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Mechanism of Vasorelaxation Caused by N-Benzylsecoboldine in Rat Thoracic Aorta

Key Words
Vasorelaxation
N-benzylsecoboldine
Ca^{2+} channel blocker
α_1-Adrenoceptor antagonist
Rat aorta

Abstract
The vasorelaxing effect of N-benzylsecoboldine on the rat thoracic aorta was investigated, and we also compare it with nifedipine and cromakalim. In high K^+ (60 m*M*) medium, Ca^{2+} (0.03–3 m*M*)-induced vasoconstriction was inhibited concentration-dependently by N-benzylsecoboldine, whereas this contraction was not altered by cromakalim. Cromakalim relaxed aortic rings precontracted with 15 but not 60 m*M* of K^+. N-benzylsecoboldine and nifedipine were more potent and effective in producing relaxation in 60 m*M* than in 15 m*M* K^+-induced contraction. N-benzylsecoboldine was found to be an α_1-adrenoceptor-blocking agent in rat thoracic aorta as revealed by its competitive antagonism of phenylephrine (PE)-induced contraction ($pA_2=6.31\pm0.04$, $pA_{10}=5.41\pm0.03$). This relaxing effect of N-benzylsecoboldine was not antagonized by indomethacin or methylene blue, and still persisted in endothelium-denuded aorta or in the presence of nifedipine (1 μ*M*). The increase of inositol monophosphate caused by PE in rat aorta was significantly suppressed by N-benzylsecoboldine, but not by nifedipine or cromakalim. High concentration of N-benzylsecoboldine (100 μ*M*) did not affect the contraction induced by B-HT 920, serotonin or $PGF_{2\alpha}$. Glibenclamide and charybdotoxin did not affect the relaxation of N-benzylsecoboldine in aortic rings precontracted with PE. Neither cGMP nor cAMP levels were changed by N-benzylsecoboldine. We suggest that N-benzylsecoboldine relaxes rat thoracic aorta by suppressing the Ca^{2+} influx and also has antagonistic effect on α_1-adrenoceptors.

Boldine is the major alkaloid present in the leaves and bark of boldo (Peumus boldus Molina), a widely distributed evergreen tree native of Chile [25]. Pharmaceutical preparations based on boldo have been widely used in South and North America, and Europe for medicinal purposes since the last century [25]. Official pharmacognostic descriptions have listed boldo preparations as diuretic, sedative and antihelminthic [24].

Recently, we found that N-benzylsecoboldine, a boldine derivative, can also act as a potent vasorelaxant [pers. observation]. In this paper, we have employed the rat thoracic aorta as an experimental system to examine in vitro the vas-

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cular smooth muscle inhibitory effect of N-benzylsecoboldine, and also compare it with nifedipine (a Ca^{2+} channel blocker) and cromakalim that is an opener or activator of K^+ channels leading to an increase in the outward K^+ current and thus to cellular hyperpolarization [9, 29].

Methods

Wistar rats of either sex 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 and mounted in organ baths containing 5 ml of Krebs solution of the following composition (m*M*): NaCl 118.2, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.7. The tissue bath solution was maintained at 37 °C and bubbled with a 95% O_2-5% CO_2 mixture. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. The aortas were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph (Model 7). In some experiments, the endothelium was removed by rubbing with a cotton ball, and the absence of acetylcholine (3 μ*M*)-induced relaxation was taken as an indicator that vessels were denuded successfully.

The contractile effect of calcium was studied in rings stabilized in high K^+ solution (60 m*M*) without Ca^{2+}. Calcium was then added from stock dilution to obtain the desired concentrations, and the effect of each Ca^{2+} concentration was recorded. The maximal tension attained at 3 m*M* Ca^{2+} was considered as 100%. The high K^+ solution was prepared by substituting NaCl with KCl in an equimolar amount. In some experiments, exposure of aortic rings to KCl (15 or 60 m*M*) or phenylephrine (PE, 3 μ*M*) caused tonic contraction maintained for 15 min; then N-benzylsecoboldine, cromakalim or nifedipine was added. After 15 min, the relaxations induced by the various drugs were expressed as percentage (as means ± SEM) of the spasmogen-induced contraction.

