskolin was strongly diminished (from $-29 \pm 2.4$ to $-32 \pm 3.7$ mV; $n = 3$). H-8 also blocked adenosine-, isoprenaline- and dibutyryl-cAMP-induced hyperpolarization (results not shown). Thus inhibition of protein kinase A prevents membrane hyperpolarization induced by cAMP-increasing autacoids. These results strongly indicate the involvement of protein kinase A in membrane hyperpolarization induced by increased cAMP.

Since agonist-induced membrane hyperpolarization was found to contribute significantly to Ca$^{2+}$ entry by providing a higher electrochemical gradient [13,20], it can be expected that cAMP amplifies, in particular, Ca$^{2+}$ entry due to its hyperpolarizing action. This hypothesis was confirmed by our experiments measuring Ca$^{2+}$ entry by the manganese-quench technique, which is based on the ability of manganese to enter endothelial cells through agonist-stimulated Ca$^{2+}$ entry pathways, thereby quenching the fura-2 fluorescence signal [21].

As shown in Figure 3, the tracing of the effect of forskolin (10 μM; preincubation for 15 min) was added as indicated. Ca$^{2+}$ entry was detected by the Mn$^{2+}$ quench technique in the presence of 250 μM Mn$^{2+}$.}

---

**Figure 2** Effect of the protein kinase inhibitor H-8 on forskolin-induced membrane hyperpolarization

Endothelial cells with and without preincubation for 15 min with H-8 (10 μM) were stimulated by forskolin (10 μM) as indicated. Membrane potential was measured as described in the Materials and methods section.

**Figure 3** Original tracing of the effect of forskolin on Ca$^{2+}$ entry into endothelial cells in the absence or presence of H-8 (10 μM; preincubation for 15 min)

Endothelial cells were preincubated with bradykinin (100 nM) or water, and forskolin (10 μM) was added as indicated. Ca$^{2+}$ entry was detected by the Mn$^{2+}$ quench technique in the presence of 250 μM Mn$^{2+}$.

**Figure 4** Original tracing of the effect of forskolin (10 μM) on bradykinin-induced [Ca$^{2+}$]$_i$ plateau under control conditions (a), after preincubation with H-8 (10 μM) for 15 min (b) and after addition of 0.7 μM okadaic acid (c)

Endothelial cells were stimulated with bradykinin (100 nM) as indicated.
further increased Mn²⁺ entry, whereas, in the absence of bradykinin, forskolin did not alter the fluorescence signal (Figure 3). In agreement with the results shown in Figure 2, the protein kinase inhibitor H-8 (10 μM) strongly reduced the effect of forskolin (10 μM) on Ca²⁺ entry in the presence of bradykinin. Similar results were observed with adenosine, isoprenaline and dibutyryl cAMP (results not shown). These results suggest (i) that cAMP-mediated membrane hyperpolarization is indeed due to activation of cAMP-dependent protein kinase, and (ii) that hyperpolarization increases cation entry, through channels, which are opened by agonists such as bradykinin. In the absence of agonist, i.e. when channels are closed, cAMP-induced membrane hyperpolarization does not affect endothelial [Ca²⁺], (Figure 1b) or Ca²⁺ entry (Figure 3).

Consistent with our findings that H-8 inhibits cAMP-mediated amplification of agonist-induced Ca²⁺ entry, it also strongly depressed the effect of forskolin (10 μM) on the bradykinin-induced Ca²⁺ plateau. Forskolin enhanced the Ca²⁺ plateau from 306 ± 13.2 to 467 ± 9.1 nM (Figure 4a; n = 8) in the absence and from 298 ± 12.0 nM to 332 ± 4.7 nM (Figure 4b; n = 7) in the presence of H-8 (10 μM). The effect of bradykinin itself, however, was not affected by H-8. Similar to the effects of forskolin, those of adenosine, isoprenaline and dibutyryl cAMP were also inhibited by H-8. The IC₅₀ values obtained for H-8 inhibition of forskolin effects [1.6 (0.8–3.2) μM] was close to that reported for inhibition of cAMP-dependent protein kinase (IC₅₀ = 1.2 μM; [22]). In agreement with these results, the phosphatase inhibitor okadaic acid, which prevents protein dephosphorylation, amplified the effect of forskolin on bradykinin-induced [Ca²⁺] plateau (Figure 4c). Addition of okadaic acid (0.7 μM) increased the extent of forskolin-induced plateau by itself from 311 ± 11.6 to 351 ± 5.3 nM (n = 11), which may reflect a high autophosphorylation in living

Figure 5 Forskolin does not affect small-conductance K⁺ channels in outside-out perforated vesicles

Upper panel shows the unitary current-to-voltage relationship (slope conductance was 9 pS). Lower panel illustrates single channel activity at 0 mV in the absence and presence of forskolin (10 μM; 1 and 2 min after bath application). Closed state of the channel is indicated by an arrow.

