The Vasorelaxing Effect of CGRP and Natriuretic Peptides in Isolated Bovine Retinal Arteries

Koen Boussery, Christophe Delaey, and Johan Van de Voorde

PURPOSE. To study the vasorelaxing effect of calcitonin gene-related peptide (CGRP) and natriuretic peptides on isolated bovine retinal arteries (BRAs) and to evaluate the possibility of the unidentified retinal relaxing factor (RRF) being one of these peptides.

METHODS. Retinal arteries were isolated from bovine eyes and mounted in a wire myograph for isometric tension recording. Concentration-response curves were generated by cumulative addition of the peptides to the organ bath.

RESULTS. In BRAs, CGRP-induced relaxation was significantly reduced by removal of the endothelium or by application of the nitric oxide synthase (NOS) inhibitor Nω-nitro-l-arginine (l-NA) or the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The nonselective K⁺ channel blocker tetraethylammoniumchloride (TEA) and the voltage-dependent K⁺ channel blocker 4-aminopyridine significantly reduced the CGRP response, whereas the Ca²⁺-activated K⁺ channel blockers apamin plus charybdotoxin, the inward rectifier K⁺ channel blocker Ba²⁺, and the adenosine triphosphate (ATP)-sensitive K⁺ channel blocker glibenclamide had no effect. The CGRP receptor antagonist CGRP (8-37) caused a small, but not significant, rightward shift in the concentration-response curve for CGRP, whereas the AM-recipient antagonist AM 22-52 had no effect. The natriuretic peptides did not induce relaxation in isolated retinal arteries.

CONCLUSIONS. Endothelium-derived NO, voltage-dependent K⁺ channels, and possibly also CGRP, receptors are involved in the CGRP response in BRAs. The natriuretic peptides do not induce vasorelaxation in isolated BRAs. No evidence was found that CGRP or a natriuretic peptide is the as yet unidentified RRF. (Invest Ophthalmol Vis Sci. 2005;46:1420–1427) DOI: 10.1167/iovs.04-1093

In many vascular beds, autonomic innervation plays a pivotal role in the regulation of arterial blood vessel tone. The retinal vasculature, however, lacks autonomic innervation, and therefore retinal arterial tone is thought to be mainly regulated by local factors. A concept previously put forth is that retinal arterial tone is thought to be mainly regulated by local factors. A concept previously put forth is that retinal arterial tone is thought to be mainly regulated by local factors. A concept previously put forth is that retinal arterial tone is thought to be mainly regulated by local factors. A concept previously put forth is that retinal arterial tone is thought to be mainly regulated by factors released from the surrounding retinal tissue. Several mediators derived from retinal tissue have been proposed as local regulators of retinal blood flow. For example, nitric oxide (NO), prostanoids, and lactate released from retinal tissue all have been described as mediators of retinal arterial tone.

In our laboratory, it has been observed that a strong vasorelaxant is released from the retina of different animal species. This vasorelaxant was given the name retinal relaxing factor (RRF) and may be essential for the maintenance of retinal circulation and in hypoxic vasodilation in retinal arteries. In three different animal models (bovine, rat, and mouse), it has been demonstrated that NO, prostanoids, and adenosine are not involved in the RRF response. Also, the involvement of most other vasoactive neurotransmitters known to be released from the retina and of the known mediators of hypoxia-induced vasodilation has been excluded. So far, the RRF does not seem to fit in any pharmacological pigeonhole, and its identity and mechanism of action remain unknown.

Ye et al. have attributed a hypothetical role in the local regulation of retinal blood flow to peptides released from retinal neurons. Although this hypothesis remains completely hypothetical, the idea of a local release of the 37-amino-acid peptide calcitonin gene-related peptide (CGRP) influencing retinal blood flow seems interesting, in that Prieto et al. demonstrated a powerful relaxing effect of CGRP in isolated bovine retinal arteries. In addition, they suggested that CGRP may be released from sensory nerves, because its effects are mimicked by capsaicin. The possible role of CGRP in the regulation of retinal arterial tone is also supported by the observation that CGRP causes a marked vasodilation in rabbit retinal arteries in vivo. More recently, Rollin et al. suggested that the natriuretic peptide (NP) family (comprising atrial natriuretic peptide [ANP], brain natriuretic peptide [BNP], and C-type natriuretic peptide [CNP]) could have a role in maintaining both the neural and vascular integrity of the mature retina and that further research is necessary to determine whether a local NP system regulates retinal blood flow. Natriuretic peptides are known to induce relaxation in different vascular beds but to our knowledge no information is available on a possible direct vasorelaxing effect of NPs in retinal arteries. In the present study, we sought to examine both the effects of NPs and the characteristics of the relaxing effect of CGRP in isolated bovine retinal arteries (BRAs), to evaluate the possibility that the as yet unidentified RRF is one of these peptides.

