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CGRP Receptors in the Gerbil Spiral Modiolar Artery Mediate a Sustained Vasodilation via a Transient cAMP-mediated Ca^{2+}-decrease

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Abstract. Alteration of cochlear blood flow may be involved in the etiology of inner ear disorders like sudden hearing loss, fluctuating hearing loss and tinnitus. The aim of the present study was to localize the vasodilator calcitonin gene-related peptide (CGRP) and to identify CGRP receptors and their signaling pathways in the gerbil spiral modiolar artery (SMA) that provides the main blood supply of the cochlea. CGRP was localized in perivascular nerves by immunocytochemistry. The vascular diameter and cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ in the smooth muscle cells were measured simultaneously with videomicroscopy and fluo-4-microfluorometry. Calcitonin receptor-like receptor (CRLR) mRNA was identified by RT-PCR as a specific 288 bp fragment in total RNA isolated from the vascular wall. The SMA was preconstricted by a 2-min application of 1 nM endothelin-1 (ET1). CGRP, forskolin, and dibutyryl-cAMP caused a vasodilation (EC_{50} = 0.1 nM, 0.3 μM, and 20 μM). CGRP and forskolin caused an increase in cAMP production and a transient decrease in the $[Ca^{2+}]_i$. The CGRP-induced vasodilation was antagonized by $CGRP_{8\text{-}37}$ (K_{DB} = 2 μM). The K^+-channel blockers iberiotoxin and glibenclamide partially prevented the CGRP- or forskolin-induced vasodilations but failed to reverse these vasodilations. These results demonstrate that CGRP is present in perivascular nerves and causes a vasodilation of the ET1-preconstricted SMA. The data suggest that this vasodilation is mediated by an increase in the cytosolic cAMP concentration, a transient activation of iberiotoxin-sensitive BK and glibenclamide-sensitive K_{ATP} K^+ channels, a transient decrease in the $[Ca^{2+}]_i$ and a long-lasting Ca^{2+} desensitization.

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Introduction

The spiral modiolar artery (SMA) is a small arteriole that surrounds the eighth cranial nerve and provides the main blood supply to the cochlea. Calcitonin gene-related peptide (CGRP) is a potent vasodilator that is released from perivascular nerve fibers (Brain et al., 1985; Kawasaki et al., 1988; Bell & McDermott, 1996). CGRP-containing neurons have been found in many cranial vessels, including the spiral modiolar artery (SMA) from guinea pigs and rats (Carlisle et al., 1990; Qiu et al., 2001). Although systemically applied CGRP has been shown to increase cochlear blood flow in rats and rabbits (Hillerdal & Andersson, 1991; Quirk et al., 1994), it is currently unknown whether CGRP causes a vasodilation of the SMA or of other elements in this vascular network. A detailed understanding of the effect of CGRP on the SMA is highly desirable, given that many inner ear disorders like sudden and fluctuating hearing, and tinnitus may be due to a dysfunction of cochlear blood flow regulation (Seidman, Quirk & Shirwany, 1999). CGRP is a 37-amino-acid peptide that is encoded by the calcitonin gene (Goodman & Iversen, 1986). CGRP receptors consist of several subunits, including a heptahelical calcitonin receptor-like receptor (CRLR), a receptor activity modifying protein (RAMP1) and a receptor component protein (RCP) (McLatchie et al., 1998; Evans et al., 2000). Two CGRP receptor types ($CGRP_1$ and $CGRP_2$) have been distinguished (Dennis et al., 1989; Juaneda,

Dumont & Quirion, 2000). These receptor types have distinct but similar affinities for the antagonist $CGRP_{8-37}$ (Dennis et al., 1989; Poyner, 1992a, 1992b; Bell & McDermott, 1996).

CGRP-induced vasodilations in different vascular beds are mediated via different cell-signaling pathways. CGRP dilates most vessels independent of the endothelium and the NO pathway, although NO and cGMP mediate CGRP-induced vasodilation of some vessels (Grace et al., 1987; Gray & Marshall, 1992). CGRP signals in many vascular beds via an increase in the cytosolic cAMP concentration, a stimulation of protein kinase A, activation of K^+-channels, a hyperpolarization of the membrane potential, closure of voltage-gated L-type Ca^{2+}-channels and a decrease of $[Ca^{2+}]_i$ (Bell & McDermott, 1996). K^+-channels that mediate CGRP-induced vasodilations include ATP-dependent (K_{ATP}) K^+ channels and large-conductance Ca^{2+}-activated (BK) K^+-channels (Nelson et al., 1990a; Sheykhzade & Berg Nyborg, 2001).

Vasodilations are commonly studied in preconstricted vessels. Endothelin 1 (ET1) is among the most potent vasoconstrictors and has been shown to mediate long-lasting vasoconstrictions via a Ca^{2+} sensitization of the myofilaments (Nishimura et al., 1992). In the SMA we have shown that ET1-induced vasoconstrictions are induced by a transient increase in $[Ca^{2+}]_i$ and maintained by a long-lasting, Rho-kinase-mediated Ca^{2+} sensitization (Scherer, Wonneberger & Wangemann, 2001, Scherer, Herzog & Wangemann, 2002). We hypothesized that CGRP mediates a vasodilation of the SMA by an inverse mechanism. In particular, we speculated that CGRP causes a decrease of the $[Ca^{2+}]_i$ and a Ca^{2+} desensitization of the myofilaments. Thus, we aimed to localize CGRP in the vascular wall, to identify functional CGRP receptors and to determine the key elements of the signaling pathways leading to a CGRP-induced vasodilation.

Parts of the present study have been presented at recent meetings (Herzog et al., 2001a; Herzog et al., 2001b; Scherer et al., 2002).

Materials and Methods

Preparation

Gerbils were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.). Animals were sacrificed by decapitation or transcardial perfusion according to a protocol approved by the Institutional Animal Care and Use Committee at Kansas State University. Lungs were isolated and the SMA was obtained by microdissection, as described previously (Wangemann & Gruber, 1998). Briefly, temporal bones were quickly separated from the skull and placed into a microdissection chamber at 4°C. The otic capsule and the cochlea were opened and the SMA was removed from the cochlear nerve to which it is loosely attached. Care was taken not to stretch the vessel.

Measurement of the Vascular Diameter

Segments of the SMA with a length of ~200 μm were transferred into a bath chamber on the stage of an inverted microscope (TE 300, Nikon, Japan). The SMA was fixed to the bottom of the chamber with two blunt glass needles, which were mounted on micromanipulators (Narashige, Japan). All experiments were conducted at 37°C. The vessel was superfused at a rate of 9 ml/min, which corresponds to a solution exchange rate of 3 times the bath chamber volume (50 μl) per sec. The basic superfusate contained (in mM): 150 NaCl, 5 HEPES, 3.6 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 5 glucose, pH 7.4. Normal contractility of the vessel was tested by raising the extracellular Ca^{2+} concentration from 1 to 10 mM. This procedure caused a reversible vasoconstriction of the SMA (Wonneberger et al., 2000). The maximal dilation was defined as the relaxation of the SMA after 1 min of superfusion with Ca-free solution. Ca-free solution contained (in mM): 150 NaCl, 5 HEPES, 3.6 KCl, 1 $MgCl_2$, 1 EGTA and 5 glucose, pH 7.4.