The content of cGMP and cAMP in aorta was assayed as described previously [11, 16]. After incubation of aortic rings with forskolin, sodium nitroprusside, N-benzylsecoboldine or dimethylsulfoxide (DMSO) for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at –80 °C until homogenized in 0.5 ml of 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 5 min and the supernatant was removed and extracted with 4×3 volumes of ether, and the cGMP or cAMP content was then assayed using RIA kits. The precipitate was used for protein assay [17]. cAMP and cGMP levels were expressed as pmol/mg protein.

The same procedure as described by Hirata et al. [10] was used to measure the [^{3}H]inositol monophosphate formation in rat aorta. Briefly, rat thoracic aortas were exposed to Krebs solution containing 10 μCi/ml of [^{3}H]inositol for 3 h and gassed with 95% O_2-5% CO_2 mixture. The tissues were then transferred to tubes containing fresh Kerbs solution with DMSO, N-benzylsecoboldine, nifedipine or cromakalim for 15 min and PE (3 μ*M*) was added and incubated for another 15 min. LiCl (10 μ*M*) was added 5 min before PE to inhibit inositol monophosphatase [1]. The aortas were then frozen in liquid nitrogen and 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 application of sample to 1 ml of Dowex-1 ion-exchange column according to the method of Neylon and Summers [21]. The tissue pellets were resuspended in 1.0 *N* NaOH and assayed for protein according to the method of Lowry et al. [17].

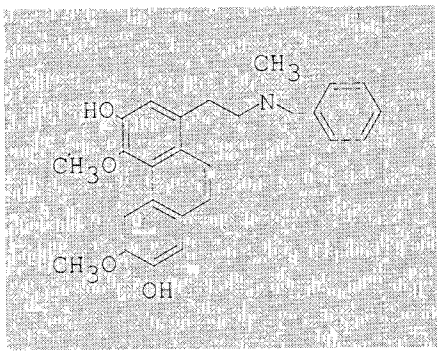


Fig. 1. Chemical structure of N-benzylsecoboldine.

Drugs. N-benzylsecoboldine (fig. 1) was synthesized from boldine, and its purity (> 99%) was confirmed by NMR, mass, IR and proton spectrophotometer. 1-PE-HCl, sodium nitroprusside, forskolin, serotonin, B-HT 920, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), charybdotoxin (Ch Tx), acetylcholine-HCl and glibenclamide were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Cromakalim was obtained from Rhône-Poulenc Ltd (Dagenham, UK). cAMP and cGMP RIA kits and myo-[2-^{3}H]inositol were purchased from Amersham (Bucks., UK). When drugs were dissolved in DMSO, the final concentration of DMSO in bathing solution did not exceed 0.1% and did not affect muscle contraction. All experiments with nifedipine were conducted in the dark.

Data Analysis. The experimental data are expressed as the means ± SEM and accompanied by the number of observations. One-way analysis of variance (ANOVA) was done for multiple comparison, and if there was a difference, then means for each antagonist were compared with the respective control by Student's t test. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of N-Benzylsecoboldine and Cromakalim on High K^+-Induced Ca^{2+}-Dependent Contractions

In Ca^{2+}-free Krebs solution containing high K^+ (60 mM), the cumulative addition of Ca^{2+} (0.03–3 mM) caused a stepwise increase of tension. After pretreatment of aorta for 15 min, N-benzylsecoboldine (1–30 μ*M*) inhibited this Ca^{2+} contraction in a concentration-dependent manner (fig. 2a)

($p<0.05$–0.001 as compared with 0.1–3 μM Ca^{2+}), whereas this contraction was not altered by cromakalim (10 μM) (fig. 2b). The ED_{50} for N-benzylsecoboldine was calculated to be about 3 μM (for Ca^{2+} concentration of 1 mM).

