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Some Characteristic Relaxant Effects of Aqueous Leaf Extract of *Andrographis paniculata* and Andrographolide on Guinea pig Tracheal Rings

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Summary: The ethnomedicinal uses of the aqueous leaf extract of *Andrographis paniculata* Nees (AP) include treatment of pain and inflammation, malaria, asthma and common cold. We designed this study to characterize some effects of AP and those of its andrographolide constituent. Guinea pig tracheal rings suspended in organ baths containing PSS were precontracted with histamine or carbachol and then exposed to cumulative concentrations of AP, andographolide or theophylline. The effect of AP was tested in Ca^{2+}-depleted tracheal rings stimulated with the EC_{50} of histamine in Ca^{2+}-free PSS. IC_{50} and E_{max} values were calculated for each relaxant. Results showed that both AP and andrographolide possessed relaxant effects on the tracheal smooth muscle. While AP was more effective on histamine-induced contraction, andrographolide and theophylline were more effective on carbachol-induced contraction. The IC_{50} values of andrographolide were significantly ($p < 0.05$) higher than those of theophylline in the two contractile agents. The presence of AP significantly ($p < 0.0001$) attenuated the contractile force produced by 6.4 x 10^{-3} M Ca^{2+} in Ca^{2+}-depleted rings. It is concluded that andographolide contributes at least in part to the relaxant action of AP on tracheal smooth muscles. The mechanism of action is related to inhibition of Ca^{2+} influx into tracheal smooth muscle cells.

Keywords: *Andrographis paniculata*, Andrographolide, Tracheal rings, Theophylline, Ca^{2+}

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INTRODUCTION

Andrographis paniculata Nees (Acanthaceae) has been used since ancient times in Asian traditional medicine (Jarukamjorn and Nemeto, 2008). The extract and its bioconstituents possess many medicinal properties including antimalarial (Rahman et al., 1999; Mishra et al., 2009), anti-inflammatory (Sheeja et al., 2006; Abu-Ghefreh et al., 2009; Chandrasekaran et al., 2010), anti-diabetic (Wibudi et al., 2008; Dandu and Inamdar, 2009), and anti-tumour (Kumar et al., 2004; Zhao et al., 2008). Others are hepatoprotective (Kapil et al., 1993; Trivedi and Rawal, 2001), analgesic (Madav et al., 1995), vasorelaxant and cardioprotective (Yoopan et al., 2007; Woo et al., 2008), uterine relaxant (Burgos et al., 2001), antiviral (Lin et al., 2001; Wiart et al., 2005), antidiarrhoeal (Gupta et al., 1990) and antibacterial (Singha et al., 2003) properties. The plant is now being cultivated in Nigeria for herbal medicinal uses (Ameh et al., 2010).

The standardized aqueous extract of the plant is marketed as KalmCold® by Natural Remedies Ltd (Bangalore, India) for the treatment of common cold. This preparation is exported to other parts of the world. There seems to be consistency in the HPLC-DAD-MS fingerprints of aerial parts of the plant obtained from different regions of the world (Arpini et al., 2008). Andrographolide and its closely related diterpines such as neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide are believed to be the main bioactive constituents of the plant (Hu and Zhou, 1982; Jarukamjorn and Nemeto, 2008).

Obstructive airway disorders are often managed with the use of herbal products (Ng et al., 2003; Rivera et al., 2004; Argüder et al., 2009), although questions have been raised about their effectiveness (Singh et al., 2007). The reasons often given for their use include relative cheapness, availability and safety profile when compared to orthodox medicines (Ernst, 2005; Cohen-Kohler, 2008). The ethnomedicinal use of AP for the treatment of common cold and obstructive airway diseases has stimulated interests in our laboratory. In this report we characterize some of the relaxant effects of the extract and andrographolide on the guinea pig tracheal smooth muscle.

