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Relaxant action mechanism of berberine identified as the active principle of *Argemone ochroleuca* Sweet in guinea-pig tracheal smooth muscle

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Abstract

In this study we investigated the relaxant effect of the aerial parts of *Argemone ochroleuca* (Papaveraceae), which is used in Mexican traditional medicine for the treatment of various respiratory diseases such as cough, bronchitis and asthma. The alkaloid berberine was identified as one of the active relaxant principles (EC50 = 118.50 ± 3.91 μM) in the dichloromethane extract of *A. ochroleuca* (EC50 = 78.03 ± 2.15 μg mL^{-1} with 95.12 ± 3.56% of relaxation). Berberine concentration-dependently relaxed the carbachol-induced precontractions but not histamine- or KCl-induced precontraction. The relaxant effect of berberine was unaffected by the presence of propranolol (3 μM), glibenclamide (10 μM) or ODQ (10 μM). However, 2′, 5′-dideoxyadenosine (10 μM) blocked the log concentration–response curves of berberine. On the other hand, berberine produced a leftward shift of the log concentration–response curves of isoproterenol, forskolin and nitroprusside. Additionally, berberine produced a parallel rightward shift of the concentration–response curve of carbachol in a competitive manner with a pA_2 of 3.87 ± 0.045. The above results suggest that the relaxant effect of berberine on tracheal muscle is due to its antagonistic effect on muscarinic acetylcholine receptors.

Introduction

In Mexican traditional medicine several *Argemone* species are locally known as Chicalote and the aerial parts of these species are used in the treatment of respiratory ailments and ocular disorders (Argueta & Cano 1994). One of these species, *Argemone ochroleuca* Sweet (Papaveraceae), is used as remedy for cough, bronchitis, asthma, cataract removal and ocular allergy and for its sedative, anticonvulsive, tranquilizing, anti-diabetic and antispasmodic properties (Argueta & Cano 1994). Previous chemical investigations on this species have revealed the presence of alkaloids, such as sanguinarine, chelerythrine, protopine, berberine, dihydrosanguinarine, dihydrochelerytrine, α-allocryptopine, heleritrine, cheilantifoline, scouletrine, reticuline and coptisine (Haisova & Slavik 1973; Israilov et al 1986; Chelombit'ko & Nazarova 1988; Takken et al 1993). Recently, Fernandez et al (2005) reported that the flavonoid isoquercitrin, isolated from *Argemone platyceras*, inhibits carbachol- and leukotriene-D4-induced contraction in guinea-pig airways. However, there are no additional reports concerning the pharmacological effects of *Argemone* species on airways smooth muscle. Therefore, the present study was undertaken to evaluate the claimed asthma-relieving effect of *A. ochroleuca*, using the guinea-pig isolated tracheal rings as an experimental model, to identify, through bioassay-guided fractionation, the active principle(s) and also to investigate the tracheal relaxant action mechanism of the main active ingredient, berberine, in leaves and flowers of *A. ochroleuca*.

Materials and Methods

Drugs

Acetylcholine chloride, histamine dihydrochloride, carbachol chloride, DL-propranolol hydrochloride, (±)-isoproterenol hydrochloride, forskolin, nitroprusside, glibenclamide, ODQ (1*H*-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one), 2′, 5′-dideoxyadenosine and ipratropium bromide were purchased from Sigma Chemical Co. (St Louis, MO). The other reagents used were of analytical grade. Glibenclamide, ODQ and forskolin were dissolved in dimethyl sulfoxide (DMSO) and diluted with water. The other drugs were dissolved in distilled water. The extracts and berberine were suspended in distilled water with traces of Tween 80. The final concentration of DMSO or Tween 80 was less than 0.1% and did not significantly affect the trachea response.

Plant material

Argemone ochroleuca Sweet aerial parts were collected in Texcoco, Estado de Mexico, Mexico during June of 2005. The specimens were identified by botanists from the Universidad Autónoma Chapingo Herbarium and a voucher sample was deposited (No. 17559).

