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The involvement of K^+ channels and $G_{i/o}$ protein in the antinociceptive action of the gallic acid ethyl ester

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Abstract

The anti-hyperalgesic action, antinociception, and also the possible mechanisms involved in the action of gallic acid ethyl ester (GAEE) isolated from the aerial part of *Phyllanthus urinaria*, have been investigated in different models of chemical, mechanical and thermal nociception in mice and rats. GAEE given by intraperitoneal (i.p.), oral (p.o.), intrathecal (i.t.) or by intracerebroventricular (i.c.v.) routes produced dose-related antinociception when assessed against chemical nociception in mice. GAEE significantly inhibited the hyperalgesia induced by bradykinin or substance P in rat paw, but did not affect the hyperalgesia caused by carrageenan or prostaglandin E_2. Furthermore, GAEE, in contrast to morphine, was completely ineffective in the hot-plate test in mice. The antinociception produced by GAEE (i.p.) in the formalin test was significantly reversed by i.c.v. treatment of animals with pertussis toxin and by i.t. administration of K^+ channel blockers such as apamin, charybdotoxin or glibenclamide, but not by tetraethylammonium. In contrast, GAEE (i.p.) antinociception was unaffected by i.p. treatment of animals with naloxone or by nitric oxide precursor, L-arginine, and this action was not secondary to its anti-inflammatory effect, nor was it associated with non-specific effects such as muscle relaxation or sedation. Thus, GAEE produces dose-dependent and pronounced systemic, spinal and supraspinal antinociception in mice, probably via activation of K^+ channels and by a $G_{i/o}$ pertussis toxin-sensitive mechanism.

Keywords: Gallic acid ethyl ester (GAEE); (*Phyllanthus*); Antinociception; K^+ channel; Pertussis toxin; (Mouse); (Rat)

1. Introduction

Previous studies from our group have shown that the hydroalcoholic extracts obtained from the leaves, stems and roots of several plants belonging to the genus *Phyllanthus* (Euphorbiaceae), such as *P. corcovadensis*, *P. urinaria*, *P. niruri*, *P. tenellus*, *P. sellowianus* and *P. caroliniensis*, and the extract of callus culture obtained from some plants of this genus administered either by intraperitoneal (i.p.) or oral (p.o.) routes, produce dose-related antinociception when assessed in chemical but not in thermal behavioural models of nociception in mice (Gorski et al., 1993; Santos et al., 1994, 1995a; Cechinel-Filho et al., 1996). In marked contrast to that reported for most non-steroidal anti-inflammatory drugs, the active principles present in such *Phyllanthus* species were largely effective in preventing the neurogenic nociception caused by formalin or by capsaicin (Santos et al., 1995a,b), by a mechanism that is still not completely understood, but is unlikely to be associated with interaction of opioid, adrenergic (either α_1 or α_2) or serotonergic systems, or with the L-arginine-nitric oxide pathway (Santos et al., 1995a,b).

Many classes of naturally-occurring secondary metabolites have been isolated and characterised in the plants of the genus *Phyllanthus* such as flavonoids, alkaloids, terpenes, lignans, tannins and phenols (Ueno et al., 1988; Bachmann et al., 1993; Foo, 1993; Santos et al., 1995c; Miguel et al., 1995, 1996; for review, see Calixto et al., 1998). We have recently shown that some steroids, flavonoids and tannins isolated from plants of the genus *Phyllanthus*, identified as β-sitosterol, stigmasterol, geraniin, furosin and quercetin, produce significant and dose-related antinociception when assessed in several

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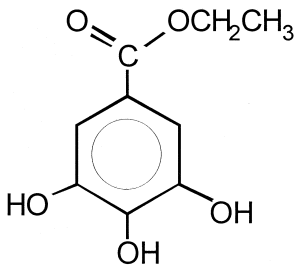

Fig. 1. Molecular structure of GAEE.

chemical models of nociception in mice (Santos et al., 1995c; Cechinel-Filho et al., 1996; Miguel et al., 1996).

In this study, we have investigated the anti-hyperalgesic and antinociceptive properties and also the possible mechanisms which underlie the antinociceptive action of gallic acid ethyl ester (GAEE) (Fig. 1), isolated from *P. urinaria* in thermal, mechanical and chemical models of nociception in mice and rats.

2. Methods

2.1. Isolation of GAEE

Roots and aerial parts of *P. urinaria* were collected in the State of Santa Catarina, Brazil, and classified by Dr. Leila da Graça Amaral and Ms. Mirian Ulyssea (Department of Botany, Federal University of Santa Catarina, UFSC). A voucher specimen was deposited in the herbarium FLOR of the Department of Botany, UFSC, Florianópolis, Brazil.

The dried plant material was powdered and extracted exhaustively by percolation with 90% ethanol at room temperature. The extracts were concentrated under reduced pressure to 1/5 of their initial volume and then, after dilution with five volumes of water, were extracted successively with hexane, dichloromethane and ethyl acetate. The GAEE (Fig. 1) was isolated from ethanol extract by chromatography on a silica gel column eluted with chloroform–methanol, as described previously (Miguel et al., 1995; Cechinel-Filho et al., 1996). The compound was identified by direct comparison with authentic samples, synthesis and by spectroscopic data (infra-red, proton- and carbon-nuclear magnetic resonance). This compound has been isolated from other species of *Phyllanthus*, such as *P. sellowianus*, *P. niruri*, *P. fraternus* and *P. carolinensis* (Miguel et al., 1995; Cechinel-Filho et al., 1996 and unpublished results).

2.2. Pharmacological analysis

2.2.1. Animals

Non-fasted male Swiss mice (25–35 g) or male Wistar rats (150–180 g), housed at 22 ± 2°C under a 12-h light/12-h dark cycle and with access to food and water ad libitum, were acclimatised to the laboratory for at least 1 h before testing and were used throughout the experiments. The experiments reported were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals according to Zimmermann (1983).

2.2.2. Acetic acid-induced abdominal constriction

The abdominal constrictions resulting from i.p. injection of acetic acid (0.6%) were produced according to the procedure described previously (Corrêa et al., 1996; Vaz et al., 1996). Animals were pre-treated with GAEE by i.p. (50.5–504.6 μmol/kg) or p.o. (252.3–1009.2 μmol/kg) routes, 30 and 60 min prior to injection of acetic acid, respectively. Control animals received a similar volume of vehicle injection. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions, i.e., the difference between the control animals (vehicle pre-treated mice) and the mice pre-treated with GAEE.

2.2.3. Formalin-induced nociception

The procedure used was essentially similar to that described previously (Vaz et al., 1996; Santos and Calixto, 1997a). Animals were injected intraplantarly with 20 μl of 2.5% formalin solution (0.92% of formaldehyde), made up in phosphate-buffer solution (concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer 10 mM), in the right hindpaw. Mice were immediately placed in a glass cylinder 20 cm in diameter and observed from 0 to 30 min following formalin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The first phase of the nociceptive response normally peaked 5 min after formalin injection and the second phase 15 to 30 min after formalin injection, representing the neurogenic and inflammatory nociceptive responses, respectively (Hunskaar and Hole, 1987). Animals were treated with GAEE by i.p. (5.0–50.5 μmol/kg) or p.o. (252.3–1009.2 μmol/kg) routes, 30 and 60 min before formalin injection, respectively. Other groups of animals were treated with GAEE intracerebroventricularly (i.c.v., 1.5–50.4 nmol/site) or intrathecally (i.t., 5.0–151.3 nmol/site) as described previously (Vaz et al., 1996; Santos and Calixto, 1997a; Beirith et al., 1998), 15 min before formalin injection. Control animals received a similar volume of vehicle systemically (i.p. or p.o., 10 ml/kg) or centrally (i.c.v. or i.t., 5 μl/site).

