Mechanisms of vasorelaxation induced by N-allylsecoboldine in rat thoracic aorta

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Abstract. N-Allylsecoboldine was shown to be the most effective of several boldine derivatives that were tested for their vasorelaxing effect on the rat aorta. In KCl (60 mmol/l) medium, Ca²⁺ (0.03 – 3 mmol/l)-induced vasoconstriction was inhibited, concentration-dependently, by N-allylsecoboldine. The IC₅₀ for N-allylsecoboldine was calculated to be about 411 μmol/l (for a Ca²⁺ concentration of 1 mmol/l). The vasorelaxant effect on KCl-induced responses was more pronounced at 60 mmol/l KCl than at 15 mmol/l KCl. Contraction of rat aorta in response to phenylephrine (0.01 – 100 μmol/l) was concentration-dependently inhibited by N-allylsecoboldine and by verapamil (3 – 30 μmol/l), while contraction in response to B-HT 920, serotonin or PGF₂α was not affected. This relaxing effect of N-allylsecoboldine persisted in endothelium-denuded aorta. In cultured A 10 vascular smooth muscle cells, N-allylsecoboldine and verapamil displaced the binding of [³H]-prazosin (Kᵦ values = 0.4±0.2 and 0.6±0.2 μmol/l, respectively). The increase of inositol monophosphate caused by phenylephrine in rat aorta was completely suppressed by chloroethylclonidine, but only slightly inhibited by N-allylsecoboldine and by verapamil. Glibenclamide or charybdo-toxin did not affect the relaxation induced by N-allylsecoboldine of aortic rings precontracted with phenylephrine. Neither the cGMP nor the cAMP content was changed by N-allylsecoboldine. We conclude that N-allylsecoboldine relaxes the rat aorta by blocking Ca²⁺ channels and that it also has an antagonistic effect at α₁-adrenoceptors.

Key words: Vasorelaxation – Boldine derivatives – Ca²⁺ channel blocker – α₁-Adrenoceptor antagonist – Rat aorta

Introduction

Boldine is the major alkaloid present in the leaves and bark of boldo (Peumus boldus) Molina, a widely distributed evergreen tree native to Chile (Speisky et al. 1991). Pharmaceutical preparations based on boldo have been widely used in South and North America, and in Europe, for medicinal purposes since the last century (Speisky et al. 1991). Official pharmacognostic descriptions have listed boldo preparations as diuretic, sedative and anthelmintic (Shamma 1972).

Recently, in a large scale screening test, we found that N-allylsecoboldine, a boldine derivative, relaxed blood vessels. In this paper, we report this previously unpublished finding, try to elucidate the mechanism of action of N-allylsecoboldine, and address the possibility that N-allylsecoboldine might exert its effects through an antagonistic action at Ca²⁺ channels and at α₁-adrenoceptors.

Materials and methods

Wistar rats of both sexes, weighing 250 – 300 g, were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. The vessels were cut into rings of about 5 mm in length which were then mounted in organ baths containing 5 ml of Krebs solution of the following composition (mmol/l): NaCl 118.2, KCl 1.9, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.7. The tissue bath solution was maintained at 37°C and bubbled with 95% O₂ – 5% CO₂. Two stainless steel hooks were inserted into the aortic lumen, one was fixed in the bath and the other was connected to a transducer. The aortic rings were equilibrated in the medium for 90 min, with three changes of the Krebs solution, and maintained under an optimal tension of 1 g before the specific experimental procedures were started. Contractions were recorded isometrically via a force-displacement transducer connected to a Gould polygraph (Model 2400). In some experiments, the endothelium was removed by rubbing with a cotton ball, and the absence of relaxation in response to acetylcholine (3 μmol/l) was taken as an indicator that the vessels were denuded of their endothelium.

The contractile effect of calcium was studied with aortic rings stabilized in a high K⁺ solution without Ca²⁺. The high K⁺ solution was prepared by substituting an equimolar amount of KCl for NaCl. Calcium was then added from a stock solution to obtain the desired concentrations (0.03 – 3 mmol/l), and the effect of each Ca²⁺ concentration
was recorded for a 3 min period. The maximal tension attained at 3 mmol/l Ca^{2+} was considered as 100%. When the maximal control response was reached, each aortic ring was washed with Ca^{2+}-containing Krebs solution for 30 min. The tissues were then incubated in KCl (60 mmol/l-Ca^{2+}) free medium together with various concentrations of N-allylsecoboldine or verapamil at 37°C for 15 min. Cumulative concentrations of Ca^{2+} (0.03–3 mmol/l) were then used to evoke contraction. In this experiment, the effects of three concentrations of N-allylsecoboldine or verapamil were obtained with a single preparation, but the tissues were washed between the additions of increasing concentrations of the drugs. In the KCl-induced contraction experiments, rat aortic rings were pre-contracted with 15 or 60 mmol/l KCl for 15 min, then increasing concentrations of N-allylsecoboldine or verapamil were added cumulatively for 18 min at 3 min intervals. In this experiment, cumulative concentration-response curves for N-allylsecoboldine or verapamil were each obtained with a single preparation. In the phenylephrine experiments, aortic rings were pre-incubated for 15 min with single concentration of N-allylsecoboldine or verapamil before generating the cumulative concentration-response curve with phenylephrine, the additions being made for 27–30 min at 3 min intervals. In these experiments, three concentrations of N-allylsecoboldine or verapamil were tested on the same preparation but the drugs were added with washout between increasing concentrations. The slopes of the resulted Schild plots were used to assess competitive antagonism.

