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Alseis yucatanensis: a natural product from Belize that exhibits multiple mechanisms of vasorelaxation

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Abstract

An aqueous extract of the bark of *Alseis yucatanensis* was studied to determine its mechanism of action in the relaxation of endothelium-denuded rat aortic tissues. The extract relaxed both norepinephrine (NE) and KCl-contracted vessels, with ED_{50}'s of 0.12 and 1.73 mg/mL, respectively. In NE-contracted vessels, two phases of relaxation were evident which were separated in both time and dose range. At high concentrations, a rapid relaxation was seen that was due to the blocking of internal (ED_{50} = 0.49 mg/mL) and external (ED_{50} = 2.34 mg/mL) calcium channels. A second, slowly developing (i.e., long-term) relaxation to baseline was seen at lower concentrations. The time to complete relaxation was dose-dependent. This long-term response was not seen in KCl-contracted vessels, was prolonged by TEA, and could be reversed by the addition of KCl to the bath. These data suggest that the long-term relaxation is due to the opening of potassium channels.

Keywords: Rat aorta; Relaxation; Calcium release channel; Receptor-operated channels; Potassium channels

1. Introduction

Alseis yucatanensis Standley (Rubiaceae) is a tree of 20–30 m in height, with a stem of 40 cm or larger in diameter, and with whitish flowers. This species grows in the secondary forests of Guatemala, Belize, and Mexico where the tree is known as "wild mamee" or "has che"; in the Yucatan of Mexico it is called "cacao-che" (Standley and Williams, 1975) or "ison" (Balick et al., 2000). The leaves of this tree are roasted and made into a powder and the powder sprinkled over skin sores after bathing.

In previous studies, an aqueous extract of the leaf or bark of *Alseis yucatanensis* was shown to completely relax endothelium-denuded rat aortic vessels that were precontracted with norepinephrine (NE; Slish et al., 1999). The purpose of the present study was to determine the biochemical mechanism of this relaxation.

2. Methodology

2.1. Plant sample collection and extraction

Alseis yucatanensis samples were collected in May 1995 in Belize and identified at the Ix Chel Tropical Research Foundation (voucher specimen number RA944); herbarium specimens were deposited at the New York Botanical Garden. The plant extract was prepared by decoction followed by lyophylization. Ground, dried *Alseis yucatanensis* bark (20 g) was added to 200 mL of boiling de-ionized water and allowed to boil for 15 min. Solid material was removed by filtration and the resultant extract was lyophilized to dryness. Dry extract yield was approximately 5% (w/w) of crude material. Working solutions of the extract were prepared daily at a concentration of 50 mg/mL in distilled water.

2.2. Tissue preparation

Measurements of the contraction of aortic smooth muscle were performed according to accepted protocols (Rapoport, 1987). Adult male Sprague–Dawley rats (175–250 g) were euthanized by 100% CO_2 inhalation followed by

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decapitation, and the thoracic aorta isolated. The aorta was cleaned of fatty deposits and the connective tissue, and was cut into four rings. The rings were denuded of endothelium by inserting forceps into the lumen of the vessel and manually rotating it. The aortic rings were attached to an isometric force transducer, placed in a 15 mL temperature controlled tissue bath, and bathed in Krebs-Ringer bicarbonate solution (KRB; 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, and 30 μM EDTA) at 37 °C. The KRB solution was continually gassed with a mixture of 95% O_2–5% CO_2.

2.3. Contraction measurements

Tissues were mounted with 3.0 g resting tension and allowed to equilibrate until the tension was maintained. Tissues were then tested by contraction with 0.3 μM norepinephrine (NE) followed by 10 μM carbachol at the peak of contraction. Previous studies (Furchgott and Zawadski, 1980) indicate that rings properly denuded of endothelium gave no response to carbachol. This procedure was repeated to insure the stability of the smooth muscle and the removal of the endothelium. Rings that perform poorly in test contractions (<1.0 g of active tension) or showed a relaxation in response to carbachol were discarded.

3. Results

3.1. Dose–response in NE- and KCl-contracted tissues

Dose–response analysis of the *Alseis yucatanensis* extract against NE- and KCl-induced contractions were performed and are shown in Fig. 1. The extract was more potent in inhibiting NE- than KCl-induced contractions, with ED_{50}'s of 0.12 and 1.73 mg/mL, respectively.