*Effects of N-Benzylsecoboldine, Nifedipine and Cromakalim on 15 or 60 m*M *KCl-Induced Contractions*

Relaxant responses to N-benzylsecoboldine, nifedipine and cromakalim were compared in aortic rings precontracted with 15 or 60 mM K^+ (in the presence of 1.9 mM Ca^{2+}). N-benzylsecoboldine and nifedipine were more potent and effective in producing relaxation in 60 mM than in 15 mM K^+-induced contraction (fig. 3a, b) ($p<0.001$), while cromakalim relaxed isolated aortic rings precontracted with 15 mM but not 60 mM K^+-induced contraction (fig. 3c) ($p<0.001$). Exposure of rat aorta to Bay K 8644 (0.1 μM) caused a tonic contraction maintained at least for 25 min. When N-benzylsecoboldine (30 μM) was added during tonic contraction (10 min after the exposure to Bay K 8644), a relaxation could also be observed (data not shown).

Effects of N-Benzylsecoboldine on PE, Serotonin, B-HT 920 and $PGF_{2\alpha}$-Induced Contractions

Increasing concentrations of PE (0.01–100 μM) evoked concentration-dependent contractions in rat thoracic aorta. N-benzylsecoboldine (3–30 μM) produced a parallel, rightward shift of the curve consistent with competitive blockade ($pA_2=6.31\pm0.04$, $pA_{10}=5{,}41\pm0.03$, slope = 1.06) (fig. 4a). However, nifedipine (0.01–1 μM) and cromakalim (0.1–3 μM) produced a nonparallel and noncompetitive shift of the concentration-response curves of PE to the right (fig. 4b, c). This relaxing action of N-benzylsecoboldine was not blocked by indomethacin (20 μM) or methylene blue (50 μM), and still persisted in endothelium-denuded aorta or in the presence of nifedipine (1 μM) (data not shown).

High concentration of N-benzylsecoboldine (10 μM) was used to test its antagonism against contraction of rat thoracic aorta caused by vasoactive agents other than α_1-adrenoceptor agonist. N-benzylsecoboldine (100 μM) did not suppress the increase in tension by serotonin (30 μM), B-HT 920 (10 μM) and $PGF_{2\alpha}$ (10 μM) (fig. 5) ($p>0.05$ as compared with the respective control).

Effects of N-Benzylsecoboldine, Nifedipine and Cromakalim on the Breakdown of Phosphoinositides

The accumulation of inositol monophosphate in rat aorta was increased in the presence of PE (3 μM). This increase was significantly suppressed by N-benzylsecoboldine (30 μM), but not inhibited by nifedipine (100 nM) or cromakalim (10 μM) (table 1).