MATERIALS AND METHODS

The extract

Sachets of standardized dried aqueous extract of *A. paniculata* were donated by Natural Remedies Ltd (Bangalore, India). The label characteristics on the sachets include batch number: RD/2114; date of manufacture: November 2008; colour: green to brownish green powder; taste: bitter; and bulk density: 0.20 to 0.80. The moisture content was 5.0% and it contained ≥ 10% andrographolide. The sachets were kept airtight and stored in a refrigerator until used for experiments.

Animals

Guinea pigs of either sex (300-400 g) were procured from the Animal House, Department of Physiology, Ambrose Alli University, Ekpoma, Nigeria and were kept in standard plastic cages for two-week acclimatization in the Animal House Unit, Department of Biochemistry, University of Benin, Benin City, Nigeria. The animals were allowed free access to pellets (Bendel Feeds and Flour Mill Ltd, Ewu, Nigeria) and tap water. They were exposed to natural lighting condition, room temperature and handled according to standard protocols for the use of laboratory animals (National Institute of Health USA, 2002). The study was approved by institutional ethical committee on the use of animals for experiments.

Tracheal Ring Experiment

Each guinea pig was sacrificed and the trachea was quickly dissected out and placed in Petri dish containing physiological salt solution (PSS). The tracheae were cleaned of adherent connective tissues as much as possible and cut into rings of 2.5 mm length. The rings were then suspended in L-shaped wire loops in 10 ml organ baths containing the PSS. The composition of the PSS was (g/l): NaCl 6.89, KCl 0.35, KH_2PO_4 0.163, $MgSO_4$ 0.295, D-glucose 2.0, $NaHCO_3$ 2.1, and $CaCl_2$ 0.235. The PSS was bubbled throughout with 95% O_2 and 5% CO_2 gas mixture (BOC Gases Nig. Plc) and temperature was maintained at 37^{o}C. Responses were measured with a force displacement transducer (FT 302) connected to a Grass model 7D polygraph (Grass instrument Co, Quincy, MA, USA). The rings were given a resting force of 1 g (Ozolua et al., 2010) and allowed an equilibration time of 45 min during which the PSS was changed four times. After equilibration, the rings were depolarized with 100 M KCl before experimental protocols.

Cumulative concentration-response relationships were obtained to histamine (1 x 10^{-10} to 4 x 10^{-4} M) and carbachol (1 x 10^{-10} to 4 x 10^{-4} M) to obtain EC_{50} values. The EC_{50} of each contractile agent (5.7 x 10^{-7} M for carbachol and 2 x 10^{-6} M for histamine) was then used to induce contraction in the rings. At the plateau phase of the contraction, cumulative concentrations of AP (0.0625 to 4 mg/ml), andrographolide (1 x 10^{-10} to 6.4 x 10^{-2} M) and theophylline (1 x 10^{-10} to 4 x 10^{-4} M) were added to the organ baths.

In another protocol to determine if the action of the extract might be related to the inhibition of extracellular influx of Ca^{2+} the protocol of Ozolua et al. (2006) was modified. The normal PSS was replaced by Ca^{2+}-free 2 mM EGTA containing PSS for 20 min. The rings were stimulated with 10^{-6} M histamine and then washed four times with Ca^{2+}-free PSS. This was repeated a second time in which 10^{-6} M histamine gave no response. Subsequently, in Ca^{2+}-free PSS (without EGTA) with 10^{-6} M histamine, cumulative concentrations of $CaCl_2$ were added to the organ baths. The protocol was repeated but with the addition of the IC_{50} of AP followed 10 min later with 10^{-6} M histamine before cumulative addition of $CaCl_2$.