Extraction and isolation

The plant aerial part, including branch, leaves and flowers but not seeds, was air-dried at room temperature under shadow. After grinding, 5 kg of plant was extracted at room temperature with hexane (3×30 L, 48 h each), then with CH_2Cl_2 (3×30 L, 48 h each) and finally with MeOH (3×30 L, 48 h each); evaporation of the solvents in vacuum gave 85 g, 33 g and 95 g of syrupy residues, respectively. The crude extracts were tested for activity at different concentrations (17.7–316 $\mu g\,mL^{-1}$) in the trachea ring assay. The CH_2Cl_2 extract showed the most relaxant activity. This extract (32 g) was subjected to percolation over silica gel (0.063–0.200 mm mesh, 200 g) using a step gradient of hexane (1.5 L; F1), hexane–CH_2Cl_2 (1:1, 1.5 L; F2), CH_2Cl_2 (1.5 L; F3), EtOAc (1.5 L; F4) and MeOH (1.5 L; F5) (Figure 1). These fractions were tested for activity (17.7–316 $\mu g\,mL^{-1}$) in the tracheal ring assay. Fraction F5 was the most active and showed a major yellow component in TLC. Ten grams of F5 were taken for a subsequent fractionation on a silica gel column (100 g) using CH_2Cl_2 (F′1), EtOAc (F′2), EtOAc−MeOH (95:5, F′3), EtOAc−MeOH (4:1, F′4) and finally MeOH (F′5) as eluents (Figure 1). The fraction F′4 (4.7 g) was the most active fraction and gave a yellowish powder, which was purified from MeOH−CH_2Cl_2 to afford a yellow solid (700 mg, 0.014%), melting point 142°C (with decomposition). This solid was identified as berberine (Figure 2) by IR, NMR and mass spectrometric analysis (Blaskó et al 1988).

Berberine: IR (KBr): $\nu_{max} = 3434, 1572, 1505, 1386\,cm^{-1}$. FAB^+MS: m/z = 336 (100); ^1H NMR (DMSO-d_6): $\delta = 9.80$ (1H, s, H-8), 8.84 (1H, s, H-13), 8.14 (1H, d, $J = 9.3$ Hz, H-11), 7.98 (1H, d, $J = 9.3$ Hz, H-12), 7.74 (s, H-1), 7.05 (s, H-4), 6.12 (2H, s, OCH_2O), 4.87 (2H, t, $J = 5.5$ Hz, H-6), 4.05 (3H, s, CH_3O), 4.03 (3H, s, CH_3O), 3.18 (2H, t, $J = 5.5$ Hz,

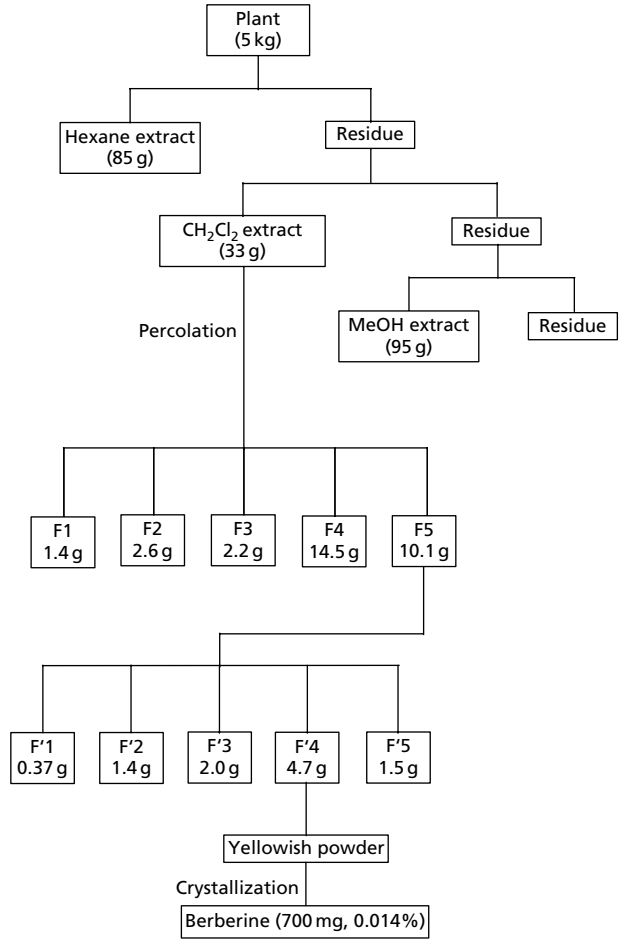

Figure 1 Bioassay-guided fractionation of *Argemone ochroleuca.*

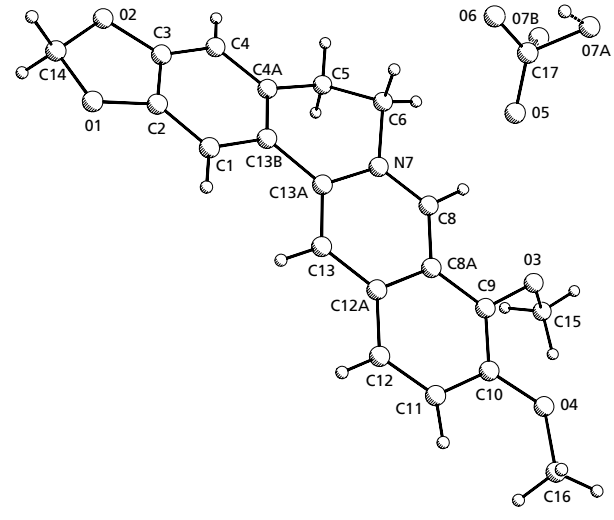

Figure 2 Perspective drawing of the X-ray structure of berberine bicarbonate.