MATERIALS AND METHODS

Tension Measurements

Bovine eyes, obtained from the local abattoir, were enucleated within half an hour after the animals were killed and were transported to the laboratory in ice-cold Krebs-Ringer bicarbonate (KRB) solution. The anterior segment and the vitreous were removed, and the eyecup was placed in cold and oxygenated (5% CO₂ in O₂) KRB solution for further preparation. A segment located between the optic disc and the first branch of the most prominent retinal artery was excised with the surrounding retinal tissue. The arterial segments were mounted in an automated dual small-vessel myograph (model 500 A; JP Trading, Aarhus, Denmark) with a tissue chamber filled with 10 mL of KRB solution. Two stainless-steel wires (40 μm in diameter) were guided through the lumen of the segments (~2 mm in length). One wire was
fixed on a holder connected to a force-displacement transducer, and the other was fixed on a holder connected to a micrometer. The adherent retinal tissue was completely removed after the first wire was fixed. After they were mounted, the preparations were allowed to equilibrate for approximately 30 minutes in the KRB solution at 37°C, bubbled with 95% O₂ and 5% CO₂ (pH 7.4). Subsequently, the optimal lumen diameter of the vessels was calculated on the basis of the passive wall tension–internal circumferences relationship. Immediately after the vessels were stretched to their optimal lumen diameter (215.6 ± 2.3 μm, n = 99), they were activated twice with a KRB solution containing 120 mM K⁺ and once with a KRB solution containing 120 mM K⁺ and 30 μM prostaglandin F (PGF)₂α to assess maximum contractility.

After mounting and preparation, the retinal arteries were contracted by adding 30 μM PGF₂α to the organ bath. When a stable contraction was reached, increasing concentrations of CGRP or of one of the NPs were added to the organ bath to generate a concentration–response curve. In the experiments on the influence of potassium ions on the CGRP response, BRAs were also contracted either by replacing the standard KRB solution in the organ bath by a KRB solution containing 30 mM K⁺ and 30 μM PGF₂α, or by a KRB solution containing 120 mM K⁺ and 30 μM PGF₂α. The characteristics of the CGRP response were studied by comparing a first concentration–response curve constructed in control conditions with a second concentration–response curve constructed in the same ring segment after a pharmacological intervention. When pharmacologically active substances were used in this intervention, an incubation time was allowed. Unless stated otherwise, this incubation time refers to the waiting time that was allowed between adding the substance and inducing the second contraction in the ring segment.

Removal of the Endothelium

The arteries were first unstretched in the myograph. Subsequently, an L-shaped micropipette was positioned at the proximal end of the vessel, and 95% O₂ and 5% CO₂ was bubbled through the lumen of the vessel for 1 minute. Subsequently, the artery was stretched by resetting the wires to their original positions, and the vessel was allowed to re-equilibrate for 0.5 hour.

