α-Adrenoceptor-Mediated Prejunctional Effects of Chloroethylclonidine in the Canine Saphenous Vein

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ABSTRACT
The present study was undertaken to look for the effect of chloroethylclonidine (CEC) on prejunctional alpha-2 adrenoceptors of the canine saphenous vein. The effect was tested on tritium overflow evoked by electrical stimulation from tissues preloaded with 0.2 µM 3H-norepinephrine. Yohimbine (3–300 nM) and CEC (1–125 µM) increased and UK-14,304 reduced the overflow of tritium evoked by 300 pulses (1 Hz). The maximal increase of tritium overflow caused by yohimbine was much higher than that caused by CEC: 3.82 and 1.74 times, respectively. CEC (5 µM) abolished both the inhibition caused by UK-14,304 and the enhancement of tritium overflow caused by yohimbine. However, when CEC was added after yohimbine, it reduced the electrically evoked overflow of tritium, the maximal effect being a reduction of tritium overflow by 35%. Prazosin (1–100 nM) did not change either the inhibitory effect of UK-14,304 or the facilitatory effect of CEC. These results suggest that CEC acts on two different subtypes of prejunctional alpha-2 autoreceptors; on one of them it acts as an antagonist and increases the electrically evoked overflow of tritium (and inhibits both the effect of UK-14,304 and yohimbine); on the other it acts as an agonist and reduces the electrically evoked overflow of tritium. Alternatively, one can admit that CEC is able to inhibit alpha-2 autoreceptors, which causes an increase of the transmitter release, and to activate a nonadrenergic inhibitory receptor thus causing a reduction of the transmitter release.

Chloroethylclonidine was described by Leclerc et al. (1980) as the first example of an alpha adrenoceptor agonist with an irreversible effect. Later on, CEC was found to bind irreversibly to alpha-1B adrenoceptors and is now used both in binding and functional studies as an antagonist in the current definition of alpha-1 subtypes (Han et al., 1987; Minne- man et al., 1988). More recently CEC was found to be an irreversible agonist at postjunctional alpha-2 adrenoceptors of the canine saphenous vein; this effect involved the activation of receptors that differ from those activated by UK-14,304 (Nunes and Guimarães, 1992, 1993; Low et al., 1994).

Apart from the postjunctional effects of CEC, there is evidence for its action on prejunctional adrenoceptors. In the rat vas deferens, CEC reduces the release of norepinephrine and of a purinergic co-transmitter by irreversible stimulation of prejunctional alpha-2 adrenoceptors, and this effect is prevented by pretreatment with rauwolscine (Bültmann and Starke, 1993). Also in vivo (in the pithed rat), CEC (25 µg/kg) significantly reduced the pressor response to electrical stimulation of spinal sympathetic nerves and this inhibitory effect was antagonized by idazoxan, which indicates that CEC activates prejunctional alpha-2 autoreceptors (Vargas et al., 1994).

The present investigation was undertaken to study the effect of CEC on prejunctional alpha adrenoceptors of the canine saphenous vein.

A preliminary report of these results was presented at the 8th Meeting on Adrenergic Mechanisms (Guimarães and Paiva, 1994).

Materials and Methods
Tissue preparations. In the municipal dog pound, mongrel dogs, 10 to 16 kg in weight, of either sex, were anesthetized with pentobarbitone sodium (30 mg/kg). Immediately after removal, the saphenous veins were placed in small vials containing aerated (95% O2 and 5% CO2) and cold Krebs-Henseleit solution of the following composition (mM): NaCl, 118.6; KCl, 4.70; CaCl2, 2.52; KH2PO4, 1.18; MgSO4, 1.23; NaHCO3, 2.50; glucose, 10; ascorbic acid, 0.57; disodium EDTA, 0.027 (Guimarães et al., 1987). The animals were sacrificed by an overdose of pentobarbitone sodium (100 mg/kg). The veins were then transported to the laboratory where they were helically cut into small strips (of about 2.5 × 25 mm) which were preincubated for 30 min in medium containing 1 mM pargyline (to inhibit monoamine oxidase), 40 µM hydrocortisone and 50 µM...
U-0521 (3,4-dihydroxy-2-methylpropiophenone) (to inhibit extraneuronal removal of norepinephrine) (Guimarães et al., 1978). Hydrocortisone and U-0521 were also kept in the medium for the remainder of the experiment. After preincubation, the vessel segments were exposed for 60 min to $^3$H-norepinephrine (0.2 μM).