Chemicals

Histamine, carbachol, and EGTA [ethyleneglycol-bis-(aminoethyl)-N, N, N', N',-tetraacetic acid] were all obtained from Sigma (UK) and prepared fresh by dissolving in distilled water. Andrographolide was donated by Natural Remedies Ltd (India). It was first dissolved in 95% ethanol before serial dilutions were made in PSS. Other chemicals and reagents were of analytical grade and were obtained from Sigma, May & Baker, BDH or Scharlau Chemie S.A. The extract was dissolved in PSS before addition to the organ bath such that the volume of bath was not altered by more than 200 µl.

Statistical Analysis

Data are presented as mean ± SEM (standard error of the mean) and *n* represents the number of guinea pigs used for a particular experiment. IC_{50} (concentration producing 50% inhibition of maximum contractile response after precontraction) and E_{max} (maximum relaxant response) values were estimated graphically. Comparisons were made where appropriate, by use of Student's t-test. All data were analyzed using GraphPad Prism software (UK). Statistical significance was set at $p < 0.05$.

RESULTS

Figure 1 shows that the extract (AP) completely relaxed tracheal rings regardless of the contractile agent. However, the E_{max} was reached with lower concentrations of AP in histamine precontracted rings compared to carbachol precontracted rings (100.0 ± 0.0% versus 67.6 ± 8.4 at 1 mg/ml, $p < 0.05$). E_{max} was reached in carbachol precontracted rings at AP concentration of 2 mg/ml. The IC_{50} values of AP for both spasmogens were not significantly different (Table 1).

The relaxant effect of andrographolide on histamine or carbachol-induced contractions is shown in Figure 2. While 1.6×10^{-3} M produced 100%

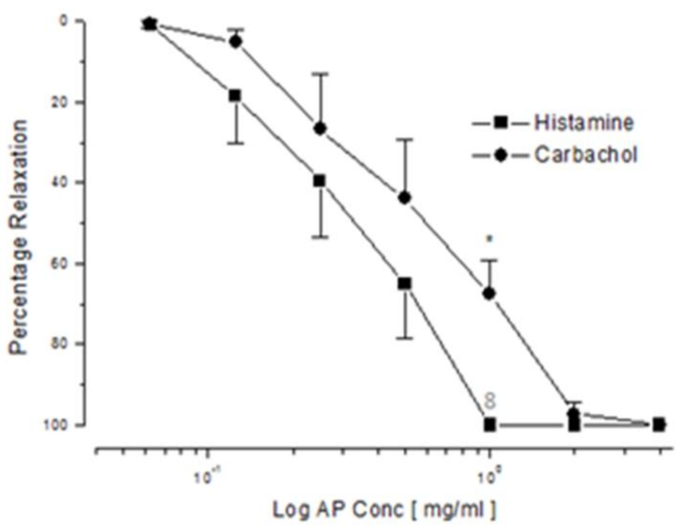

Figure 1.
The relaxant effect of aqueous leaf extract of *A. paniculata* (AP) on histamine and carbachol precontracted tracheal rings. Complete relaxation was produced in both cases. *$p < 0.05$ compared to the corresponding value. n = 5 per group.

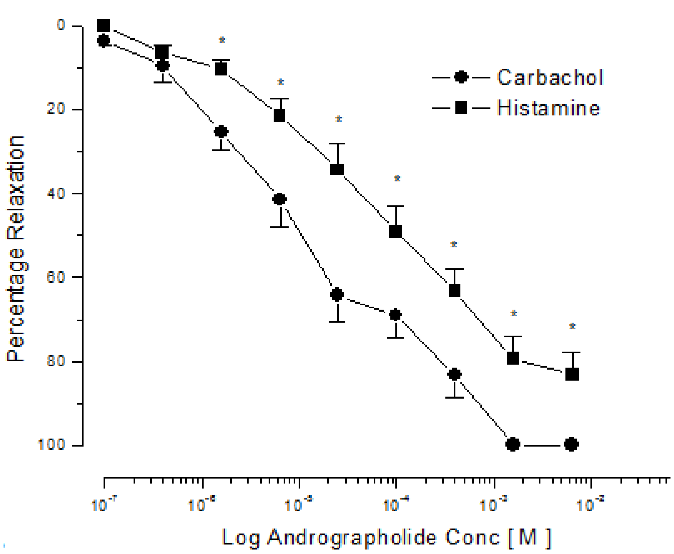

Figure 2.
The relaxant effect of andrographolide on histamine and carbachol-induced contractions of tracheal rings. Complete relaxation was not produced in histamine-contracted rings. *$p < 0.05$ compared to the corresponding value. n = 5 per group.