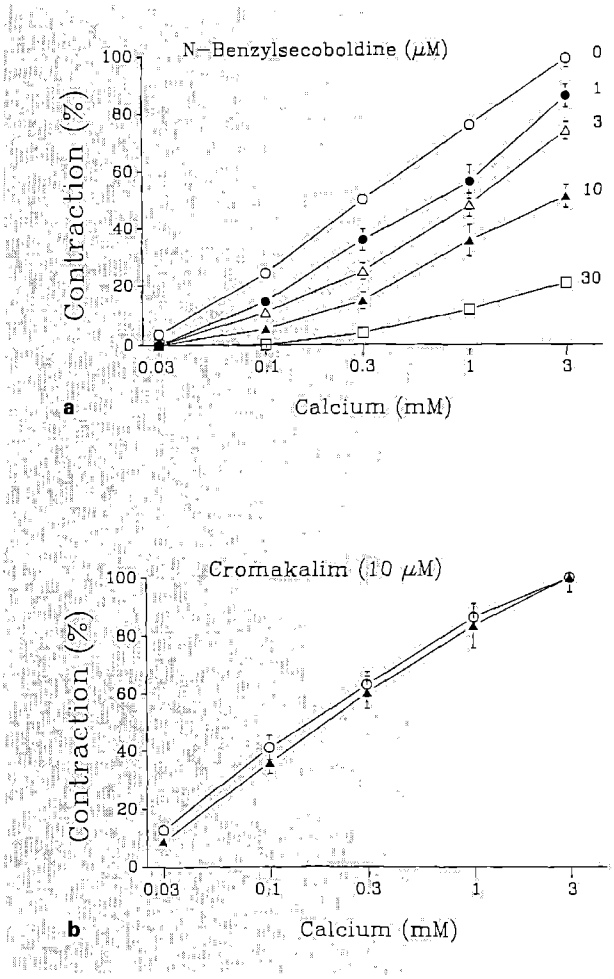
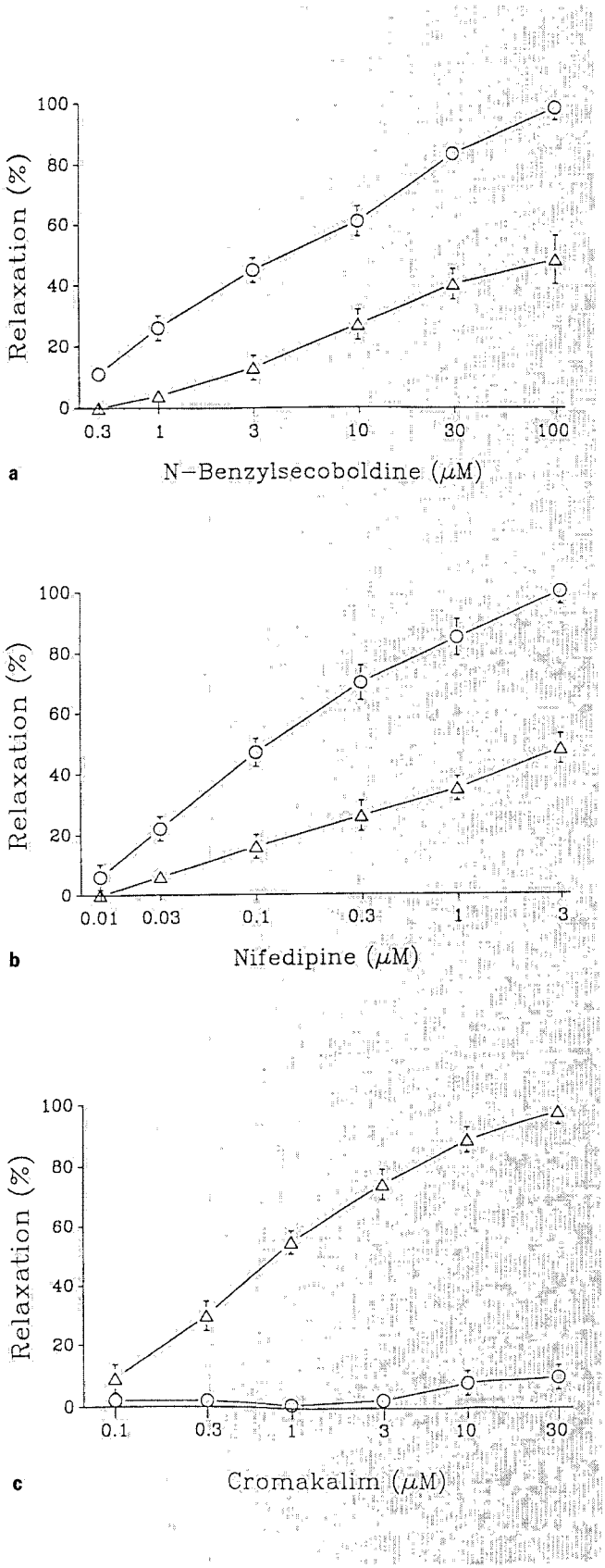