**Perifusion experiments.** The vessel segments were mounted in 1-ml glass chambers between two platinum electrodes and perfused with amine-free medium (aerated and at 37°C) moving from bottom to top at a flow rate of 0.8 ml/min. From $t = 100$ min (at which the onset of the perfusion) the perfusion fluid was collected continuously as 5-min samples. Transmural electrical stimulation (1 Hz, 2 ms, 100 V, for 5 min; Stimulator II X, Hugo Sachs Elektronik, March-Hugstetten, Germany) was applied at min 120 ($S_1$), 170 ($S_2$), 220 ($S_3$), 270 ($S_4$) and 320 ($S_5$). In addition to hydrocortisone and U-0521, cocaine (12 μM) was also present in the perfusion fluid from 90 min onward.

To test the effect of UK-14,304 [5-bromo-6-(imidazoline-2-ylamino)quinoxaline], chloroethylclonidine or yohimbine, each of these drugs was used alone, being added cumulatively to the perfusion fluid in concentrations increasing by about half-log increments. These additions were made 20 min (UK-14,304 or yohimbine) or 30 min (chloroethylclonidine) before $S_1$, $S_3$ and $S_5$. To study the influence of yohimbine on the effect of UK-14,304 or CEC, yohimbine was added to the perfusion fluid 20 min before $S_1$ and left in this fluid for the remainder of the experiment. Likewise, to study the influence of CEC on the effect of UK-14,304 or yohimbine, CEC was added to the perfusion fluid 30 min before $S_1$ and left in this fluid for the remainder of the experiment.

For the calculation of the overflow elicited by electrical stimulation, those samples were taken into account in which the overflow of tritium exceeded that in the last prestimulation sample; usually this applied to the three or four samples collected during and after stimulation. The spontaneous overflow measured in the last prestimulation sample was assumed to represent the spontaneous outflow in subsequent samples; it was subtracted from the overflow determined in stimulation and poststimulation samples. Fractional $^3$H-efflux/min was calculated by dividing $^3$H-efflux in each 5-min sample by tritium present in the tissue at the onset of the respective collection period and by 5. The fractional release was calculated by dividing evoked tritium overflow by tritium present in the tissue at the beginning of the stimulation period. Drug effects are expressed as percentage of the fractional release of tritium evoked by $S_0$ (or $S_4$ or $S_5$) over that evoked by $S_2$. Each result was corrected for tissue-dependent changes as determined in parallel drug-free control experiments.

IC$_{50%}$ represents the concentration of the agonist that reduces the evoked overflow of tritium by 50% and EC$_{50%}$ represents the concentration of the antagonist that increases the evoked overflow by 50%. Both IC$_{50%}$ and EC$_{50%}$ values were determined by interpolation between the two nearest points from the 50% values of the concentration-response curves.

**Determination of tritium in the overflow and in the tissue.** Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta LKB Wallac, Turku, Finland) in 2-ml aliquots of perifusate or 0.5 ml of tissue extract + 1.5 ml of Krebs-Henseleit solution. The extraction was made in 3 ml of 0.1 M perchloric acid during 18 h.

**Statistical analysis.** Results are expressed as arithmetic means ± S.E.M. or as geometric means with 95% confidence limits. One-way analysis of variance was used to test differences between unpaired results. A probability level of .05 or less was considered statistically significant.