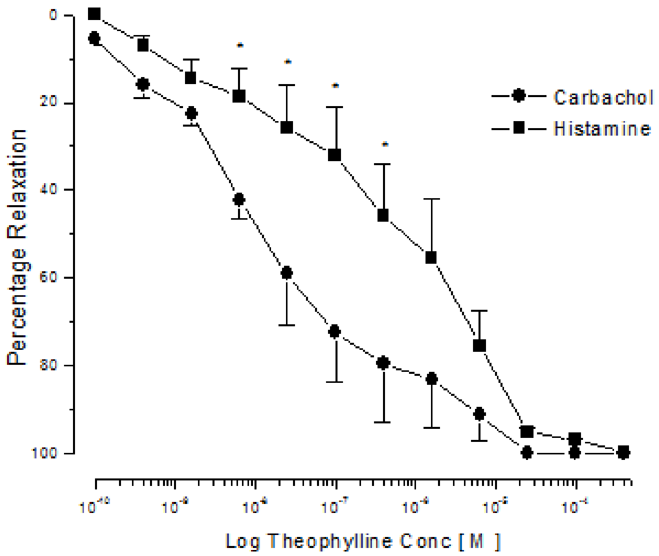

Figure 3.
The relaxant effect of cumulative concentrations of theophylline on histamine and carbachol-induced contractions of tracheal rings. Maximum relaxation was observed in contractile both agents. *$p < 0.05$ compared to the corresponding value. n = 5 per group

Table 1.
Maximum relaxant responses (E_{max}) and concentrations producing 50% relaxant effect (IC_{50}) of AP, andrographolide and theophylline.

	Histamine		Carbachol	
	IC_{50}	E_{max}	IC_{50}	E_{max}
AP	$37.0 \pm 7.8 \times 10^{-2}$ mg/ml	100.0 ± 0.0	$6.4 \pm 1.2 \times 10^{-1}$ mg/ml	100.0 ± 0.0
Andrographolide	$2.3 \pm 0.8 \times 10^{-4}$ M	83.1 ± 5.2	$2.1 \pm 1.5 \times 10^{-5}$ M*	100.0 ± 0.0
Theophylline	$11.0 \pm 4.3 \times 10^{-5}$ M$^{\#}$	100.0 ± 0.0	$2.1 \pm 1.9 \times 10^{-7}$ M*$^{\#}$	100.0 ± 0.0

*$p < 0.05$ compared to corresponding value in row; $^{\#}p < 0.05$ compared to andrographolide value in column. AP = *A. paniculata*. n = 5 per group.

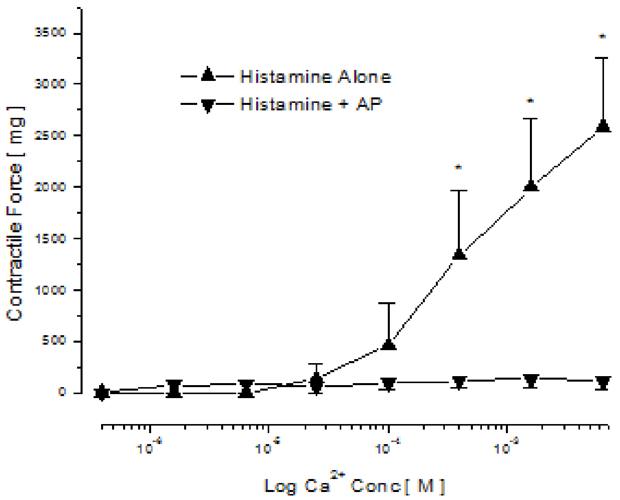

Figure 4.
The contractile force produced by cumulative concentrations of Ca^{2+} in Ca^{2+}-depleted histamine-stimulated tracheal rings suspended in Ca^{2+}-free PSS with or without aqueous leaf extract of *A. paniculata* (AP). *p< 0.05 compared to the corresponding value. n = 5 per group.