Drugs

The experiments were performed in a KRB solution of the following composition (mM): NaCl 135, KCl 5, NaHCO₃ 20, glucose 10, CaCl₂ 2.5, MgSO₄ 1.3, KH₂PO₄ 1.2, and EDTA 0.026 in H₂O. KRB solutions containing 30 mM K⁺ (K₃₀) and 120 mM K⁺ (K₁₂₀) were prepared by equimolar replacement of NaCl by KCl. N⁵-nitro-L-arginine (L-NA), 1H-[1,2]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin, acetylcysteine, cimetidine, N⁵-tetraethy lammoniumchloride (TEA), glibenclamide, 4-aminopyridine (+AP), adrenomedullin fragment 22-52 (AM 22-52), atrial natriuretic peptide (ANP, rat) and brain natriuretic peptide-32 (BNP, rat) were obtained from Sigma-Aldrich (St. Louis, MO); CGRP (rat), ibertotoxin, levocromakalim, and CGRP 8-37 (rat) from Tocris (Bristol, UK); apamin and charybdotoxin from Latoxan (Valence, France); sodium nitroprusside (SNP) and BaCl₂ from Merck (Darmstadt, Germany); PGF₂α (Dinolytic) from Upjohn (Puurs, Belgium); and Ctype natriuretic peptide (CNP, human and porcine) from Calbiochem-Novabiochem (Lauffelfingen, Switzerland). Stock solutions were made in water, except for ODQ, glibenclamide, and levromakalim (dissolved in dimethylsulfoxide), indomethacin (dissolved in ethanol), acetylcysteine chloride (dissolved in phthalate buffer, pH 4.0), and charybdotoxin (dissolved in 0.9% NaCl). The final concentration of both ethanol and dimethylsulfoxide in the organ bath never surpassed 0.1%.

Statistical Methods

The data are expressed as the mean ± SEM and were evaluated with Student’s t-test for paired samples or with repeated-measures ANOVA with the Bonferroni post hoc test, when appropriate. Two groups of data were considered to be significantly different at P < 0.05. Relaxations are expressed as the percentage of decrease in tone (n = number of preparations tested). The estimated pEC₅₀ and estimated maximum response (E₅₀) were calculated by using a non-linear-regression curve fit.

RESULTS

Effect of CGRP on PGF₂α-Induced Contractions in BRAs

After PGF₂α (30 μM) induced a stable contraction (5.96 ± 0.14 mN, n = 95), cumulative addition of CGRP (10 pM to 30 nM) caused a concentration-dependent relaxation in the BRAs (Fig. 1A). The estimated pEC₅₀ was 8.80 ± 0.04, and the E₅₀ was 80.06% ± 2.46% (n = 95). In a first series of experiments, three consecutive concentration–response curves were constructed in the same ring segments to assess the reproducibility of the relaxing effect of CGRP. No significant difference in CGRP response was observed in these repeated trials (Fig. 1B). A small decline in PGF₂α-induced tone was noted in the repeated trials. The mean contractions were, respectively, 5.85 ± 0.33, 5.57 ± 0.21 (P > 0.05 vs. trial 1, n = 5), and 5.38 ± 0.23 mN (P < 0.05 vs. trial 1, n = 5) in the first, second, and third trials. Thorough and repeated replacement of the KRB solution in the organ bath between the trials did not prevent this reduction in PGF₂α-induced tone.

Influence of Endothelium Removal, L-NA, ODQ, and Indomethacin on CGRP Response

The role of the vascular endothelium in the CGRP response was studied by comparing the CGRP-induced relaxations before and after removal of the endothelium of the BRAs. After removal of the endothelium, the pEC₅₀ of CGRP was significantly reduced, whereas E₅₀ was not significantly affected.
Table 1. Influence of Endothelium Removal or the Application of l-NA, ODO, or Indomethacin on PGF$_{2\alpha}$-Induced Contraction and on pEC$_{50}$ and $E_{\text{max}}$ for CGRP in the BRAs

<table>
<thead>
<tr>
<th></th>
<th>PGF$_{2\alpha}$ (mN)</th>
<th>pEC$_{50}$</th>
<th>$E_{\text{max}}$ (% Relaxation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>Endothelium (n = 11)</td>
<td>5.85 ± 0.54</td>
<td>5.73 ± 0.33</td>
<td>8.98 ± 0.17</td>
</tr>
<tr>
<td>+ l-NA (n = 6)</td>
<td>6.25 ± 0.48</td>
<td>6.50 ± 0.40</td>
<td>8.46 ± 0.07</td>
</tr>
<tr>
<td>+ ODO (n = 8)</td>
<td>5.93 ± 0.61</td>
<td>6.45 ± 0.65$^*$</td>
<td>8.58 ± 0.08</td>
</tr>
<tr>
<td>+ Indomethacin (n = 6)</td>
<td>6.07 ± 0.38</td>
<td>5.28 ± 0.41$^*$</td>
<td>9.14 ± 0.12</td>
</tr>
</tbody>
</table>

