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ANTINOCICEPTIVE PROPERTIES OF MIXTURE OF α- AMYRIN AND β - AMYRIN TRITERPENES: EVIDENCE FOR PARTICIPATION OF PKC AND PKA PATHWAYS

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ABBREVIATIONS: L-NOARG, N^G-nitro-L-arginine; TPA, 12-O-tetraradecanoylphorbol-13-acetate; NO, nitric oxide; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; NMR, nuclear magnetic resonanse; PCPA, DL-p-chloro phenylalanine methyl ester; i.p., intraperitoneal; p.o., oral; i.c.v., intracerebroventricular; i.t., intrathecal; i.pl., intraplantar; 8-Br-cAMP, 8-Brome- cyclic AMP.

ABSTRACT

The mixture of two pentacyclics triterpenes α-amyrin and β-amyrin isolated from the resin from *Protium kleinii*, given by i.p.(intraperitoneal) or p.o.(oral) routes, caused dose-related and significant antinociception against the visceral pain in mice produced by i.p. injection of acetic acid. Moreover, i.p., p.o., intracerebroventricular (i.c.v.) or intrathecal (i.t.) administration of α,β-amyrin also inhibited both neurogenic and inflammatory phases of the overt nociception caused by intraplantar (i.pl.) injection of formalin. Likewise, α,β-amyrin given by i.p., p.o., i.t. or i.c.v. routes inhibit the neurogenic nociception induced by capsaicin. Moreover, i.p. treatment with α,β-amyrin was able to reduce the nociception produced by 8-Br-cAMP and by TPA. On the other hand, in contrast to morphine, α,β-amyrin failed to cause analgesia in thermal models of pain. The antinociception caused by the mixture of compounds seems to involve mechanisms independent of opioid, α-adrenergic, serotoninergic and nitrergic system mediation, since it was not affected by naloxone, prazosin, yohimbine, PCPA or L-arginine. Interestingly, the i.p. administration of α,β-amyrin reduced the mechanical hyperalgesia produced by i.pl injection of carrageenan, capsaicin, bradykinin, substance P, PGE_2, 8-Br-cAMP and TPA in rats. However, the mixture of compounds failed to alter the binding sites of [^{3}H]bradykinin, [^{3}H]resiniferatoxin or [^{3}H]glutamate in vitro. It is concluded that the mixture of triterpene α-amyrin and β-amyrin produced consistent peripheral, spinal and supraspinal antinociception in rodents, especially when assessed in inflammatory models of pain. The mechanisms involved in their action are not completely understood, but seem to involve the inhibition of PKA and PKC sensitive pathways.

Introduction

Natural products, since ancient times, have contributed to the development of modern therapeutic drugs, especially those derived from higher plants. Futhermore, they have also contributed to our current understanding about the mechanisms envolved in neurotransmitter actions, especially regarding the process of pain transmission and treatment (see for review Calixto et al., 2000) Species of the genus *Protium* (Burseracea) are known for the production of oleoresin exudates that occur as a result of insect stings, broken branches, or other acts injurious in their bark (Siqueira et al.,1995). The resins and leaves of some species of *Protium* are commonly used in folk medicine for healing of ulcers and as anti-inflammatory agent (Corrêa, 1984).

In early pharmacological studies it has been demonstrated an immunostimulant (Delaveau et al.,1980) and anti-inflammatory activities for the aqueous extract from resins of Burseraceae species (Duwiejua et al., 1993), along with anti-inflammatory activity of the essential oil, obtained from the leaves, and resin of some species of *Protium* (Siani et al., 1999).

Chemical analysis carried out with the resin from *Protium kleinii* reveled the presence of a mixture of two triterpenes belonging to the ursane (α-amyrin) and oleanane (β-amyrin) series. These triterpenes have been reported to exert anti-inflammatory effect when assessed in animal models of inflammation (Akihisa et al.,1996; Recio et al., 1995; Kweifio-Okai et al., 1994). Furthermore, some in vitro studies have shown that α-amyrin is relatively specific inhibitor of the catalytic

subunit (cAK) of cyclic AMP-dependent protein kinase (Hasmeda et al., 1999). In addition, α-amyrin and its palmitate and linoleate esters are found effective to inhibit collagenase (Kweifio-Okai et al., 1994) while β-amyrin palmitate inhibits α_1-adrenoceptors (Subarnas et al., 1993).