To investigate whether the antinociceptive activity of GAEE in formalin-induced nociception was associated with anti-oedematogenic activity, we measured the paw oedema by comparing the difference in weight between the formalin-treated paw and the control paw (treated with vehicle). For this purpose, animals were sacrificed 30 min after formalin injection by cervical dislocation, and the paw was cut at the knee joint and weighed on an analytical balance.

2.2.4. Capsaicin-induced nociception

In an attempt to provide more direct evidence concerning the possible antinociceptive effect of the GAEE on neurogenic nociception, we also investigated whether GAEE antagonised capsaicin-induced licking in the mouse paw. The procedure used was similar to that described previously (Santos and Calixto, 1997a,b). After the adaptation period, 20 μl of capsaicin (1.6 μg/paw made in phosphate-buffer solution) was injected intraplantarly in the right hindpaw. Animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. Animals were treated with GAEE by i.p. (15.1–151.3 μmol/kg) or p.o. (252.3–1009.2 μmol/kg) routes, 30 and 60 min before capsaicin injection, respectively. Control animals received a similar volume of vehicle injected by i.p. or p.o. routes.

2.2.5. Hyperalgesia in the rat paw

The procedures used were similar to those described previously (De Campos et al., 1996). The animals were pre-treated i.p. with the GAEE (15.1–151.3 μmol/kg) 30 min before injection of 0.1 ml of bradykinin (3 nmol/paw), substance P (10 nmol/paw), carrageenan (300 μg/paw), prostaglandin E_2 (10 nmol/paw) or phosphate-buffer solution alone, into the right hindpaw. The hyperalgesia was evaluated 0.5 h later, except for carrageenan, which was assessed at 3 h. The nociceptive threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure to the dorsal site of inflamed or control paws, using a Basile analgesy meter (Ugo Basile, Milan, Italy) according to the method of Randall and Selitto (1957), with minor modifications. The weight on the analgesy meter ranged from 0 to 750 g, and the threshold was expressed as load (g) tolerated. When bradykinin was used, animals were pre-treated with the angiotensin-converting enzyme-inhibitor, captopril (5 mg/kg, s.c.), 1 h prior to experiments, to prevent its degradation (Corrêa and Calixto, 1993).

2.2.6. Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37) was maintained at 56 ± 1°C. Animals were placed into a glass cylinder and the time between placement and shaking or licking of the paws or jumping was recorded as the index of response latency. An automatic 30 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Animals were treated with GAEE (up to 151.3 μmol/kg, i.p.), morphine (6.6 μmol/kg, s.c.) or with vehicle (10 ml/kg, i.p.) 30 min before testing.

2.2.7. Measurement of motor performance

In order to evaluate the possible non-specific muscle-relaxant or sedative effects of GAEE, the mice were tested on the rota-rod (Vaz et al., 1996; Beirith et al., 1998). The apparatus consisted of a bar, 2.5 cm in diameter, subdivided into six compartments by disks 25 cm in diameter (Ugo Basille, Model 7600). The bar rotated at a constant speed of 22 revolutions per min. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 60 s. Animals were treated with GAEE (151.3 μmol/kg, i.p.) or with the same volume of vehicle (10 ml/kg, i.p.) 30 min before the experiments. The results are expressed as the time (s) for which animals remained on the rota-rod. The cut-off time used was 60 s.

2.2.8. Analysis of the possible mechanism of action of GAEE

To investigate the participation of opioid system in the antinociceptive effect of the GAEE, mice were pre-treated with naloxone (a non-selective opioid receptor antagonist, 13.7 μmol/kg, i.p.), 15 min before administration of GAEE (50.5 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or vehicle (10 ml/kg, i.p.). The other groups of animals received GAEE, morphine, naloxone or vehicle, 30 min before the formalin injection (Vaz et al., 1996; Santos and Calixto, 1997a). To assess the possible participation of $G_{i/o}$ protein (sensitive to pertussis toxin) in the antinociceptive action of GAEE, mice were pre-treated with pertussis toxin (1 μg/site, i.c.v.) 7 days before the administration of GAEE (50.5 μmol/kg, i.p.) or with morphine (13.3 μmol/kg, s.c.), used as positive control. Other groups of animals were treated with saline (5 μl/site, i.c.v), and 7 days after received GAEE, morphine or the vehicle, 30 min before the formalin injection (Beirith et al., 1998; Santos et al., 1999).

In a separate series of experiments, we also investigated the role played by the L-arginine-nitric oxide pathway in the antinociceptive effect caused by GAEE. To this end, mice were pre-treated with L-arginine (3444.0 μmol/kg, i.p.), and after 15 min they received GAEE (50.5 μmol/kg, i.p.), N^G-nitro-L-arginine (342.0 μmol/kg, i.p.) or vehicle (10 ml/kg, i.p.). The algesic responses of the first and the second phase of the formalin test were recorded 30 min after administration of GAEE, N^G-nitro-L-arginine or vehicle. Other groups of animals received only GAEE (50.5 μmol/kg, i.p.), N^G-nitro-L-arginine (342.0 μmol/kg, i.p.) or vehicle (10 ml/kg, i.p.), 30 min before formalin injection, respectively (Santos et al., 1995b; Vaz et al., 1996; Beirith et al., 1998; Santos et al., 1999).

We next investigated the possible role played by various K^+ channel blockers in the antinociceptive effect caused by GAEE. For this purpose, mice were pre-treated with apamin (50 ng/site, i.t.; a blocker of small (or low)-conductance calcium-gated K^+ channels), charybdo-

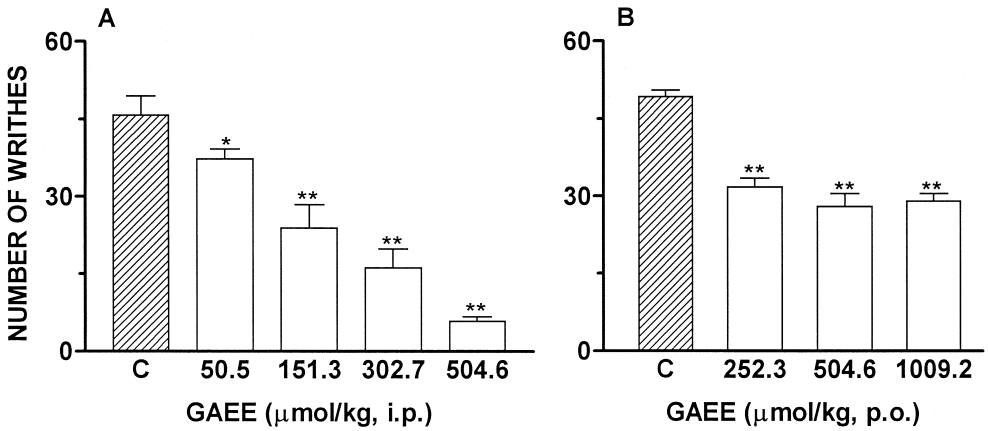

Fig. 2. Effect of i.p. (panel A) or p.o. (panel B) injection of the GAEE on acetic acid-induced writhing in mice. Each column represents the mean for 8 to 10 animals and the vertical lines indicate the S.E.M. The hatched columns indicate the control values (C, animals treated with the vehicle) and the asterisks denote significance levels, when compared with control groups, $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

