Rat Retinal Tissue Releases a Vasorelaxing Factor

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PURPOSE. To investigate whether the retina of the rat exerts a vasodilatory influence by the release of a relaxing factor and to characterize the retinal relaxing factor (RRF).

METHODS. The relaxing influence of the rat retina was investigated by placing the retina in close proximity with a precontracted isolated rat carotid artery ring segment, mounted for isometric tension measurements.

RESULTS. Application of rat retina relaxed the artery in a reliable and reproducible way. The nitric oxide (NO)-synthase inhibitor Nω-nitro-l-arginine (L-NA), the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-b]quinoxalin-1-one (ODQ), and the removal of the endothelium of the artery all failed to affect the RRF response. The RRF response was not decreased; in contrast, it increased after treatment with a cyclooxygenase (COX) inhibitor (indomethacin or sodium diclofenac). Acute hypoxia profoundly enhanced retina-induced relaxation. Several potential mediators of hypoxia-induced vasodilation were excluded as candidates for the RRF or for mediating the enhanced response to RRF in hypoxia. Inhibition of the plasma membrane Ca\(^{2+}\)-adenosine triphosphatase (ATPase) with vanadate significantly affected the RRF response.

CONCLUSIONS. The release of an as yet unidentified relaxing factor(s) from the rat retina was demonstrated. Acute hypoxia profoundly enhances the RRF response. None of the known mediators of hypoxia-induced vasodilation nor NO, prostanooids, or endothelial factors mediate the RRF response. Activation of the plasma membrane Ca\(^{2+}\)-ATPase seems to be involved in the RRF response. (Invest Ophthalmol Vis Sci. 2002;43:3279–3286)

Retinal function is strongly dependent on an adequate supply of oxygen and nutrients through both the retinal and choroidal circulation. In contrast to choroidal circulation, retinal circulation is characterized by a relatively low level of flow and a high level of oxygen extraction.\(^{1-3}\) Therefore, mainly the retinal blood flow must adapt closely to changes in retinal metabolism. Recently, it has been reported that bovine retina continuously releases a strong vasorelaxant that may be important in the maintenance of retinal circulation.\(^{4}\) Isolated bovine retinal arteries that were completely free of surrounding retinal tissue contracted much more strongly in response to different vasoconstrictors than preparations with adherent retinal tissue. Moreover, bovine retinal artery first contracted with, for example, prostaglandin (PGF\(_{2\alpha}\)) profoundly relaxed when a piece of bovine retinal tissue was brought in proximity with the artery, indicating that the retina releases a strong vasorelaxant. The name retinal relaxing factor (RRF) was coined for this vasorelaxant.\(^{4}\)

Many questions remain to be answered in relation to the RRF—for example, identity, physiological role, regulatory mechanisms for its release, mechanism of action on vascular smooth muscle cells, potential involvement in ophthalmic diseases. The present study was undertaken to find out whether RRF research, as yet performed mainly on bovine tissues,\(^{4,5}\) can be extended to a small laboratory animal such as the rat. Herein we report that the release of a relaxing factor from rat retinal tissue can be demonstrated by using a bioassay. Furthermore, we investigated whether this RRF corresponds to nitric oxide (NO) or a prostanooid, whether the influence of RRF is mediated by endothelial cells, and whether hypoxia influences the RRF response. Finally, we determined the influence of some blockers of intracellular calcium ([Ca\(^{2+}\)]. reducing mechanisms on the RRF response.

MATERIALS AND METHODS

Rats used in the experiments presented in this study were handled in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

TENSION MEASUREMENTS

In preliminary experiments, we found that several types of precontracted blood vessels (rat aorta, rat carotid artery, rat mesenteric artery, rat femoral artery) relax when covered with rat retinal tissue. From these arteries, the carotid artery was selected for use as RRF-detector preparation in further experiments. This preparation provided reliable relaxation. Carotid arteries and retinas were taken from female Wistar rats (160–260 g), after cervical dislocation. The arteries and the retinas were isolated from the surrounding tissues in cooled and oxygenated (5% CO\(_2\) in O\(_2\)) Krebs-Ringer bicarbonate (KRB) solution. Isolated retinas were kept in a Warburg incubator in a small container with 10 mL KRB solution at constant temperature (37°C) and oxygenated with 5% CO\(_2\) in O\(_2\). Segments of the carotid arteries were mounted in an automated dual small-vessel myograph (model 500 A; J. P. Trading, Aarhus, Denmark) by transferring them to the tissue chamber filled with 10 mL KRB solution. Two stainless steel wires (40 μm in diameter) were guided through the lumen of the segments (±2 mm). 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. After they had been mounted, the preparations were allowed to equilibrate for approximately 30 minutes in the KRB solution at 37°C, bubbled with 95% O\(_2\) and 5% CO\(_2\). The distance between the two steel wires was gradually increased with the micrometer until a stable preload of 0.5 g (or 4,905 mN) was obtained. Subsequently, the preparations were contracted by adding a contractile agent to the standard KRB solution in the organ bath, or by replacing the standard KRB solution in a KRB solution containing 30 mM K\(^{+}\) and 30 μM PGF\(_{2\alpha}\). Retina-induced relaxation was obtained by putting the rat retina on top of the contracted artery.