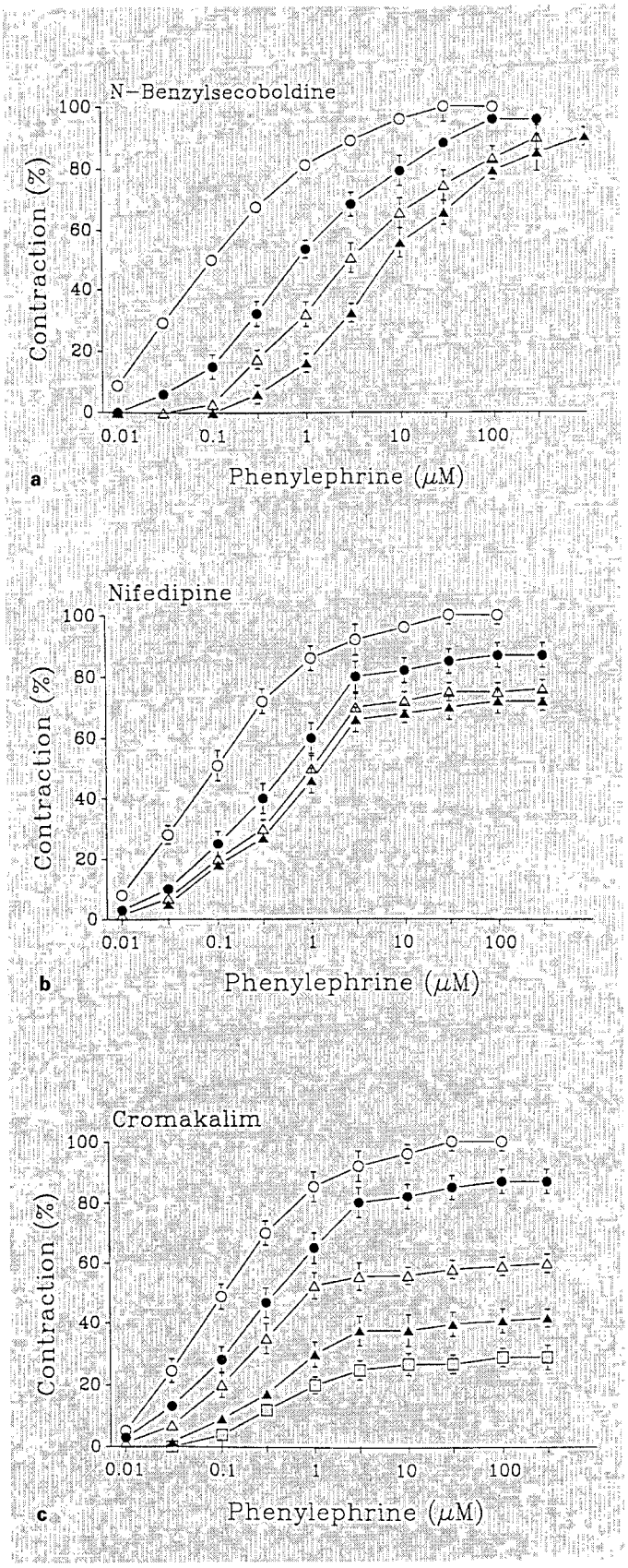
Fig. 2. Effects of N-benzylsecoboldine and cromakalim on th Ca^{2+}-dependent contraction of rat aorta induced by KCl. In high K^+ (6 mM) medium, various concentrations of N-benzylsecoboldine (**a**) c cromakalim (10 μM) (**b**) were preincubated with aorta at 37 °C for 1 min, then cumulative concentrations of Ca^{2+} (0.03–3 mM) were use to evoke the contraction. The values (% of maximum contraction) ar presented as means ± SEM (n = 8).

Fig. 3. Cumulative concentration-dependent relaxation by N benzylsecoboldine, nifedipine and cromakalim on vascular response to KCl. Rat aortic rings were precontracted with 15 (△) or 60 (○) mM K^+ for 15 min; then various concentrations of N-benzylsecoboldin (**a**), nifedipine (**b**) or cromakalim (**c**) were added. Relaxations are pre sented as percentages (means ± SEM, n = 8) of the K^+-induce contraction.

Fig. 4. Antagonism of the concentration-response curves to P by 15 min pretreatment of aorta with N-benzylsecoboldine (●, 3; △ 10; ▲, 30 μM) (**a**), nifedipine (●, 0.01; △, 0.1; ▲, 1 μM) (**b**) and cro makalim (●, 0.1; △, 0.3; ▲, 1; □, 3 μM) (**c**) or DMSO (○, 0.1%). Th values (% of maximum contraction) are presented as means ± SEM (n = 6).