In our earlier study we have shown that the eter extract and the isolated pentacyclic triterpene brein obtained from *Protium kleinii* caused pronounced and dose-related inhibition when tested in several models of nociception in mice (Otuki et al., 2001). The purpose of the present study was, therefore, to evaluate the peripheral, spinal and supraspinal antinociceptive effects and some of the mechanisms underlying the action of the mixture of α-amyrin and β-amyrin (1:1) on chemical, mechanical and thermal models of nociception in mice and rats.

Methods

Isolation and chemical identification of the active compound

Botanical material was collected in morro do Bau, state of Santa Catarina, Brazil, and was classified by Dr. Ademir Reis as beeing *Protium kleinii*, a plant of the family Burseraceae. A voucher of this planta (excicata number VC Filho 019) was deposited in the herbarium FLOR at the Federal University of Santa Catarina.

The resinous bark of P. kleinii (50 g) was powdered and extracted with diethylic ether in the proportion of 1:10 (w/v), being stirred and macerated at room temperature (21 ± 3ºC) for approximately two weeks. The solvent was fully evaporated under reduced pressure and the extract (33.42 g) was chromatographed (14.42 g) on a silica gel column eluted successively with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, methanol and water,

respectively. The fraction eluted with hexane-ethyl acetate 1:1 gave a crystalline solid (120 mg), which was identified as being the triterpene α- amyrin and β - amyrin (Fig. 1), the major naturally compound present in this plant. This mixture was obtained in a 1:1 proporcion by gas cromatography spectra. The chemical identification was obtained by using the ^{1}H and ^{13}C NMR spectra.

Animals

Male Swiss mice (25-35 g) or male wistar rats (200-300 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 used. Experiments were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2h before testing and were only used once. Experiments reported in this study were carried out in accordance with current guidelines for the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

Abdominal constriction induced by acetic acid

The abdominal constriction induced by intraperitoneal injection of dilute acetic acid (0.6%) was carried according to the procedures described previously (Vaz et al., 1996). Animals were pre-treated with α,β-amyrin given i.p. (0.1-10 mg/kg) or p.o. (25-100 mg/kg) 30 and 60 min before testing, respectively. The control group received the same volume of 0.9 % of NaCl (10ml / kg). After challenge, pairs of mice were placed in separate boxes and the number of abdominal constriction was cumulatively counted over a period of 20 min.

Formalin test

The procedure used was essentially similar to that described previously (Vaz et al., 1996). Twenty microliters of 2.5% formalin solution (0.92% of formaldehyde), made up in PBS (NaCl 137 mM, KCl 2.7 mM and phosphate buffer, 10 mM), were injected intraplantarly under the surface of the right hindpaw. Animals were treated with α,β-amyrin, or vehicle (10 ml/kg) by i.p. (0.1-10 mg/kg) or p.o. (5-100 mg/kg) routes, 0.5 and 1 h before formalin injection, respectively. Other groups of animals were treated with α,β-amyrin or with vehicle (5 μl/site) by i.c.v. (1-10 μg/site) or i.t. (1-30 μg/site) routes, as reported previously (Hylden and Wilcox, 1980; Vaz et al., 1996), 10 min before formalin injection. After intraplantar injection of formalin, the animals were immediately placed in a glass cylinder 20 cm in diameter and the time spent licking the injected paw was timed with a chronometer and considered indicative of nociception.