The presence of the nitric oxide (NO) signaling pathway was evaluated by using the NO donor DEA-nonoate (Calbiochem, La Jolla, CA or Cayman, Ann Arbor, MI), which releases NO with a half-life of 2 min. Solutions containing DEA-nonoate were prepared from an alkaline stock solution in which DEA-nonoate is stable. Fresh DEA-nonoate solutions were applied to the SMA within 25 sec. DEA-nonoate stock solutions that were depleted of NO by acidification were used in control experiments.

The vascular diameter was monitored as described previously (Wangemann & Gruber, 1998). Briefly, the image of the vessel was monitored by a black/white video-camera (WV-1410, Panasonic) and mixed with a time signal (Time Code Generator, Fast Forward Video, Irvine, CA). Images were displayed on a monitor (PVM-122, Sony) and recorded on videotape (AG-1960, Panasonic). The outer diameter of the vessel was measured by two video-edge detectors (Crescent Electronics, East Sandy, UT). The calibrated analog signal encoding the vascular diameter was digitized at a rate of 12 Hz and recorded by a data acquisition program (Axoscope 8.0, Axon Instruments, Union City, CA).

Simultaneous Measurement of the $[Ca^{2+}]_i$ and the Vascular Diameter

Simultaneous measurements were performed as described previously (Scherer et al., 2001). Briefly, the smooth muscle cells of vessel segments were loaded with the Ca^{2+}-indicator dye fluo-4 by incubation for 35 min at 37°C in basic superfusion solution (*see* above) containing 5 μM fluo-4-AM (Molecular Probes, Eugene, OR). Vessels were mounted in the superfusion chamber on the stage of the inverted microscope (Diaphot, Nikon) and the preparation was alternately illuminated with light of 600 and 488 nm (Deltascan, Photon Technology, South Brunswick, NJ). Epi-illumination was chosen for the 488-nm light path and transluminination for the 600-nm light path. The 488-nm light was reflected by a 515-nm dichroic mirror (Omega Optical, Brattleboro, VT) to reach the preparation. The fluorescence (500–550 nm) emitted in response to 488 nm excitation was transmitted through the 515-nm dichroic mirror, reflected by a 576-nm dichroic mirror (Omega Optical) and limited with a band-pass filter to a wavelength between 518 and 542 nm (Omega Optical). The fluorescence signal was detected by a photon counter and digitally recorded at a rate of 10 Hz (Felix 1.4, Photon Technology). Changes in the emission intensity were taken as measures of changes in $[Ca^{2+}]_i$. The image observed with the 600-nm light was transmitted through the 515- and 576-nm dichroic mirrors, detected by a chilled CCD camera (C5985-02, Hamamatsu) at a rate of 2.5 Hz. The vascular diameter was measured with two video edge detectors as described above. The analog

signal encoding the vascular diameter was digitized at a rate of 10 Hz and recorded by a data acquisition program (Felix 1.4, Photon Technology).

Data Analysis

Experiments were bracketed by 1-min superfusions with Ca-free solution that were performed to induce a maximal vasodilation and reduce $[Ca^{2+}]_i$ to a minimal level. The vascular diameter or fluorescence intensity in the absence of Ca^{2+} was considered as baselines. The magnitude of the vascular tone or fluorescence intensity was determined as the difference between the recorded values and the baseline. Measurements were averaged over a period of 1 min to reduce noise that was mainly due to an intense vasomotion. Drug-induced effects were normalized to the magnitude of the vascular tone or fluorescence intensity during 1 min immediately prior to the application of drugs.

cAMP Assay

SMA was isolated from animals that were transcardially perfused with a NaCl solution containing (in mM) 150 NaCl, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, and 5 glucose, pH 7.4. Blood-free segments of SMA from one animal were divided into 3–7 samples (2–4 mm SMA) of apparently equal amounts of tissue. Samples were transferred into NaCl solution (in mM: 150 NaCl, 5 HEPES, 3.6 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 5 glucose, pH 7.4) containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) and equilibrated for 6 min with agitation at 37°C. The samples (46 µl) were then incubated for 12 min with agitation at 37°C with 0.1 to 100 nM CGRP or 10 µM forskolin. Samples of SMA that were not stimulated served as controls. The reaction was stopped by addition of a lysis reagent containing 2.5% dodecyltrimethylammonium bromide and sonication for 30 min at 4°C. Tissue fragments were removed by centrifugation and cAMP was measured in the supernatant with a colorimetric immunoassay according to the manufacturer's protocol (RPN 225, Amersham, Piscataway, NJ). The detection range of this assay was between 12.5 and 3200 fmol cAMP per well. Data were expressed as cAMP production per total SMA from one ear (estimated to consist of ~10 µg wet weight of tissue).

Pharmacological Analysis

The agonist concentration that caused a half-maximal effect (EC_{50}) was obtained by fitting data to the equation $E_{max} = C^h/(EC_{50}^h + C^h)$, where E_{max} is the maximal effect, C is the concentration of the agonist and h defines the slope. The affinity of the antagonist $CGRP_{8\text{-}37}$ for the receptor (K_{DB}) was obtained from cumulative dose-response curves in the absence and presence of $CGRP_{8\text{-}37}$. K_{DB} was determined by the Schild equation $p(K_{DB}) = \log(y) - \log(DR-1)$, where y is the concentration of the antagonist and DR is the dose ratio. The DR was obtained according to $DR = EC_{50\ antagonist}/EC_{50\ agonist}$, where $EC_{50\ antagonist}$ is the EC_{50} of CGRP in the presence of $CGRP_{8\text{-}37}$ and $EC_{50\ agonist}$ is the EC_{50} in the absence of the antagonist. All nonlinear curve fits were obtained by a least-squares algorithm using programmable spreadsheet and plotting software (Origin 6.0, Microcal, Northhampton, MA).

Statistical analysis was performed by using paired and unpaired t-tests as appropriate (Sigma Stat 1.0, Jandel Corperation, San Rafael, CA). Error probabilities of $P < 0.05$ were considered as significant. Data are given as mean ± standard error of the mean (SEM).

RNA Preparation

Gerbil lung tissue was rapidly frozen in liquid nitrogen, pulverized and transferred into TRIzol for isolation of total RNA according to the manufacturer's procedures (GIBCO BRL, Life Technologies). Total lung RNA was dissolved in RNase free water to a concentration of 1 µg/µl. Eight SMA from transcardially perfused gerbils were isolated by microdissection and pooled in TRIzol. The tissue was disrupted by one freeze/thaw cycle and sheer stress induced by a 25-gauge hypodermic syringe needle. Total RNA from the vascular wall of the SMA was isolated in the presence of 20 µg/µl glycogen according to manufacturer's procedures and dissolved to a concentration of 0.25 µg/µl. Prior to analysis by RT-PCR, RNA samples were treated for 15 min at room temperature with 2 units of amplification-grade DNase I (GIBCO BRL, Life Technologies) in the presence of 0.25 µl RNasin (Promega) and DNase I buffer. DNase I was inactivated by heating the samples to 75°C in the presence of 2.3 mM EDTA.

cDNA Synthesis and PCR Amplification

Sense (5′-TTT CAA GAG CCT AAG TTG CC-3′) and antisense (5′-CCC CAG CCA AGA AAA TAA TAC C-3′) primers for the gerbil CRLR ($CGRP_1$ receptor) were designed based upon conserved regions of the rat and human CRLR nucleotide sequences (GenBank accession numbers X70658 and L76380). Primers spanned 288 nucleotides of the $CGRP_1$ receptor mRNA sequence. Total RNA (250 ng) was reverse transcribed into cDNA in a 10-µl reaction aliquot containing PCR buffer (GIBCO BRL, Life Technologies), 20 units RNasin (Promega), 1 mM dNTP (GIBCO BRL, Life Technologies), 5 mM $MgCl_2$ (GIBCO BRL, Life Technologies), 25 pmoles of the antisense primer, and 25 units Moloney Murine Leukemia Virus reverse transcriptase (Perkin-Elmer). Control reactions to detect genomic DNA contamination were performed without reverse transcriptase. The reaction aliquot was incubated for 50 min at 42°C, 5 min at 99°C, and 5 min at 5°C (2400 thermocycler, Perkin Elmer).