toxin (250 pg/site, i.t.; a blocker of large (or fast)-conductance calcium-gated K^+ channels), tetraethylammonium (1 μg/site, i.t.; a blocker of voltage-gated K^+ channels) or glibenclamide (100 μg/site, i.t.; a blocker of ATP-gated K^+ channels), and after 15 min they received GAEE (50.5 μmol/kg, i.p.) or vehicle (10 ml/kg, i.p.) (Gimemez-Gallego et al., 1988; Strong, 1990; Aronsen, 1992; Welch and Dunlow, 1993; Welch et al., 1995). The nociceptive responses caused by formalin were recorded 30 min after administration of GAEE or vehicle. Other groups of ani-

Table 1
Comparison of the mean ID_{50} values for the antinociceptive actions of GAEE, aspirin, acetaminophen, morphine, and dipyrone in several models of nociception in mice

Acetic acid test	Route	ID_{50} [a]	Inhibition (%)		
GAEE	i.p. (μmol/kg)	164.2 (128.7–212.5)	88.0 ± 2.0		
	p.o. (μmol/kg)	nd	43.0 ± 5.0		
Aspirin[b]	i.p. (μmol/kg)	133.1 (73.0–243.3)	83.0 ± 2.0		
Acetaminophen[b]	i.p. (μmol/kg)	125.0 (104.0–150.0)	88.0 ± 1.0		
Formalin test		**First phase (ID_{50})[a]**	**Inhibition (%)**	**Second phase (ID_{50})[a]**	**Inhibition (%)**
GAEE	i.p. (μmol/kg)	nd	26.0 ± 5.0	18.6 (14.6–24.7)	82.0 ± 2.0
	p.o. (μmol/kg)	nd	22.0 ± 8.0	nd	26.0 ± 6.0
	i.c.v. (nmol/site)	17.6 (8.6–36.2)	55.0 ± 5.0	4.9 (0.7–13.1)	66.0 ± 9.0
	i.t. (nmol/site)	nd	35.0 ± 4.0	23.4 (14.7–36.8)	74.0 ± 4.0
Morphine[b]	s.c. (μmol/kg)	3.7 (2.4–5.4)	88.0 ± 3.0	4.7 (3.3–7.0)	100.0
	i.c.v. (nmol/site)	4.2 (3.5–5.0)	100.0	4.1 (3.1–5.0)	100.0
	i.t. (nmol/site)	2.4 (1.8–5.0)	94.0 ± 2.0	1.0 (0.4–2.6)	100.0
Aspirin[b]	i.p. (μmol/kg)	nd	17.0 ± 3.0	123.0 (77.0–209.0)	85.0 ± 4.0
Acetaminophen[b]	i.p. (μmol/kg)	nd	11.0 ± 4.0	120.4 (90.0–161.0)	88.0 ± 3.0
Dipyrone[c]	i.p. (μmol/kg)	154.5 (99.9–238.8)	74.0 ± 2.0	263.7 (234.3–296.9)	91.0 ± 1.0
	i.c.v. (μmol/site)	0.4 (0.3–0.7)	68.0 ± 7.0	0.4 (0.3–0.5)	82.0 ± 8.0
	i.t. (μmol/site)	1.3 (0.9–1.8)	61.0 ± 3.0	0.9 (0.6–1.4)	86.0 ± 7.0
Capsaicin test	**Route**	**ID_{50} [a]**	**Inhibition (%)**		
GAEE	i.p. (μmol/kg)	nd	44.0 ± 4.0		
	p.o. (μmol/kg)	nd	37.0 ± 9.0		
Morphine[b]	s.c. (μmol/kg)	2.6 (2.0–3.4)	92.0 ± 6.0		
Dipyrone[c]	i.p. (μmol/kg)	207.6 (179.5–240.0)	70.0 ± 8.0		

[a] 95% confidence limits.
[b] Data from Vaz et al. (1996).
[c] Data from Beirith et al. (1998).
nd: not determined.

mals received vehicle (5 μl/site) by i.t. route, 15 min prior to the administration of GAEE or vehicle and 30 min after received the formalin injection.

2.2.9. Drugs

The following substances were used: acetic acid, formalin, morphine hydrochloride (Merck, Darmstadt, Germany), L-arginine, N^G-nitro-L-arginine, bradykinin, substance P, prostaglandin E_2, carrageenan (grade IV, lambda), apamin, tetraethylammonium chloride, charybdotoxin, pertussis toxin, phosphate-buffer solution and capsaicin (Sigma, St. Louis, USA), naloxone hydrochloride and glibenclamide (Research Biochemicals International, Natick, MA, USA). Drugs were dissolved in 0.9% of NaCl solution, with the exception of GAEE, capsaicin and glibenclamide which were dissolved in tween 80, absolute ethanol and dimethyl sulfoxide, respectively. All drugs were prepared just before use in 0.9% w/v of NaCl solution. The final concentration of Tween, ethanol and dimethyl sulfoxide did not exceed 5% and did not cause any effect ''per se''.

2.2.10. Statistical analysis

The results are presented as mean ± S.E.M., except the ID_{50} values (i.e., the dose of drugs reducing the nociceptive response by 50% relative to control value) which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance between groups was calculated by analysis of variance followed by Dunnett's multiple comparison test or by Newmann–Keuls' test when appropriate. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance. The ID_{50} values were determined by linear regression from individual experiments using linear regression ''GraphPad'' software.

3. Results

3.1. Acetic acid-induced abdominal constriction

The results in Fig. 2 show that the GAEE, given by i.p. or p.o. routes, produced dose-related inhibition of the acetic acid-induced abdominal constrictions. Given p.o., the GAEE was less potent than when it was given by i.p. route. The ID_{50} (and 95% confidence limit) values (μmol/kg) and the inhibitions (%) for these effects are presented in Table 1.