**Drugs.** Chloroethylclonidine hydrochloride (RBI, Natick, MA); cocaine hydrochloride (Uquapa, Lisboa, Portugal); hydrocortisone 21-hemisuccinate (Sigma, St. Louis, MO); $^3$H-T-4-(-)-norepinephrine (12.4 Ci mmol$^{-1}$) (New England Nuclear, Dreieich, Germany); paroxetine hydrochloride (Sigma); U-0521 (3,4-dihydroxy-2-methylpropiophenone; Upjohn Kalazamoo, MI); UK-14,304 [5-bromo-6-(imidazoline-2-ylamino)quinoxaline)] (Pfizer, Seixal, Portugal); yohimbine hydrochloride (Sigma)

**Results**

In the first series of experiments, vein strips prelabeled with $^3$H-norepinephrine were stimulated electrically five times; each stimulation period ($S_1$ to $S_5$) consisted of a train of 300 pulses (1 Hz). In the absence of drugs, the spontaneous outflow of tritium decreased slowly with time. However, the fractional rate of loss (spontaneous outflow per min divided by tritium content of the tissue) remained constant as in previous studies (Guimarães et al., 1978). The fractional rate of spontaneous tritium outflow immediately before $S_2$ was $0.000162 ± 0.000006$ min$^{-1}$ ($n = 30$) and the overflow elicited by $S_2$ amounted to 0.332 ± 0.017% ($n = 42$) of tritium content of the tissue. In the absence of drugs, the evoked overflow expressed in % of tissue content (see “Materials and Methods”) remained approximately constant from $S_1$ to $S_5$ in control experiments (for example, ratio $S_5/S_2 = 0.99 ± 0.10$; $n = 5$).

**Effect of UK-14,304.** In most of the experiments, UK-14,304 was added cumulatively to the perfusion fluid; three concentrations were used per strip: 3, 10 and 30 nM or 10, 30 and 100 nM. The first, the second and the third addition were made 20 min before $S_3$, $S_4$ and $S_5$, respectively. UK-14,304 caused concentration-dependent reductions of tritium overflow evoked by electrical stimulation with an IC$_{50%}$ value of 16.9 (14.6; 19.7) nM ($n = 5$). The maximal effect of UK-14,304 was a reduction of tritium overflow by 77.0 ± 5.3% ($n = 6$) (fig. 1).

**Effect of CEC.** CEC did not change the fractional rate of spontaneous tritium overflow. In some experiments cumulative concentration-response curves were determined for the effect of CEC (1–125 μM) on the electrically evoked overflow of tritium. As for UK-14,304, only three concentrations were used per experiment: 1, 5 and 25 μM or 5, 25 and 125 μM. The first addition of CEC (1 or 5 μM) was performed 30 min before $S_3$, the second (5 or 25 μM) 30 min before $S_4$ and the third (25 or 125 μM) 30 min before $S_5$. As shown in figure 2, CEC (1–125 μM) caused a concentration-dependent increase of the electrically evoked overflow of tritium, its EC$_{50%}$ being 14.1 (5.1; 39.3) μM ($n = 6$). The maximal effect of CEC was obtained with 125 μM and increased the evoked overflow by a factor of 1.74 (1.45; 2.09) ($n = 6$).

In some experiments, the influence of CEC on UK-14,304 effects was studied. In these experiments CEC was added to the perfusion fluid 30 min before $S_1$ and left in this fluid for the remainder of the experiment. Under these conditions, 5 μM CEC abolished the inhibitory effect of UK-14,304 (3–100 nM) (fig. 1).

**Effect of yohimbine.** When used alone, yohimbine (3–300 nM) was added cumulatively to the perfusion fluid according to the schedule which was similar to that for UK-14,304 and CEC; 3, 10 and 100 nM were used in some experiments and 10, 30 and 300 nM in others. The first addition of yohimbine to the perfusion fluid was made 20 min before $S_3$ and the subsequent ones 20 min before $S_4$ and $S_5$. As shown in figure 3, yohimbine increased the overflow of tritium evoked by electrical stimulation in a concentration-dependent manner. The EC$_{50%}$ of yohimbine was 3.1 (1.2; 7.9) nM ($n = 4$) and its maximal effect increased the electrically evoked overflow of
tritium by a factor of 3.82 (3.06; 4.39) (fig. 3). When CEC (5 μM) was added to the perifusion fluid before yohimbine (see “Materials and Methods”) it abolished this enhancing effect of yohimbine (fig. 3).