Analysis of Possible Mechanism of Action of α,β-amyrin

To investigate the possible participation of the opioid system in the antinociceptive effect of α,β-amyrin, animals were pre-treated with naloxone (a non selective antagonist of opioid receptors, 5 mg/kg, i.p.), 15 min before the administration of α,β-amyrin (10 mg/kg, i.p.), morphine (5 mg/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.). The other groups of animals received only α,β-amyrin, morphine, naloxone or saline 30 min before the formalin injection (Vaz et al., 1996). To explorere the possible contribuition of serotonin to the antinociceptive effect of

α,β-amyrin, animals were pre-trested with DL-p-chlorophenylalanine methyl ester (PCPA), (an inhibitor of serotonin synthesis, 100 mg/kg, i.p.) once a day for 4 consecutive days, prior to administration of α,β-amyrin (10 mg/kg, i.p.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.).

To examine the possible contribuition of α_1- and α_2-adrenoreceptors in the antinociceptive effect caused by α,β-amyrin, animals were pretreated with prazosin (0.15 mg/kg, i.p.) or with yohimbine (0.15 mg/kg, i.p.), and after 15 min the animals received α,β-amyrin (10 mg/kg, i.p.), phenylephrine (10 mg/kg, i.p.), clonidine (0.1 mg/kg, i.p.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.). We further investigated the possible participation of nitric oxide-L-arginine pathway in the analgesic effect caused by α,β-amyrin. Animals were pre-treated with L-arginine (600mg/kg), and after 15 min they received α,β-amyrin (10 mg/kg, i.p.), N^G-nitro-L-arginine (nitric oxid synthase inhibitor, L-NOARG, 75 mg/kg, i.p.), or saline (0.9% NaCl solution, 10 ml/kg, i.p.).

Algogen-induced overt nociception in mice

The procedure used was similar to that described previously (Siebel et al., 2004 Sakurada et al., 1992). Twenty microliters of capsaicin (5.2 nmol/paw), TPA (12-O-tetradecanoylphorbol-13-acetate) (50 pmol/paw) or 8-Br-cAMP (10 nmol/paw) were injected intraplantarly (i.pl.) under the surface of the right hindpaw. Before challange, the animals were placed individually in transparent glass cylinders 20 cm in diameter, serving as observation chambers. Animals were observed individually for 5 min after capsaicin, 10 min after 8-Br-cAMP or 15 to 45

min after TPA injection. The amount of time spent licking the injected paw timed with a chronometer was considered indicative of nociception. Animals were treated either with i.p. (0.3-30 mg/kg) or p.o. (5-100 mg/kg) injection of vehicle (10 ml/kg) or α,β-amyrin, 0.5 and 1h before algogen injection. Other groups of animals were treated with α,β-amyrin or with vehicle (5 µl/site) by i.c.v. (0.3-3 µg/site) or i.t. (0.3-3 µg/site) routes 10 min before capsaicin injection.

Effect of α,β-amyrin on the hyperalgesia in the rat paw

The procedures used were similar to those described previously (Randal and Sellitto, 1957; Corrêa et al., 1996; Taiwo and Levine, 1991). The animals were pre-treated i.p. with the α,β-amyrin (30 mg/kg) 30 min before injection of 0.1 ml of bradykinin (3 nmol/paw), substance P (10 nmol/paw), capsaicin (20 nmol/paw), carrageenan (300 µg/paw), PGE_2 (10 nmol/paw), 8-Br-cAMP (1 nmol/paw), TPA (0,1 nmol/paw) or only with phosphate-buffer solution alone (control group), into the right hindpaw. The used dose of α,β-amyrin was defined in pilot experiments. The hyperalgesia was evaluated 0.5 h later, except for carrageenan, which was assessed at 3 h later. The nociception 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 Randal and Selitto (1957). 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 et al., 1996).