REMOVAL OF THE ENDOTHELIUM

Before this procedure, the arteries were unstretched in the myograph. Subsequently, an L-shaped micro pipette was positioned at the proximal end of the vessel, and 95% O\(_2\)+5% CO\(_2\) was bubbled through the lumen for 2 minutes. Subsequently, the artery was again stretched by...
Hypoxia

Acute hypoxia was induced in the organ baths by switching the gas mixture used to bubble the organ bath from 95% O_{2}-5% CO_{2} to 95% N_{2}-5% CO_{2}. This coincides with a reduction of the oxygen tension in the organ bath from 558.0 ± 35.3 to 61.3 ± 3.18 mm Hg, as was described in a previous publication.

Statistical Methods and Drugs

The data were computed as the mean ± SEM and evaluated statistically with Student’s t-test for paired samples. Two groups of data were considered to be significantly different when P < 0.05. Relaxation is expressed as the percentage decrease in tone (n = number of preparations tested).

The experiments were performed in a KRB solution of the following composition (in mM): NaCl 135, KCl 5, NaHCO_{3} 20, glucose 10, CaCl_{2} 2.5, MgSO_{4} 1.3, KH_{2}PO_{4} 1.2, and EDTA 0.026 in H_{2}O. A KRB solution containing 30 mM K^+ (K_{0}) was prepared by equimolar replacement of NaCl by KCl. Norepinephrine, serotonin, N^{N}-nitro-L-arginine (L-NA), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin, sodium dicyclofenac, acetylsalicylic acid, adenosine, glutamic acid, aspartic acid, lactic acid, taurine, thapsigargin, cyclopiazonic acid, sodium metavanadate, amiloride, ouabain, and nifedipine were obtained from Sigma (St. Louis, MO); PGF_{2a} (Dinoltyc) from Upjohn (Puurs, Belgium); sodium nitroprusside (SNP) from Merck (Darmstadt, Germany); y-aminobutyric acid (GABA) from Interlaboratoire (Wezembeek, Belgium); and 1,3-dimethyl-2-thiourea from Sigma-Aldrich (Steinheim, Germany). Stock solutions were made in water, except for ODQ, cyclopiazonic acid, amiloride, and nifedipine (dissolved in dimethyl sulfoxide), for indomethacin and thapsigargin (dissolved in ethanol), and for acetylsalicylic acid (dissolved in phthalate-buffer, pH 4.0). The final concentration of both ethanol and dimethyl sulfoxide in the organ bath never surpassed 0.1%.

RESULTS

Influence of Rat Retina: The Protocol

A segment of the rat carotid artery was mounted in the organ bath of the myograph, and the vessel was contracted in a KRB solution containing 30 mM potassium and 30 μM PGF_{2a}. When a stable contraction was obtained, the rat retina was put on top of the carotid artery. This elicited a profound and stable relaxation (mean: 33.24% ± 0.33 minutes (n = 156)) within a mean time of 7.95 ± 0.33 minutes (n = 156). Other tissues—for instance, samples of cornea, bladder wall, or mesenterium of the rat—failed to relax the carotid artery under the same conditions. When the retina was removed, the relaxing influence disappeared and tone returned to its original level within a mean time of 6.51 ± 0.25 minutes (n = 156). In the next series of experiments, we investigated whether this relaxing effect could be elicited in a reproducible way with the use of the same donor and detector preparations. Therefore, the carotid artery was contracted, and the retina was put on top of the artery. When a stable relaxation was obtained, the retina was removed. This procedure was repeated three times, and thereafter the organ bath was washed. The same procedure was then repeated. An original tracing of such an experiment is shown in Figure 1. The mean of the first series of relaxations was not different from the mean of the second series (38.79% ± 5.48% vs. 40.17% ± 4.06%, n = 8, P > 0.05).