$^*$ $P < 0.05.$

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The relaxing effect of acetylcholine (Ach) was used to evaluate the effectiveness of endothelium removal. After endothelium denudation, the acetylcholine-induced relaxation was significantly decreased (Ach 0.1 mM: 31.98% ± 2.48% relaxation before and 9.23% ± 1.22% after removal of the endothelium; $n = 11$, $P < 0.05$). Treatment with the NO-synthase inhibitor l-NA (0.1 mM, 10 minutes) significantly reduced both the pEC$_{50}$ and $E_{\text{max}}$ of CGRP (Table 1, Fig. 2B). Also the relaxation induced by acetylcholine was significantly diminished after treatment with l-NA (Ach 0.1 mM: 34.37% ± 3.79% relaxation in the absence and 6.08% ± 1.65% in the presence of l-NA; $n = 6$, $P < 0.05$). Treatment of the BRAs with the soluble guanylyl cyclase inhibitor ODQ 21,22 (1 $\mu$M, 20 minutes) significantly reduced the $E_{\text{max}}$ of CGRP, but had no significant effect on the pEC$_{50}$ of CGRP, but caused a significant increase in $E_{\text{max}}$ (Table 1, Fig. 2D). The PGF$_{2\alpha}$-induced contraction was not significantly different after endothelium removal or treatment with l-NA, whereas it was significantly increased in the presence of ODQ and significantly decreased after treatment with indomethacin (Table 1).

Influence of Increasing Concentrations of Potassium Ions on the CGRP Response

In these experiments, the BRAs were contracted successively by adding 30 $\mu$M PGF$_{2\alpha}$ to the standard KRB solution in the organ bath (containing 5 mM K$^+$) or to a KRB solution containing 30 mM K$^+$ or 120 mM K$^+$. During the steady state of each one of these contractions, a concentration–response curve for CGRP was constructed. The results of these experiments are presented in Table 2 and Figure 3A. In the presence of 30 mM K$^+$, both the PGF$_{2\alpha}$-induced contraction and the pEC$_{50}$ of CGRP were unaffected. The $E_{\text{max}}$ of CGRP, in contrast, was significantly reduced in the presence of 30 mM K$^+$.  

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/ on 06/26/2017)
In the presence of 120 mM $K^+$, the PGF$_{2\alpha}$-induced contraction was significantly increased, and the $E_{max}$ of CGRP was significantly decreased. The pEC$_{50}$ of CGRP appeared to be unaffected.

**Influence of $K^+$-Channel Blocking on CGRP Response**

The nonselective potassium channel blocker TEA (3 mM, 15 minutes)$^{23}$ significantly reduced both the pEC$_{50}$ and $E_{max}$ of CGRP (Table 3, Fig. 3B), but increased relaxations induced by SNP (0.1 mM: 21.91% ± 3.51% relaxation in the absence and 29.00% ± 5.58% in the presence of TEA; $n = 4$, $P < 0.05$).

Treatment of the BRAs with a combination of the Ca$^{2+}$-activated $K^+$ channel ($K_{Ca}$) blockers apamin$^{24}$ (0.1 $\mu$M, 15 minutes, a blocker of small-conductance $K_{Ca}$) and charybdotoxin$^{24}$ (0.1 $\mu$M, 15 minutes, a blocker of large- and intermediate-conductance $K_{Ca}$) had no effect on the CGRP response (Table 3, Fig. 4A). Also, treatment of the BRAs with the selective large-conductance $K_{Ca}$ blocker iberiotoxin$^{25}$ (0.1 $\mu$M, 15 minutes) or with the inward rectifier potassium channel blocker Barium chloride$^{26}$ (Ba$^{2+}$, 30 $\mu$M, 10 minutes) had no effect on CGRP-induced relaxation (Table 3, Fig. 4B). The adenosine triphosphate (ATP)-sensitive potassium channel blocker glibenclamide$^{25}$ (1 $\mu$M, 30 minutes) did not decrease, but in contrast significantly increased, the $E_{max}$ and pEC$_{50}$ of CGRP (Table 3, Fig. 4C). In the experiments with glibenclamide, the relaxing effect of 1 $\mu$M levcromakalim in the BRAs was significantly attenuated after treatment with glibenclamide (67.30% ± 17.69% relaxation in control conditions versus 0.40% ± 0.26% after glibenclamide treatment; $n = 6$, $P < 0.05$). Treatment of the BRAs with the voltage-dependent potassium channel blocker 4-AP$^{27}$ (1 mM, 15 minutes), in contrast, significantly reduced the $E_{max}$ of CGRP, but had no significant effect on the pEC$_{50}$ (Table 3, Fig. 4D). Relaxations induced by SNP in the BRAs were not significantly affected by 4-AP (0.1 mM: 23.02% ± 5.08% relaxation in the absence and 19.73% ± 4.86% in the presence of 4-AP; $n = 4$). In all these experiments, contraction in the presence of the $K^+$ channel blocker was smaller than in control conditions, although this decrease was not statistically significant in the experiments with Ba$^{2+}$ or with the combination of apamin and charybdotoxin.

