Presynaptic Effects of Moxonidine in Isolated Buffer Perfused Rat Hearts: Role of Imidazoline-1 Receptors and $\alpha_2$-Adrenoceptors

ULRICH SCHÄFER, CHRISTOF BURGDORF, ASTRID ENGELHARDT, THOMAS KURZ, and GERT RICHARDT
Medizinische Klinik II, Medizinische Universität zu Lübeck, Lübeck, Germany
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ABSTRACT
Numerous studies support the concept that centrally acting antihypertensive drugs, such as imidazolines, mediate sympathoinhibition not only via activation of central nervous $\alpha_2$-adrenoceptors ($\alpha_2$-AR) but also via imidazoline-1 receptors ($I_1$-R). An additional presynaptic involvement in sympathetic neurotransmission of imidazolines, via $I_1$-R independent of $\alpha_2$-AR, is still controversial and remains to be clarified in the heart. Concentration response curves on endogenous norepinephrine (NE) overflow evoked by stimulation of epicardial postganglionic sympathetic nerves in isolated buffer-perfused rat hearts were performed for brimonidine, moxonidine, rauwolscine, and efaroxan. To unmask an $I_1$-R-mediated effect of moxonidine, hearts were pre-exposed in additional experiments with brimonidine or rauwolscine with or without AGN192403 or efaroxan. This effect was also totally inhibited with pre-exposure with indomethacin (10$^{-7}$ M) and tricyclodecan-9-yl-xanthogenate (D-609), an inhibitor of phosphatidylinositol-selective phospholipase C (PC-PLC; 10$^{-7}$ M). Conversely, moxonidine was without modulating efficacy under $\alpha_2$-AR-blockade by rauwolscine. In summary, we demonstrate that moxonidine reduces NE release independently of $I_1$-R, demonstrating the prominent effect of $\alpha_2$-AR in cardiac tissue under basal conditions. We also demonstrate that $I_1$-R are involved in NE release under conditions of $\alpha_2$-AR-stimulation involving both a pathway of prostaglandins and PC-PLC.

Numerous studies support the concept that centrally acting antihypertensive drugs such as clonidine and related imidazoline derivatives mediate sympathoinhibition not only via activation of central nervous $\alpha_2$-adrenoceptors ($\alpha_2$-AR) but also via imidazoline-1 receptors ($I_1$-R). Recently, radioligand binding studies have identified $I_1$-R in the heart (El-Ayoubi et al., 2002). In addition, another presynaptic inhibitory subclass of imidazoline binding sites (I-BS), namely the non-$I_1$-/non-$I_2$-BS, sharing different pharmacological properties of the well known $I_1$- and $I_2$-BS has been characterized in cardiovascular tissue (Molderings and Göthert, 1999, Göthert et al., 1999). Moreover, imidazoline derivatives have been demonstrated to interact with the sympathetic neurotransmission via these nonadrenergic presynaptic receptors in different experimental models. I-BS-mediated inhibition of electrically evoked [3H]NE overflow was observed in the tissue of rabbit pulmonary artery/aorta (Göthert and Molderings, 1991; Molderings et al., 1991; Molderings and Göthert, 1995) and human right atrium (Molderings et al., 1997, 1999). Inhibition of endogenous NE release...
release from sympathetic nerve endings through activation of I-R has been further described in isolated perfused rabbit hearts (Fuder and Schwarz, 1993). Conversely, under α2-AR autoinhibition-free conditions, typical I-R ligands have failed to influence NE release via putative I-R (Gaiser et al., 1999). Moreover, the α2A-AR has been identified as the major presynaptic autoinhibitory receptor subtype regulating NE release from sympathetic nerves (Altman et al., 1999). These observations indicate that the significance of presynaptic I-R in cardiac tissue is disputable.