Relaxation (%)
100
80
60
40
20
0
0.3
1
3
10
30
100
a
N−Benzylsecoboldine (μM)
Relaxation (%)
100
80
60
40
20
0
0.01
0.03
0.1
0.3
1
3
b
Nifedipine (μM)
Relaxation (%)
100
80
60
40
20
0
0.1
0.3
1
3
10
30
c
Cromakalim (μM)

3


N−Benzylsecoboldine
Contraction (%)
100
80
60
40
20
0
0.01
0.1
1
10
100
a
Phenylephrine (μM)
Nifedipine
Contraction (%)
100
80
60
40
20
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0.01
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b
Phenylephrine (μM)
Cromakalim
Contraction (%)
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Phenylephrine (μM)

4

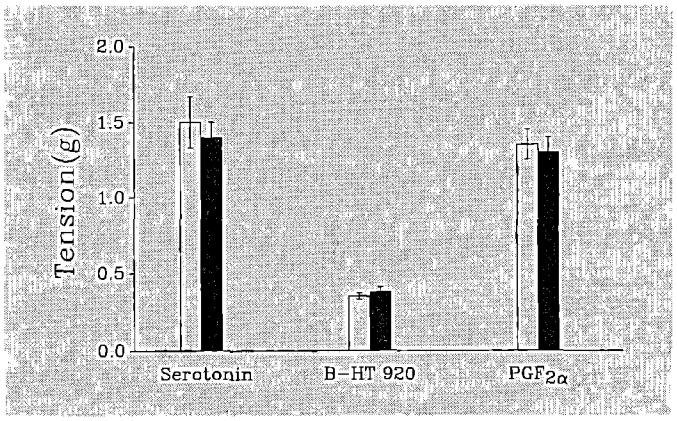

Fig. 5. Effects of N-benzylsecoboldine on the contractile responses of rat aorta to serotonin, B-HT 920 and PGF_{2a}. Aortic rings were preincubated with 0.1% DMSO (□, control) or N-benzylsecoboldine (100 μ*M*, ■) at 37 °C for 15 min, then serotonin (30 μ*M*), B-HT 920 (10 μ*M*) or PGF_{2a} (10 μ*M*) was used to trigger the contraction. The values are presented as means ± SEM (n = 6).

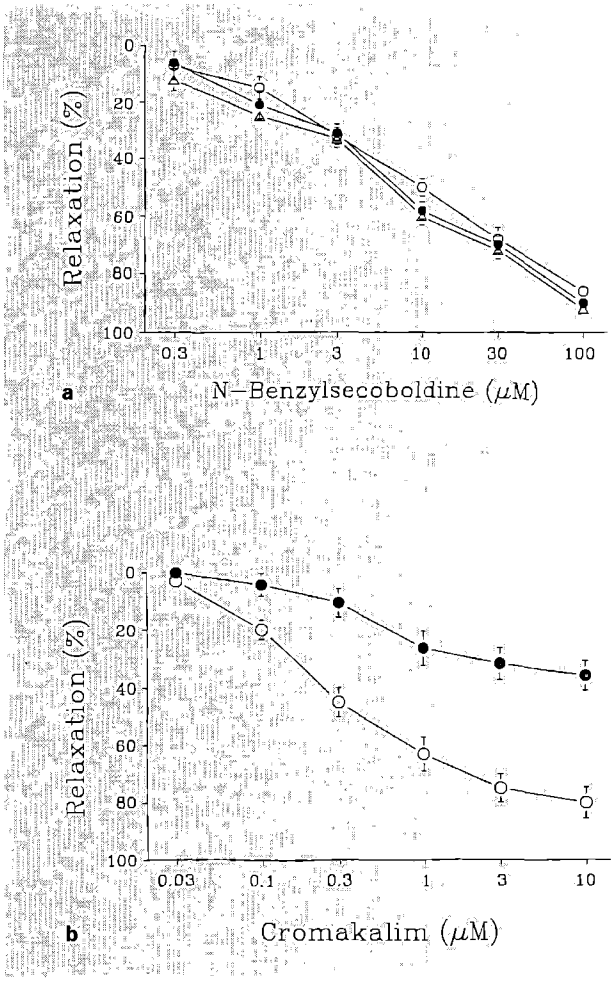

Fig. 6. Effects of glibenclamide and ChTx on the vasorelaxing effect of N-benzylsecoboldine in rat aorta. After pretreatment of rat aorta with glibenclamide (10 μ*M*, ●) or ChTx (100 n*M*, △) for 15 min, PE (3 μ*M*) was used to induce muscle contraction. Various concentrations of N-benzylsecoboldine (**a**, ○) or cromakalim (**b**, ○) were added 15 min after the exposure of aorta to PE. Relaxations are presented as percentages (means ± SEM, n = 8) of the PE-induced contraction.