Glutamate-induced thermal hyperalgesia

To test the hypothesis whether or not the excitatory amino acid glutamate was involved in the α,β-amirin antinociception, we assessed the effect of α,β-amyrin given by oral route on the hyperalgesic response caused by spinally administered glutamate (100 nmol/site, i.t.) in mice. The analgesic effect was assessed in the hot plate test as described previously (Ferreira et al 1999) in control group (saline treated) or in mice pre-treated with α,β-amyrin (0.03 – 5 mg/kg, p.o., 60 min prior). The response to the thermal stimuli was measured on the hot-plate apparatus (Ugo Basile, model-DS 37) maintained at 50 ± 1 ºC as described in item 2.6. The maximal hyperalgesic response caused by i.t. injection of glutamate was observed at 5 min after the injection, and this time-point was used in all future experiments. A cut-off of 30 s was used for the hot plate. The maximal possible effect (MPE) of glutamate-induced hyperalgesia was calculated as follows:

$$\% \text{ MPE} = \frac{\text{Postdrug - predrug}}{\text{30 – predrug}} \times 100$$

Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described previously by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37) was maintained at 56 ± 1 ºC. Animals were placed into a glass cylinder with 24 cm

diameter on the heated surface, 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. Control animals (0.9% NaCl solution, 10 ml/kg, i.p.), or mice pre-treated with α,β-amyrin (30 mg/kg, i.p.) 30 min earlier. Other groups of animals were treated with morphine (10 mg/kg, s.c., 30 min prior).

Tail-flick test

A radiant heat tail-flick analgesiometer was used to measure response latencies according to the method described previously by D'Amour and Smith (1941), with minor modifications. Animals responded to a focused heat stimulus by flicking or removing their tail, exposing a photocell in the apparatus immediately below it. The reaction time was recorded for control mice (0.9% NaCl solution, 10 ml/kg, i.p.) and for animals pre-treated 30 min before with α,β-amyrin (30 mg/kg, i.p.) or with morphine (10 mg/kg, s.c.). An automatic 20-s cut-off was used to minimise tissue damage. Animals were selected 24 h previously on the basis of their reactivity in the test. To determine the baseline, each animal was tested before administration of drugs.

Measurement of motor performance

In order to evaluate possible non-specific muscle relaxant or sedative effects of α,β-amyrin, mice were tested on the rota-rod (Rosland et al., 1990). The apparatus

consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 revolutions per minute. 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 α,β-amyrin (30 mg/kg, i.p.) or with the same volume of 0.9% NaCl solution (10 ml/kg, i.p.) 30 min before being tested. The results are expressed as the time (s) for which animals remained on the rota-rod. The cut-off time used was 60 s.

[^{3}H] glutamate binding assay

Cerebral cortices obtained from rats (killed by decapitation) were dissected and homogenised in 20 volumes of ice-cold 0.32 M sucrose containing 10 mM Tris HCl buffer, pH 7.4, and 1 mM $MgCl_2$. The homogenate was centrifuged at 1000×g, and the pellet was re-homogenised and centrifuged again. The second pellet was discarded, and both supernatants were pooled and centrifuged at 27000×g for 15 min. The resulting pellet was dissolved in 1 mM Tris HCl, pH 7.4, for 30 min. The pellet was washed three times in 10 mM Tris HCl buffer, pH 7.4, at 27000×g for 15 min (Beirith et al., 1998). The final pellet was diluted in 10 mM Tris HCl and frozen at -70°C until use. On the day of the experiment, the membranes were thawed and incubated with 0.04% Triton X-100 at 37°C for 30 min and were then washed three times in 10 mM Tris HCl buffer, pH 7.4, at 27000×g for 15 min. The final pellet was resuspended in 10 mM Tris HCl and the suspensions were assayed for [^{3}H]glutamate binding. Assays of [^{3}H]glutamate binding were carried out in triplicate in a total

volume of 0.5 ml containing 0.1 ml membrane (0.2-0.3 mg protein), 50 mM Tris HCl pH 7.4, 40 nM radioactive ligand ([^{3}H]glutamate, 53 Ci/mmol), in the presence or absence of α,β-amyrin (concentration in the range 1-100 μg/ml). Unspecific binding was assayed similarly, except that 40 M non-radioactive glutamate (displacer) was added to the incubation medium. Centrifugation at 12000×g for 25 min was used to separate [^{3}H]glutamate not bound to membranes. The supernatant was discarded, and the walls of the Eppendorf tubes and the surfaces of the pellets were quickly and carefully rinsed with cold deionized water, followed by processing for radioactivity. Specific binding was calculated as the difference between binding values in the absence and the presence of the displacer. The results represent the means of three independent experiments.