The contents of cGMP and cAMP in aortic tissue were assayed as described previously (Kaufman et al. 1987; Yu et al. 1993). After incubation with forskolin, sodium nitroprusside, N-allylsecoboldine or DMSO for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 0.5 ml 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at 10000 g for 5 min, the supernatant was removed and extracted 4× with 3 volumes of ether. The cGMP or cAMP content was then assayed using RIA kits. The precipitate was used for protein assay (Lowry et al. 1951). cAMP and cGMP levels were expressed as pmol/mg protein.

The procedure described by Hirata et al. (1990) was used to measure the [3H]inositol monophosphate formation in rat aorta. Briefly, rat thoracic aortae were placed in Krebs solution containing 10 μCi/ml of [3H]inositol for 3 h and gassed with a 95% O2, 5% CO2 mixture. The tissues were then transferred to tubes containing fresh Krebs solution containing DMSO, N-allylsecoboldine, verapamil or chlorothiazide for 15 min. Saline or phenylephrine (3 μmol/l) was then added and the tissues were incubated for further 15 min. LiCl (10 mmol/l) was added 5 min before phenylephrine to inhibit inositol monophosphatase (Berridge et al. 1982). The aortae were frozen in liquid nitrogen and then homogenized in 1.3 ml of 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was collected and the trichloroacetic acid was removed by washing with 4×3 volumes of ether. The inositol monophosphate in the aqueous phase was analyzed by separation on a 1 ml volume Dowex-1 ion-exchange column according to the method of Neylon and Summers (1987). The tissue pellets were resuspended in 1.0 mol/l NaOH and assayed for protein according to the method of Lowry et al. (1951).

Rat A10 vascular smooth muscle cells (VSMC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of air/CO2 (19:1). For binding assays, cells were seeded in 24 well plates (5×10³ cells/ml). The binding assay for α1-adrenoceptors was done with [3H]-prazosin as described previously (Hornung et al. 1979). The equilibrium saturation binding of [3H]-prazosin and competition between [3H]-prazosin and N-allylsecoboldine were determined. Specific [3H]-prazosin binding was determined from the difference between counts in the presence and absence of 100 μmol/l phentolamine. Confluent cells were washed with 0.5 ml Krebs-Henseleit solution (KHS) containing (mmol/l): NaCl 117.5, KCl 5.6, MgSO4 1.18, NaH2PO4 1.2, NaHCO3 25.0, glucose 5.5, HEPES 25.0 and CaCl2 2.5; then 250 μl containing various concentrations of [3H]-prazosin (0.01–8 mmol/l) were added to each of the 24 wells. The assay plate was shaken for 30 min at 37°C. The reaction was terminated by rapid filtration under vacuum through a Whatman GF/B filter, using a cell harvest manifold. The filter was washed three times with 5 ml KHS. Each filter was then placed in a vial containing 4 ml of scintillation fluid and the radioactivity was determined by liquid scintillation counting (Beckman 5000 TC).

Drugs. N-allylsecoboldine, N-benzylsecoboldine, N-acetyl-OO-diacyt-secoboldine, N-allyl-secoglaucine and secoboldine (Fig. 1) were prepared from boldine by one of the authors (Lee), and purity (>99%) as confirmed by mass spectrometry, IR, NMR and proton spectroscopy. 1-Phenylephrine-HCl, boldine, verapamil-HCl, sodium nitroprusside, forskolin, serotonin, B-HT 920, PGF2α, charybdotoxin, acetylcholine-HCl and glibenclamide were obtained from Sigma Chem. Co. (St. Louis, Mo., USA). Cromakalim was obtained from Rhône-Poulenc Ltd. (Dagenham, UK). cAMP and cGMP RIA kits and myo-[2-3H]inositol were purchased from Amersham. When drugs (N-allylsecoboldine, N-benzylsecoboldine, N-acetyl-OO-diacyt-secoboldine, N-allyl-secoglaucine, secoboldine and verapamil) were dissolved in dimethylsulfoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1% and did not affect muscle contraction.

Data analysis. The experimental data are expressed as means ±SEM. A one-way analysis of variance (ANOVA) was done for multiple comparisons. Differences between means were compared by Student’s t-test and P values of less than 0.05 were considered to show statistical significance. The pA2 values were calculated for N-allylsecoboldine and verapamil according to the equation:

\[ pA_2 = -\log(\text{dose ratio}) \]  

(Mackay 1978)

Results

Phenylephrine (3 μmol/l) and KCl (60 mmol/l) caused a contraction of rat thoracic aorta (1.70 ± 0.15 g for phenylephrine, 1.50 ± 0.14 g for KCl) which was maintained for at least 30 min. The vasorelaxing effects of boldine derivatives, including N-allylsecoboldine, N-benzylsecoboldine, N-acetyl-OO-diacyt-secoboldine, N-allyl-secoglaucine and secoboldine, on KCl (60 mmol/l) or phenylephrine (3 μmol/l)-induced contraction of rat thoracic aorta were compared by pre-treating the aortic tissues with the boldine derivatives (50 μmol/l) for 15 min. N-allylsecoboldine was the most potent vasorelaxant (Table 1) and was used in the following experiments to study the mechanism of action of these vasorelaxing drugs.

![Fig. 1. Chemical structures of boldine derivatives](image-url)