Since the possible existence of a presynaptic action of moxonidine independent of α2-AR remains to be clarified in the heart, we performed experiments in isolated buffer perfused rat hearts to discriminate between α2-operated and I-R-mediated effects. To unmask a partial I-R-mediated effect of moxonidine, rat hearts were pre-exposed with the α2-AR-agonist brimonidine or the α2-AR-antagonist rauwolscine in additional experiments. Endogenous NE overflow evoked by stimulation of epicardial postganglionic sympathetic nerves was investigated with different protocols of pharmacological intervention.

Materials and Methods

Isolated Perfused Heart. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Male Wistar rats (mean weight 238.5 g; Charles River, Sulzdorf, Germany) were anesthetized with thiopental sodium (100–200 mg/kg i.p.; Trapanal; thiopental sodium; ALTANA-Pharma, Konstanz, Germany). A medial laparotomy was carried out, and 0.1 ml of heparin sodium (500 U; Liqueum; heparin sodium; Roche, Grenzach-Whylen, Germany) was injected into the inferior vena cava. The thorax was opened, and the heart was rapidly removed, weighed (mean weight 0.89 g), and placed in cold perfusion buffer. Isolated hearts were perfused via the ascending aorta at a constant flow of 8 ml/min/g heart weight with a modified Krebs-Henseleit solution (consisting of 142 mM NaCl, 1.85 mM Ca2+, 1.1 mM Mg2+, 135 mM Cl−, 0.22 mM H2PO4−, 16.7 mM HCO3−, 11 mM glucose. EDTYA 0.027). The Krebs-Henseleit solution was saturated with 95% O2/5% CO2. The gas flow was adjusted to achieve a pH of 7.4. All experiments were performed in the presence of desipramine (10−7 M) to inhibit neuronal uptake and non-exocytotic release of NE.

Exocytotic NE Release. Two 13 × 8-mm, concavely shaped, metal paddles were placed in opposite positions on each heart, touching the heart in such a manner that the interventricular septum was located between both paddles. NE release was induced by two electrical field stimulations at 20 and 35 min during perfusion (S1 and S2; each 1 min, 5 V, 6 Hz, 2-ms pulse width). The effect of each pharmacological intervention on NE release, which started either at 10 min (S1) or at 23 min (S2) of perfusion, was intrinsically compared with the release after S1 (expressed as ratio S2/S1). Stimulation-induced NE release as described has been characterized previously to be exocytotic (Schömig et al., 1987).

Experimental Procedures. After the aorta was cannulated, each heart was perfused for 23 min without intervention (equilibration period). Baseline NE release (S1) was induced in the 21st min of perfusion and S2 in the 36th min. Concentration response curves regarding NE release were performed for brimonidine, moxonidine, rauwolscine, AGN192403, and efaroxan. The infusion of the substance to be tested was started after an equilibration period and continued until the end of the experiment. In combination experiments, the equilibration period was 10 min, and each drug was given during S1 and S2, whereas moxonidine was present only during S2.

Statistical Analysis. The results in the figures are expressed as ratio of S2/S1 (percentage of baseline NE release, mean ± S.E.M.). The relative inhibition of evoked overflow in relation to a corresponding group of control experiments and the concentration of agonist causing 50% reduction (IC50) were calculated by curve fitting.

In the absence of stimulation, NE concentrations in the effluent were below the detection limit (< 0.1 pmol/g). None of the drugs that were used induced a detectable NE release before S1 and S2. Electrically stimulated exocytotic NE release was calculated as cumulative overflow during and 2 min following each stimulation period. Control experiments with two electrical field stimulations in the same heart performed 14 min apart showed a comparable amount of NE release (S1: 201 ± 26 pmol/g; S2: 196 ± 21 pmol/g; n = 14).

Effects of Brimonidine and Efaroxan. Application of the α2-AR antagonist rauwolscine (10−8–10−5 M; S2) concentration dependently enhanced NE release from sympathetic nerve endings compared with control (Fig. 1A). Low concentrations of rauwolscine markedly increased NE release (log EC50 = −8.43 ± 0.04). AGN 192403 (10−7–10−4 M; S2), characterized as highly selective I-R ligand, did not affect transmitter release compared with control hearts without drug infusion (Fig. 1A). Conversely, the mixed antagonist (at α2-AR and I-R) efaroxan, infused at concentrations of 10−6–10−5 M (S2), increased dose dependently NE release with a log EC50 of −6.32 ± 0.13 (Fig. 1A).