The relaxing influence of the rat retinal tissue was also examined after precontraction of the detector preparation with an adrenergic agonist. Norepinephrine (0.3 μM) induced a mean contraction of 12.88 ± 1.64 mN in the rat carotid artery. When the rat retina was put on top of the detector preparation, it elicited a mean relaxation of 39.50% ± 5.51% (n = 12). Precontraction with serotonin resulted in a mean contraction of 11.8 ± 1.71 mN and a mean retina-induced relaxation of 32.54% ± 9.81% (n = 6).

For a reliable protocol, it is important to have a sustained and stable contraction during each part of the experiment. When only PGF_{2α}, noradrenaline, or serotonin was used as contracting agent, the precontraction level tended to decrease toward the end of the experiment. Therefore, a KRB solution containing 30 mM K^+ and 30 μM PGF_{2α} was used to contract the carotid arteries. The presence of 30 mM K^+ also had a stabilizing effect on retina-induced relaxation. Whether the presence of 30 mM K^+ influences the strength of the RRF response was investigated by performing a first series of three relaxations on carotid artery contracted in a KRB solution containing 30 mM K^+ and 30 μM PGF_{2α}, (mean relaxation: 54.71% ± 4.44%, n = 8) and a second series of three relaxations on the same carotid artery contracted in a standard KRB solution containing only 30 μM PGF_{2α}, (mean relaxation: 41.96% ± 4.87%, n = 8). Although the relaxations tended to diminish in the presence of K^+, responses in the absence and presence of 30 mM K^+ were not significantly different (P > 0.05). The contraction, however, increased from a mean of 9.75 ± 0.73 mN in the absence of 30 mM K^+ to a mean of 13.84 ± 0.64 mN in the presence of 30 mM K^+ (P < 0.05, n = 8).

Influence of l-NA and ODQ

The NO-synthase inhibitor l-NA and the soluble guanylyl cyclase inhibitor ODQ were used to investigate the potential involvement of NO in the relaxation caused by the rat retina. In a first series of experiments, both the retina and the carotid artery were treated with l-NA (0.1 mM) for 10 minutes before the artery was contracted in the second part of our protocol. The results of these experiments are shown in Figure 2. l-NA induced an increase of 12.64% ± 1.88% (P < 0.05, n = 8) in the contractile tone of the carotid artery in response to 30 mM K^+ and 30 μM PGF_{2α}. The mean of the first series of relaxations was 21.58% ± 1.25%, and the mean of the second series was 23.71% ± 5.60% (n = 8, P > 0.05). Under the same experimental conditions the effect of 10 μM of acetylcholine
In a second series of experiments, the soluble guanylyl cyclase inhibitor ODQ 1 μM was added after the second contraction in the protocol was stabilized, 20 minutes before the second series of relaxations in response to application of the retina was performed. ODQ increased contraction with 35.11% ± 16.94% (n = 8). In the absence of ODQ, the retina induced a mean relaxation of 31.50% ± 2.60%. In the presence of ODQ, the retina induced a not significantly different relaxation of 24.17% ± 3.92% (n = 8, P > 0.05; Fig. 3). The response caused by adding SNP (1 μM), however, was significantly decreased after treatment of the carotid artery with ODQ (41.17% ± 4.01% before and 4.25% ± 0.41% after treatment with ODQ; n = 8, P < 0.05; Fig. 3).

Influence of Indomethacin and Sodium Diclofenac

In these experiments, the involvement of PGs in the relaxation caused by the rat retina was investigated. Both the retina and the carotid artery were treated with the cyclooxygenase (COX) blocker indomethacin (10 μM), added 20 minutes before the second contraction in our protocol. The mean relaxation in the absence of indomethacin was 34.57% ± 4.81%. The mean relaxation in the presence of indomethacin was significantly larger (48.4% ± 3.83%; n = 10, P < 0.05; Fig. 4). These results were confirmed using another COX-inhibitor, sodium diclofenac (10 μM). In these experiments, the mean relaxation in the presence of sodium diclofenac (46.06% ± 4.04%) was also significantly larger than the mean relaxation in the absence of sodium diclofenac (29.56% ± 2.24%; n = 6, P < 0.05; Fig. 4).