The 10 µl of cDNA from the above reaction was amplified by PCR in a 100-µl reaction that contained 20 mM Tris-HCl and 50 mM KCl, 50 pmole of antisense primer, 50 pmole of sense primer, 3 mM $MgCl_2$, cDNA and 2.5 units Taq DNA polymerase (GIBCO BRL, Life Technologies). The reaction sample was incubated as follows: denaturation for 5 min at 98°C; 40 amplification cycles: 45 sec denaturation at 98°C, 45 sec annealing at 50°C, and 45 sec extension at 72°C; and final extension for 7 min at 72°C (2400 thermocycler, Perkin Elmer). PCR products were analyzed by horizontal gel electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. The 288 bp PCR products obtained from SMA and lung were cloned and sequenced for identification.

Immunocytochemistry

SMA was isolated from gerbils that were transcardially perfused with PBS (containing in mM: 137 NaCl, 1.8 K_2HPO_4, 2.7 KCl, 10.1 Na_2HPO_4, pH 7.4) followed by PBS containing 4% paraformaldehyde. The SMA was isolated and postfixed in cold 4% paraformaldehyde for 1 hr at 4°C. After washing with PBS the SMA was permeabilized with 0.1% Triton-X 100 for 30 min and washed again in PBS. The SMA was then incubated overnight at 4°C with primary antibodies (monoclonal mouse anti-CGRP, C-226, Sigma, St. Louis, MO; polyclonal rabbit anti-neuron-specific enolase, AB951, Chemicon, Temecula, CA). After careful washing in PBS, the SMA was incubated for 1 hr at room temperature with secondary antibodies (Alexa Fluor 488 goat anti-mouse, A-1001, Molecular Probes; Alexa Fluor 568 goat anti-rabbit, A-11036,

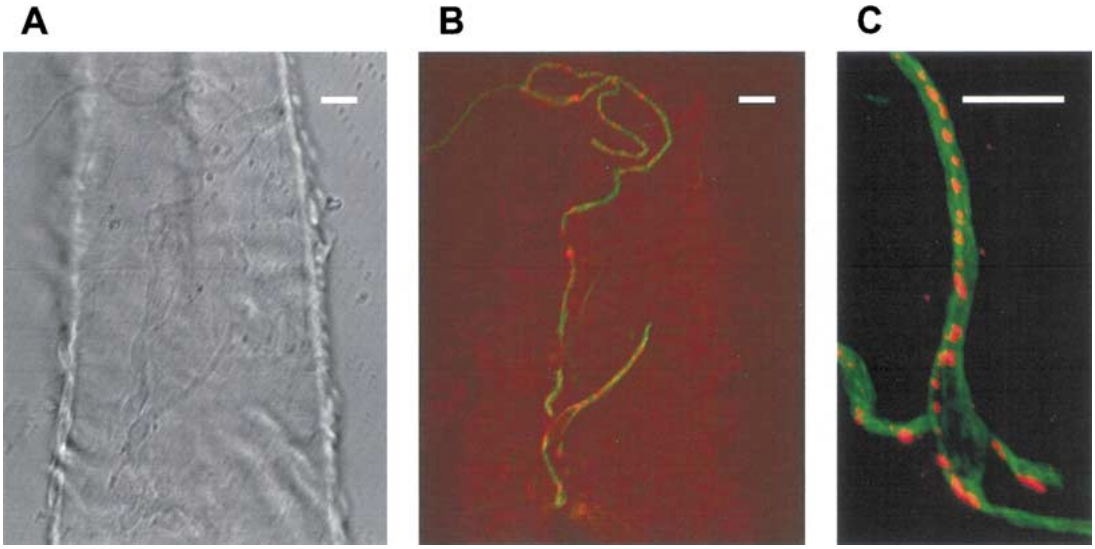

Fig. 1. Perivascular nerves of the spiral modiolar artery (SMA) contain CGRP. (*A*) Laser-scanning brightfield image of the SMA. (*B*) Confocal epifluorescence image of the SMA. Double-labeling of neuron specific enolase (*green*) and CGRP (*red*). (*C*) Enlarged confocal epifluorescence image of the SMA (compare to *B*). Note that CGRP appeared to be packaged in vesicles and that CGRP and neuron-specific enolase were not colocalized. Calibration bars indicate 10 μm.

Molecular Probes). Vessel segments were finally washed with PBS and placed on a slide, coverslipped and visualized by confocal microscopy (Zeiss, LSM 410).

Drugs

Drugs were obtained from Sigma (St. Louis, MO) unless noted otherwise and dissolved in solution either directly or via DMSO, as appropriate.

Results

Perivascular Nerves of the SMA Contain CGRP

CGRP was localized in the vascular wall of the SMA by immunocytochemistry. Immunoreactivity for CGRP was located in a varicose pattern along nerve fibers that were immunopositive for neuron-specific enolase (Fig. 1). Neuron-specific enolase is a cytosolic protein that is not expected to be present in neurotransmitter-containing vesicles. Consistently, no evidence for colocalization of CGRP and neuron-specific enolase was observed. Staining for CGRP was considered specific since it was absent when the anti-CGRP antibody was preabsorbed with the antigenic peptide CGRP (*data not shown*).

CGRP Mediates a Vasodilation of the SMA

A stable vasoconstriction was induced by a 2-min exposure to 1 nM ET1 (Fig. 2*A*). Maximal dilation was defined as the dilation induced by superfusion with Ca-free solution for 1 min. The vascular tone was found to be stable for at least 8 minutes (92 ± 7%, $n = 7$). CGRP (10 pM–100 nM) caused significant vasodilations of ET1-preconstricted SMA (Fig. 2*B*). These vasodilations were only partially reversible upon removal of CGRP from the superfusion medium.

SMA Contains CGRP Receptors

The agonist CGRP and the antagonist $CGRP_{8\text{-}37}$ were used to demonstrate that the SMA contains functional CGRP receptors. The EC_{50} for CGRP in the absence of an antagonist was 0.1 nM ($pEC_{50} = 9.86 \pm 0.16$, $n = 14$; Fig. 2*C*) and in the presence of 10 μM of the antagonist $CGRP_{8\text{-}37}$, it was 0.8 nM ($pEC_{50} = 9.11 \pm 0.23$, $n = 9$). This shift in the EC_{50} corresponds to a K_{DB} value of 2 μM ($pK_{DB} = 5.7 \pm 0.4$). These observations demonstrate that the SMA contains functional CGRP receptors.