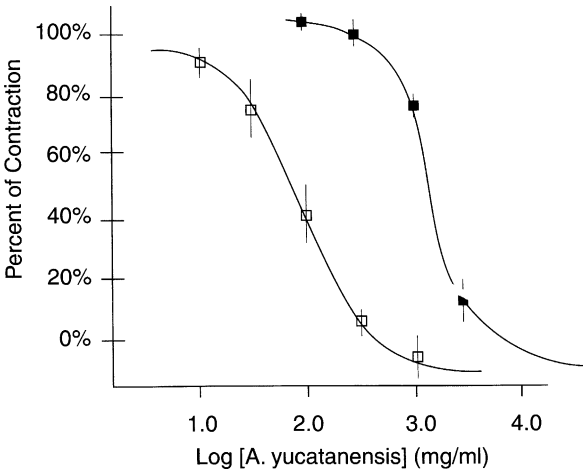


Fig. 1. Effect of *Alseis yucatanensis* on NE- and KCl-induced contractions. Tissues were contracted with either 3×10^{-7} M NE (open squares) or 55 mM KCl (filled squares) and a cumulative dose response was performed ($n = 12$ and 16, respectively).

3.2. Differentiation of cell surface and internal calcium responses

Since *Alseis yucatanensis* was more potent against NE-induced contractions, studies were performed to differentiate between its effects on two components of NE signal transduction pathway, namely, internal release of calcium from the endoplasmic reticulum (ER) and cell surface calcium channels. In control experiments, tissues were incubated in Ca^{2+}-free Krebs-Ringer buffer containing 1 mM EGTA for 15 min and then exposed to 3×10^{-7} M NE. This resulted in phasic contractions due to ER calcium release (Fig. 2A). When this response reached a plateau, 2 mM $CaCl_2$ was added to the bath, causing a tonic