Effects of Brimonidine and Moxonidine. Infusion of the α2-AR agonist brimonidine (10−8–10−5 M; S2) and moxonidine (10−8–10−5 M; S2) concentration dependently suppressed NE release from sympathetic nerve endings compared with control (Fig. 1A). Moxonidine at low concentrations (10−8–10−7 M) did not affect transmitter re-
lease, whereas a NE release suppression was observed at concentrations higher than $10^{-7}$ M. The inhibitory potency of brimonidine was 1.36-fold ($10^{-7}$ M), 1.47-fold ($10^{-6}$ M), and 1.68-fold ($10^{-5}$ M) higher compared with moxonidine at equimolar concentrations. The EC$_{50}$ of moxonidine was only mildly lower compared with brimonidine (log EC$_{50}$: $-5.75 \pm 0.49$ versus $-6.15 \pm 0.14$), indicating a similar receptor-mediated effect of both substances (Fig. 1B).

Effects of Moxonidine in Combination with AGN192403. The application of AGN194203 ($10^{-6}$ M) throughout the experiment (S1 + S2) was without effect on moxonidine-induced (log EC$_{50}$: $-6.01 \pm 0.25$) modulation of NE release compared with control (moxonidine at S2; Fig. 2).

Effects of Moxonidine in Combination with Rauwolscine. The coinfusion of rauwolscine ($10^{-5}$ M; S1 + S2) at a concentration sufficient to block all $\alpha_2$-AR as indicated by
reaching a plateau in the concentration response curve (Fig. 1A) completely abolished any modulating efficacy of moxonidine (10^{-6} M) (Fig. 3A). AGN192403 in combination with rauwolscine (S1 + S2) did not further influence the absence of an effect of moxonidine (Fig. 3B). The S2-stimulation in this set of experiments revealed significant (p < 0.001) lower NE concentrations compared with S1 (control; i.e., rauwolscine 10^{-5} M without moxonidine), indicating depletion of NE stores in the presynaptic nerve terminals.

Effects of Moxonidine in Combination with Brimonidine and AG0N192403 or Efaroxan. Moxonidine (10^{-6} M; S2) decreased stimulated NE release under continuous application of brimonidine (10^{-5} M; S1 + S2) by 55.2% (Fig. 3C). Surprisingly, the inhibition of transmitter release was 2.3-fold stronger with moxonidine (10^{-6} M) under brimonidine compared with the concentration response curves of moxonidine without brimonidine. Moreover, this 2.3-fold potentiating efficacy of moxonidine was completely preventable after coinfusion of AGN192403 (10^{-5} M; S1 + S2; Fig. 3D). The combination of brimonidine with AGN192403 revealed a significant reduction of the inhibitory action (i.e., S2 > S1) of brimonidine (p = 0.04). To further clarify these findings, we performed concentration response curves of moxonidine (10^{-8}-10^{-4} M; S2) after pre-exposure with brimonidine (10^{-6} M; S1 + S2) alone and brimonidine (10^{-6} M) either in combination with AGN1192403 (10^{-6} M) or efaroxan (10^{-6} M), respectively. In lower concentrations, moxonidine decreased the inhibitory action of brimonidine (S2/S1 > 100%), whereas higher concentrations tended to decrease the S2/S1 ratio. Again, after pre-exposure with brimonidine in combination to AGN192403 or efaroxan, respectively, the sigmoidal dose-response curve of moxonidine (Fig. 4A) was abolished with AGN192403 (Fig. 4B) and efaroxan (Fig. 4C).