When yohimbine (100 nM) was added to the perifusion fluid before the other drugs under study (see “Materials and Methods”) it abolished the inhibitory effect of UK-14,304 (3–100 nM) (fig. 1) and reversed that of CEC which became inhibitory (fig. 2). The maximal inhibitory effect of CEC after yohimbine reduced tritium overflow by 35.2% ± 5.8% (n = 2).

Effect of prazosin. When used alone, prazosin (1–100 nM) did not change either the spontaneous efflux of tritium or its overflow elicited by electrical stimulation. In concentrations up to 100 nM, prazosin also did not change either the inhibitory effect of UK-14,304 (3–100 nM) or the facilitatory effect of CEC (5–125 μM). At the concentration of 1 μM, prazosin caused a small but significant displacement of the concentration-response curve of CEC to the right (fig. 4).

Discussion

In the first study of the effects of CEC at the prejunctional level it was shown that in the rat vas deferens CEC reduced the release of noradrenaline by irreversible activation of prejunctional α-2 adrenoceptors (Bültmann and Starke, 1993). In the present study it was shown that CEC, in a concentration-dependent way, increased the overflow of tritium evoked by electrical stimulation in the canine saphenous vein, which indicates that CEC inhibits prejunctional α-2 adrenoceptors in this tissue. Furthermore, CEC abolished the concentration-dependent reduction of the electrically evoked overflow of tritium caused by the selective α-2 adrenoceptor UK-14,304 confirming that CEC inhibits prejunctional α-2 autoreceptors in the canine saphenous vein. This is the first report of an antagonistic action of CEC at prejunctional α-2 adrenoceptors.

Surprisingly, in the presence of the classical selective α-2 adrenoceptor antagonist yohimbine, the effect of CEC was reversed, a reduction of the overflow evoked by electrical stimulation being observed. This unexpected finding may be
explained on the basis of the existence of more than one kind of receptor for CEC. The first example of such a reversal phenomenon, the conversion of a pressor effect to exogenous epinephrine into a depressor one by ergotoxine, was observed in vivo and was described by Dale (1906). Only 42 years later this reversal phenomenon was interpreted by Ahlquist (1948) on the basis of the existence of two different types of adrenoceptors. Some years later, such a reversal phenomenon was also shown in isolated organs. In strips of saphenous vein previously contracted by prostaglandin F2α in the presence of phentolamine, epinephrine caused a concentration-dependent relaxation; similarly, in strips preloaded with epinephrine and previously contracted by prostaglandin F2α in the presence of phentolamine, electrical stimulation caused frequency-dependent relaxations. This inhibitory response to either exogenous or "endogenous" epinephrine was also interpreted on the basis of the existence of two different kinds of receptors (Guimarães and Paiva, 1981a, b). The reversal observed in the present study may also show the existence of two different subtypes of alpha-2 adrenoceptors at prejunctioinal level of the canine saphenous vein. When we compare the reversal obtained in the present study with those referred to above, we have to take into account a disturbing factor, the increase in norepinephrine concentration at the biophase caused by yohimbine in the present experiments. However, it is not easy to explain how this increase contributes to the reversal observed. The addition of the same drugs (yohimbine + CEC) by a reversed order (CEC first) gave no place to any reduction of the electrically evoked overflow despite the increase in norepinephrine concentration at the biophase.

Based on functional studies with different agonists and antagonists in several tissues, evidence has been accumulated which supports the hypothesis that more than one subtype of alpha-2 adrenoceptors exist prejunctionally. For example, Akers et al. (1991) showed that the antagonist potency of the compound SK&F 104078 at prejunctional alpha-2 adrenoceptors is agonist- and tissue-dependent. Furthermore, Oriowo et al. (1991) proposed the existence of two prejunctional alpha-2 adrenoceptor subtypes in the rat vas deferens, one being sensitive to SK&F 104078 and another being insensitive to this compound.