3.2. Formalin-induced nociception

The results in Fig. 3(A and B) show that the GAEE given by i.p. route caused a dose-related and significant

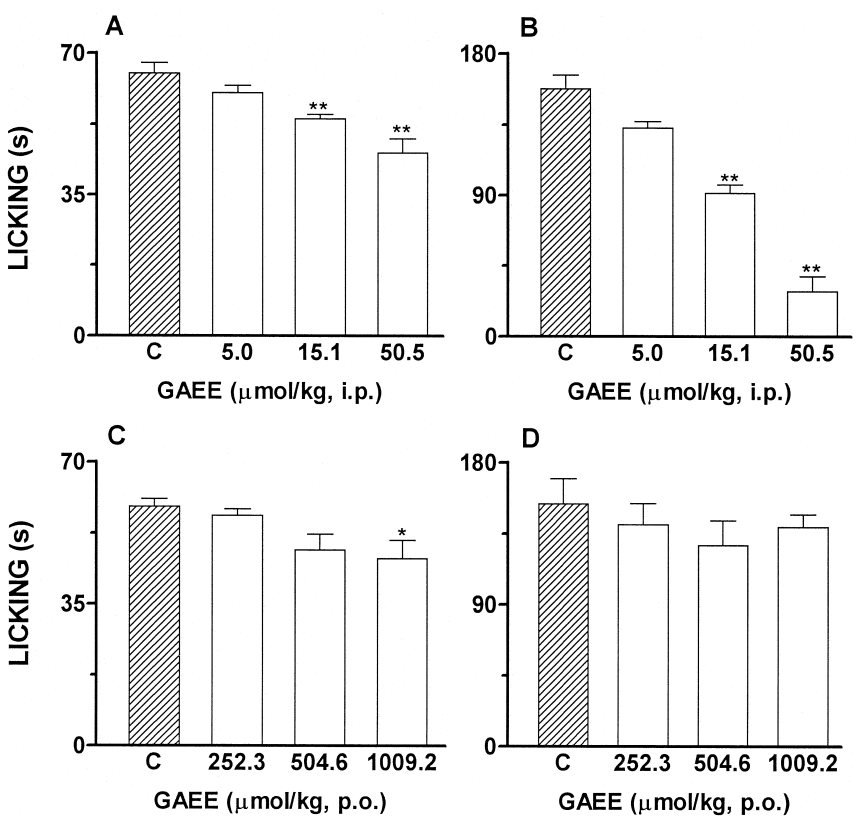

Fig. 3. Effect of i.p. (upper panels) or p.o. (lower panels) injection of the GAEE on formalin-induced nociception in mice. The total time spent licking the hindpaw was measured in the early (0–5 min, panels A and C) and the late phase (15–30 min, panels B and D), after intraplantar injection of formalin. Each column represents the mean for 8 to 10 animals and the vertical lines indicate the S.E.M. The hatched columns indicate the control values (C, animals treated with the vehicle) and the asterisks denote significance levels, when compared with control groups, $^*P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

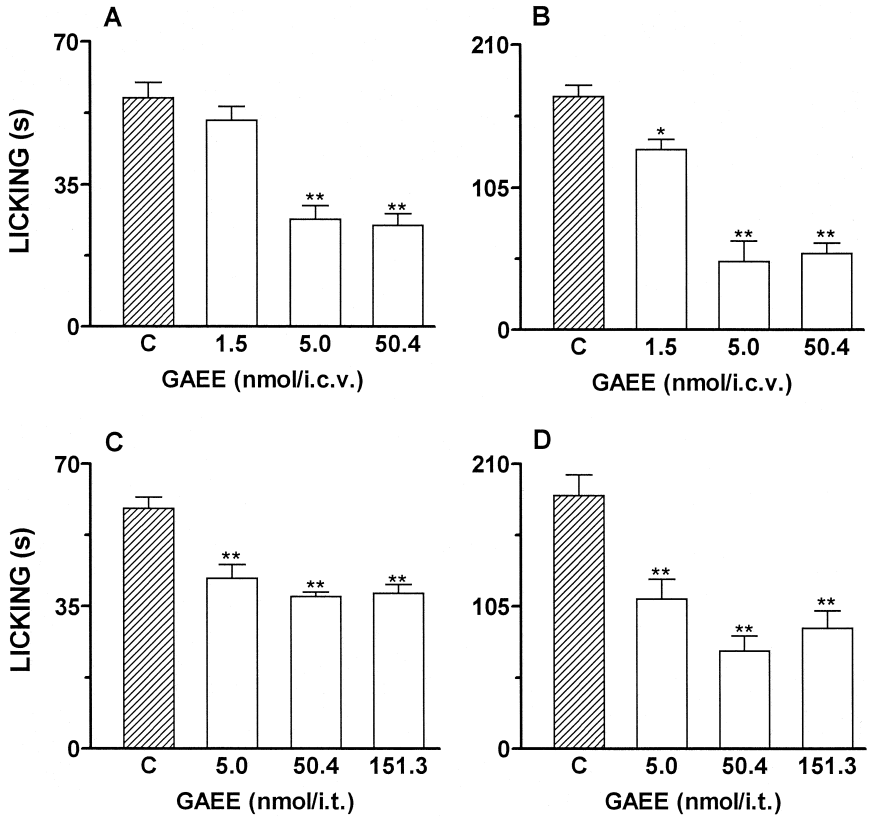

Fig. 4. Effect of i.c.v. (upper panel) or i.t. (lower panels) injection of the GAEE on formalin-induced nociception in mice. The total time spent licking the hindpaw was measured in the early (0–5 min, panels A and C) and the late phase (15–30 min, panels B and D), after intraplantar injection of formalin. Each column represents the mean for 8 to 10 animals and the vertical lines indicate the S.E.M. The hatched columns indicate the control values (C, animals treated with the vehicle) and the asterisks denote significance levels, when compared with control groups, $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

inhibition of the early (0 to 5 min) and the late phase (15 to 30 min) of the formalin-induced licking. Given orally, the GAEE caused significant inhibition against the first but not the late phase of the formalin test (Fig. 3C and D). The i.p. administration of GAEE was, however, more active in preventing the inflammatory than the neurogenic component of the formalin nociceptive response. The calculated mean ID_{50} values and the inhibitions (%) for these effects

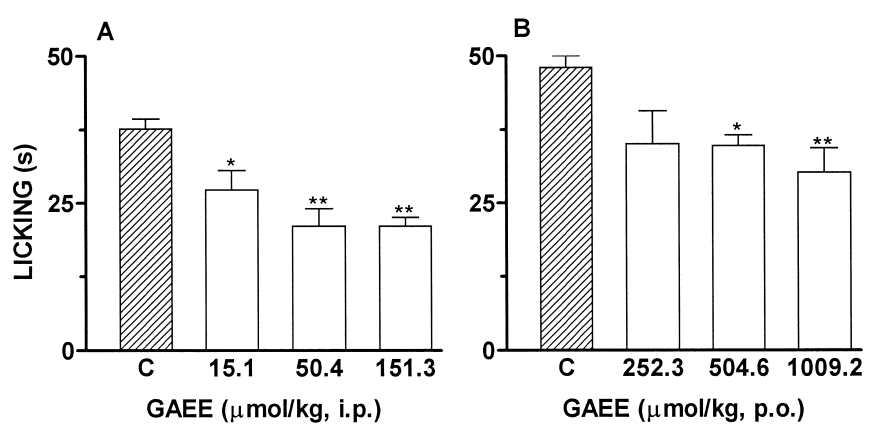

Fig. 5. Effect of i.p. (panel A) or p.o. (panel B) injection of the GAEE on capsaicin-induced nociception in mice. The total time spent licking the hindpaw (0–5 min) was measured after intraplantar injection of capsaicin. Each column represents the mean for 8 to 10 animals and the vertical lines indicate the S.E.M. The hatched columns indicate the control values (C, animals treated with the vehicle) and the asterisks denote significance levels, when compared with control groups, $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