H-5). ^{13}C NMR (DMSO-d_6): $\delta = 150.7$ (C-10), 150.1 (C-3), 148.0 (C-2), 145.7 (C-8), 143.9 (C-9), 137.8 (C-13a), 133.3 (C-12a), 131.0 (C-4a), 127.0 (C-11), 123.9 (C-12), 121.7 (C-8a), 120.7 (C-13b), 120.4 (C-13), 108.7 (C-4), 105.7 (C-1), 102.2 (OCH_2O), 62.2 (C_{10}-OCH_3), 57.3 (C_9-OCH_3) 55.6 (C-6), 26.6 (C-5). These data corresponded with those in the literature (Blaskó et al 1988).

Unequivocal identification of berberine structure, as berberine bicarbonate ($C_{21}H_{19}NO_7$) in this plant, was determined by X-ray diffraction analysis (Figure 2). X-ray data were collected on an Enraf-Nonius CAD4 with a wavelength of 0.71073 Å (molybdenum) using a yellow crystal grown from CH_2Cl_2–MeOH. The structure was solved and refined by using SHELXL97. Data collection was conducted at 293°K on a monoclinic crystal, $P2_1/n$; $a = 7.0340(18)$ Å, $b = 15.760(2)$ Å, $c = 16.295(2)$ Å, $\beta = 100.185(12)^o$, $V = 1777.9(6)$ Å^3, $Z = 4$; $R_1 = 4.9\%$, $wR_2 = 11.5\%$, GOF = 0.998.

Animals and guinea-pig trachea preparation

Male guinea-pigs, 300–450 g, bred under conventional conditions and fed with standard diet (Purina Pellets) and drinking water, were used. Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999, Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio) and in compliance with international rules on the care and use of laboratory animals.

The guinea-pigs were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (95 mg kg^{-1}). The chest was opened to obtain the trachea, which was transferred to a dish containing warm Krebs solution. After removal of excess connective tissue and fat the trachea was divided into eight small rings of about 2-mm length containing two or three cartilaginous segments. Each tracheal ring was hung between two hooks inserted into the lumen, and placed in a 10-mL organ bath containing Krebs solution with composition (in mM): NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 25 and glucose 11.1. This solution was maintained at 37°C and bubbled with 5% CO_2–95% O_2. Isometric tension was recorded through an eight-channel Biopack System polygraph MP100 via a Grass FT 03E force transducer. The data were digitalized and analysed by mean of software for data acquisition (Acknowledge 3.7.3). Tissues were placed under a resting tension of 1.5 g and allowed to stabilize for 60 min and they were washed with fresh Krebs solution at 15-min intervals before starting the experiments. After a stabilization period the tracheal rings were contracted with acetylcholine chloride (30 μM) twice at 30-min intervals and washed after stimulation with fresh Krebs solution. Thirty minutes after the tissues were contracted with carbachol chloride (3 μM), cumulative additions of crude extracts (17.7, 31.6, 56.2, 100, 177 or 316 μg mL^{-1}), fractions or reference drugs were made to the bath to yield the required tracheal relaxant effects and allowed to reach a steady state at each concentration. The relaxant potencies of crude extracts or reference drugs were expressed as EC50 (half-maximal effective concentration). In another set of experiments the tissues were precontracted with carbachol (3 μM), histamine (30 μM) or KCl (30 mM) and cumulative concentration–response curves for dichloromethane extract or berberine were constructed.

To evaluate the participation of ATP-sensitive potassium channel and β-adrenergic receptors, glibenclamide (10 μM), propranolol (3 μM), or their vehicles, were incubated for 5 min, after the carbachol (3 μM)-precontracted tissue reached a steady state, then berberine was added at different concentrations (26, 47, 84, 151, 268 476 μM). Similarly, 2′, 5′-dideoxyadenosine (10 μM) or ODQ (10 μM) were added 5 min before the addition of berberine (26, 47, 84, 151, 268, 476 μM), or its vehicle, after carbachol (3 μM)-induced precontraction reached a steady state. To observe the effect of berberine (151, 268, 476 μM) on the relaxant responses of isoproterenol (10^{-10} to 3.16×10^{-6}M), forskolin (10^{-8} to 10^{-6} M) and nitroprusside (10^{-10} to 10^{-5} M) to carbachol (3 μM)-induced precontractions, trachea rings were incubated with berberine after the precontraction reached a steady state for 5 min before the first addition of relaxant drug.