The presence of transcripts coding for the heptahelical subunit CRLR of the CGRP receptor was determined by RT-PCR analysis of total RNA isolated from blood-free gerbil lung and blood-free SMA, using primers specific for the gerbil CRLR. A product of the expected size of 288 bp was found in gerbil lung and SMA (Fig. 3). Cloning, sequencing and comparison to known sequences confirmed the identity of the product (GenBank Accession # AF340235).

CGRP Receptors Signal via cAMP

CGRP increased cAMP production consistent with the concept that CGRP receptors signal via stimula-

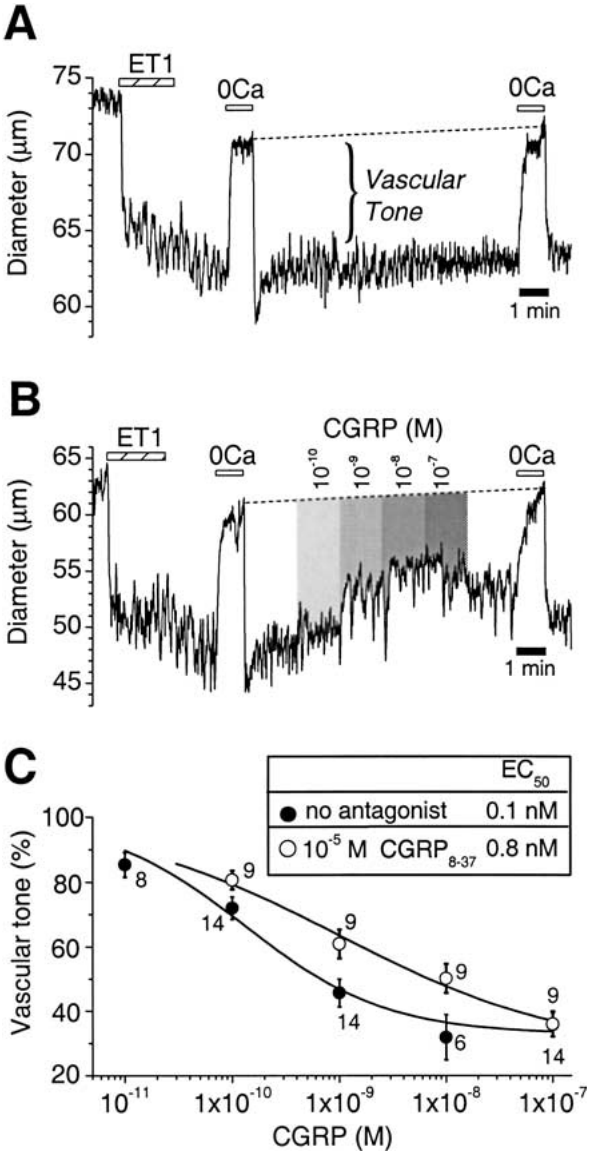

Fig. 2. CGRP receptors mediate a vasodilation of the SMA. (*A*) Control experiment. Superfusion for 2 min with 1 nM ET1 caused a long-lasting vasoconstriction. The maximal vasodilation was determined by 1-min exposures to Ca-free solution (0 Ca). The vascular tone was stable for at least 8 min and was defined as the difference between the measured vascular diameter and the interpolated baseline spanning the two determinations of the maximal vasodilation. (*B*) CGRP caused a dose-dependent vasodilation of ET1-preconstricted vessels. (*C*) Dose-response curves for the effect of CGRP in the absence and presence of the CGRP-receptor antagonist 10 µM $CGRP_{8-37}$. Numerals next to the symbols depict the number of experiments.

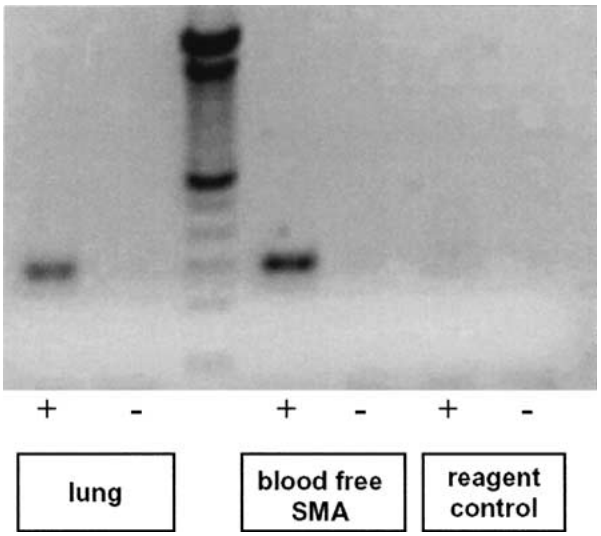

Fig. 3. The SMA contains transcripts for the CGRP receptor-subunit CRLR. Agarose gel electrophoresis of reverse-transcriptase polymerase chain reaction (RT-PCR) products. RT-PCR was performed on total RNA obtained from lung and SMA with gene-specific primers for CRLR. The reaction yielded a product of the expected size of 288 bp. Reagent control represents reactions without RNA sample. Lanes marked + and − indicate the presence or absence of reverse transcriptase, respectively. The third lane is a ladder of DNA fragments. Cloning and sequencing revealed 95–97% homology with known rat, mouse and human amino-acid sequences. The gerbil sequence is available in GenBank, accession number #AF340235.

tion of adenylyl cyclase. Maximal stimulation was achieved at 10 nM CGRP (Fig. 4*A*). Forskolin (10 µM) increased cAMP production from 1.7 ± 0.2 to 6.5 ± 0.7 pM per total SMA from one ear ($n = 9$). If cAMP would be the mediator between activation of the CGRP receptor and vasodilation, it should be possible to induce a vasodilation via the membrane-permeable cAMP analog dibutyryl-cAMP (db-cAMP) or by direct stimulation of adenylyl cyclase with forskolin. Indeed, db-cAMP and forskolin caused dose-dependent vasodilations of ET1-preconstricted vessels with EC_{50} values of 20 µM ($pEC_{50} = 4.71 \pm 0.26$, $n = 12$) and 0.3 µM ($pEC_{50} = 6.49 \pm 0.07$, $n = 7$), respectively (Fig. 4*B* and *C*).

CGRP Receptors Signal via cAMP and a Transient Decrease in $[Ca^{2+}]_i$

Simultaneous measurements of $[Ca^{2+}]_i$ and the vascular diameter were performed to investigate whether the vasodilation of ET1-preconstricted vessels is associated with a decrease in $[Ca^{2+}]_i$. CGRP (0.1 µM) caused a transient decrease of the $[Ca^{2+}]_i$ and a sustained vasodilation (Fig. 5*A–C*). Similar observations were made with forskolin (10 µM; Fig. 5*D–F*) and with db-cAMP, although the transient nature of the db-cAMP-induced $[Ca^{2+}]_i$ decrease was less pronounced. Taken together, these observations suggest that the CGRP-induced vasodilation is initiated by a decrease of the $[Ca^{2+}]_i$ and sustained by a reduction of the Ca^{2+} sensitivity of the myofilaments, which can be described as a Ca^{2+} desensitization.