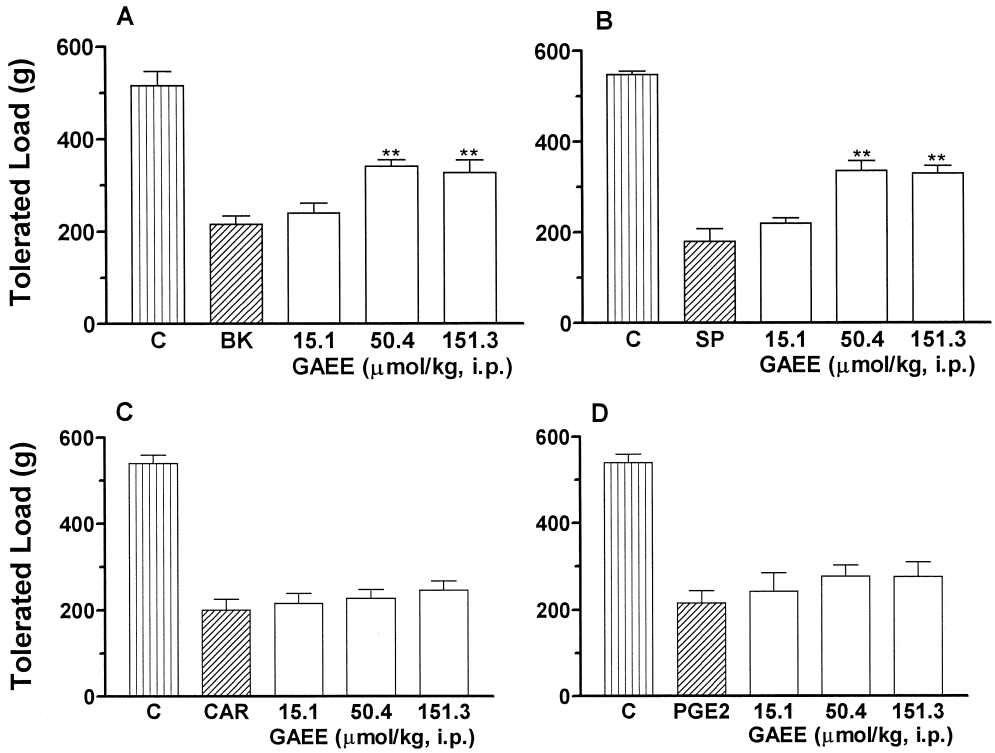

Fig. 6. Effect of i.p. injection of the GAEE on bradykinin (3 nmol/paw, panel A), substance P (10 nmol/paw, panel B), carageenan (300 μg/paw, panel C) or prostaglandin E_2 (10 nmol/paw, panel D)-induced hyperalgesia in the rat paw. The vertically-hatched column indicates the control values (C, phosphate-buffer solution-injected paws) and the diagonally-hatched column indicates the bradykinin (BK), substance P (SP), carrageenan (CAR) or prostaglandin E_2 (PGE_2)-injected paws, in the absence of the GAEE. Each column represents the mean of five to eight animals and the vertical lines indicate the S.E.M. The diagonally-hatched columns represent the control group and the asterisks denote significance levels, when compared with control group, $^{**}P < 0.01$ (Dunnett's multiple comparison test).

are presented in Table 1. Independent of the route of administration used, GAEE failed to affect the paw oedema associated with the second phase of the formalin test (results not shown).

The i.c.v. or i.t. injection of the GAEE produced dose-dependent and significant inhibition of both phases of the formalin-induced licking (Fig. 4A–D). At the ID_{50} level, GAEE was about 1.2- to 23.4-fold less potent than mor-

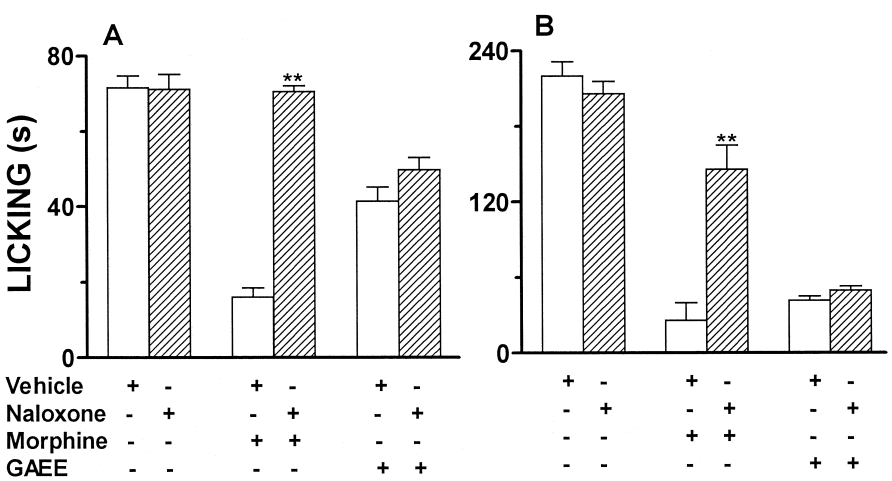

Fig. 7. Effect of pre-treatment of animals with naloxone (13.7 μmol/kg, i.p. hatched column) on the antinociceptive action caused by morphine (13.3 μmol/kg, s.c.) and the GAEE (50.5 μmol/kg, i.p.) on formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min, panel A) and against the second phase (15–30 min, panel B) after intraplantar injection of formalin into the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the S.E.M. The open columns represent the control values (animals treated with the vehicle) and the asterisks denote the significance levels, when compared with control groups, $^{**}P < 0.01$ (Newmann–Keuls' multiple comparison test).

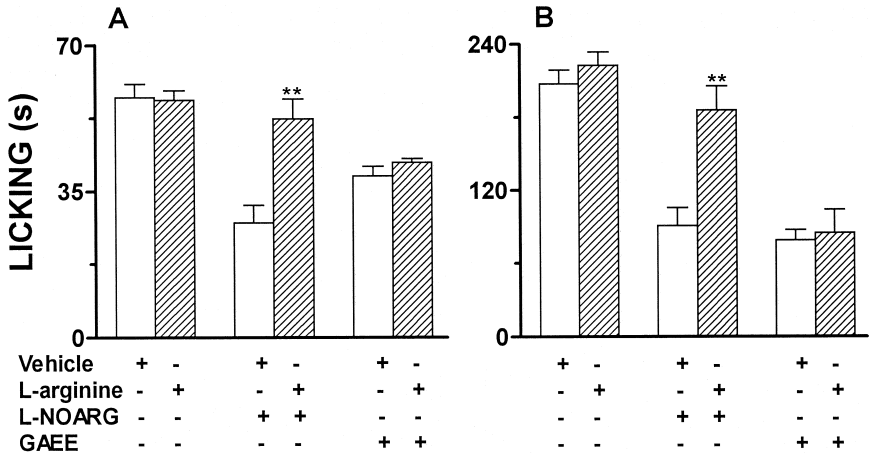

Fig. 8. Effect of pre-treatment of animals with L-arginine (3444 μmol/kg, i.p., hatched column) on the antinociceptive action caused by N^G-nitro-L-arginine (L-NOARG, 342.0 μmol/kg, i.p.) and the GAEE (50.5 μmol/kg, i.p.) on formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min, panel A) and against the second phase (15–30 min, panel B) after intraplantar injection of formalin into the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the S.E.M. The open columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels, when compared with control groups, $^{**}P < 0.01$ (Newmann–Keuls' multiple comparison test).

phine (Table 1). The calculated mean ID_{50} values (nmol/site) and the inhibitions (%) for the GAEE given by i.c.v. and i.t. are presented in Table 1.