To determine the antagonistic effects of berberine against the contractions elicited by carbachol, cumulative concentration–response curves for carbachol (10^{-10} to 10^{-2} M) were constructed in the absence or in the presence of different concentrations of the berberine, as follows: after the tissues were incubated with different concentrations of berberine bicarbonate (151, 268, 476 μM), ipratropium bromide (1.77×10^{-3}, 3.16×10^{-3}, 5.62×10^{-3}, 1×10^{-2} μM, used as reference drug) or their vehicles (distilled water with Tween 80 in traces, for berberine and distilled water for ipratropium) for 15 min, carbachol was cumulatively added into normal Krebs solution. The antagonistic potentials of berberine and ipratropium were expressed as pA_2 values.

Data analysis

The EC50 (for relaxant effect and antagonistic effect experiments) values were calculated by linear regression (Tallarida 2000). All the values are shown as mean ± s.e.m. of at least six experiments. The differences among these values were statistically calculated by one-way analysis of variance, and then determined by Dunnett's *t*-test (Montgomery 1991). $P < 0.05$ was considered statistically significant. The pA_2 value was calculated according to the Schild equation: $\log((A'/A) - 1) = \log B - \log K_B$, where A is the EC50 of carbachol in absence of the antagonist, A′ is the EC50 of carbachol in the presence of antagonist, K_B is the affinity constant for the antagonist and B is the concentration (M) of the antagonist. The value of pA_2 is the abscissa to the origin when $A'/A = 2$ (Tallarida & Murray 1987).

Results

Hexane, dichloromethane and methanol extracts obtained from aerial parts without seeds of *Argemone ochroleuca*, concentration-dependently ($P < 0.05$) relaxed the carbachol (3 μM)-induced precontractions (Figure 3); the values of

maximal relaxation were 59.13 ± 4.86, 95.12 ± 3.56 and $31.87 \pm 3.76\%$ respectively. The dichloromethane extract was also the most potent relaxant extract (EC50 = $78.03 \pm 2.15\,\mu g\,mL^{-1}$). This extract showed a more potent relaxant effect on the carbachol ($3\,\mu M$)-induced precontrations than when the precontractions were elicited by $30\,\mu M$ histamine ($38.6 \pm 5.4\%$ of relaxation), but it was unable to relax the guinea-pig trachealis muscle when it was precontracted with 30 mM KCl. These results encouraged us to perform the bio-guided fractionation of the dichloromethane extract of *Argemone ochroleuca*. The scheme of fractionation and identification of the tracheal smooth muscle relaxing compound from this extract is showed in Figure 1. After percolation of dichloromethane extract with different solvents, the activity was found in two fractions: one obtained with EtOAc (F4, Figure 1; EC50 = 143.9 $7.3\,\mu g\,mL^{-1}$) and the other with methanol (F5, Figure 1; EC50 = $110.2 \pm 5.8\,\mu g\,mL^{-1}$). The methanol fraction was used for further fractionation using open column chromatography. The fraction eluted with EtOAc−MeOH (4:1), obtained as a yellowish semi-solid, showed the highest activity (F4′, Figure 1; EC50 = $56.00 \pm 4.15\,\mu g\,mL^{-1}$). The major component of this mixture was precipitated from MeOH−CH_2Cl_2 (1:1) as a yellow solid (700 mg, 0.014%), whose melting point began with decomposition at 142°C, and it was identified as berberine bicarbonate (Figure 2) by comparison of its spectral data (IR, ^{1}H NMR, ^{13}C NMR and MS) with the literature data (Blaskó et al 1988) and by X-ray diffraction analysis. This pure compound concentration-dependently relaxed the carbachol-induced precontractions, but it was unable to relax the guinea-pig trachealis muscle when it was precontracted with $30\,\mu M$ histamine (25% relaxation only; $P > 0.05$ vs control) or 30 mM KCl. The EC50 value for berberine bicarbonate was $118.50 \pm 3.91\,\mu M$ ($46.8 \pm 1.55\,\mu g\,mL^{-1}$) while the EC50 value for ipratropium bromide, used as reference drug, was $0.0032 \pm 0.00015\,\mu M$.

The relaxant effect of berberine was not affected by pretreatment with glibenclamide ($10\,\mu M$), an ATP-sensitive potassium-channel blocker. Also, the β-adrenoceptor antagonist propranolol at $3\,\mu M$ did not antagonize the relaxant effect of berberine, a concentration that significantly blocked the effect of isoproterenol (3.16×10^{-10} to 10^{-6} M) to relax carbachol-induced precontractions. In the same form ODQ ($10\,\mu M$), a soluble guanylate cyclase inhibitor, blocked the effect of nitroprusside (10^{-10} to 10^{-5} M), but did not affect the log concentration–response curves of berberine. However, 2′, 5′-dideoxyadenosine ($10\,\mu M$), an adenylate cyclase inhibitor, blocked the log concentration–response curves of berberine, in the same manner that it blocked the effect of forskolin (1×10^{-8} to 1.77×10^{-6} M) to relax carbachol-induced precontractions (Table 1).

On the other hand, berberine significantly reduced the EC50 values of isoproterenol, forskolin and nitroprusside (Table 2).