Glibenclamide Antagonism of Cromakalim but not N-Benzylsecoboldine in PE-Induced Contraction

Cromakalim (0.03–10 μ*M*) produced concentration-dependent relaxation of rat aortic rings precontracted with PE (3 μ*M*). Addition of glibenclamide (10 μ*M*) to PE-contracted aortic rings produced no change in basal tension. However, glibenclamide shifted the concentration-dependent relaxation curve of cromakalim to the right (fig. 6b). N-benzylsecoboldine (0.3–100 μ*M*) produced concentration-dependent relaxation of rat aortic rings precontracted with PE (3 μ*M*). After pretreatment of aorta with glibenclamide for 15 min, N-benzylsecoboldine still inhibited this PE-induced contraction in a concentration-dependent manner (fig. 6a) ($p > 0.05$). Similarly, the concentration-dependent relaxation curve of N-benzylsecoboldine was not affected by ChTx (100 n*M*) (fig. 6a) ($p > 0.05$).

Effects of N-Benzylsecoboldine on the cGMP and cAMP Formations in Rat Aorta

The cyclic nucleotides content of aorta as measured by radioimmunoassay showed that neither cGMP nor cAMP level was changed significantly by N-benzylsecoboldine (30 μ*M*) (table 2).

Discussion

The present experiments have demonstrated that N-benzylsecoboldine, a boldine derivative, depressed the contractile responses of rat aorta to KCl depolarization and α_1-adrenoceptor stimulation by PE.

It is widely accepted that contraction of vascular smooth muscle requires the increase of cytosolic free Ca^{2+}. The high K^+-induced contraction of smooth muscle is the result of an increase in Ca^{2+} influx through voltage-dependent Ca^{2+} channels [14, 15]. Bay K 8644 also promotes Ca^{2+} in-

flux through these channels in vascular smooth muscle [7, 26]. N-benzylsecoboldine (1–30 μ*M*) inhibited high K^+ (60 m*M*)- and Bay K 8644-induced contractions; thus it may be a blocker of voltage-dependent Ca^{2+} channel. Like nifedipine, its vasorelaxant effect on KCl-induced responses was more prominent at 60 m*M* KCl than at 15 m*M* KCl. This result indicates that N-benzylsecoboldine may exert the same potential-dependent inhibition of Ca^{2+} channels. This observation was further supported by the same potential-dependent suppression of Ca^{2+} current in cardiac cells by N-benzylsecoboldine and nifedipine [unpubl. observation]. Cromakalim (10 μ*M*) inhibited contractions elicited by low concentrations of K^+ (15 m*M*) but was ineffective against high concentrations of K^+ (60 m*M*). The mechanism of this effect is related to opening or activation of K^+ channels leading to an increase in the outward K^+ current and thus to cellular hyperpolarization [9, 29]. It is clear that a 'Ca^{2+} channel blocker' may have various mechanisms and sites of action [23]. For example, pinacidil is a new antihypertensive agent which may relax vascular smooth muscle [4]. Its mechanisms of action are thought to be due to (a) inhibiting of Ca^{2+} influx in K^+-contracted arterial strips [18, 28] and (b) opening of K^+ channels in smooth muscles, thus increasing the K^+ permeability and hyperpolarizing the cell membrane [5]. Unlike cromakalim, N-benzylsecoboldine also relaxed the glibenclamide (10 μ*M*)-treated aorta precontracted with PE. Thus, the vasorelaxation caused by N-benzylsecoboldine was not related to opening of ATP-sensitive K^+ channels since these channels are inhibited by glibenclamide [2, 3, 18]. ChTx, a peptide produced by the scorpion *Leirus quinquestriatus*, is a selective and potent blocker of large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel in smooth muscle [13]. Again, N-benzylsecoboldine also relaxed the ChTx (100 n*M*)-treated aorta precontracted with PE. Thus, large-conductance Ca^{2+}-activated K^+ channel was not affected by N-benzylsecoboldine.