Effects of Moxonidine in Combination with Brimonidine and D-609 or Indomethacin. Both D-609 (10^{-7} M; S2) and indomethacin (10^{-7} M; S2) were without significant effect on transmitter release (Fig. 5, A and B). Under continuous application (S1 + S2) again, neither D-609 nor indomethacin influenced the inhibitory action of moxonidine (S2) on stimulated NE release. Interestingly, during continuous application of brimonidine (10^{-5} M; S1 + S2), both inhibitors D-609 and indomethacin were able to abolish the prominent inhibitory action of moxonidine (10^{-6} S2; Fig. 5, A and B).

Discussion

Moxonidine remains the most selective and potent agonist at the I1-R described to date with a 30- to 700-fold selectivity for I1-R over \( \alpha_2 \)-AR. Furthermore, its marked potency in lowering blood pressure relative to its weakness as a pure \( \alpha_2 \)-AR-agonist is well known. In the present study, we wanted to find out whether moxonidine is able to decrease NE release independent of \( \alpha_2 \)-AR in isolated buffer-perfused rat hearts. With the availability of a new pharmacological tool, AGN192403 (the selective ligand at I1-R), we performed experiments to discriminate between \( \alpha_2 \)-AR- and I1-R-mediated effects.

The main findings in the present study were: 1) moxonidine was able to reduce stimulated NE overflow in isolated buffer-perfused rat hearts; 2) AGN192403, a selective ligand at I1-R, had no influence on the dose-response curve of moxonidine, demonstrating the prominent effect of \( \alpha_2 \)-AR-mediated presynaptic autoinhibition of NE release in cardiac tissue; 3) moxonidine was without modulating efficacy under \( \alpha_2 \)-AR-blockade by rauwolscine; 4) conversely, moxonidine strongly enhanced the reduction of NE release under pre-stimulation of \( \alpha_2 \)-AR by brimonidine, a selective agonist at \( \alpha_2 \)-AR; this effect was completely abolished by AGN192403 and efaroxan, indicating an interdependence of both receptors (\( \alpha_2 \)-AR + I1-R) under conditions of \( \alpha_2 \)-AR-stimulation/desensitization; and 5) this interdependence of both receptors seems to involve a pathway of prostaglandins and phosphatidylcholine-selective phospholipase C since both indomethacin and D-609 were able to block the potentiating inhibitory efficacy of moxonidine.

Previous studies have characterized moxonidine to act mainly as a selective agent at \( \alpha_2 \)-AR and less as an agonist at I-B5, because of its lacking effect on evoked NE release under \( \alpha_2 \)-AR blockade (Molderings et al., 1991; Molderings and Göhert 1995). Despite the possible displacement of rauwolscine from \( \alpha_2 \)-AR by moxonidine, we could not find any modulating activity in NE overflow either by the imidazoline-derivative moxonidine alone or in combination with...
Fig. 3. Influence of moxonidine (10⁻⁶ M) versus control on endogenous-stimulated norepinephrine overflow (S2) after pre-exposure with rauwolscine (10⁻⁶ M) (A), rauwolscine (10⁻³ M) and AGN192403 (10⁻⁵ M) (B), brimonidine (10⁻⁵ M) (C), and brimonidine (10⁻⁵ M) and AGN192403 (10⁻⁵ M) (D) administered throughout the experiment (during S1 and S2). The effect of each pharmacological intervention on norepinephrine release (S2) is intraindividually compared with the release during control condition (S1) and expressed as ratio of S2/S1 (percentage of control release). Mean ± S.E.M., n = 5–8/group, * denotes p < 0.05 by Student’s t test. Depicted in the small inserted graphs is the influence on endogenous-stimulated norepinephrine overflow during control stimulation. The effect of each pharmacological intervention in the control group on norepinephrine release (S2) is intraindividually compared with the release during baseline condition (S1) and expressed as ratio of S2/S1.
AGN192403. Moreover, we could clearly demonstrate that the inhibitory action of moxonidine on NE release under electrically stimulated basal conditions is solely dependent on α2-AR since pretreatment with AGN192403 was without effect.