Influence of Removing the Endothelium

In this series of experiments, we investigated the potential involvement of the endothelium in rat RRF-induced relaxation. Therefore, the RRF response was examined before and after removal of the endothelium of the carotid artery. The retina caused a relaxation of 33.67% ± 3.13% before and of 25.83% ± 4.34% after removal of the endothelium (n = 4, P > 0.05; Fig. 5). Acetylcholine (10 μM)-induced relaxation, however, was significantly decreased by removing the endothelium (25.50% ± 4.41% before and 6.50% ± 1.19% after removal of the endothelium; n = 4, P < 0.05).

Influence of Acute Hypoxia

The effect of acute hypoxia on smooth muscle tone of the rat carotid artery was studied both in the presence and the absence of a rat retina placed nearby. An original recording of such an experiment is shown in Figure 6. After a stable contraction was reached (mean relaxation: 11.76 ± 0.53 mN, n = 14), acute hypoxia was induced. This resulted in a small decrease in tone to a mean of 10.47 ± 0.48 mN (n = 14), or a relaxation of 10.82% ± 1.93%. Reoxygenation of the organ bath rapidly and completely restored contraction. Subsequently, the organ bath was washed three times, and the arteries were contracted again. This time, a rat retina was put on top of the artery as soon as a stable contraction was reached. This elicited a decrease in tone from 11.64 ± 0.58 to
When in the organ bath pH was measured at decreasing distance from the retina, no change in pH was detected. Even in close proximity with the retina, pH was stable at 7.4. Glutamic acid, GABA, aspartic acid, taurine, and glycine (all tested in a concentration range of 1 mM to 0.1 mM, n = 4) failed to relax rat carotid artery contracted in a KRB solution containing 30 mM K⁺ and 30 μM PGF₂α. Adenosine induced only a small relaxation (7.26% ± 2.25%, n = 4) at the highest concentration (0.1 mM). Lactic acid induced a small (14.83% ± 3.70%, n = 6) and transient relaxation at the highest concentration (1 mM).

Furthermore, the potential influence of these hypoxic mediators on the synthesis and/or release of the RRF from the retina was tested by incubating the retina in a Warburg apparatus with one of the possible mediators for 1 hour between the two series of retina-induced relaxations in our protocol. The mediator was also present in the organ bath in the same concentration during the second series of relaxations. In that way, the RRF response after incubation with the possible mediator (second series of relaxations) can be compared with the RRF response under control conditions (first series of relaxations). The RRF response was not significantly influenced after a 1-hour incubation of the retina with 1 mM lactic acid (30.67% ± 2.67% relaxation before and 36% ± 4.64% relaxation after incubation, n = 6, P > 0.05), 0.1 mM adenosine (32.67% ± 2.07% relaxation before and 36% ± 4.74% after incubation, n = 6, P > 0.05), 1 mM GABA (29.78% ± 3.00% relaxation before and 33.39% ± 2.64% after incubation, n = 6, P > 0.05), 10 mM glutamic acid (32.29% ± 3.39% relaxation before and 34.17% ± 3.13% after incubation, n = 6, P > 0.05), 0.1 mM aspartic acid (24.89% ± 3.81% relaxation before and 34.44% ± 4.95% after incubation, n = 6, P > 0.05), 1 mM taurine (24.17% ± 4.75% relaxation before and 33.28% ± 2.82% after incubation, n = 6, P > 0.05), or 1 mM glycine (29.50% ± 3.61% relaxation before and 33.94% ± 6.08% after incubation, n = 6, P > 0.05).

The possibility that PGs mediate the enhanced RRF response in acute hypoxia was investigated by comparing the effect of hypoxia on the RRF response in the presence and absence of the COX inhibitor indomethacin. Hypoxia induced a mean relaxation of 45.32% ± 6.94% (compared with the tone of the artery in the presence of the rat retina, before hypoxia was induced) in the absence of indomethacin and of 46.57% ± 8.41% in the presence of indomethacin (n = 6, P > 0.05).