In other experiments, berberine produced a parallel rightward shift of the concentration–response curve of carbachol (EC50 = $0.10 \pm 0.014\,\mu M$) in a competitive manner at concentration of $151\,\mu M$ (EC50 = $0.34 \pm 0.099\,\mu M$), $268\,\mu M$ (EC50 = $2.18 \pm 0.388\,\mu M$) and $476\,\mu M$ (EC50 = $55.9 \pm 13.6\,\mu M$; Figure 4A), with a pA_2 of 3.87 ± 0.045. Ipratropium bromide also produced a parallel rightward shift of the concentration response curve of carbachol (Figure 4B), but this displacement was more potent than berberine, showing a pA_2 of 9.05 ± 0.057 (Figure 5).

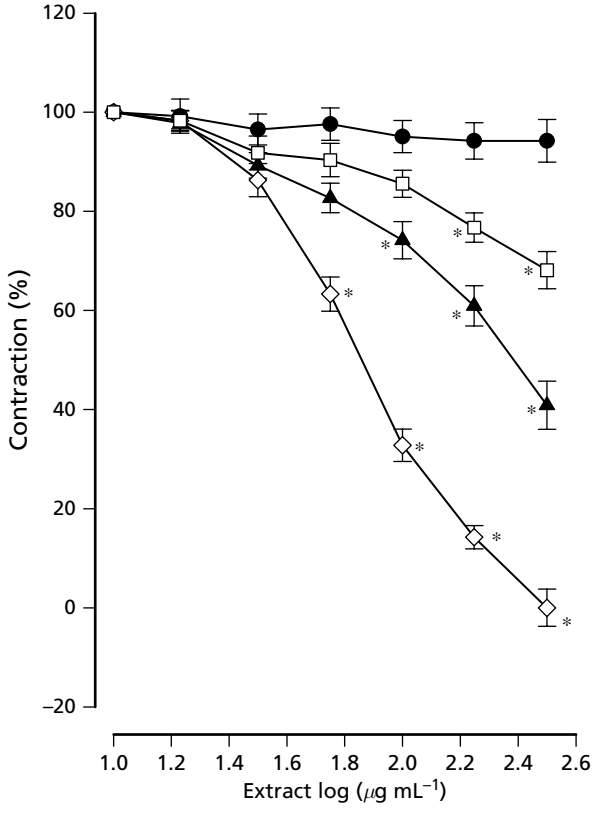

Figure 3 The relaxant effects of hexane (▲), dichloromethane (◊) and methanol (□) extracts of *A. ochroleuca* ($17.7–316\,\mu g\,mL^{-1}$) or control (●) on carbachol ($3\,\mu M$)-induced precontraction in guinea-pig trachea. Each point value represent the mean ± s.e.m., n = 6. $^*P < 0.05$, vs respective control at the same time; Dunnett's *t*-test after analysis of variance test.

Table 1 EC50 (μM) values of berberine and forskolin against carbachol ($3\,\mu M$)-induced precontractions in guinea-pig tracheal smooth muscle in the absence and presence of 2′,5-dideoxyadenosine

2′,5′-Dideoxyadenosine (μM)	Berberine bicarbonate EC50 (μM)	Forskolin EC50 (μM)
0	118.50 ± 3.91	0.262 ± 0.045
10	194.88 ± 76.05*	0.947 ± 0.121*

Values are presented as the means ± s.e.m., n = 6 (where n is the number of experiments). $^*P < 0.05$, vs respective control; Student's no-paired *t*-test.

Table 2 EC50 (μM) values of isoproterenol, forskolin and nitroprusside against carbachol (3 μM)-induced precontractions in guinea-pig trachealis in the absence and presence of berberine bicarbonate

Berberine (μM)	Isoproterenol (μM)	Forskolin (μM)	Nitroprusside (μM)
0	0.064 ± 0.0069	0.219 ± 0.018	0.816 ± 0.149
151	0.0081 ± 0.0036*	0.038 ± 0.0038*	0.014 ± 0.0043*
268	0.0027 ± 0.0003*	0.017 ± 0.0082*	0.0086 ± 0.0022*
476	0.0009 ± 0.0002*	0.0117 ± 0.0028*	0.0019 ± 0.0007*

Values are presented as the means ± s.e.m., n = 6 (where n is the number of experiments). *$P < 0.05$ versus respective control; Dunnett's *t*-test after analysis of variance test.