Comparing the release-modifying effects of efaroxan and AGN192403, the dose-response curve of efaroxan revealed a prominent increase in NE (+350%), whereas AGN192403 was without any modulating activity. This observation supports the concept of a predominant presynaptic autoinhibition of NE release by α2-AR, whereas 1-R tend to play a minor role under electrically stimulated-basal conditions. Since AGN192403 has been shown to be highly selective for 1-R (Munk et al., 1996), the presynaptic blockade of the autoinhibitory α2-AR by efaroxan seems to be, at the first glance, the sole receptor type to be involved in NE release after exposure to efaroxan. The greater magnitude in NE release under efaroxan (concentrations exceeding 10⁻⁶ M) compared with rauwolscine is somehow surprising, however, since the EC₅₀ of rauwolscine is 75-fold lower compared with efaroxan, despite similar affinity at α₂-AR (Ernsberger et al., 1997). A recent article indicated that 1-R ligands such as moxonidine, efaroxan, and benzazoline, the former described as high-affinity 1-R ligands, exhibited only low affinities at 1-R labeled with [¹²⁵I]LNP 911, a new highly selective radiiodinated probe for 1-R (Greney et al., 2002). Furthermore, this article suggests the existence of an allosteric modulation site at the level of the 1-R, probably sensitive to moxonidine, accelerating the rate of dissociation of [¹²⁵I]LNP 911. In contrast, allosteric activation of the rat α₂⁺AR by agmatine, a proposed endogenous ligand at I-BS, has also been suggested as a possible mechanism to influence NE release since agmatine dose dependently increased the inhibitory effect of moxonidine and clonidine at segments of rat vena cava (Molderings et al., 2000). Additionally, influence on the rate of association and dissociation of clonidine and rauwolscine binding has been demonstrated with agmatine. Therefore, allosteric interactions might be an explanation for the observed differences between rauwolscine and efaroxan but remains unproven in our study.

Since there is still a broad evidence that NE release is regulated exclusively through an activation of prejunctional α₂-AR (Gaiser et al., 1999), we wanted to know if moxonidine might interfere with the selective agonist brimonidine at α₂-AR in a simple additive manner (i.e., shifting the dose-response curve to the left). We tried to unmask a potential effect at 1-R of moxonidine upon pre-exposure to α₂-AR stimulation with brimonidine and coinfused AGN192403 or efaroxan throughout these experiments to elucidate whether 1-R are involved in addition to α₂-AR.

In experiments with pre-exposure to brimonidine (10⁻⁶ M), we could not find a left shift of the dose-response curve but rather found a biphasic profile of transmitter modulation by moxonidine. During control-stimulation (brimonidine at S1 and S2), we found a S2/S1 ratio of 102%, ruling out a homol-gous desensitization with brimonidine (10⁻⁶ M). The inhibitory action of moxonidine (reduction of brimonidine-mediated inhibition of transmitter-release) might again be explained by allosteric interactions at the receptor level resulting in that biphasic profile of transmitter-modulation. The interesting finding in subsequent combination experiments was that this effect was blocked with AGN192403 and the mixed antagonist efaroxan, respectively.

In another set of experiments pre-exposure of brimonidine at submaximal concentrations (83% of ECmax), we found a strong potentiation of the inhibitory effect of moxonidine. Since this effect was again blocked with the selective 1-R-ligand AGN192403, an 1-R-mediated effect is suggested, and there is some evidence for a mutual cross activation between both receptor types. Interestingly, the combination of AGN192403 and brimonidine induced a distinct loss of the inhibitory action of brimonidine, again indicating an inter-
dependency between both receptor types. Nevertheless, moxonidine was without any inhibitory efficacy after pretreatment with AGN192403 and brimonidine. This surprising phenomenon indicates the possibility of a presynaptic agonistic inhibitory property at I1-R being unmasked after submaximal \(\alpha_2\)-AR-activation (a high degree of \(\alpha_2\)-AR-occupancy seems to be required). Nevertheless, the question to the exact mechanistic cross talk between both presynaptic receptors arises. The intraneuronal signal transduction pathway used by \(\alpha_2\)-AR has been suggested to involve the adenyl cyclase-cAMP system (Schoffelmeer et al., 1986) to rely on a G protein-mediated blockade of voltage-gated Ca\(^{2+}\) channels (Boehm and Huck, 1997), as well as other heteroreceptors (opioid, A1-receptors), and to be linked to pertussis toxinsensitive G proteins (Gobel et al., 2000).