**Influence of Hypoxic Metabolites**

The results of the previous experiments suggest that hypoxia promotes the release of vasodilator molecules from the retina. Several substances have been suggested as possible mediators of the hypoxic vasodilation.⁸⁻¹⁴ In this series of experiments, we tested the potential involvement of some hypoxia-related substances in RRF response.
Involvement of [Ca\textsubscript{i}]-Reducing Mechanisms

To further characterize the RRF response, we investigated the influence of several blockers of intracellular calcium ([Ca\textsubscript{i}])-reducing mechanisms on the RRF response. The role of calcium uptake into intracellular stores during retina-induced relaxation was studied by treating the carotid artery with the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor thapsigargin (2 \( \mu \text{M} \)), added to the organ bath 45 minutes before the second contraction in our protocol. In these experiments, thapsigargin did not change the resting tone of the carotid artery, and also contraction induced by 30 mM K\textsuperscript{+} and 30 \( \mu \text{M} \) PGF\textsubscript{2}\alpha was not altered in the presence of thapsigargin. The mean relaxation in control conditions (20.50\% ± 2.29\%) was not different from the mean relaxation in the presence of thapsigargin (21.17\% ± 3.70\%; n = 6, P > 0.05).

To characterize the RRF released from rat retinal tissue, a reliable protocol was established. First, we investigated whether reproducible relaxation could be obtained when a rat retina is put repetitively in close proximity with a precontracted rat carotid artery. This provides evidence that retina-induced contractions were too unstable to perform reliable experiments.

Two inhibitors of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger were used: 1,3-dimethyl-2-thiourea (DMTU) and amiloride. DMTU (25 mM) was added to the organ bath 30 minutes before the second contraction in our protocol. It caused a significant decrease in the second K\textsuperscript{+} and PGF\textsubscript{2}\alpha-induced contraction (5.48 ± 0.52 mN in the presence of DMTU vs. 11.60 ± 0.65 mN in the absence of DMTU). However, retina-induced relaxations were not blocked (mean relaxation: 49.83\% ± 7.94\%, n = 6). Amiloride (0.1 mM) was added to the organ bath 20 minutes before the second contraction in our protocol. It caused a small, but not significant decrease in contractile tone (8.30 ± 1.58 mN in the presence of amiloride vs. 10.60 ± 0.72 mN under control conditions, P > 0.05, n = 4). The RRF response was certainly not blocked by the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange inhibitor (22.5\% ± 7.90\% relaxation in the absence of amiloride vs. 31.42\% ± 10.42\% in the presence of amiloride, P > 0.05, n = 4).

To evaluate the potential involvement of closure of L-type Ca\textsuperscript{2+} channels, the RRF response was examined after relaxing the carotid artery with the L-type Ca\textsuperscript{2+} channel blocker nifedipine. Nifedipine (1 \( \mu \text{M} \)), added when the second contraction in the protocol was stabilized, induced a mean relaxation of 54.97\% ± 2.29\% (n = 4), resulting in a mean tone of 6.08 ± 1.06 mN (n = 4). In these conditions, the retina still caused an important relaxation of the carotid artery (mean relaxation: 76.17\% ± 2.74\%, n = 4).

The plasma membrane Ca\textsuperscript{2+}-ATPase can be inhibited with sodium vanadate.\textsuperscript{15} Vanadate (1 mM) was added to the organ bath 90 minutes before the second contraction, and induced a transient contraction (maximal contraction: 2.51 ± 0.31 mN after 11.75 ± 0.63 minutes, n = 6) in the carotid artery. In the presence of vanadate, the second K\textsuperscript{+} and PGF\textsubscript{2}\alpha-induced contraction was significantly increased (12.91 ± 0.45 mN in the presence vs. 9.94 ± 0.54 mN in the absence of vanadate, P < 0.05, n = 6). The RRF response was significantly diminished in the presence of vanadate (10.44\% ± 3.37\% vs. 35.11\% ± 0.75\% relaxation under control conditions, P < 0.05, n = 6; Fig. 7).

**FIGURE 7.** Relaxations (expressed as the percentage decrease of the tone induced by 30 mM K\textsuperscript{+} and 30 \( \mu \text{M} \) PGF\textsubscript{2}\alpha) of carotid artery in response to application of rat retina in the presence and absence of sodium metavanadate (n = 6; *P < 0.05).