**Influence of AM and CGRP Receptor Antagonists on CGRP Response**

Incubation of the BRAs with the proposed CGRP$_1$ receptor antagonist CGRP 8-37 (1 $\mu$M, added after stabilization of the second contraction, 5 minutes before adding 10 $p$M CGRP) caused a small shift in the CGRP concentration-response curve (Fig. 5A), but the pEC$_{50}$ and the $E_{max}$ of CGRP were not significantly reduced (pEC$_{50}$: 8.75 ± 0.23 in the absence and 8.06 ± 0.26 in the presence of CGRP 8-37, $n = 6$; $E_{max}$: 90.70% ± 7.78% in the absence and 94.19% ± 3.67% in the presence of CGRP 8-37, $n = 6$). Incubation of the BRAs with the proposed AM receptor antagonist AM 22-52 (1 $\mu$M, added after stabilization of the second contraction, 5 minutes before adding 10 $p$M CGRP) did not significantly affect the CGRP response (Fig. 5B). The pEC$_{50}$ and $E_{max}$ of CGRP were respectively 8.55 ± 0.07 and 86.40% ± 12.20% in control conditions, and 8.59 ± 0.16 and 96.57% ± 1.87% in the presence of AM 22-52 ($n = 4$). In both the experiments with CGRP 8-37 and with AM 22-52, PGF$_{2\alpha}$-induced tone in the second concentration-response curve was slightly, but not significantly, smaller than PGF$_{2\alpha}$-induced tone in the first concentration-response curve.

**Influence of NPs on PGF$_{2\alpha}$-Induced Contractions in BRA**

The relaxing effects of ANP, BNP, and CNP (all three in the concentration range of 1 $p$M to 0.1 $\mu$M) were tested in BRAs contracted with 30 $\mu$M PGF$_{2\alpha}$. At the highest concentration tested (0.1 $\mu$M), a mean relaxing effect was noted of 3.83% ± 1.72%, 1.50% ± 1.19%, and 3.50% ± 1.50% for ANP, BNP, and CNP, respectively ($n = 6$, Fig. 6).

**DISCUSSION**

The present study confirms the observation of Prieto et al.$^{11}$ that CGRP has a powerful relaxing effect on retinal arterial smooth muscle. In our experiments in which three consecutive concentration-response curves for CGRP were constructed in isolated BRAs, the CGRP response was reproducible. In contrast, a decrease in PGF$_{2\alpha}$-induced tone was noted in these repeated trials. This decrease was rather limited, and only the difference between the first and the third contractions was statistically significant. Nevertheless, the contractile response of the BRAs clearly tended to diminish after a concentration-response curve for CGRP was constructed. This de-

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**Table 2. Influence of Increasing Concentrations of K$^+$ in the Organ Bath on PGF$_{2\alpha}$-Induced Contraction and on pEC$_{50}$ and $E_{max}$ for CGRP in the BRAs**

<table>
<thead>
<tr>
<th>K$^+$ Concentration in the Organ Bath</th>
<th>Contraction (mN)</th>
<th>pEC$_{50}$ (%)</th>
<th>$E_{max}$ (%) Relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM (standard KRB)</td>
<td>6.30 ± 0.76</td>
<td>8.55 ± 0.08</td>
<td>78.14 ± 12.65</td>
</tr>
<tr>
<td>30 mM</td>
<td>6.38 ± 0.68</td>
<td>8.49 ± 0.08</td>
<td>36.15 ± 4.74*</td>
</tr>
<tr>
<td>120 mM</td>
<td>6.85 ± 0.78*</td>
<td>8.31 ± 0.13</td>
<td>17.30 ± 1.40</td>
</tr>
</tbody>
</table>

n = 4. * P < 0.05 vs. 5 mM K$^+$. 