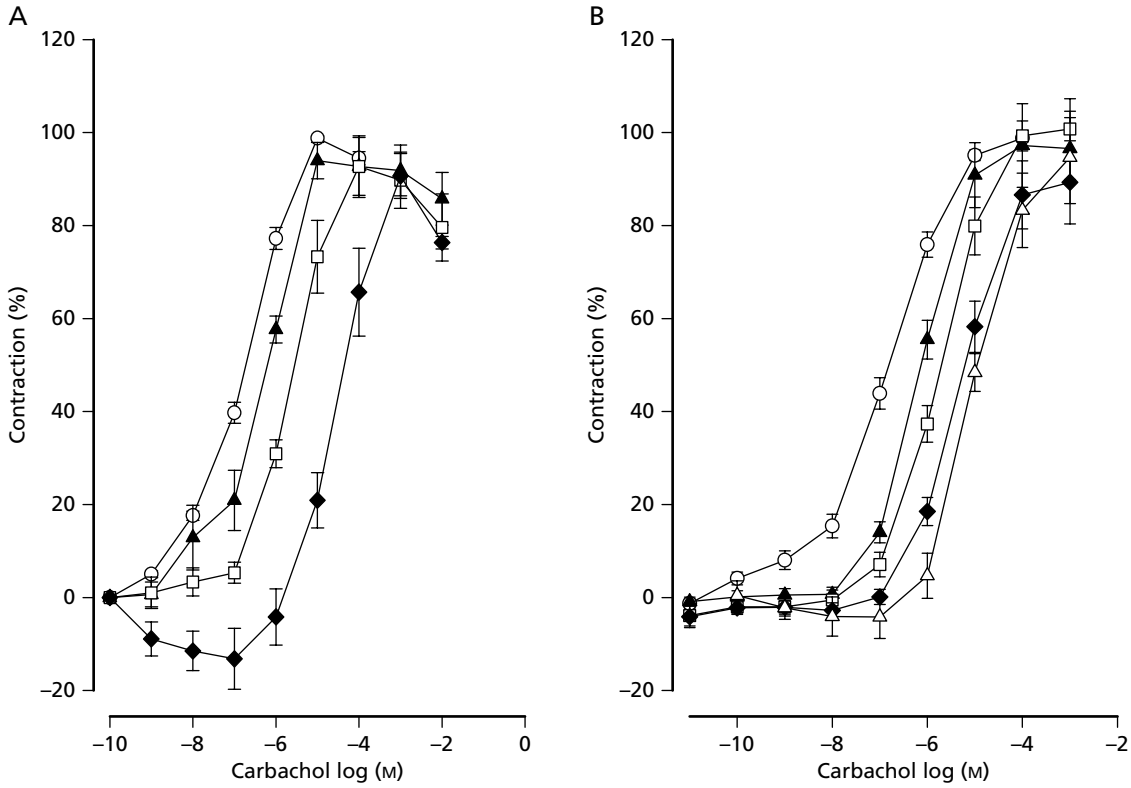

Figure 4 The inhibitory effects of berberine bicarbonate (A: 151 μM (▲), 268 μM (□), 476 μM (◊)) or ipratropium bromide (B: 1.77×10^{-3} μM (▲), 3.16×10^{-3} μM (□), 5.62×10^{-3} μM (♦), 1×10^{-2} μM (△)) on the contractile response of carbachol (○) on guinea-pig trachea. Each point value represents the mean ± s.e.m. of 5–9 experiments.

Discussion

In this study, we found strong evidence that *Argemone ochroleuca* elicits a relaxant effect in tracheal smooth muscle. The relaxant EC50 value for the dichloromethane extract of aerial parts of this plant was significantly lower than the EC50 values obtained for the methanol and hexane extracts (Figure 3). This suggested that the dichloromethane extract contains active relaxant compounds. These results provide experimental support for the use of this plant as a remedy for asthma in Mexican traditional medicine (Argueta & Cano 1994). In addition, from the bioassay-guided fractionation of the dichloromethane extract, berberine bicarbonate was isolated as one of the active principles responsible for the relaxant effect.

Berberine has been reported before in *Argemone ochroleuca* and other *Argemone* species (Haisova & Slavik 1973; Israilov et al 1986; Chelombit'ko & Nazarova 1988; Takken et al 1993). Berberine has been reported to have several pharmacological actions, including anti-tumour (Iizuka et al 2003), anxiolytic (Peng et al 2004), analgesic (Yesilada & Küpeli 2002), in-vitro antioxidant potential (Shirwaikar et al 2006), acetylcholinesterase inhibition (Ingkaninan et al 2006) and anti-diarrhoeal activity. Further-