**DISCUSSION**

The present study clearly demonstrates the release of an as yet unknown relaxing substance from the rat retina that is enhanced under hypoxic conditions. This can be demonstrated not only in retina from large animals such as cows, sheep, dogs, and pigs,\textsuperscript{1} but also in a small laboratory animal such as the rat. Because the retinal relaxing factor is not selective for retinal arteries, it is possible to further investigate the characteristics of the RRF, by using, for example, rat carotid arteries as the detector of the RRF. It should be noted, however, that there are important physiological and anatomic differences between rat carotid and retinal arteries. For example, carotid arteries are elastic conducting arteries, and therefore the tunica media in these arteries contains more elastic fibers and less smooth muscle than in retinal arteries; carotid arteries are innervated, whereas sympathetic innervation is absent in the retinal vessels; and retinal vascular endothelial cells are part of the blood-retinal barrier. Despite these differences, this bioassay offers the opportunity of continuously exploring the characteristics and the identity of the RRF in a small laboratory animal, which provides opportunities for in vivo interventions. In our experimental conditions, similar sized samples of other tissues such as the cornea, bladder wall, or mesenteric tissue, failed to relax the rat carotid artery. This provides evidence that retina-induced relaxation is specific and certainly not due to a mechanical artifact.

To characterize the RRF released from rat retinal tissue, a reliable protocol was established. First, we investigated whether reproducible relaxation could be obtained when a rat retina is put repetitively in close proximity with a precontracted rat carotid artery. In preliminary experiments, we found that the rat retina elicits relaxation in rat carotid artery precontracted with norepinephrine, PGF\textsubscript{2}\alpha, or serotonin. Soon it became clear that serotonin- or norepinephrine-induced contractions were too unstable to perform reliable experiments. Also, PGF\textsubscript{2}\alpha-induced contractions tended to decrease toward the end of the experiment. Therefore, a KRB solution containing 30 mM K\textsuperscript{+} and 30 \( \mu \text{M} \) PGF\textsubscript{2}\alpha was used to contract the carotid arteries. The presence of 30 mM K\textsuperscript{+} had a stabilizing effect on both contraction and relaxation. Because it is well known that increased concentrations of K\textsuperscript{+} can have a profound influence on arterial tone,\textsuperscript{16,17} the potential influence of the presence of 30 mM K\textsuperscript{+} on the effect of the application of
the retina had to be investigated. Therefore, some experiments were performed in which the effect of application of the retina was measured in preparations precontracted with or without 30 mM K⁺. Because no significant difference was seen, all further experiments were performed with a precontracting solution containing 30 mM K⁺ and 30 μM PGF₂α, allowing reproducible experiments. In experiments in which two identical series of three consecutive applications of the retina were performed, we found that the mean of the first series of three relaxations was similar to the mean of the second series of relaxations. This protocol with reproducible RRF-induced relaxations offers prospects for pharmacologic interventions between the two series of relaxations to characterize the RRF and the RRF-induced relaxation.

Donati et al.⁶ suggested that NO released from the retina could control retinal arterial tone. These in vivo experiments in the intact retina clearly show that NO is an important possible candidate for the RRF. Therefore, we examined whether NO could be involved in RRF-induced relaxation. NO is formed out of l-arginine by NO synthase and induces a relaxation through cyclic guanosine monophosphate (cGMP) by activating soluble guanylyl cyclase. The formation of NO is blocked by the NO synthase inhibitor L-NA. The relaxing effect of NO can be blocked by the guanylyl cyclase inhibitor ODQ. In the present study, these two blockers were used to investigate the involvement of NO in the relaxation caused by the rat retina. The NO synthesis inhibitor L-NA was unable to abolish the relaxing influence of a rat retina placed on a rat carotid artery. The acetylcholine-induced relaxation, in contrast, was completely blocked by L-NA. It was therefore concluded that inhibition of the formation of NO in both the retina and the carotid artery does not affect the RRF response. Treatment of the rat carotid artery with the soluble guanylyl cyclase inhibitor ODQ caused a small but not significant decrease in RRF response. The relaxation in response to SNP however, was significantly decreased by ODQ. Both ODQ and L-NA caused an increase in contractile tone. An inhibition of the influence of the basal release of NO from the endothelium of the carotid artery is probably responsible for this increase. Both the experiments with L-NA and ODQ show that it is very unlikely that the rat RRF, studied in our experimental conditions, is NO or that its effect is NO dependent.

Pournaras et al.¹² suggested that PGs derived from retinal tissue are mediators of retinal arterial dilation. In the present study, both the retina and the carotid arteries were treated with the COX inhibitors indomethacin or sodium diclofenac. This treatment did not decrease, but in contrast even increased, the relaxation induced by a retina placed in proximity. This increase could be explained by a shift in the arachidonic acid metabolism after inhibition of COX. Further research is necessary to test this hypothesis. We can conclude, however, that the RRF response is certainly not diminished after inhibition of the production of PGs in the carotid artery and the retina. These experiments show that it is very unlikely that the rat RRF is a prostamoid or that its effect is prostamoid dependent.