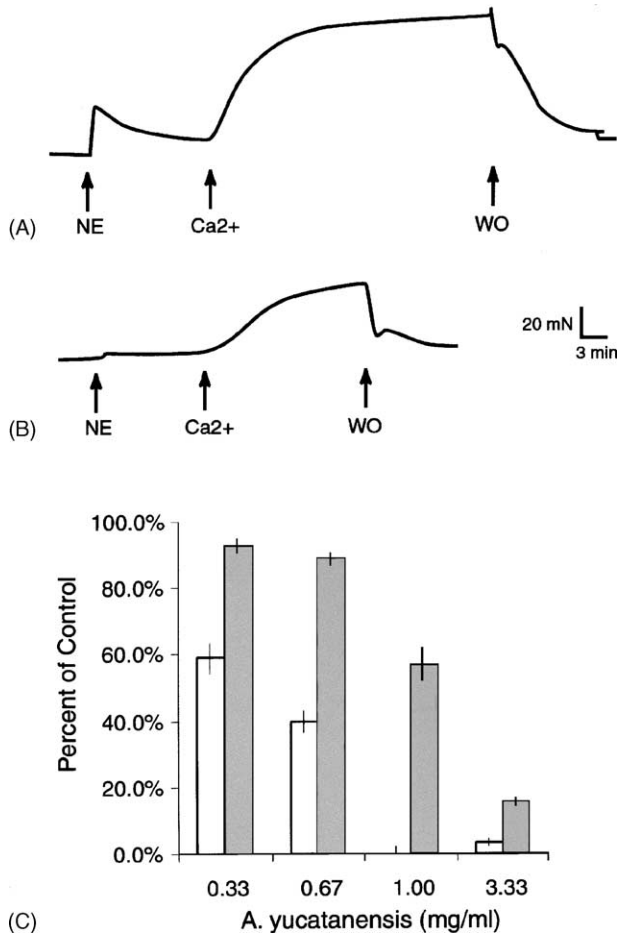


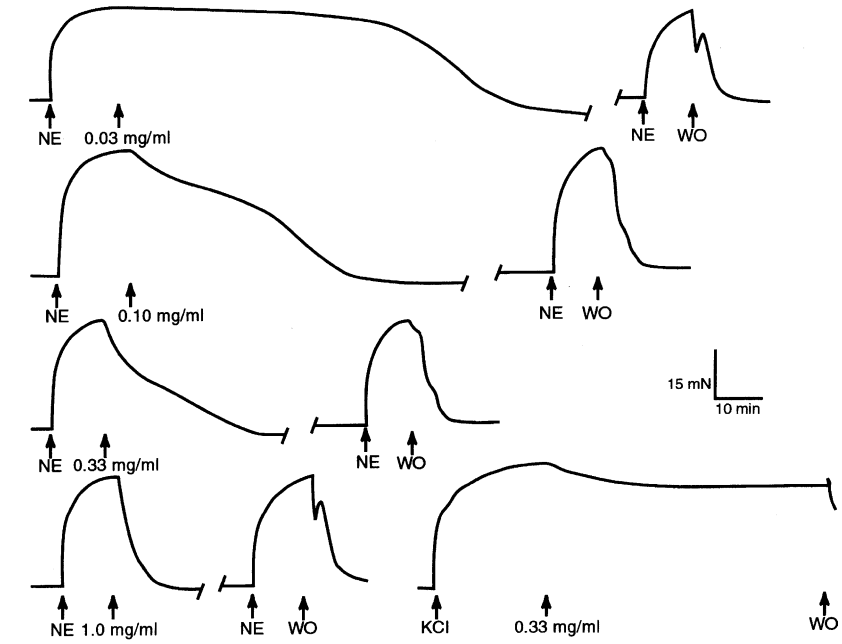
Fig. 2. Effect of *Alseis yucatanensis* on calcium entry and ER calcium release. Tissues were incubated in Ca^{2+}-free Krebs-Ringer buffer containing 1 mM EGTA for 15 min and then exposed to 3×10^{-7} M NE. After reaching plateau, 2 mM $CaCl_2$ was added to the bath. (A) Representative control contraction. (B) After pretreatment with 1.0 mg/mL *Alseis yucatanensis* for 15 min. (C) Dose response of the effect on NE-induced phasic contraction (open columns) and calcium-induced tonic contraction (shaded columns) (0.33 mg/mL, $n = 12$; 0.67 mg/mL, $n = 14$; 1.00 mg/mL, $n = 12$; 3.33 mg/mL, $n = 7$).

contraction, which is due to calcium entry through voltage- and receptor-operated channels. This procedure effectively separates the effect of NE on ER calcium channels versus cell surface calcium channels (Huang and Ho, 1996; Nakai, 1994).

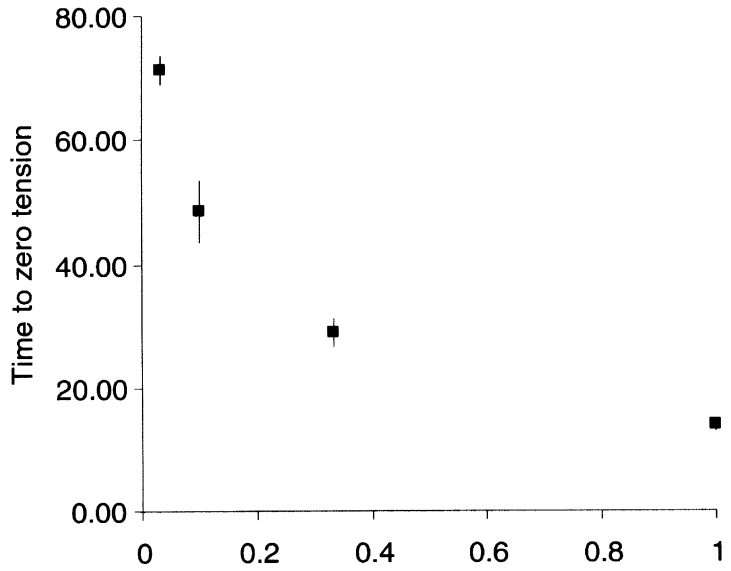
This procedure was repeated in the same tissue after pretreatment with *Alseis yucatanensis* for 15 min (Fig. 2B). At 1.0 mg/mL, the *Alseis yucatanensis* extract eliminated the phasic contraction, while the contraction due to calcium entry was 57.1 ± 4.9% of the control. Fig. 2C shows a dose–response of the effect of the extract on ER and cell surface calcium channels; the ED_{50} of the effect on ER channels was 0.49 mg/mL compared to 2.34 mg/mL for the effect on cell surface calcium channels.

3.3. Characterization of long-term relaxation

Subsequent observations revealed that there were two phases to the relaxation produced by *Alseis yucatanensis* in NE-contracted tissues. The dose–response results described above were due to short-term exposure of the tissues to the extract (<30 min). Upon longer exposure at low doses, the



(A)



(B)

Fig. 3. Long-term relaxation of NE-induced contractions by *Alseis yucatanensis*. (A) Representative relaxations followed by control contractions in the same tissue (after washout of extract). Control contraction with NE maintained maximal tension >1.5 h. (B) Dose–response of the time from addition of *Alseis yucatanensis* to the return to baseline tension (0.03 mg/mL, $n = 6$; 0.10 mg/mL, $n = 8$; 0.33 mg/mL, $n = 10$; 1.00 mg/mL, $n = 12$).