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**Figure 3. Concentration-response curves for CGRP in BRAs (A) in the presence of increasing concentrations of K$^+$ in the organ bath (n = 4), and (B) in the absence (control) and presence of 3 mM TEA (n = 7). Relaxations are expressed as the percentage of relaxation of the tone induced by 30 $\mu$M PGF$_{2\alpha}$ (P < 0.05).**
crease in tone is not a time-dependent effect, but could be specific for successively constructed CGRP concentration–response curves, because no reduction in tone was found in similar experiments with another vasorelaxant. As this reduction in tone did not affect the reproducibility of the CGRP response, we used a protocol in which repeated concentration–response curves were constructed in the same BRA ring segment, to characterize pharmacologically the CGRP response in isolated BRAs.

Although in most of the tissues examined so far, relaxation in CGRP response appears to be endothelium independent, in some blood vessels such as rat aorta and rat pulmonary artery, the CGRP response has been shown to be endothelium dependent. In our experiments, removal of the endothelium of the BRA resulted in a significant decrease in the CGRP response. Dysfunction of the endothelium was illustrated by the fact that the acetylcholine-induced relaxation, which is known to be endothelium dependent, was significantly reduced. It should be noted, however, that although the reduction was statistically significant, a substantial fraction of the vasorelaxing influence of CGRP remained unaffected after removal of the endothelium and can therefore be regarded as endothelium independent. It is well known that the vascular endothelium can mediate the regulation of vascular tone through the release of several smooth muscle relaxants such as nitric oxide (NO), PGs, and the endothelium-derived hyperpolarizing factor (EDHF). Both the NO-synthase inhibitor L-NA and the soluble guanylyl cyclase inhibitor ODQ had an inhibitory effect on the relaxations induced by CGRP in BRAs. These results suggest that the endothelium-dependent part of the CGRP response in BRAs is mediated by the release of NO from the vascular endothelium. The effectiveness of L-NA and ODQ in our experiments was demonstrated by the fact that, respectively, the acetylcholine- and the SNP-induced relaxation in BRAs were significantly reduced in their presence. In comparison with the SNP response in the experiments designed to

![Figure 4](image-url)

**Figure 4.** Concentration–response curves for CGRP in BRAs in the absence (control) or presence of (A) 0.1 μM apamin + 0.1 μM charybdotoxin (n = 6), (B) 30 μM Ba²⁺ (n = 5), (C) 1 μM glibenclamide (n = 6), or (D) 1 mM 4-aminopyridine (n = 7). Relaxations are expressed as the percentage of relaxation of the tone induced by 30 μM PGF₂α (*P < 0.05).

<p>| Table 3. Influence of Several K⁺ Channel Blockers on PGF₂α-Induced Contraction and on pEC₅₀ and E₅₀ for CGRP in the BRAs |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>PGF₂α (mN)</th>
<th>pEC₅₀</th>
<th>E₅₀ (% Relaxation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>-----------------</td>
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</tr>
<tr>
<td>TEA (n = 7)</td>
<td>7.14 ± 0.37</td>
<td>6.14 ± 0.46*</td>
<td>8.99 ± 0.07</td>
</tr>
<tr>
<td>Apam + charyb (n = 6)</td>
<td>5.87 ± 0.35</td>
<td>5.36 ± 0.42</td>
<td>8.74 ± 0.11</td>
</tr>
<tr>
<td>Iberiotoxin (n = 6)</td>
<td>5.53 ± 0.48</td>
<td>4.95 ± 0.58*</td>
<td>8.83 ± 0.13</td>
</tr>
<tr>
<td>Ba²⁺ (n = 5)</td>
<td>4.35 ± 0.57</td>
<td>3.99 ± 0.69</td>
<td>8.80 ± 0.03</td>
</tr>
<tr>
<td>Glibenclamide (n = 6)</td>
<td>4.64 ± 0.39</td>
<td>3.65 ± 0.41*</td>
<td>8.88 ± 0.10</td>
</tr>
<tr>
<td>4-AP (n = 7)</td>
<td>5.80 ± 0.26</td>
<td>5.41 ± 0.20*</td>
<td>8.86 ± 0.08</td>
</tr>
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</table>

Apam, apamin; charyb, charybdotoxin.