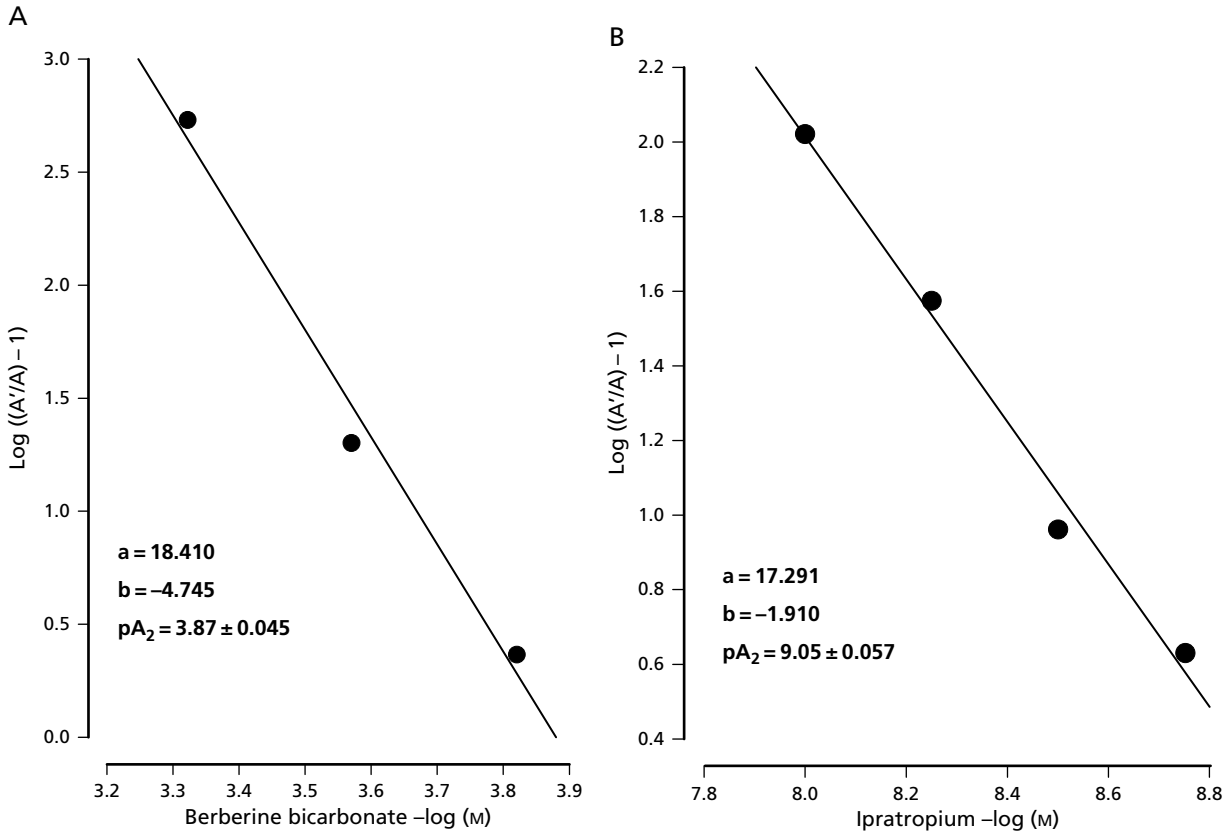

Figure 5 Schild plot for berberine bicarbonate (A) or ipratropium bromide (B).

more, berberine has cardiovascular effects (Lau et al 2001) and in isolated vascular preparations causes relaxation (Ko et al 2000).

The effect of berberine on the isolated guinea-pig trachealis muscle has been studied by Abdel-Haq et al (2000) and it was found that the berberine relaxant effect is not antagonized either by the β-blocker timolol or by xanthine amine congener, an adenosine antagonist. In our study, the cumulative concentration–relaxing response curve of berberine to carbachol-induced precontraction was not modified by propranolol (3×10^{-6} M), a non selective β-adrenoceptor blocker, suggesting that its relaxant effect is not through the activation of β-adrenoceptors. These results are in agreement with those obtained by Abdel-Haq et al (2000). Despite berberine potentiating isoproterenol relaxation (Table 2), it is not possible to consider that berberine acts on β-adrenoceptors, because it has been reported that the relaxant effect of berberine is not antagonized by timolol (Abdel-Haq et al 2000) and propranolol (this study), two very well known β-adrenoceptor antagonists. Hence the synergistic effect should be due to other mechanisms.

It is known that the opening of K^+_{ATP} channels mediates the relaxation of isolated guinea-pig trachea (Thirstrup et al 1997). To investigate whether K^+_{ATP} channels are involved in the guinea-pig trachea relaxation response to berberine, the preparation was pretreated with glibenclamide, a K^+_{ATP}-channel blocker; however, glibenclamide did not affect its relaxant response, suggesting that berberine's relaxant effect is not through the opening of ATP-sensitive potassium channels. Several NO-releasing compounds, such as sodium nitroprusside and endogenous NO, activate soluble guanylate cyclase (sGC) that increases cyclic GMP (cGMP) and relaxes airway smooth muscle (Hall 2000). Berberine produced a parallel leftward shift of the log concentration–response curves of nitroprusside to carbachol (3 μM)-induced precontractions of the trachealis, and significantly reduced the EC50 values (Table 2). However the tracheal relaxant effect was not inhibited by pretreatment with the sGC inhibitor ODQ. These results suggest that berberine induced relaxation of the guinea-pig trachea by a mechanism of action that is unlike to the activation of sGC/cGMP pathways, although it has been suggested that endothelial NO is likely different to be involved in the berberine vasodilating activity (Chiou et al 1998; Ko et al 2000).