3.3. Capsaicin-induced nociception

The results in Fig. 5(A and B) show that GAEE, given by i.p. or p.o. routes, produced partial but significant inhibition of the capsaicin-induced nociception. The inhibition values (%) were 44.0 ± 4.0 and 37.0 ± 9.0, for i.p. or p.o. administration, respectively (Table 1).

3.4. Hyperalgesia in the rat paw

When assessed in the Randall–Selitto model, the GAEE partially but significantly reversed the hyperalgesia caused by intraplantar injection of both bradykinin (3 nmol/paw) and substance P (10 nmol/paw) ($P < 0.01$). The inhibitions observed were 42.0 ± 5.0 and 49.0 ± 7.0%, respectively (Fig. 6A and B). However, the GAEE, at the same doses had no significant effect on the hyperalgesic responses caused by either carrageenan or prostaglandin E_2 in the rat paw (Fig. 6C and D).

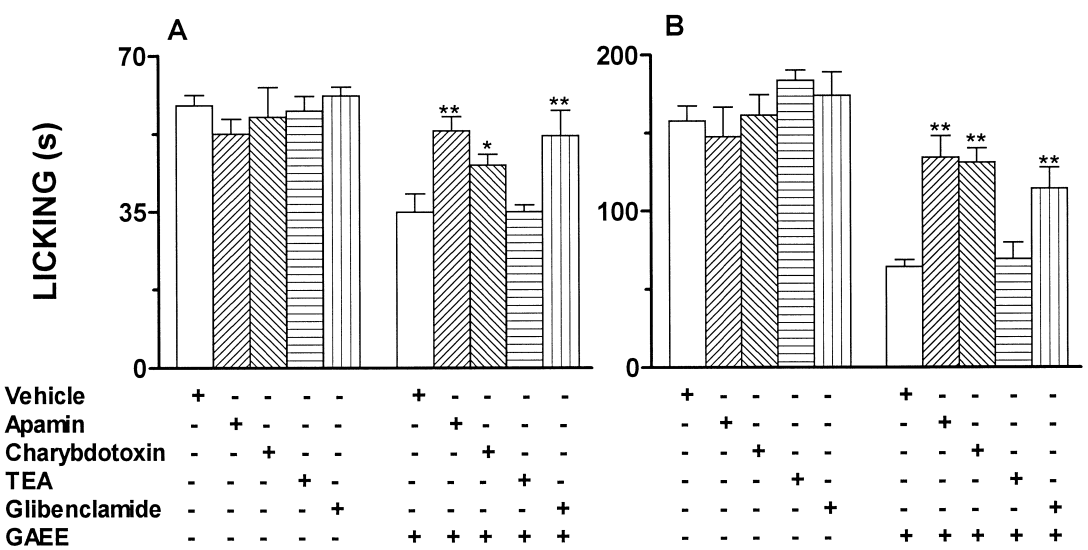

Fig. 9. Effect of i.t. treatment with apamin (50 ng/site), charybdotoxin (250 pg/site), tetraethylammonium (TEA, 1 μg/site) or glibenclamide (100 μg/site) on the antinociceptive profile caused by GAEE (50.5 μmol/kg, i.p.) on the formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min, panel A) and against the second phase (15–30 min, panel B) after intraplantar injection of formalin into the hindpaw. Each column represents the mean for five to eight animals and the vertical lines indicate the S.E.M. The open columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels, when compared with control groups (ANOVA), $^{*}P < 0.05$, $^{**}P < 0.01$ (Newmann–Keuls' multiple comparison test).

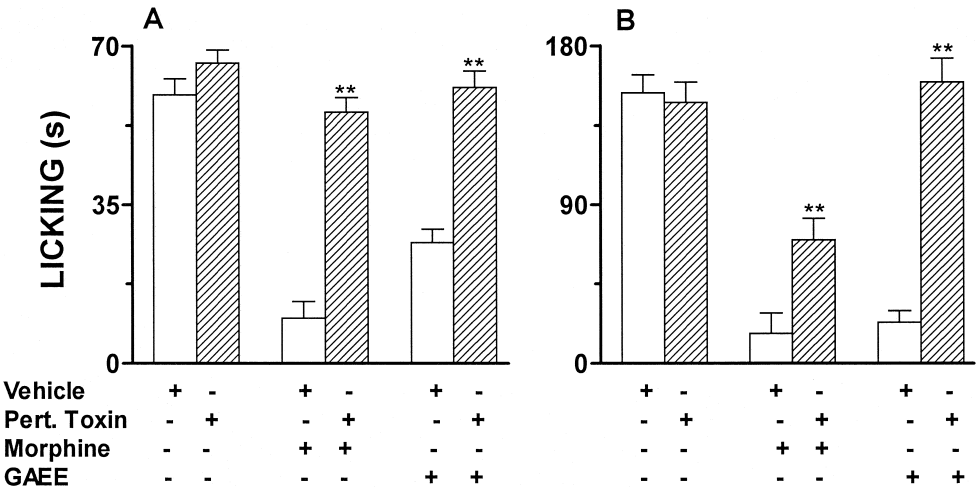

Fig. 10. Effect of pre-treatment of animals with pertussis toxin (1.0 μg/i.c.v., hatched column) on the antinociceptive action caused by morphine (13.3 μmol/kg, s.c.) and the GAEE (50.5 μmol/kg, i.p.) on the formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min, panel A) and against the second phase (15–30 min, panel B) after intraplantar injection of formalin into the hindpaw. Each column represents the mean for five to eight animals and the vertical lines indicate the S.E.M. The closed columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels, when compared with control groups (ANOVA), $^{**}P < 0.01$ (Newmann–Keuls' multiple comparison test).

3.5. Hot-plate test

The GAEE did not cause any significant change in the latency response in the hot-plate assay (control response 6.2 ± 0.4 s vs. GAEE-treated animals 5.6 ± 0.5 s). Under similar conditions, morphine caused a significant ($P < 0.01$) and marked increase in the latency response in the algesiometer assay (control response 6.2 ± 0.4 s vs. morphine-treated group 10.7 ± 0.7 s, $N = 9$).

3.6. Rota-rod test

The GAEE did not significantly affect the motor response of animals. Control response in the rota-rod test was 59.1 ± 0.7 s vs. 58.5 ± 1.0 s in the presence of the tested compound ($N = 7$).

3.7. Analysis of the antinociceptive mechanism of action of GAEE

The pre-treatment of animals with naloxone given 15 min before injection of morphine largely reversed the antinociception caused by morphine when analysed in both phases of the formalin-induced licking, without affecting the antinociception caused by GAEE (Fig. 7A and B). The pre-treatment of animals with L-arginine, given 15 min prior, completely reversed the antinociceptive effect caused by N^G-nitro-L-arginine, but did not reverse the antinociception caused by GAEE against either phases of the formalin test (Fig. 8A and B).