relaxation in carbachol precontracted tracheal rings, it was 79.4 ± 5.3% relaxation in histamine precontracted rings. The IC_{50} values for carbachol precontracted rings were also significantly lower than values for histamine precontracted rings (Table 1).

In Figure 3 the relaxant effect of theophylline on histamine or carbachol precontractions is shown. Like andrographolide, relaxation was more easily attained in carbachol precontracted rings than histamine precontracted rings. Although the IC_{50} values were significantly different (p< 0.05) E_{max} were not (Table 1).

The presence of AP in the organ baths significantly inhibited Ca^{2+} loading in Ca^{2+}-depleted tracheal rings stimulated with histamine (Figure 4). At the maximum concentration of $CaCl_2$ to which the rings were exposed (6.4 x 10^{-3} M), the contractile force in presence of AP was 112.5 ± 82.6 mg compared to 2587.0 ± 674.7 mg in the absent of AP (p< 0.0001).

DISCUSSION

This study has shown that both AP and andrographolide relax the isolated tracheal rings. Since andrographolide is a constituent of the extract (Hu and Zhou, 1982; Jarukamjorn and Nemeto, 2008), it can be inferred that the relaxant action of AP on tracheal smooth muscles is dependent at least in part on its andrographolide constituent. While AP was more effective in histamine precontracted rings, andrographolide-like theophylline was more effective in carbachol precontracted rings. Histamine and carbachol induce contraction in tracheal smooth muscles by stimulating H_1 and M_2 receptors respectively both resulting in the activation of receptor-operated channels and influx of Ca^{2+} into the smooth muscle (Walsh, 2002; Ehler, 2008; Jude et al.,2008). However, theophylline acts by inhibiting phosphodiesterase enzyme and consequently facilitating cAMP-mediated increase in intracellular Ca^{2+} levels (Persson, 1986). It is not immediately known if the other constituents of the extract possess H_1 receptor blocking action that would explain the greater effect in histamine-induced contractions.

The relaxant properties of the extract on uterine and vascular smooth muscles have been reported (Burgos et al., 2001; Yoopan et al., 2007; Woo et al., 2008). In both uterine and vascular smooth muscles, the mechanisms of action have been related to inhibition of calcium utilization. From the present study it seems likely that a culminating point in the mechanism of action of the extract and andrographolide is the inhibition of Ca^{2+} influx. This may involve receptor (e.g. H_1 and M_2) blockade (Walsh, 2002; Ehler, 2008), inhibition of cyclic nucleotide metabolism (Persson, 1986), β_2-adrenoceptor activation (Johnson, 2006) or opening of potassium channels (Small et al., 1992).

While the relaxant effects of AP and andrographolide explain the antiasthmatic use of the extract in ethnomedicine, other properties of the plant may also be involved. Since airway inflammation and viruses are often implicated in chronic asthma, the anti-inflammatory (Sheeja et al., 2006; Abu-Ghefreh et al., 2009; Chandrasekaran et al., 2010) and antiviral (Lin et al., 2001; Wiart et al., 2005) properties of AP may accentuate its relaxant action.

In conclusion, this study has shown that both the aqueous leaf extract of *A. paniculata* and its andrographolide both relax tracheal smooth muscles. This effect is due at least partly to the inhibition of extracellular Ca^{2+} influx into the smooth muscles.

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