[^{3}H] resiniferatoxin binding assay

Binding assays were carried out as described (Szallasi et al., 1998). To obtain membranes for the binding studies, spinal cord of rats were removed and disrupted with the aid of a tissue homogenizer in an ice-cold buffer A (pH 7.4) which contained (mM) KCl, 5, NaCl, 5.8, MgCl2, 2; CaCl2, 0.75; glucose, 12; sucrose, 137; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10. The homogenate was first centrifuged for 10 min at 1,000 g in 4 ºC; the low speed pellets were discarded; the supernatants were further centrifuged for 30 min at 35,000 g in 4 ºC; and the resulting high speed pellets, resuspended in buffer A, were stored at –70 ºC until assayed.

Binding assays were carried out in duplicate with a final volume of 500 µl, containing buffer A, supplemented with 0.25 mg/ml bovine serum albumin, membranes (100 µg/ protein) and 50 pM of [^{3}H]-RTX. For the measurement of the non-specific binding, 100 nM of non-radioactive RTX were included in some tubes.

Assay mixtures were set up on ice and the binding reaction was then initiated by transferring the assay tubes to a 37ºC water bath. Following a 60 min incubation period, cooling the mixtures on ice terminated the binding reaction, and then 100 µg of bovine α_1-acid glycoprotein was added to each tube (to reduce non-specific binding). Finally, the bound and free membranes [^{3}H]-RTX were separated by centrifuging for 15 min at 20 000 g in 4 º C. The pellet was quantified by scintillation counting. Specific binding was calculated as the difference of the total and non-specific binding.

[^{3}H] bradykinin binding assay

The specific binding of [^{3}H]-bradykinin (a high-affinity bradykinin B_2 receptor ligand) was assayed according to the method previously described (Manning et al., 1986) with minor modifications. The ilea from guinea pigs were removed and homogenized in ice-cold buffer (50 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and 1 mM 1,10-phenanthroline, pH 6.8) with a Polytron. The homogenate was centrifuged to remove cellular debris (1000xg, 20 min, 4°C) and the supernatant was centrifuged (100,000xg, 60 min, 4°C). Then, the pellet was resuspended in ice-cold assay buffer (50 mM TES, 1 mM 1,10-phenanthroline, 140 g/ml bacitracin, 1 mM dithiothreitol, 1 M captopril and 0.1% bovine serum albumin, pH 6.8) and used.

In the binding assay, membranes were incubated with [^{3}H]-bradykinin (final concentration 0.5 nM), in the absence or presence of α,β-amyrin (100 µg/ml) at room temperature for 90 min. For the measurement of the non-specific binding, 1 µM of non-radioactive bradykinin was included in some tubes. Receptor-bound [^{3}H]bradykinin was harvested by filtration through Whatman GF/B glass fibre filters under reduced pressure and the filter was washed 4 times with 2 ml of ice-cold buffer (50 mM Tris–HCl). The radioactivity retained on the washed filter was measured with a liquid scintillation counter. The experiments were performed three times in duplicate.

Drugs

The following substances were used: formalin, morphine hydrochloride, acetic acid (Merck, AG, Darmstadt, Germany), naloxone hydrochloride (Dupont, Garden City, USA), capsaicin (Calbiochen, San Diego, California, USA), PCPA, glutamate, L-arginine, L-NOARG, yohimbine, clonidine, L-phenylephrine, prazosin, TPA, 8-Br-cAMP, PGE_2, substance P and carrageenan (Sigma, St Louis, USA). [^{3}H]BK and [^{3}H]glutamate (Amersham) and [^{3}H]RTX (Perkin-Elmer). Morphine and naloxone were dissolved in 0.9% NaCl solution just before use while capsaicin and α,β-amyrin were dissolved in ethanol and tween 80 plus 0.9% NaCl solution, respectively. The final concentration of tween 80 or ethanol did not exceed 5% and did not cause any effect per se.