The i.t. administration of various K^+ channel blockers, including apamin, charybdotoxin or glibenclamide, given 15 min beforehand, significantly prevented the antinociception caused by GAEE against both phases of formalin-induced nociception (Fig. 9A and B). However, tetraethylammonium, a blocker of voltage-gated K^+ channels, failed to affect the antinociceptive action of the GAEE against the formalin test (Fig. 9A and B). The i.c.v. administration of pertussis toxin, an inactivator of $G_{i/o}$ protein, caused a significant inhibition of morphine-induced antinociception when assessed against both phases of formalin-induced nociception (Fig. 10A and B). Under the same conditions, pertussis toxin also significantly and completely antagonised the antinociceptive action caused by GAEE against the first and second phases of the formalin test (Fig. 10A and B).

4. Discussion

The results presented here show for the first time that GAEE, a small molecule isolated from the ethanolic extract of the plants belonging to the genus *Phyllanthus* (Euphorbiaceae), when given by oral, intraperitoneal or by spinal and supraspinal sites, is effective in preventing significantly the nociception elicited by acetic acid, formalin and capsaicin in mice, and also the hyperalgesia caused by bradykinin and substance P in rats. However, GAEE, independent of the dose used, is largely ineffective in preventing the pain response when assessed in thermal models of nociception such as the hot plate test, a very sensitive opioid assay. Very similar results have been documented previously in relation to the ethanolic extract of *Phyllanthus* species (Gorski et al., 1993; Santos et al., 1995a,b; Cechinel-Filho et al., 1996). Thus, besides other antinociceptive constituents previously reported for the *Phyllanthus* species such as furosin, geraniin, stigmasterol and β-sitosterol (Santos et al., 1995c; Miguel et al., 1996),

GAEE probably accounts for the antinociceptive properties shown in the ethanolic extract obtained from different plants of this genus (Gorski et al., 1993; Santos et al., 1995a,b; Cechinel-Filho et al., 1996).

When compared with some standard analgesic drugs, GAEE, depending on the route of administration employed, was found to be equipotent or about 1- to 23-fold less potent that morphine, but it was about to 6- to 81-fold more active than aspirin, acetoaminophen or dipyrone (Vaz et al., 1996; Beirith et al., 1998). Contrasting with most non-steroidal anti-inflammatory drugs, GAEE was found to be effective in preventing the neurogenic nociception elicited by formalin (early phase) and capsaicin in mice, but its efficacy was in most cases partial in such models. Essentially, the same results have been previously reported for different ethanolic extracts of certain *Phyllanthus* species (Gorski et al., 1993; Santos et al., 1995a,b; Cechinel-Filho et al., 1996).

The results of the present study also indicate that, despite the lack of antioedematogenic action of the GAEE revealed by analysis against the paw oedema associated with the second phase of the formalin test, GAEE produced significant inhibition of the bradykinin and substance P, without interfering with carrageenan- and prostaglandin E_2-induced hyperalgesia. One possible explanation for the anti-hyperalgesic action of the GAEE against the neuropeptide-mediated hyperalgesia might be that it acts directly on sensory C-fibres at the release and/or the action of such algesic peptides.

An additional objective of the present study was to evaluate by use of several pharmacological procedures, some of the mechanisms by which GAEE produces antinociception in chemical models of nociception. We have recently demonstrated that the ethanolic extract and GAEE isolated from *P. urinaria* produce direct and concentration-dependent relaxation in both guinea pig trachea and rat portal vein ''in vitro'' pre-contracted by histamine or endothelin. These relaxant responses caused by GAEE in both preparations are significantly antagonised in the presence of either charybdotoxin or, to a lesser extent, glibenclamide, strongly suggesting the involvement of large-conductance calcium-gated K^+ channels besides ATP-gated K^+ channels (unpublished results). Data of the present study extend these previous results and indicate that activation of small- or large-conductance calcium-gated K^+ channels and ATP-gated K^+ channels, but not tetraethylammonium-sensitive voltage-gated K^+ channels, significantly accounts for the antinociceptive action of GAEE. This notion is based on the fact that i.t. treatment of animals with apamin, charybdotoxin or glibenclamide significantly prevented its antinociception according to the formalin test. However, tetraethylammonium (a blocker of the voltage-gated K^+ channels) failed to affect the action of the GAEE. Another interesting aspect of the present study is the demonstration that i.c.v. treatment of animals seven days prior with pertussis toxin completely reversed the GAEE antinociception against both phases of the formalin test. Very similar action of pertussis toxin has been demonstrated for morphine antinociception (Hernandez et al., 1995; Beirith et al., 1998; Santos et al., 1999, and present study). These findings are consistent with the notion that GAEE antinociception depends on the interaction with $G_{i/o}$ protein sensitive to ''in vivo'' treatment with pertussis toxin (Hernandez et al., 1995; Shah et al., 1994, 1997; Standifer and Pastenak, 1997). It has been reported that the calcium-gated K^+ channels are activated by alteration in intracellular calcium and are believed to be coupled to G proteins or other second-messenger systems (Welch et al., 1995). Thus, we cannot discard the possible participation of calcium channels or modulators of calcium in the antinociception caused by GAEE. Further studies are required to clarify this hypothesis.

Although GAEE antinociception revealed some similarity with the action of morphine, its antinociceptive actions do not involve any interaction with opioid receptor, as revealed by the complete lack of effect of naloxone, in conditions where morphine antinociception was completely reversed. Finally, data of the present study show that L-arginine-nitric oxide pathway seems unlikely to be involved in the antinociceptive action of GAEE, evident by the fact that L-arginine, a precursor of nitric oxide, under conditions where it consistently reversed the antinociception caused by N^G-nitro-L-arginine (a nitric oxide synthase inhibitor) (Santos et al., 1995b; Vaz et al., 1996; Beirith et al., 1998), had no significant effect on the GAEE antinociception. The present data also show that the antinociception properties here demonstrated for GAEE in different assays of chemical nociception is not the consequence of any non-specific action such as motor dysfunction of animals, as revealed by the lack of any detectable effect in the rota-rod test.

5. Conclusion

The results of the present study show for the first time that the small molecule GAEE, isolated from certain species of *Phyllanthus* plants, produces systemic, spinal and supraspinal antinociception when assessed in several chemical assays of nociception in mice. Its antinociceptive actions are not related with opioid system interaction, with L-arginine-nitric oxide pathway, or with CNS-related motor inco-ordination. However, GAEE antinociception seems likely to be associated with activation of both small- and/or large-conductance calcium-gated K^+ channels as well as with ATP-gated K^+ channels and by pertussis toxin-sensitive $G_{i/o}$ protein mechanisms.

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References

Aronsen, J.K., 1992. Potassium channels in nervous tissue. Biochem. Pharmacol. 43, 11–14.

Bachmann, T.L., Ghia, F., Torssell, K.B.G., 1993. Lignans and lactones from *Phyllanthus anisolobus*. Phytochemistry 33, 189–191.

Beirith, A., Santos, A.R.S., Rodrigues, A.L.S., Creczynski-Pasa, T.B., Calixto, J.B., 1998. Spinal and supraspinal antinociceptive action of dipyrone on formalin, capsaicin and glutamate tests. Study of the mechanism of action. Eur. J. Pharmacol. 345, 233–245.