Many stimuli are known to relax isolated blood vessels in an endothelium-dependent way.¹⁹–²¹ Removal of the endothelium of the rat carotid artery did not significantly decrease the relaxing influence of a retina placed in proximity. The acetylcholine response, however, which is known to be endothelium dependent, was significantly decreased after the endothelium was removed. These results show that the effective removal of the endothelium did not diminish the rat RRF response. That the rat RRF relaxes the artery in an endothelium-dependent way is therefore very unlikely.

These conclusions that rat RRF responses are not mediated by NO, prostamoids, or the endothelium are consistent with the findings in experiments in a previous study of bovine RRF.¹⁴ In that study, the original observation that bovine retinal tissue exerts a marked inhibitory influence on the contractile tone of isolated retinal arterial was described. Besides excluding NO, prostamoids, and the endothelium from mediating the bovine RRF response, the study also eliminated the possible involvement of the major retinal neurotransmitters. Glutamate, glycine, GABA, dopamine, and melatonin did not induce relaxation of the bovine retinal artery. Involvement of proteins or polypeptides was found to be unlikely, because addition of trypsin did not alter the relaxing influence of an incubation solution. Because the RRF response was found to be endothelium independent, involvement of endothelium-dependent vasodilators, such as acetylcholine or histamine, could be excluded. Because the presence of an adenosine receptor blocker failed to affect the relaxation, adenosine was also excluded as a candidate for the bovine RRF.

Hypoxia is a well-known vaso dilatory stimulus in the retinal arterial vascular bed,²² reflecting a physiological regulatory process that adapts blood flow to the metabolic needs of the tissue. Previous research on bovine retinal tissue revealed that hypoxic vasodilation is mediated by the retina.²³ This led us to investigate whether vasodilation of rat carotid artery by application of rat retina is sensitive to hypoxia. It was indeed observed that acute hypoxia largely enhanced retina-induced relaxation. This cannot be explained by a direct effect of hypoxia on precontracted rat carotid artery, because acute hypoxia applied in the absence of retina had only a small relaxing influence. Thus, the response to RRF is largely enhanced in hypoxic conditions, which may be responsible for hypoxic retinal vasodilation. It remains to be determined whether this increased response is due to enhanced release of RRF or another mechanism (e.g., increased sensitivity of the artery to RRF or concomitant release of potentiaing vasodilator).

Considering the increased RRF response in hypoxia, the hydrogen ions, lactic acid, K⁺, PGs, adenosine, and excitatory amino acids such as GABA, glutamic acid, aspartic acid, glycine, and taurine, all described as potential mediators of hypoxia-induced vasodilation²⁴–²⁶ were investigated, either as candidates for the RRF or for mediating the enhanced response to RRF. Hydrogen ions were excluded from being the RRF, because no pH-changes could be measured at the surface of the retina. GABA, glutamic acid, aspartic acid, glycine, and taurine all failed to relax the carotid artery in our experiments. Furthermore, treatment of the retina for 1 hour with one of these metabolites did not change the retina-induced relaxation of the rat carotid artery. Only a high concentration of lactic acid induced a small and transient relaxation of the carotid artery. Treatment of the retina with lactate for 1 hour did not affect the retina-induced relaxation. Also adenosine induced only a small and transient relaxation of the carotid artery in the highest concentration and had no influence on retina-induced relaxation after 1 hour of treatment. Therefore, GABA, glutamic acid, aspartic acid, glycine, taurine, lactic acid, and adenosine can be excluded from playing the role of rat RRF, and from being the mediator of the enhanced response to RRF in hypoxic conditions. The possibility that the RRF is a PG is excluded by the experiments with the COX inhibitors indomethacin and sodium diclofenac, as described earlier in this discussion. Furthermore, the possibility that PGs mediate the enhanced response to RRF in hypoxic conditions is excluded, because the influence of hypoxia is not affected by indomethacin. Because there is no blockade of retina-induced relaxation in the presence of 30 mM K⁺, we also excluded the possibility that potassium ions were the RRF or enhanced the RRF response in hypoxia.
In this study, we also evaluated the potential involvement of several \([\text{Ca}^{2+}]\)-reducing mechanisms in the RRF response. Vasorelaxation in response to the RRF may involve a decrease in intracellular \(\text{Ca}^{2+}\) concentration \((\text{[Ca]}_{\text{L}})\) due to the activation of the SERCA, the plasma membrane \(\text{Ca}^{2+}\)-ATPase, and the \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger. In addition, \([\text{Ca}]\) reduction may be due to the closure of \(\text{L-type Ca}^{2+}\) channels.\(^{23–26}\)