Statistical analysis

The results are presented as means ± S.E.M., except the ID_{50} values (i.e. the dose of α and β amyrin reducing the pain responses by 50% relative to the control value) which are reported as geometric means accompanied by their respective 95% confidence limits. Data were analyzed by analysis of variance (ANOVA) or *t*-test and complemented by Dunnett's or Newman Keul's post-hoc test. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance. The ID_{50} values were determined by linear regression analysis from individual experements using "GraphPad Software" (San Diego, USA), and are reported as geometric means accompanied by their respective 95% confidence limits. In the glutamate-induced hyperalgesia the ID_{50} value was calculated on the peak (5 min) of glutamate response

Results

Abdominal constriction induced by acetic acid

Results of Fig. 2 show that α,β-amyrin, given i.p. (0.1-10 mg/kg) or by p.o. (25-100 mg/kg) route, dose dependently inhibited acetic acid-induced abdominal constrictions. The calculated mean ID_{50} values and the inhibitions obtained were: 0.79 (0.63-1.01) mg/kg and 41.0 (26.10-66.01) mg/kg and 84 ± 3 and 83 ± 7%, respectively.

Formalin test

The results of Fig. 3 (A and C) show that α,β-amyrin, given i.p. (0.1-10 mg/kg) caused a significant and dose-related inhibition of the neurogenic (0 to 5 min) and the inflammatory phase (15 to 30 min) of the formalin-induced licking. The calculated mean ID_{50} values for these effects were > 10 and 0.16 (0.07-0.37) mg/kg, respectively. I.p. administration with α,β-amyrin was significantly more active to inhibit the inflammatory phase than the neurogenic phase of the formalin response. The inhibitions obtained were 45 ± 6 and 99 ± 1%, respectively.

When administered by p.o route α,β-amyrin (5.0-100 mg/kg) produced dose-related inhibition of both the neurogenic and the inflammatory phase of the formalin test, being more potent (about 3-fold) and efficacious against the second phase (Fig. 3 A and C). The calculated mean ID_{50} values and the inhibitions observed for the early and the late phase were 70.0 (63.0 -78.0) and 19.7 (8.7- 44.0) mg/kg with inhibitions of 56 ± 4 and 99± 1%, respectively.

Given i.c.v. (1.0-10 μg/site) α,β-amyrin produced dose-dependent inhibition of both phases of the formalin-induced licking (Fig. 3 B and D). The calculated mean ID_{50} values for these effects (μg/site) were 4.96 (2.55 - 9.62) and 0.72 (0.14 - 3.6), against the early and the late phase of the formalin response, respectively. The inhibitions observed for the early and the late phase were 67 ± 7 and 84 ± 4%, respectively.

Given i.t. (1.0-30 μg/site) α,β-amyrin produced dose-dependent inhibitition of both phases of the formalin-induced licking (Fig. 3 B and D). The calculated mean ID_{50} values for these effects (μg/site) were: 4.71 (1.28-17.33) and 7.84 (4.74-12.95), against the early and the late phase of the formalin response, respectively with inhibitions of 66 ± 3 and 67 ± 3%, respectively.

Analysis of possible mechanism of action of α,β-amyrin

The pre-treatment of animals with naloxone given 15 min before injection of morphine largely reversed the antinociception caused by morphine when analysed for both phases of the formalin-induced licking, leaving the antinociceptive effect of α,β-amyrin unaffected (Table 1). The pre-treatement of animals with L-arginine, given 15 min prior, completely reversed the antinociception effect caused by N^G-nitro-L-arginine, but did not reverse the antinociception caused by α,β-amyrin against either phases of the formalin test (Table 1). Treatment of the animals with prazosin or yohimbine, 10 min before, significantly reversed the antinociception caused by phenylephrine and clonidine, respectively, but failed to interfere significantly with the antinociception caused by α,β-amyrin against both phases of formalin-induced

nociception (Table 1). The pretreatement of animals with PCPA (once a day for 4 days) did not significantly modify the antinociceptive effect caused by α,β-amyrin against both phases of formalin-induced nociception (Table 1).