CEC caused an enhancement of the overflow evoked by electrical stimulation which was smaller than that caused by yohimbine (1.74- and 3.82-fold, respectively). This difference between the maximal effects of these two antagonists may be explained if one admits that CEC acts simultaneously on two different populations of prejunctional alpha adrenoceptors: as antagonist on one of them and as agonist on the other one. The final enhancement of the electrically evoked overflow of
Vanhoutte (1986), the pA2 values for prazosin against facilitatory influence of CEC. According to Flavahan and change either the inhibitory influence of UK-14,304 or the other has already been reported. The above discussed reversal of CEC effect, from a facilitatory (in the absence of yohimbine) into an inhibitory one (in the presence of yohimbine), may be explained assuming that yohimbine blocks only one part of the presynaptic alpha-2 adrenoceptors in this tissue. Unpublished results (S. Guimaraes and M.Q. Paiva) indicate that both oxymetazoline and UK-14,304 cause a reduction of the electrically evoked overflow of tritium in this tissue. However, these selective alpha-2 adrenoceptor agonists are differentially antagonized by yohimbine, exactly as alpha-2 adrenoceptors in the rat vas deferens, a fraction of which is sensitive to SKF 104078 and another is insensitive (Bylund and Iversen, 1990; Oriowo et al., 1991). Alternatively, one may speculate that, under control conditions, CEC inhibits alpha-2 autoreceptors (thus causing an increase in the overflow of tritium) and that in the presence of the alpha-2 adrenoceptor antagonist yohimbine it activates some nonadrenergic receptor (imidazoline-, 5-hydroxytryptamine-, dopamine receptor) the activation of which causes a decrease of tritium-evoked overflow.

The hypothesis that prejunctional alpha-1 adrenoceptors might be involved in the effect of CEC can be ruled out because prazosin, in concentrations up to 100 nM, did not change either the inhibitory influence of UK-14,304 or the facilitatory influence of CEC. According to Flavahan and Vanhoutte (1986), the pA2 values for prazosin against alpha-1 adrenoceptor-mediated responses in isolated blood vessels ranged from 10 pM to 10 nM. In earlier experiments carried out in the saphenous vein, at postjunctional level the pA2 values of prazosin were 7.65 and 6.02 for alpha-1 and alpha-2 adrenoceptor-mediated responses, respectively (Guimaraes and Nunes, 1990). In the present study, the concentrations of prazosin required to antagonize CEC are as high as those required to block alpha-2 adrenoceptors.

Which kind (kinds) of alpha-2 adrenoceptors are involved in these responses to UK-14,304, CEC and yohimbine in the saphenous vein is a question to which the present results give no answer. In the human saphenous vein, prejunctional alpha-2 adrenoceptors have been characterized as alpha-2A adrenoceptors (Molderings and Gothert, 1995). However, there are interesting differences between human and canine saphenous veins at the prejunctional level. For example, whereas the canine saphenous vein is endowed with prejunctional beta adrenoceptors mediating a facilitatory influence on the transmitter release (Guimaraes et al., 1978), the human saphenous vein is devoid of these receptors (Molderings et al., 1988); Moreover, although oxymetazoline did not act as an agonist, inhibiting the electrically evoked overflow of tritium in the human saphenous vein (Molderings and Gothert, 1995), it acted as a potent agonist which concentration-dependently reduced the overflow of tritium evoked by electrical stimulation in the saphenous vein (M.Q. Paiva, A. Mota, D. Moura, S. Guimaraes, unpublished results).

In conclusion, the present results suggest that, at prejunctional levels of the canine saphenous vein, there is more than one kind of alpha-2 adrenoceptor which both participate in the feedback regulation of norepinephrine release evoked by electrical stimulation; alternatively, it may be that the canine saphenous vein is endowed with alpha-2 adrenoceptors mediating an inhibitory influence which is blocked by CEC and also with some kind of nonadrenergic receptors also mediating an inhibitory effect which is activated by CEC. These results also show that CEC cannot be taken as a "pure" alpha-1B antagonist, because in the canine saphenous vein, it inhibits alpha-2 adrenoceptor-mediated responses.

References


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