* P < 0.05.
evaluate the specificity of the effects of ODQ, the SNP response in the experiments with ODQ seemed to be unusually high. This apparent inconsistency is probably due to a high variability in the SNP response in different isolated BRAs. A high variability in the response of BRAs to both SNP and acetylcholine has been described by Benedito et al.22 Treatment of the BRAs with the COX inhibitor indomethacin caused no decrease, but, in contrast, even an increase in the CGRP response. Previous research revealed that the relaxations induced in BRAs by other vasorelaxants, such as adenosine (our unpublished results, 2002), SNP (unpublished results, 2002), and adrenomedullin28 were increased in the presence of indomethacin, suggesting that this effect is not specific for CGRP. The involvement of vasorelaxing prostanoids released from the vascular endothelium can be excluded, as no inhibitory effect on CGRP response could be achieved by COX inhibition. The decrease in PGF$_{2\alpha}$-induced contraction in the presence of indomethacin may be because PGF$_{2\alpha}$-induced contraction, as was described earlier, tends to be decreased when a second concentration–response curve for CGRP is constructed in the same ring segment. However, also in previous research, indomethacin has been shown to have a small but significant inhibitory effect on PGF$_{2\alpha}$-induced contractions in BRAs.28

The CGRP response’s being reduced in the presence of 30 mM of K$^+$ could be regarded as an argument in favor of the involvement of EDHF, since the presence of 30 mM of K$^+$ is known to abolish the EDHF response.24 However, several other arguments refute this suggestion. First, L-NA has been shown to inhibit the CGRP response to an extent that may account for the total reduction in CGRP response after removal of the endothelium. Another endothelium-derived mediator with a major role in the endothelium-dependent part of the relaxation therefore seems rather unlikely. Second, the CGRP response was unaffected by treatment of the BRAs with the combination of apamin and charybdotoxin, a mixture that is known to inhibit EDHF-mediated vasorelaxation.34,35 Consequently, we conclude that EDHF is probably not involved in the CGRP-induced relaxation in BRAs.

The observation that the relaxations in CGRP response were reduced in the presence of increased concentrations of K$^+$ could also be consistent with the involvement of K$^+$ channel activation in the CGRP response, since an increase in K$^+$ concentration is known to reduce the vasorelaxing effect of K$^+$ channel openers.36 This hypothesis is confirmed by the observation that the CGRP response was significantly reduced by TEA, which is generally considered as a rather nonspecific K$^+$ channel blocker.29-31 A nonspecific effect of TEA can be excluded, as the relaxing effect of SNP was not reduced but rather was enhanced in the presence of TEA. Four distinct types of K$^+$ channels are functionally important in the vasculature: (1) voltage-dependent K$^+$ (Kv) channels, (2) Ca$^{2+}$-activated K$^+$ (KCa) channels, (3) inward rectifier K$^+$ (Kir) channels, and (4) ATP-sensitive K$^+$ (KATP) channels.39,40 As CGRP-induced relaxations in BRAs were not affected by the combination of apamin (a blocker of small-conductance K$_{Ca}$) and charybdotoxin (a blocker of large- and intermediate-conductance K$_{Ca}$) or by iberiotoxin (a selective large-conductance K$_{Ca}$ blocker), the involvement of K$_{Ca}$ channels in the CGRP response in BRA can be excluded. Also the K$_{ir}$ blocker Ba$^{2+}$ had no effect on the CGRP-induced relaxations, and therefore the involvement of K$_{ir}$ channels can be excluded as well. The presence of the K$_{ATP}$ blocker glibenclamide did not reduce but in contrast significantly increased the CGRP response. The effectiveness of glibenclamide in our experiments was demonstrated by the fact that the relaxations induced by the known K$_{ATP}$ opener levcromakalim34 were significantly reduced in the presence of glibenclamide. There seems to be no obvious explanation for the increase in CGRP response caused by glibenclamide. Nevertheless, it seems very unlikely that K$_{ATP}$ channels could be involved in the CGRP-induced relaxations, as glibenclamide had no inhibitory effect on these relaxations. Treatment of the BRAs with the K$_{ir}$ channel blocker 4-AP did significantly reduce the $E_{\text{max}}$ of CGRP in our experiments. A nonspecific effect of 4-AP can be excluded, as SNP-induced relaxations were not affected by 4-AP. Although a substantial part of the relaxing effect of CGRP remained unaffected in the presence of 4-AP, these experiments suggest that K$_{ir}$ channels...
could, at least in part, be involved in the relaxing effect of CGRP in isolated BRAs. A role for KCa channel activation in the vasorelaxing effect of CGRP in isolated blood vessels has been suggested before, although in most of these blood vessels CGRP is thought to activate K<sub>ATP</sub> channels or large-conductance K<sub>Ca</sub> channels. To our knowledge, isolated BRA is the first type of blood vessel in which a role for KCa channel activation in the vasorelaxing CGRP response is suggested.