Calixto, J.B., Santos, A.R.S., Cechinel Filho, V., Yunes, R.A., 1998. A review of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. Med. Res. Rev. 18, 225–258.

Cechinel-Filho, V., Santos, A.R.S., De Campos, R.O.P., Miguel, O.G., Yunes, R.A., Ferrari, F., Messana, I., Calixto, J.B., 1996. Chemical and pharmacological studies of *Phyllanthus caroliniensis* in mice. J. Pharm. Pharmacol. 48, 1231–1236.

Corrêa, C.R., Calixto, J.B., 1993. Evidence for participation of B_1 and B_2 Kinin receptors in formalin-induced nociceptive response in the mouse. Br. J. Pharmacol. 110, 193–198.

Corrêa, C.R., Kyle, D.J., Chakravarty, S., Calixto, J.B., 1996. Antinociceptive profile of the pseudopeptide B_2 bradykinin receptor antagonist NPC 18688 in mice. Br. J. Pharmacol. 117, 552–558.

De Campos, R.O.P., Alves, R.V., Kyle, D.J., Chakravarty, S., Mavunkel, B.J., Calixto, J.B., 1996. Antioedematogenic and antinociceptive actions of NPC 18521, a novel bradykinin B_2 receptor antagonist. Eur. J. Pharmacol. 316, 227–286.

Eddy, N.B., Leimbach, D., 1953. Synthetic analgesics: II. Dithienylbutenyl and dithienylbutylamines. J. Pharmacol. Exp. Ther. 107, 385–393.

Foo, L.Y., 1993. Amariin, a di-dehydrohexahydroxydiphenoyl hydrolysable tannin from *Phyllanthus amarus*. Phytochemistry 33, 487–491.

Gimemez-Gallego, G., Navia, M.A., Reuben, J.P., Katz, G.M., Kaczorowski, G.J., Garcia, M.L., 1988. Purification, sequence and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. Proc. Natl. Acad. Sci. U.S.A. 85, 3329–3333.

Gorski, F., Corrêa, C.R., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 1993. Potent antinociceptive activity of the hydroalcoholic extract from *Phyllanthus corcovadensis*. J. Pharm. Pharmacol. 45, 1046–1049.

Hernandez, A., Soto-Moyano, R., Mestre, C., Eschalier, A., Pelissier, I., Paeile, C., Contreras, E., 1995. Intrathecal pertussis toxin but not cyclic AMP blocks kappa opioid-induced antinociception in rat. Int. J. Neurosci. 81, 193–197.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.

Miguel, O.G., Cechinel-Filho, V., Pizzolatti, M.G., Santos, A.R.S., Calixto, J.B., Ferrari, F., Messana, I., Yunes, R.A., 1995. A triterpene and phenolic compounds from leaves and stems of *Phyllanthus sellowianus*. Planta Med. 61, 391.

Miguel, O.G., Calixto, J.B., Santos, A.R.S., Messana, I., Ferrari, F., Cechinel-Filho, V., Pizzolatti, M.G., Yunes, R.A., 1996. Chemical and preliminary analgesic evaluation of geraniin and furosin isolated from *Phyllanthus sellowianus*. Planta Med. 62, 97–102.

Randall, L.O., Selitto, J.J., 1957. A method for measurement of analgesic activity on inflamed tissue. Arch. Int. Pharmacodyn. Ther. 111, 409–419.

Santos, A.R.S., Calixto, J.B., 1997a. Further evidence for the involvement of tachykinin receptor subtypes in formalin and capsaicin models of pain in mice. Neuropeptides 31, 381–389.

Santos, A.R.S., Calixto, J.B., 1997b. Ruthenium red and capsazepine antinociceptive effect in formalin and capsaicin models of pain in mice. Neurosci. Lett. 235, 73–76.

Santos, A.R.S., Cechinel-Filho, V., Niero, R., Viana, A.M., Moreno, F.N., Campos, M.M., Yunes, R.A., Calixto, J.B., 1994. Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in mice. J. Pharm. Pharmacol. 46, 755–759.

Santos, A.R.S., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 1995a. Further studies on the antinociceptive action of the hydroalcoholic extracts from plants of the genus *Phyllanthus*. J. Pharm. Pharmacol. 47, 66–71.

Santos, A.R.S., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 1995b. Analysis of the mechanisms underlying the antinociceptive effect of the extracts of plants from the genus *Phyllanthus*. Gen. Pharmacol. 26, 1499–1506.

Santos, A.R.S., Niero, R., Cechinel-Filho, V., Yunes, R.A., Pizzolatti, M.G., Delle Monache, F., Calixto, J.B., 1995c. Antinociceptive properties of steroids isolated from *Phyllanthus corcovadensis* in mice. Planta Med. 61, 329–332.

Santos, A.R.S., Miguel, O.G., Yunes, R.A., Calixto, J.B., 1999. Antinociceptive properties of the new alkaloid, *cis*-8,10-di-*N*-propyllobelidiol hydrochloride dihydrate isolated from *Siphocampylus verticillatus*: evidence for the mechanism of action. J. Pharmacol. Exp. Ther. 289, 417–426.

Shah, S., Duttaroy, A., Davis, T., Yoburn, B.C., 1994. Spinal and supraspinal effects of pertussis toxin on opioid analgesia. Pharmacol. Biochem. Behav. 49, 773–776.

Shah, S., Breivogel, C., Selly, D., Munirathinam, G., Childers, S., Yoburn, C., 1997. Time-dependent effects of in vivo pertussis toxin on morphine analgesia and G-proteins in mice. Pharmacol. Biochem. Behav. 56, 465–469.

Standifer, K.M., Pastenak, G.W., 1997. G proteins and opioid receptor-mediated signalling. Cell Signalling 9, 237–248.

Strong, P.N., 1990. Potassium channel toxins. Pharmacol. Ther. 46, 137–162.

Ueno, H., Horie, S., Nishi, Y., Shagawa, H., Kawasaki, M., Suzuki, S., Hayashi, T., Arisawa, M., Shimizu, M., Yoshizaki, M., Morita, N., 1988. Chemical and pharmaceutical studies on medicinal plants in Paraguay. Geraniin, an angiotensin converting enzyme inhibitor from ''Parapai Mi'', *Phyllanthus niruri*. J. Nat. Prod. 51, 357–359.

Vaz, Z.R., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 1996. Antinociceptive action of 2-(4-bromobenzoyl)-3-methyl-4,6-dimethoxy benzofuran, a novel xanthoxyline derivative on chemical and thermal models of nociception in mice. J. Pharmacol. Exp. Ther. 278, 304–312.

Welch, S.P., Dunlow, L.D., 1993. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: a common mechanism of action? J. Pharmacol. Exp. Ther. 267, 390–399.

Welch, S.P., Dunlow, L.D., Patrick, G.S., Razdan, R.K., 1995. Characterization of anandamide- and fluoroanandamide-induced antinociception and cross-tolerance to Δ^9-THC after intrathecal administration to mice: blockade of Δ^9-THC-induced antinociception. J. Pharmacol. Exp. Ther. 273, 1235–1244.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.