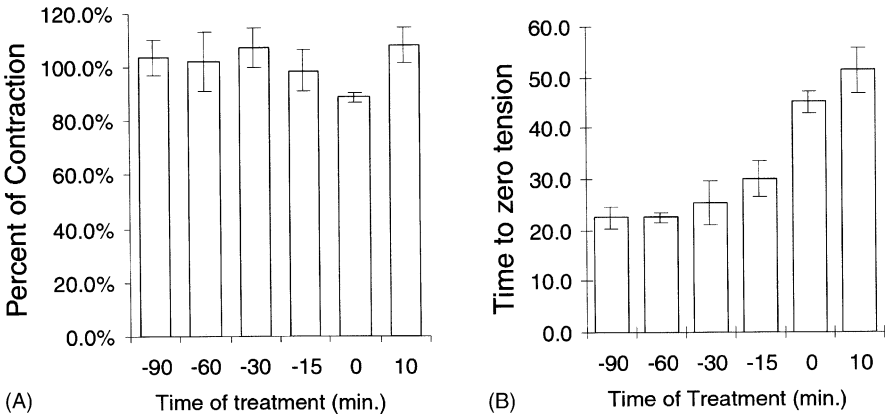


Fig. 4. Pretreatment of tissues with *Alseis yucatenensis*. Tissues were pretreated with 0.10 mg/mL *Alseis yucatanensis* for the times shown. (A) The peak of the NE induced contraction after pretreatment with *Alseis yucatanensis* as compared to a control contraction in the same tissue immediately prior to the experiment ($n = 4$). (B) The effect of pretreatment with *Alseis yucatanensis* on the time from the addition of NE to the return to baseline tension ($n = 4$).

extract was capable of producing 100% relaxation in the NE-contracted tissues (Fig. 3A). The two phases of relaxation were most pronounced at 0.10 and 0.33 mg/mL. This relaxation was not observed in tissues contracted with KCl. The relaxation was rapidly reversible, as shown by subsequent contractions in the same tissue.

The length of time needed to produce 100% relaxation was dose-dependent (Fig. 3B). A high dose of *Alseis yucatanensis* extract (1.00 mg/mL) produced relaxation in 14.0 ± 0.9 min, while 33 μg/mL extract produced 100% relaxation in 71.3 ± 2.2 min. In one experiment an extremely low dose (10 μg/mL) produced 50% relaxation after 2 h of incubation (data not shown), however, control tissues had also begun to lose tension at this time.

3.4. Effect of pretreatment on long-term relaxation

The effect of pretreatment of the tissues with *Alseis yucatanensis* on the NE-induced contraction was studied. Tissues were pretreated with 0.10 mg/mL of the extract for 0, 15, 30, 60, and 90 min before addition of NE. Pretreatment with *Alseis yucatanensis* had no effect on the peak of the NE contraction as compared to a control contraction performed in the same tissue (Fig. 4A).

However, pretreatment significantly decreased the length of time required to produce relaxation (Fig. 4B). Relaxation produced by the addition of 0.10 mg/mL of the extract after the peak of NE contraction (+10 min) required 41.2 ± 4.4 min to return to baseline tension. Simultaneous addition of NE and the extract (0 min) resulted in a contraction time (45.1 ± 2.0 min) similar to the time for relaxation when added after the peak of NE. Pretreating the tissues with this dose of *Alseis yucatanensis*, and then adding NE, resulted in a contraction to peak tension followed by a return to relaxation to baseline tension. Pretreatment of the tissues for a period shorter than 41.2 min (15 and 30 min) resulted in a shorter contraction/relaxation time than that seen with simultaneous addition (30.0 ± 3.4 min and 25.4 ± 4.3 min, respectively). Pretreatment of the tissues for periods longer than 41.2 min (60 and 90 min) resulted in a minimum contraction/relaxation time (22.5 ± 2.1 min and 22.4 ± 1.1 min) after addition of NE.