Inhibition of SERCA with thapsigargin and cyclopiazonic acid did not influence \(\text{K}^{+}\)- and \(\text{PGF}_{2\alpha}\)-induced contraction in the carotid artery, nor did it influence retina-induced relaxation. These results suggest that SERCA activation is not involved in the relaxing effect of the RRF.

The involvement of the \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger was assessed with two inhibitors DMTU and amiloride. Both these inhibitors reduced contraction, although the decrease caused by amiloride was not statistically significant. This observation is consistent with previous reports showing that a reverse mode of the \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger may contribute to \(\text{Ca}^{2+}\)-entry produced by a contractile agent.\(^{27,28}\) Because of the significant difference in contractile tone before and after treatment of the carotid artery with DMTU, the mean results of the two series of relaxations were not statistically compared. The mean relaxations, expressed as the percentage of relaxation, seem to be very high in the presence of DMTU, because of the significant decrease in preexisting tone in the second contraction. This decrease caused an increase in the relaxation percentages, without increase in the absolute relaxing influence of the retinal tissue. It was clear, however, that the retina still relaxed the carotid artery after treatment with DMTU. Therefore, it was concluded that DMTU did not abolish the RRF response. Because amiloride caused a less significant decrease in contractile tone, the results of these experiments were statistically analyzed. The RRF response was increased, although this increase was not statistically significant. It was concluded that neither DMTU nor amiloride was able to block retina-induced relaxation. These results suggest that it is very unlikely that the \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger plays an important role in RRF-induced relaxation.

To evaluate the potential involvement of \(\text{L-type Ca}^{2+}\) channels, the RRF response was examined after adding the \(\text{L-type Ca}^{2+}\) channel blocker nifedipine. As expected, nifedipine caused a significant decrease of the contraction, but in the presence of nifedipine, application of the retina still induced an important extra relaxation of the carotid artery. Because of the significant difference in preexisting tone, mean relaxations were not statistically compared. Again, the apparent increase in RRF response can be attributed to the significant decrease in preexisting tone. It is clear, however, that the relaxing influence of the RRF is not diminished by the preceding inhibition of the \(\text{L-type Ca}^{2+}\) channels, suggesting that blocking the \(\text{L-type Ca}^{2+}\) channels does not play an important role in the RRF response.

Inhibition of the plasma membrane \(\text{Ca}^{2+}\)-ATPase with vanadate resulted in a small but significant increase in preconstrictile tone. The retina-induced relaxations, however, were significantly antagonized by vanadate. Although vanadate may also influence other mechanisms,\(^{29,30}\) these results suggest that \(\text{Ca}^{2+}\)-extrusion by the plasma membrane \(\text{Ca}^{2+}\)-ATPase may play an important role in the RRF-induced relaxation of the rat carotid artery. However, because no selective plasma membrane \(\text{Ca}^{2+}\)-ATPase blocker is available, we were not able to differentiate between the different action mechanisms that may be influenced by vanadate. Therefore, these experiments rather suggest than conclusively prove the involvement of the plasma membrane \(\text{Ca}^{2+}\)-ATPase in retina-induced relaxation. Further research is necessary to fully understand the mechanism by which vanadate blocks the rat RRF response.

The present study confirms the existence of an as yet unidentified relaxing factor(s) released from rat retina, as previously shown to be released from retina of cows, dogs, sheep, and pigs and to be very potent on retinal arteries.\(^4\) That a small laboratory animal like the rat can be used for research on the retinal relaxing factor(s) greatly extends the possibilities for RRF research. It offers opportunities to perform in vivo interventions and to use pathologic animal models (e.g., diabetes). Such studies are important because the RRF may be of physiological and pathophysiologival relevance. It may provide an explanation for hypoxic retinal vasodilation and for contracted retinal arteries in diseases associated with retinal cell loss (i.e., retinitis pigmentosa, panretinal photoocoagulation, and descending optic atrophy).

Acknowledgments

The authors thank Eric Tack and Elie Behaeghe for excellent technical assistance.

References