The involvement of the activation of adenylate cyclase/cyclic AMP (AC/cAMP) pathways in the relaxant action induced by berberine was drawn from the fact that berberine produced a significant reduction of the EC50 of forskolin (Table 2), an activator of adenylate cyclase (Seamon et al 1983). Furthermore, in the presence of the adenylate cyclase inhibitor 2′, 5′-dideoxyadenosine (Sabouni et al 1991), the relaxant effect of berberine was reduce significantly (Table 1). This experiment suggested that the relaxant effect of berberine is in part via the stimulation

of adenylate cyclase. However, more experimental data are necessary to confirm this postulation.

On the other hand, berberine concentration-dependently relaxed the carbachol-induced precontractions but not histamine- or KCl-induced precontraction. The CH_2Cl_2 extract of *A. ochroleuca* was also able to relax more efficiently the carbachol-induced precontractions than histamine-induced precontraction and it was unable to relax the KCl-induced precontractions. These results suggested that cholinergic receptors are involved in the relaxant effect of berberine and the extract. Considering the last observation, an additional experiment was designed to investigate the anticholinergic effect of berberine, using as reference drug ipratropium, a non-selective muscarinic receptor antagonist with clinical uses in the treatment of asthma and chronic obstructive pulmonary disease (Eglen et al 1999). In these experiments, berberine produced a clear parallel rightward shift of the concentration–response curve of carbachol in a competitive manner (Figure 4A) with a pA_2 of 3.87 ± 0.045 (Figure 5A). The antagonistic effect of berberine was considerably lower than the antagonistic effect of ipratropium, with a pA_2 value of 9.05 ± 0.057 (Figure 5A). These experiments provided additional support that the relaxant effect of berberine is through an antagonistic effect on muscarinic receptors. This experiment is in agreement with the antimuscarinic effect reported for berberine in other tissues (Tsai & Ochillo 1991). It has been established that stimulation of muscarinic M_3 receptors mediates smooth muscle contraction and although 50–80% of muscarinic receptors on airways smooth muscle are M_2 type receptors, the function of these receptors is still unclear. Stimulation of M_2 muscarinic receptors in airways smooth muscle leads to inhibition of activation of adenylate cyclase, which results in an acute lowering of intracellular cAMP levels (Fryer & Jacoby 1998). Other studies provide evidence of the contractile role of the M_2 receptor when M_3 muscarinic receptors are blocked (Ehlert 2003; Walker et al 2004). Other reports have suggested that prolonged stimulation of muscarinic M_2 receptors may sensitize adenylate cyclase and thus the M_2 receptor present on airway smooth muscle may play two roles: an acute inhibition of adenylate cyclase, and in the long term, due to homoeostatic regulation, the upregulation of adenylate cyclase expression, thus counteracing the action of muscarinic agonists at M_3 receptors coupled to phospholipase C (Hall 2000; Michal et al 2001; Mistry et al 2005). The results obtained in this study provide a key point for the identification of the subtypes of muscarinic receptor associated with the action mechanism of the relaxant effect of berberine.

The antagonist muscarinic effect of *Argemone platyceras*, another *Argemone* species known also as Chicalote, has already been reported (Fernández et al 2005). However, the flavonoid isoquercitrin was identified as the active principle (Fernández et al 2005), and that work did not report the presence of berberine.

It has been reported that the in-vivo administration of berberine induces IL-12 production in macrophages, and it has been suggested that this response is importantly involved in the immunotherapy of asthma (Kim et al 2003). Thus, the identification of berberine as the active relaxant principle in *A. ochroleuca* has a relevant role, because this effect can be related to the traditional use of this medicinal plant by the Mexican people in the treatment of asthma.

Finally we cannot discount the presence of other active relaxant compounds in *A. ochroleuca*, because in the fractionation process we obtained two active fractions (F4 and F5, Figure 1) with a similar level of activity ($EC50 = 143.9 \pm 7.3\,\mu g\,mL^{-1}$ for fraction F4 and $EC50 = 110.2 \pm 5.8\,\mu g\,mL^{-1}$ for fraction F5), although these values did not match with the abundant presence of berberine in F5.

Conclusion

In conclusion, this study demonstrated that *Argemone ochroleuca* produces a clear relaxant effect on guinea-pig trachealis muscle, where one of the active principles was identified as the alkaloid berberine. This compound has the ability to produce a weak antagonistic effect on the muscarinic receptors, and its action mechanism involves the activation of adenylate cyclase/cyclic AMP (AC/cAMP) pathways. Finally, this study provides experimental support for the traditional use of this medicinal plant in the treatment of asthma.

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