Algogen-induced overt nociception

The results of Fig. 4 (A and B) show that α,β-amyrin, given orally (5-100 mg/kg), i.p. (0.3-30 mg/kg), i.t. (0.3-3.0 μg/site) or by i.c.v. (0.3-3.0 μg/site) route, dose dependently inhibited capsaicin-induced licking. The calculated mean ID_{50} values and the inhibitions observed were: 8.04 (1.98-32.68) mg/kg, 1.5 (0.7-3.0) mg/kg, 0.3 (0.07-1.32) μg/site and 0.89 (0.69-1.15) μg/site and 89 ± 2, 82 ± 4, 84 ± 2 and 88 ± 2%, respectively. Moreover, i.p. treatment with α,β-amyrin are able to reduce the overt nociception produced by TPA or 8-Br-cAMP (Fig. 4, C and D). The calculated inhibitions observed were 77 ± 4 % and 59 ± 3 % for 8-Br-cAMP and TPA, respectively.

Hyperalgesia in the rat paw

When assessed in the Randal-Selitto model of nociception, the α,β-amyrin (30 mg/kg, i.p.) partially, but significantly reversed the hyperalgesia caused by intraplantar injection of bradykinin (3 nmol/paw), substance P (10 nmol/paw), capsaicin (20 nmol/paw), carragenan (300 μg/paw), PGE_2 (10 nmol/paw), 8-Br-cAMP (1 nmol/paw) or TPA (0,1 nmol/paw). The inhibitions observed were 85 ± 26, 29 ± 5, 47 ± 5, 52 ± 17 , 75 ± 9 %, 93 ± 8 %,and 83 ± 7 % respectively (Fig. 5).

Glutamate-induced hyperalgesia

Results of Fig. 6 show that α,β-amyrin, given orally (0.03-5.01 mg/kg), dose dependently inhibited glutamate-induced hyperalgesia. The calculated mean ID_{50} value (estimated at 5 min) was 1.14 (0.82-1.6) mg/kg and the maximal inhibition was 100%. Given alone, α,β-amyrin, over the same range of doses where it was effective in inhibiting glutamate-induced hyperalgesia, had no effect in the hot-plate assay (results not shown).

Hot-plate and tail-flick tests

α,β-amyrin (30 mg/kg, i.p.), given 30 min prior, did not cause any significant increase change in the latency response in either hot-plate or the tail-flick assays. Under similar conditions, morphine (10 mg/kg, s.c.), used as a reference drug and given 30 min before, caused significant and marked analgesic effect in both models (Table 2).

Rota-rod test

α,β-amyrin (30 mg/kg), given i.p., 30 min beforehand, did not significantly affect the motor response of the animals. The control response in the rota-rod test was 60 s versus 60 s in the presence of α,β-amyrin (n= 8) (results not show).

[^{3}H]BK, [^{3}H]RTX, [^{3}H]Glutamate binding studies

High concentratios of α,β-amyrin (up to 100 μg/ml) were not able to alter either [^{3}H]bradykinin, [^{3}H]RTX, [^{3}H]glutamate specific binding to guinea pig ilium , rat

spinal cord membranes or cerebral cortex in vitro (results not show). In the same conditions, Hoe140 (100 nM), capsaicin (30 μM) or non-radioactive glutamate (30 μM) blocked the specific binding of [3H]bradykinin, [^{3}H]RTX, [^{3}H]glutamate to membranes (inhibition of 100%).