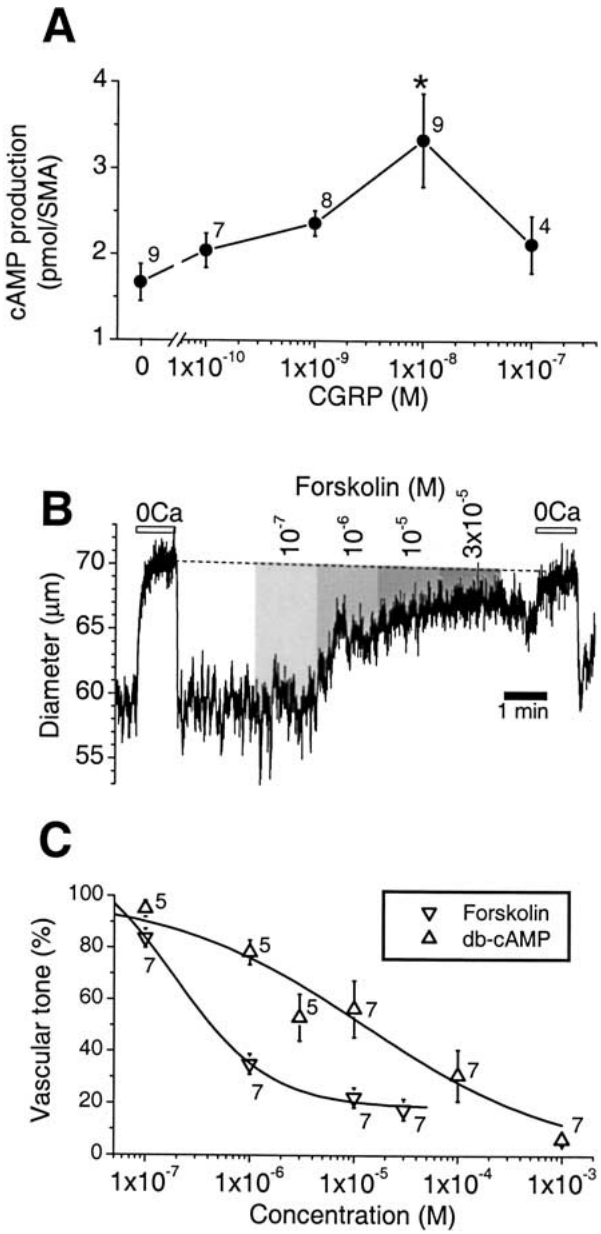

Fig. 4. CGRP receptors in the SMA signal via cAMP. A) CGRP increased cAMP-production. Values are given as cAMP-production per total SMA from one ear. B) Forskolin caused a dose-dependent vasodilation of the ET1-preconstricted SMA. C) Dose-response curve for the forskolin-induced vasodilation. Significant changes are marked (*).

Iberiotoxin- and Glibenclamide-Sensitive K^+ Channels Contribute to the Initial but not the Sustained Phase of the CGRP-induced Vasodilation

The K^+-channel blockers iberiotoxin (10 µM), TEA (20 mM) and glibenclamide (3 µM) had no significant effect on the vascular tone of ET1-preconstricted vessels (Fig. 6). CGRP- (10 nM) and forskolin-induced (10 µM) vasodilations were partially prevented by K^+-channel blockers. For example, iberiotoxin had no significant effect on vascular tone (97 ± 6%, $n = 8$) and CGRP in the presence of iberiotoxin reduced the vascular tone to 64 ± 8% ($n = 8$, Fig. 6). A further reduction of the vascular tone to 43 ± 7% was observed upon removal of iberiotoxin. Thus, iberiotoxin prevented 39% $[(64\%-43\%) \times 100/(97\%-43\%)]$ of the CGRP-induced vasodilation. Similar observations were made with other K^+-channel blockers. TEA and glibenclamide prevented 52 ± 9% ($n = 5$) and 63 ± 6% ($n = 8$) of CGRP-induced vasodilation. Further, iberiotoxin and glibenclamide prevented 38 ± 8% ($n = 8$) and 15 ± 3% ($n = 9$) of forskolin-induced vasodilation. Interestingly, K^+-channel blockers could not reverse CGRP-induced dilations. CGRP reduced the vascular tone to 46 ± 5% ($n = 5$, Fig. 7). Addition of glibenclamide caused no significant change in the vascular tone (38 ± 4%, $n = 5$). Similar observations were made with TEA. These observations suggest that CGRP activates iberiotoxin- and glibenclamide-sensitive K^+ channels and that these K^+ channels play a role during the initial but not during the sustained phase of the vasodilation.

CGRP Receptors Do not Signal via Nitric Oxide (NO)

If the CGRP-mediated vasodilation involved the NO signaling pathway, it would be expected that vasodilations are prevented by the NO synthase inhibitor 100 µM L-NAME or the guanylyl cyclase inhibitor 10 µM 1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ). Neither L-NAME nor ODQ had a significant effect on the vascular tone or CGRP-induced vasodilation (Fig. 8). These findings suggest that it is unlikely that the NO pathway mediates the CGRP-induced vasodilation.

NO Mediates a Vasodilation of the SMA

The observation that the NO pathway does not mediate the CGRP-induced vasodilation raises the question whether NO by itself could mediate a vasodilation of the SMA. If the SMA would contain the NO pathway, it would be expected that a fresh preparation of the NO-donor DEA-nonoate causes a dose-dependent vasodilation and that a depleted preparation has no significant effect. Fresh DEA-nonoate caused a vasodilation with an EC_{50} of 0.03 µM ($pEC_{50} = 7.56 \pm 0.40$, $n = 6$, Fig. 9). In contrast, depleted DEA-nonoate caused a significantly smaller vasodilation, suggesting that the effect of DEA-nonoate was mediated by NO. These findings suggest that NO mediates a vasodilation of the SMA.