3.5. Mechanism of long-term relaxation

The long-term relaxation can be partially reversed by the addition of 50 mM KCl to the bath. In Fig. 5A, 50 mM

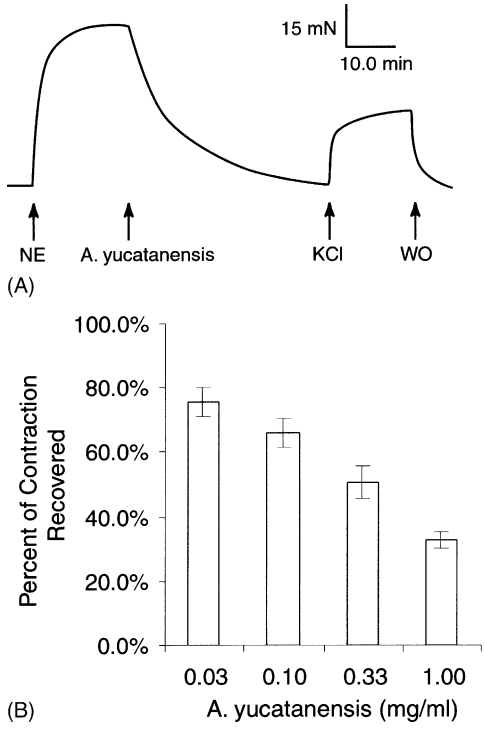


Fig. 5. KCl can partially reverse long-term relaxation by *Alseis yucatanensis*. KCl (50 mM) was added to the bath after the tissue relaxed to baseline tension. (A) Representative experiment of the effect of 50 mM KCl on the relaxation produced by 1.00 mg/mL *Alseis yucatanensis*. (B) Dose–response of the percentage of the original contraction recovered by KCl. (0.03 mg/mL, $n = 6$; 0.10 mg/mL, $n = 10$; 0.33 mg/mL, $n = 14$; 1.00 mg/mL, $n = 11$).

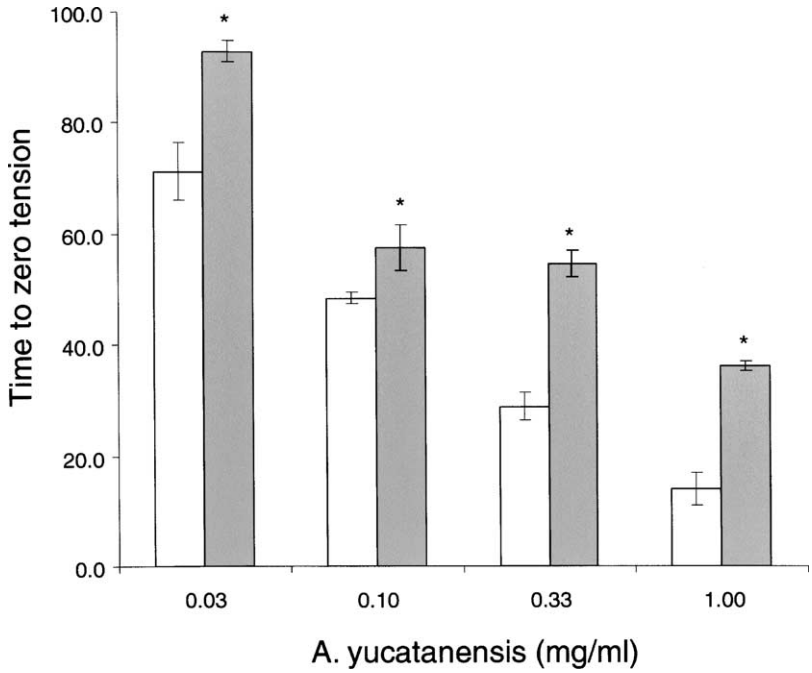


Fig. 6. Effect of TEA on long-term *Alseis yucatanensis* relaxation. Tissues were pretreated for 15 min with 1.0 mM TEA-Cl before NE-induced contraction followed by long-term *Alseis yucatanensis* relaxation. Open columns are control (0.03 mg/mL, $n = 6$; 0.10 mg/mL, $n = 8$; 0.33 mg/mL, $n = 10$; 1.00 mg/mL, $n = 12$). Shaded columns are in the presence of 1.0 mM TEA-CLC* represents $P < 0.01$, (0.03 mg/mL, $n = 7$; 0.10 mg/mL, $n = 13$; 0.33 mg/mL, $n = 13$; 1.00 mg/mL, $n = 9$).

KCl was added to the bath after complete relaxation was induced by 1.0 mg/mL *Alseis yucatanensis*; this resulted in the recovery of 32.4 ± 2.5% of the original contraction. The dose-dependency of this effect is shown in Fig. 5B. The KCl reversed a higher proportion of the relaxation produced by lower concentrations of *Alseis yucatanensis* than that caused by larger concentrations.