It has been reported that activation of α_1-adrenoceptors by PE stimulates phosphoinositide breakdown to increase the concentration of inositol trisphosphate and diacylglycerol, which mediate vasoconstriction [19]. As shown in figure 4 and table 1, N-benzylsecoboldine produced a parallel, rightward shift of the PE-induced contraction curve consistent with competitive blockade, and the inositol monophosphate formation induced by PE was also inhibited. However, nifedipine and cromakalim produced antagonism of the concentration-response curve of PE in a noncompetitive manner, and the inositol monophosphate formation was not affected. Since PE-induced contraction is little dependent upon voltage-dependent Ca^{2+} channel opening [19], addition of nifedipine (1 μ*M*) eliminates the influence of voltage-dependent Ca^{2+} entry, N-benzylsecoboldine still produced a parallel, rightward shift of the curve with competitive blockade. These results imply that N-benzylsecoboldine may possess α_1-adrenoceptor-blocking properties. Wong et al. [30] reported that verapamil (a Ca^{2+} channel blocker) also has been shown to block α_1-adrenoceptors. High concentration of N-benzylsecoboldine had no apparent effect on B-HT 920 (an α_2-adrenoceptor agonist) [6], serotonin and $PGF_{2\alpha}$-induced contraction.

Endothelial cells respond to a variety of neurohumoral and physical stimuli to release endothelium-dependent vasodilators such as endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) [12, 27]. It is generally ac-

Table 1. Effects of N-benzylsecoboldine, nifedipine and cromakalim on the accumulation of inositol monophosphate in rat aortas by PE

Treatment	Inositol monophosphate, cpm/mg protein
Resting	1,400±140
Control	2,395±9
N-benzylsecoboldine	1,540±85*
Nifedipine	2,290±40
Cromakalim	2,345±67

Rat aortic rings were preincubated with N-benzylsecoboldine (30 μ*M*), nifedipine (100 n*M*), cromakalim (10 μ*M*) or DMSO (0.1%, control) for 15 min, followed by the stimulation with PE (3 μ*M*) for 15 min. Resting: aortas without any treatment. Data are presented as total inositol monophosphates accumulated (cpm/mg protein) and expressed as means ± SEM (n = 6). Data were analyzed by ANOVA test and then by Student's t test. *$p < 0.001$ as compared with the control value.

Table 2. Effects of N-benzylsecoboldine on the cGMP and cAMP levels of rat aorta

Treatment	cAMP, pmol/mg protein	cGMP, pmol/mg protein
Control	4.2±0.4	6.3±0.7
N-benzylsecoboldine	4.3±0.3	6.0±0.4
Forskolin	8.5±0.4*	–
Na nitroprusside	–	12.5±1.2*

After preincubation of aortic rings with N-benzylsecoboldine (30 μ*M*), forskolin (1 μ*M*), sodium nitroprusside (1 μ*M*) or DMSO (0.1%, control) for 2 min, the reaction was stopped by immersing the tissue into liquid nitrogen. The cGMP and cAMP contents in rat aorta were then measured by RIA kits. The results are expressed as the means ± SEM (n = 8). Data were analyzed by ANOVA test and then by Student's t test. *$p < 0.001$ as compared with the respective control.

cepted that the release of EDRF and PGI_2 may have important physiological roles as dilating mediators in certain vessels. The relaxing action of N-benzylsecoboldine persisted in endothelium-denuded aorta, or in intact aorta in the presence of indomethacin or methylene blue. Thus, the vasorelaxation caused by N-benzylsecoboldine was independent of endothelium and not mediated by either EDRF or PGI_2.

Other important mediators for relaxing the vascular smooth muscle are cyclic nucleotides [20]. Sodium nitroprusside has been shown to be a potent relaxing agent in vascular smooth muscles. It produces prompt, dose-dependent increases in the level of cGMP which has a direct activating effect on guanylate cyclase [8]. Forskolin can produce increase in cAMP levels via activation of adenylate cyclase [22]. Neither the cAMP nor the cGMP content was changed by N-benzylsecoboldine (table 2). This indicates that the inhibitory effects of N-benzylsecoboldine on the contractile responses caused by high K^+ or PE are not due to the increase of cellular cyclic nucleotide concentrations.

In conclusion, these results suggest that N-benzylsecoboldine relaxes rat thoracic aorta by suppressing the Ca^{2+} influx and also has an antagonistic effect on a1-adrenoceptors.

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