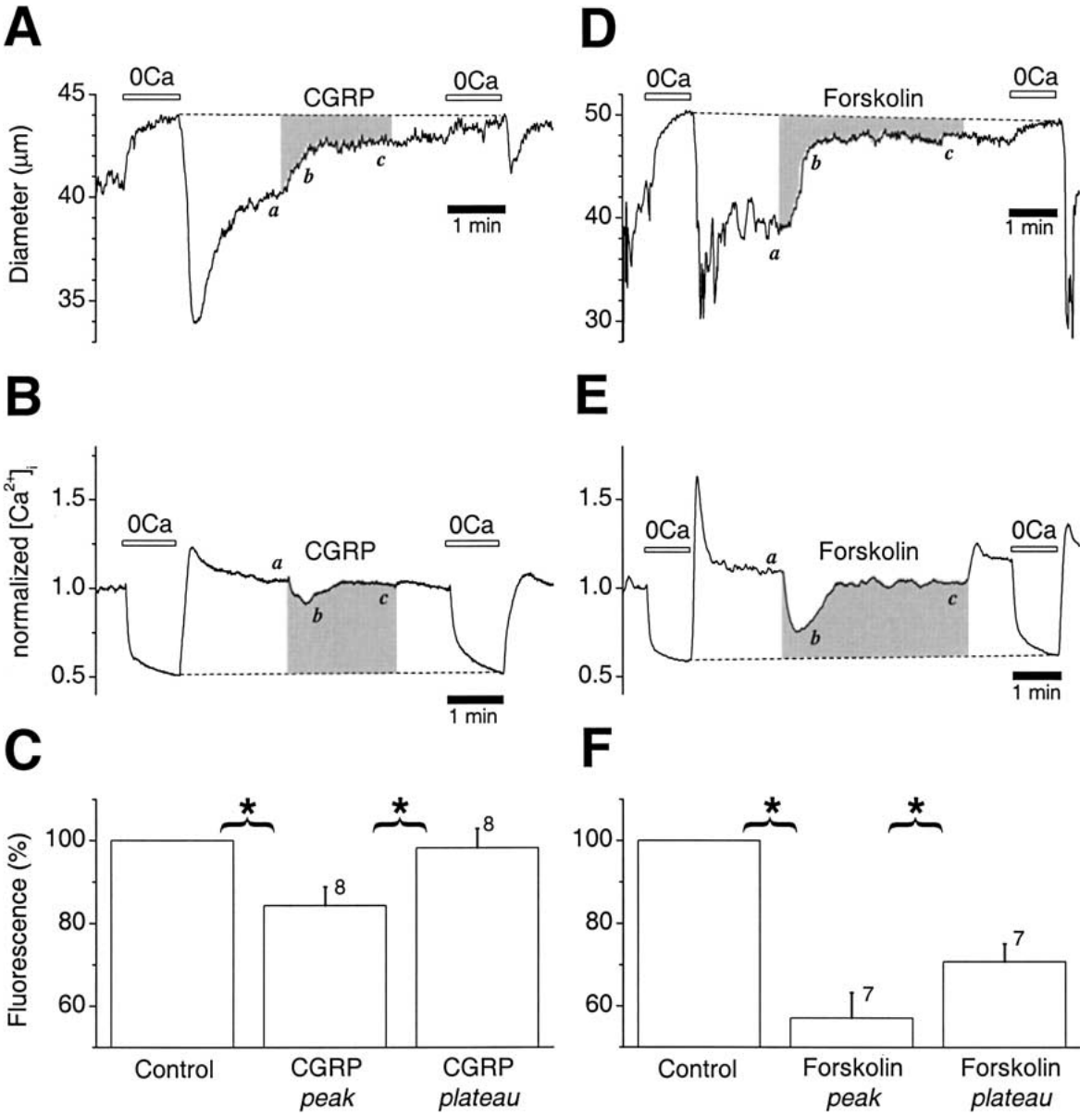

Fig. 5. CGRP receptors signal via cAMP and a transient decrease in $[Ca^{2+}]_i$. (*A–C*) 0.1 µM CGRP caused in paired experiments of ET1-preconstricted SMA a sustained vasodilation (A) and a transient decrease of $[Ca^{2+}]_i$ (B). Effects of $[Ca^{2+}]_i$ are summarized in (C). (*D–F*) 10 µM forskolin caused in paired experiments of ET1-preconstricted SMA a sustained vasodilation (*D*) and a transient decrease of $[Ca^{2+}]_i$ (*E*). Effects on the $[Ca^{2+}]_i$ are summarized in (*F*). The CGRP- and forskolin-induced transient decreases of the $[Ca^{2+}]_i$ and sustained increases of the vascular diameter are emphasized (*a, b, c*). Significant changes are marked (*).

Discussion

The major findings of the present study are 1) CGRP is present in perivascular nerves of the gerbil SMA; 2) CGRP mediates a vasodilation via CGRP receptors; 3) the CGRP-induced vasodilation is mediated via cAMP and a transient decrease in $[Ca^{2+}]_i$ and not via the NO pathway although NO causes a vasodilation of the SMA. 4) BK and K_{ATP}-channels contribute to the initial but not to the sustained phase of the CGRP-induced vasodilation. 5) The sustained phase of the CGRP-induced vasodilation appears to be due to a Ca^{2+} desensitization of the myofilaments.

CGRP and CGRP Receptors are Present in the SMA

CGRP is present in the perivascular nerves of the gerbil SMA (Fig. 1) consistent with findings in other species (Carlisle et al., 1990; Qiu et al., 2001). The present study, however, is the first to demonstrate

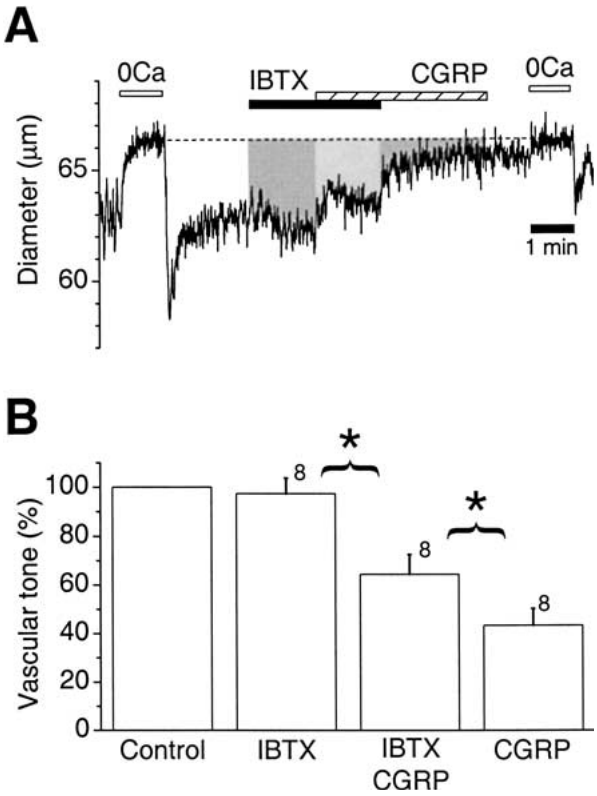

Fig. 6. Iberiotoxin attenuates the initial phase of the CGRP-induced vasodilation in the SMA. (*A*) 10 μM iberiotoxin, a selective blocker of BK-channels, had no significant effect on the vascular diameter of ET1-preconstricted vessels but partially prevented vasodilation induced by 10 nM CGRP. Note that a further relaxation was achieved by CGRP after removal of iberiotoxin. (*B*) Data summary. Significant changes are marked (*).

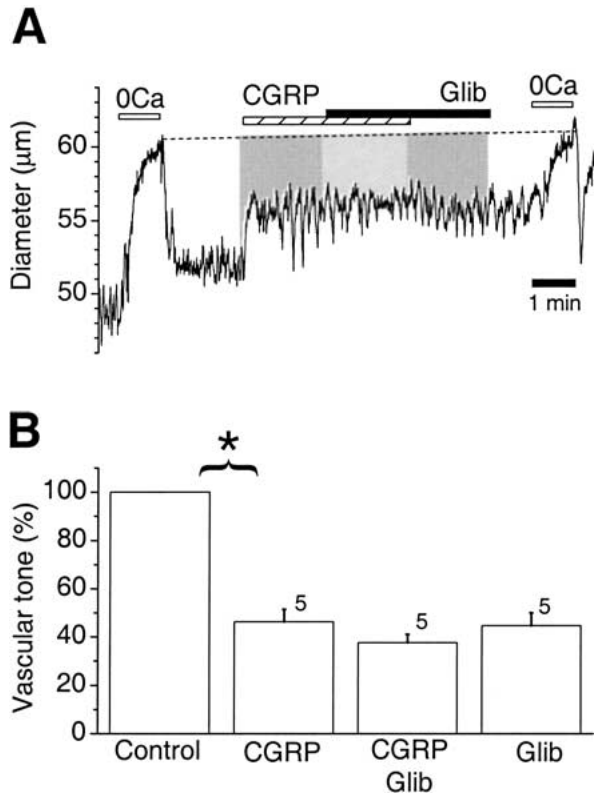

Fig. 7. Glibenclamide had no significant effect on the sustained phase of the CGRP-induced vasodilation in the SMA. (*A*) 3 μM glibenclamide, a blocker of K_{ATP} channels, had no significant effect on the vascular diameter during the sustained phase of the vasodilation induced by 10 nM CGRP. (*B*) Data summary. Significant changes are marked (*).