The long-term relaxation could be inhibited by the addition of 1.0 mM TEA-Cl to the bath (Fig. 6). TEA-Cl significantly increased the time to relaxation at each dose of *Alseis yucatanensis* tested ($P < 0.01$). However, this long-term relaxation was not affected by pretreatment either with 100 μM *N*-ω-nitro-L-arginine or with 30 μM indomethacin (data not shown).

4. Discussion and conclusions

The present study showed that an aqueous extract of *Alseis yucatanensis* was more potent in relaxing NE-induced contractions ($ED_{50} = 0.12$ mg/mL) than KCl-induced contractions ($ED_{50} = 1.73$ mg/mL) of rat aortic tissue. The extract blocked NE-induced contraction by inhibiting both calcium release from the endoplasmic reticulum (0.49 mg/mL) and the entry of calcium through receptor-operated and voltage-dependent channels (2.34 mg/mL). The potency of these responses may explain the difference in its potency for NE- and KCl-contracted vessels.

The *Alseis yucatanensis* extract also produced profound relaxation at lower doses that took a longer period of time. The long-term effect was readily reversible, which showed that it was not due to damage to the tissue. This long-term relaxation was somewhat unique in that it was an all-or-nothing response. Yet it was dose-dependent; lower doses required longer times to reach baseline. This was a very potent effect; 33 μg/mL of the extract produced 100% response. Pretreatment showed that the relaxation seen at lower doses did not affect the ability of the tissue to contract, but rather it inhibited its ability to maintain tension in the contracted state. Also, the time dependency of the relaxation was independent of exposure to NE.

The mechanism of the long-term relaxation was probably due to the opening of potassium channels. This causes hyperpolarization of the cell membrane and closure of voltage-dependent calcium channels (Jackson, 2000; Nelson and Quayle, 1995). This was suggested by the data; the long-term relaxation was not seen in KCl-induced contractions (Fig. 3), was inhibited in the presence of the potassium channel blocker TEA-Cl (Fig. 6; Nakai, 1994; Huang et al., 1999), and was reversed by the addition of 50 mM KCl to the bath (Fig. 5). The greater reversal by KCl at lower doses of *Alseis yucatanensis* (Fig. 5B) may be due to the fact that at the higher concentrations the extract also blocks calcium channels.

In conclusion, an aqueous extract of *Alseis yucatanensis* produced a potent relaxation of rat aortic tissue. At high concentrations *Alseis yucatanensis* produced a rapid relaxation by blocking cell surface and internal calcium channels, with a higher affinity for the internal calcium release channels. At lower doses, *Alseis yucatanensis* produced a profound

relaxation on a longer time scale that may be due to the opening of potassium channels.

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References

Balick, M.J., Nee, M.H., Atha, D.E., 2000. Checklist of the vascular plants of Belize, with common names and uses. Memoirs of the New York Botanical Garden 85, 1–204.

Furchgott, R., Zawadski, J., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373–376.

Huang, Y., Ho, I.H.M., 1996. Separate activation of intracellular Ca^{2+} release, voltage-dependent and receptor-operated Ca^{2+} channels in rat aorta. Chinese Journal of Physiology 39, 1–8.

Huang, Y., Lau, C.W., Chan, F.L., Yao, X.Q., 1999. Contribution of nitric oxide and K+ channel activation to vasorelaxation of isolated rat aorta induced by procaine. European Journal of Pharmacology 367, 231–237.

Jackson, W.F., 2000. Ion channels and vascular tone. Hypertension 35, 173–178.

Nakai, T., 1994. Effects of diazoxide on KCl- and norepinephrine-induced contractions in isolated aorta. Journal of Pharmaceutical Sciences 83, 838–841.

Nelson, M.T., Quayle, J.M., 1995. Physiological roles and properties of potassium channels in arterial smooth muscle. American Journal of Physiolology 268, C799–C822.

Rapoport, R.M., 1987. Effects of norepinephrine on contraction and hydrolysis of phosphatidylinositides in rat aorta. Journal of Pharmacology and Experimental Therapeutics 242, 188–194.

Slish, D.F., Ueda, H., Arvigo, R., Balick, M.J., 1999. Ethnobotany in the search for vasoactive herbal medicines. Journal of Ethnopharmacology 66, 159–165.

Standley, P.C., Williams, L.O., 1975. Flora of Guatemala. Fieldiana (Botany) 24, 1–274.