It is generally accepted that vasodilation induced by CGRP is mediated through the CGRP<sub>1</sub> receptor and is competitively blocked by the proposed CGRP<sub>1</sub> receptor blocker CGRP 8-37. There are, however, some conflicting data, in that Wisskirchen et al. demonstrated that relaxations induced by CGRP in rat aorta were not affected by CGRP 8-37. Champion et al. reported that AM 22-52, proposed to be an AM receptor antagonist, inhibited vasodilator responses to CGRP in the hindlimb vascular bed of the cat. Whereas AM 22-52 did not affect the CGRP response in our experiments, the concentration-response curve for CGRP was slightly shifted to the right in the presence of CGRP 8-37. Neither the pEC<sub>50</sub> nor E<sub>max</sub> of CGRP was significantly reduced by CGRP 8-37, and only the decrease in the relaxation caused by 3 nM of CGRP was statistically significant. Even though the concentration of CGRP 8-37 used in these experiments has been shown to inhibit CGRP-induced relaxations in other vascular preparations, it should be taken into account that some doubts have been expressed concerning the potency and selectivity of both AM 22-52 and CGRP 8-37 as receptor antagonists. Although no clear-cut conclusions can be drawn from our observations with AM 22-52 and CGRP 8-37, they at least suggest that CGRP receptors could be involved in the CGRP response, whereas AM receptors are probably not involved. Development of more potent and selective blockers is necessary to establish conclusively the exact role of these receptors in the vasorelaxing effect of CGRP in isolated BRAs.

The observation that CGRP is a potent vasodilator in BRAs raises the question of whether the RRF—an as yet unidentified vasorelaxant, known to be released from retinal tissue and known to have a powerful relaxing effect on BRAs—could be CGRP released from the retinal tissue. We demonstrated that the CGRP response in BRAs was, at least in part, dependent on the release of NO from the vascular endothelium. However, previous research on the characteristics of the relaxing effect of the RRF revealed that the response to the RRF is completely unaffected by removal of the endothelium or by treatment of the BRAs with the NOS inhibitor l-NA. These findings suggest that the hypothesis that CGRP is the unidentified RRF can be rejected. NPs ANP, BNP, and CNP can also be excluded as possible candidates for the RRF, as none of these peptides induced a substantial relaxation in PGF<sub>2α</sub>-contracted isolated BRAs. These observations also suggest that it is very unlikely that one of these NPs, which are expressed in the retina, could act as a modulator of retinal blood flow through a direct vasorelaxing effect on retinal arteries.

In conclusion, the present study confirmed that CGRP is a potent vasodilator in isolated BRAs and demonstrated that the vascular endothelium, activation of voltage-dependent potassium channels and possibly also CGRP receptor stimulation are involved in the CGRP-induced relaxations. The present study also showed that the NPs ANP, BNP, and CNP do not relax isolated BRAs. The observations in this study clearly demonstrate that neither CGRP nor one of the NPs could be the as yet unidentified RRF.

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References

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