that CGRP causes via CGRP receptors a vasodilation of the SMA (Fig. 2). In general, CGRP receptors have been shown to consist of three subunits, CRLR, RCP, and RAMP1 (McLatchie et al., 1998; Evans et al., 2000). CRLR was unambiguously identified in the vascular wall of the SMA by the presence of transcripts (Fig. 3). Two different receptor subtypes ($CGRP_1$ and $CGRP_2$) have been described according to their affinity for the antagonist $CGRP_{8\text{-}37}$ (Dennis et al., 1989; Juaneda et al., 2000). $CGRP_1$ receptors are characterized by pK_{DB} values for $CGRP_{8\text{-}37}$ that are larger than 6.0 (Poyner, 1992a) and larger than 6.2 or 7.0 (Bell & McDermott, 1996a), whereas $CGRP_2$ receptors are characterized by smaller pK_{DB} values. The observation that the pK_{DB} for $CGRP_{8\text{-}37}$ in the gerbil SMA was 5.7 ± 0.4 and not significantly different from 6.2 or 6.0 precludes an unambiguous determination as to which receptor subtype is present. Ambiguity in the identification of CGRP receptor subtypes originates from the observations that affinity values may be species-dependent and are known to differ with the method of preconstriction (Waugh et al., 1999).

CGRP Receptors Signal via cAMP

Several observations suggest that CGRP mediates the vasodilation of the SMA via an increase in the cytosolic cAMP concentration. Forskolin and CGRP caused an increase in the cytosolic cAMP concentration (Fig. 4), a transient activation of BK and K_{ATP} channels, a transient decrease of the $[Ca^{2+}]_i$ and a sustained vasodilation (Fig. 5). The observation that the effects of forskolin and db-cAMP are similar to that of CGRP (Fig. 4 and 5) suggest that cAMP is the main second messenger of the CGRP signaling pathway in the SMA. This finding is consistent with observations in other vascular beds (Fiscus et al., 1991; Jansen et al., 1992).

CGRP Receptors Signal via cAMP and K^+ Channels

The observation that neither iberiotoxin nor TEA or glibenclamide had a significant effect on the vascular diameter of the ET1-preconstricted SMA suggests that BK and K_{ATP} channels are not active or do not contribute to the vascular diameter under these con-

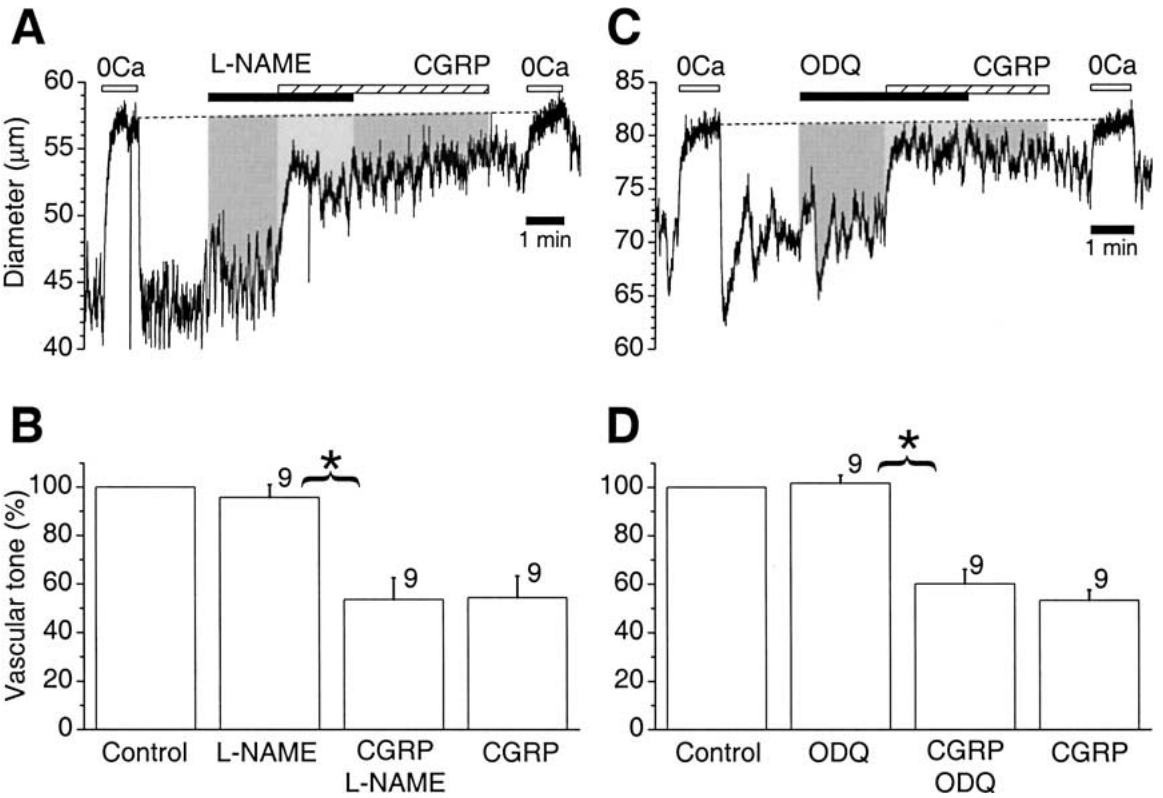

Fig. 8. CGRP receptors do not signal via nitric oxide (NO). The nitric oxide synthase inhibitor L-NAME (100 μM) (*A*) and the guanylyl cyclase inhibitor ODQ (10 μM) (*C*) had no significant effect on the initial phase of the CGRP-induced vasodilation in the SMA. (*B* and *D*) Data summaries. Significant changes are marked (*).

ditions. BK channels may be closed since these channels require an elevated $[Ca^{2+}]_i$ for opening and since $[Ca^{2+}]_i$ of ET1-preconstricted SMA is near resting levels. Indeed, ET1 has been shown to cause a transient increase of $[Ca^{2+}]_i$, after which $[Ca^{2+}]_i$ returns to near resting values (Scherer et al., 2001). The finding that BK and K_{ATP} channels, at least under our experimental conditions, do not contribute to the vascular diameter of the SMA, sets this vessel apart from other cranial vessels where BK and K_{ATP} channels have been shown to contribute to the membrane potential and thereby to the vascular tone (Nelson & Quayle, 1995; Quayle, Nelson & Standen, 1997). K_{ATP}- channels in the ET1-preconstricted SMA may be closed, since ET1 is likely to activate PKC, which has been shown to block BK and K_{ATP} channels in other preparations (Minami, Fukuzawa & Nakaya, 1993; Bonev & Nelson, 1996).