Figure 6 Forskolin enhances open-state probability of large-conductance K⁺ channels in outside-out vesicles

(a) Time-dependent increase in channel activity in the presence of 10 μM forskolin. Upper panel illustrates channel activity in the absence (control) as well as in the presence (1 and 2 min) of forskolin. Lower panel shows the time course of channel activity, given as mean number of open channels during 10 s intervals. (b) Unitary current-to-voltage relationship of forskolin-sensitive channels (slope conductance was 113 pS at 0 mV). (c) Stimulation of channel activity at potentials of −40 mV and +40 mV. Closed state of channels is indicated by arrows.
cells [23]. In the presence of okadaic acid, the effect of forskolin on the bradykinin-induced Ca\(^{2+}\) plateau was strongly enhanced (653 ± 16.9 nM, followed by a plateau at 591 ± 8.9 nM; Figure 4c; n = 11), compared with its effect in the absence of okadaic acid (from 306 ± 13.2 to 467 ± 9.1 nM, followed by a plateau at 372 ± 14.7 nM; Figure 4a). Evaluation of combined effects, by the method of Pöch [24], suggested evidence for a synergistic interaction of okadaic acid and forskolin.

Among the various membrane transport systems which may be involved in cAMP-induced hyperpolarization of the endothelial cell membrane, primary candidates are endothelial K\(^{+}\) channels. We have recently reported on the existence of two distinct types of K\(^{+}\) channels in pig endothelial cells, and demonstrated the functional significance of agonist-induced K\(^{+}\)-channel activation in terms of amplifying agonist-induced Ca\(^{2+}\) entry [13]. In an attempt to test whether or not forskolin activates one or both types of K\(^{+}\) channels, we studied the effects of forskolin on endothelial K\(^{+}\) channels in perforated outside-out vesicles, i.e. a recording configuration which preserves intracellular signal transduction such as cyclic-nucleotide-dependent phosphorylation. The predominant K\(^{+}\) channel in pig endothelial cells is Ca\(^{2+}\)-dependent and of small conductance [13]. Forskolin did not affect this channel, as illustrated in Figure 5. Besides this small-conductance K\(^{+}\) channel, endothelial cells express in addition a Ca\(^{2+}\)-dependent K\(^{+}\) channel of large conductance, as reported by several groups [25,26]. This K\(^{+}\) channel is present at a rather low density in the membrane [13,25], but nevertheless may contribute significantly to the control of membrane potential, owing to its high conductance. We found this type of channel in 4 out of 95 patches. As illustrated in Figure 6, large-conductance K\(^{+}\) channels were activated upon bath administration of forskolin (10 \(\mu\)M). Significant stimulatory effects were detectable even at potentials as negative as −40 mV, which is close to the cells’ resting potential (Figure 6c). Thus activation of large-conductance Ca\(^{2+}\)-dependent K\(^{+}\) channels may well represent a mechanism underlying the hyperpolarizing effect of forskolin. The suggested involvement of protein kinase A is in agreement with the finding that large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels are regulated via phosphorylation by protein kinase A as demonstrated in smooth muscle [27].

In summary, our results suggest that cAMP-mediated amplification of agonist-induced Ca\(^{2+}\) entry results from membrane hyperpolarization, which is based on activation of large-conductance K\(^{+}\) channels. cAMP-induced hyperpolarization involves activation of protein kinase A and enhances the electrochemical gradient for Ca\(^{2+}\), consequently amplifying agonist-induced Ca\(^{2+}\) entry and NO formation [8]. This mechanism may serve as a physiologically important link between the endothelial adenylate cyclase system and NO biosynthesis.

This work is supported by a grant (No. P 8561) from the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich.

REFERENCES


Received 29 October 1992/13 November 1992; accepted 20 November 1992