Since the transmembrane signaling pathway and intracellular second messengers linked with I1-R have been suggested to involve a phosphatidylycholine-selective phospholipase C (PC-PLC) and the accumulation of diacylglycerol (Separovic et al., 1996) as well as the accumulation of free arachidonic acid and the release of prostaglandins (Ernberger et al., 1997), we performed experiments using selective blockers of PC-PLC and cyclooxygenase. Interestingly, both substances, D-609 and indomethacin, were able to completely block the inhibitory action of moxonidine under \(\alpha_2\)-AR-stimulation with brimonidine, confirming the aforementioned hypothesis of this mutual cross talk between I1-R and \(\alpha_2\)-AR. A hypothetical model of neuronal pathways with I1-R located upstream of \(\alpha_2\)-AR within the central nervous system was first proposed by Head (1995). This model offered an explanation for the observed phenomenon, i.e., that the effects of moxonidine can be antagonized by I1-R-antagonists (upstream) but only with high doses of \(\alpha_2\)-AR-antagonists (downstream).

Since our results clearly suggest an \(\alpha_2\)-AR-dependent I1-R-mediated mechanism to decrease the stimulated NE overflow, the question of the clinical significance of this cross talk between both presynaptic receptors arises. Recently, I1-R have been characterized in the heart under pathophysiological conditions. Interestingly, I1-R are up-regulated in the presence of hypertension or heart failure, suggesting their involvement in cardiovascular regulation (El-Ayoubi et al., 2002). Moreover, I1-R have been shown to be involved in the release of atrial natriuretic peptide (Gutkowska et al., 1997; Mukaddam-Daher et al., 1997) and prostaglandins (Ernberger et al., 1995). Prostaglandins (Starke and Montel, 1973; Wennmalm and Junstad, 1976) and atrial natriuretic peptide (Zukowska-Grojec et al., 1986; Drewett et al., 1988) in turn were found to reduce NE release from sympathetic nerves. Therefore, it is possible that moxonidine reduces NE release under \(\alpha_2\)-AR-stimulation via such an I1-R-mediated indirect mechanism. Imidazoline derivatives like moxonidine might be of clinical interest under conditions of hyperadrenergic states that causes enhanced NE spill over, as observed in hypertension, congestive heart failure, and myocardial infarction with a consecutive activation of presynaptic \(\alpha_2\)-AR. The great magnitude of sympatholytic activity (Floras, 2002) was recently demonstrated for moxonidine in two heart failure trials (Swedberg et al., 2002; MOXCON). Unfortunately, moxonidine-treatment in very high dosages was associated with adverse outcome due to increased mortality and worsening of heart failure (MOXCON), probably due to insufficient residual sympathetic outflow to support cardiac output or peripheral resistance. One fundamental question is whether this excessive sympatholysis might be attributable to the synergistic efficacy of moxonidine at \(\alpha_2\)-AR and I1-R, under increased presynaptic \(\alpha_2\)-autoreceptor activation (hyperadrenergic circumstances), as observed in our study.

In summary, we have demonstrated in vitro that moxonidine reduces NE release independently of I1-R under electrically stimulated-basal conditions. But we could also demonstrate that I1-R are involved in NE release under conditions of \(\alpha_2\)-AR-stimulation comprising prostaglandins and PC-PLC.
References


Address correspondence to: Dr. Ulrich Schäfer, Medizinische Klinik II, Universitätsklinikum Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany. E-mail: uSchaefer@t-online.de.