The closed BK and K_{ATP} channels of ET1-preconstricted SMA appeared to open in response to CGRP. Opening of K^+ channels most likely caused a hyperpolarization of the membrane potential and closure of voltage-gated L-type Ca^{2+} channels. Closure of L-type Ca^{2+} channels may have reduced the influx of Ca^{2+} from the extracellular compartment and may have led to the observed decrease of the $[Ca^{2+}]_i$. Evidence for the presence of this signaling pathway in the SMA comes from the finding that iberiotoxin and glibenclamide attenuated the CGRP-induced vasodilation and from the observation that inhibition of L-type Ca^{2+} channels causes a vasodilation of the SMA (Wangemann et al., 1998). This conclusion is consistent with observations in a variety of other blood vessels (Nelson et al., 1990b; Resler-ova & Loutzenhiser, 1998; Wellman, Quayle & Standen, 1998). Interestingly, opening of BK and K_{ATP} channels in response to CGRP appeared to be transient in the SMA, since iberiotoxin and glibenclamide could partially prevent the CGRP-induced vasodilation but failed to reverse it. A transient opening of these K^+ channels would be expected to lead to a transient hyperpolarization and a transient closure of L-type Ca^{2+} channels. The observation that CGRP caused a transient decrease of the $[Ca^{2+}]_i$ supports the hypothesis of a transient rather than a sustained opening of BK and K_{ATP} channels. This finding sets the SMA apart from other vessel preparations where CGRP has been found to cause a sustained activation of K_{ATP} channels that led to a sustained decrease of the $[Ca^{2+}]_i$ (Kageyama, Yanagisawa & Taira, 1993; Sheykhzade & Berg Nyborg, 2001). It is apparent that BK and K_{ATP} channels in the SMA play an important role during the initial but not the sustained phase of the vasodilation and that

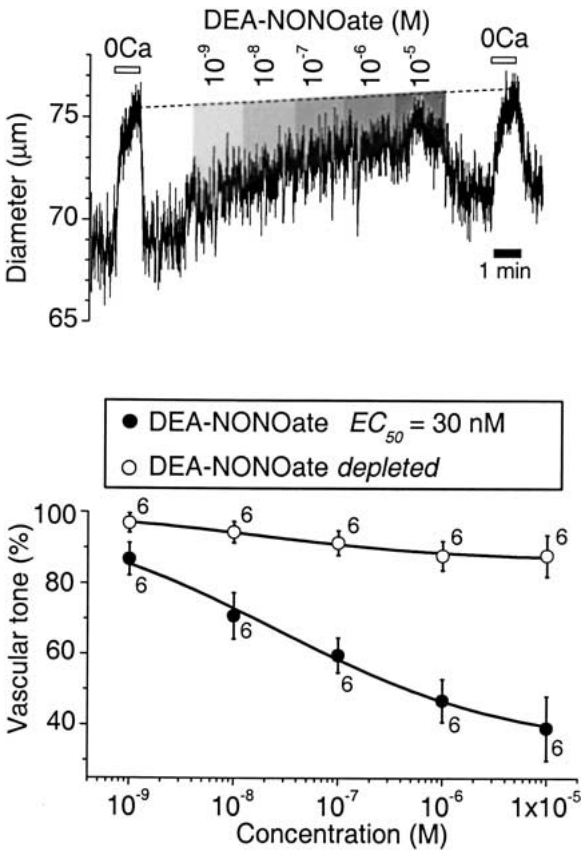

Fig. 9. NO mediates a vasodilation of the SMA. (*A*) The NO donor DEA-NONOate caused a dose-dependent vasodilation of the ET1-preconstricted SMA. (*B*) Dose-response curves for the vasodilation induced by fresh and depleted DEA-NONOate. The numbers next to the symbols depict the number of experiments.

the sustained vasodilation cannot be explained by a simple decrease of the $[Ca^{2+}]_i$.

CGRP Receptors via cAMP and K^+ Channels Cause a Transient Decrease of $[Ca^{2+}]_i$

Tension or length of vascular smooth muscle cells depends on $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ promotes activation of myosin light chain kinase (MLCK) and Ca^{2+}-dependent phosphorylation of myosin light chain (MLC) that leads to a vasoconstriction. Inversely, a decrease of $[Ca^{2+}]_i$ leads to a dephosphorylation of MLC and a vasodilation. The initiation of the CGRP- and forskolin-induced vasodilations appears to be mediated simply via a decrease in the $[Ca^{2+}]_i$ (Fig. 5).

CGRP Receptors via cAMP Mediate a Ca^{2+} Desensitization

The Ca^{2+} sensitivity of the myofilament is the result of a balance between the activities of the MLCK that promotes Ca^{2+}-dependent phosphorylation and constriction and the myosin light chain phosphatase (MLCP) that reverses phosphorylation and promotes vasodilation (Somlyo et al., 1999). An increase in the Ca^{2+} sensitivity can be achieved via an increase of the activity of MLCK or a decrease in the MLCP. Such a Ca^{2+} sensitization results in vasoconstrictions that are not due to increases of the $[Ca^{2+}]_i$. Inversely, a Ca^{2+} desensitization results in vasodilations that are not due to decreases of the $[Ca^{2+}]_i$. The finding that CGRP and forskolin caused a transient decrease of the $[Ca^{2+}]_i$ and a sustained vasodilation (Fig. 5) suggests that cAMP reduced the Ca^{2+} sensitivity of the contractile apparatus. The present study is to our knowledge the first to demonstrate that CGRP causes a Ca^{2+} desensitization in vascular smooth muscle cells.

This Ca^{2+} desensitization could be due to a decrease in the MLCK activity or an increase in the MLCP activity (Somlyo et al., 1999; Somlyo & Somlyo, 2000). On the one hand, cAMP has been shown to decrease MLCK activity via protein kinase A-mediated phosphorylation of MLCK (Garcia et al., 1997). On the other hand, cAMP has been shown to increase MLCP activity via inhibition of RhoA or Rho-kinase (Essler et al., 2000). Recent observations demonstrate that ET1 causes a Ca^{2+} sensitization that is sensitive to the Rho-kinase inhibitor Y-27632 (Scherer et al., 2002). These findings suggest that ET1 activates Rho-kinase in the SMA. It is conceivable that CGRP via cAMP decreases this Rho-kinase activity, which would lead to an increase in MLCP activity, a Ca^{2+} desensitization of the myofilaments and the observed maintenance of the vasodilation in the presence of an increasing $[Ca^{2+}]_i$. The observations that ET1 causes a Ca^{2+} sensitization and that CGRP causes a Ca^{2+} desensitization points toward a functional antagonism between these two vasoactive peptides.

CGRP Receptors Do not Signal via NO although NO Mediates a Vasodilation in the SMA

The NO pathway has been shown to mediate CGRP-induced vasodilations in preparations such as renal and pulmonary arteries (Gray & Marshall, 1992) but not in others, including rat irideal arterioles and thoracic aorta (Grace et al., 1987). The finding that neither the nitric oxide synthase inhibitor L-NAME nor the guanylyl cyclase inhibitor ODQ had a significant effect on the CGRP-induced vasodilation of the SMA makes it unlikely that the NO pathway is mediating the vasodilation (Fig. 8). The NO pathway, however, appeared to be present in the SMA since the NO donor DEA-nonoate induced a vasodilation (Fig. 9).

Conclusion

CGRP dilates ET1-preconstricted gerbil SMA by increasing cAMP production, activating BK and K_{ATP} channels, a transient decrease of the $[Ca^{2+}]_i$ and a Ca^{2+} desensitization of the contractile apparatus. To our knowledge, this study is the first to suggest that CGRP causes a Ca^{2+} desensitization of the myofilaments of the vascular smooth muscle cells. Further, the present study extends the understanding of mechanisms that regulate cochlear blood flow and might contribute to the foundation on which to base a rational pharmacological therapy of inner ear diseases.

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