Discussion

The results presented in the current study show that the mixture of α,β-amyrin triterpenes administered systemically, spinally and supraspinally to mice produces pronounced and dose-related antinociception. Furthermore, this effect seems to be related with its ability to interfere with PKC and PKA-sensitive pathways.

The antinociception elicited by α,β-amyrin also appears to be independent of the activation of important endogenous analgesic systems, namely opioidergic, serotoninergic and noradrenergic. In fact, the treatment of animals with PCPA, at a dose known to inhibit serotonin synthesis (Vaz et al, 1996; Beirith et al, 1998), fails to interfere with α,β-amyrin-induced antinociception when assessed in the formalin model of pain. Furthermore, the α1- and α2-adrenoceptors seem unlikely to be involved in the antinociceptive action of α,β-amyrin, evidenced by the fact that selective antagonists of these receptors fail to alter the antinociception caused by α,β-amyrin, in conditions where they produce significant inhibition of the antinociception provoked by the selective agonists. Finally, the mechanism underlying the antinociceptive action of α,β-amyrin seems to be unrelated to activation of the opioid system. The antinociceptive action of α,β-amyrin, in

contrast to that reported for morphine, was not reversed by naloxone, a non-selective opioid antagonist.

α,β-Amyrin was devoid of analgesic action when assessed in thermal models of nociception, the tail flick and hot-plate tests, under conditions where morphine had a marked antinociceptive effect. The hot-plate and tail-flick tests are commonly used to assess narcotic analgesic or other centrally acting drugs, including sedatives and psychomimetics (Vaz et al., 1996; Beirith et al., 1998). However, these thermal tests are not sensitive to the analgesic action of some drugs that act in the central nervous system, including weak agonists of opioid receptors. Apart from a its lack of action in these thermal models of pain, the antinociceptive effect of α,β-amyrin may possess a central component, since i.t. and i.c.v. injection of amyrin was just as efficacious as systemically-administered α,β-amyrin in producing antinociception.

Also revelant are our findings showing that α,β-amyrin was able produce dose-dependent systemic inhibition of the hyperalgesia induced by i.t. injection of glutamate in mice. However, α,β-amyrin was not able to alter the [^{3}H]-glutamate binding sites to cerebral cortex membranes. These results suggest that the antinociceptive effect of α,β-amyrin is unrelated with a direct interaction with glutamate receptors. Some nociceptive actions produced by glutamate are mediated by nitric oxide (NO)-cGMP pathway activation (Ferreira et al., 1999; Beirith et al., 2002). NO is amodulator of the nociceptive processes, being able to produce analgesic or antinociceptive effects, depending on the experimental model and the dose or site of administration tested (Aley and Levine, 1999). However, the

systemic treatment with NO-synthase inhibitors usually produces an antinociceptive effect in the formalin model (Beirith et al 1998). Our results show that the antinociception caused by α,β-amyrin is unlikely to involve any interaction with nitric oxide, since the treatment of animals with L-arginine, a precursor of nitric oxide, in conditions where it consistently reversed the antinociception caused by N^G-nitro-L-arginine (a nitric oxide synthase inhibitor) (Vaz et al, 1996; Beirith et al, 1998), failed to interfere with α,β-amyrin-induced antinociception.

In addition, α,β-amyrin also failed to directly interact with vanilloid or with kinin B_2 receptor binding sites, apart from its inhibitory activity in capsaicin-, bradykinin- or carrageenan-induced mechanical hyperalgesia. Several inflammatory mediators produce nociception by peripheral and spinal sensory fiber sensitisation through protein kinase activation, including PKC, PKA and mitogen activated kinases (Scholz and Woolf, 2002). Evidence now suggests that the mechanical hyperalgesia produced by PGE_2 could to be mediated by peripheral PKA stimulation (Malmberg et al., 1997; Aley and Levine, 1999). Moreover, bradykinin-induced overt nociception and mechanical hyperalgesia is mediated by peripheral activation of PKC and vanilloid receptor (Ferreira et al., 2004). On the other hand, intraplantar capsaicin seems to induce nociceptive action via direct activation of peripheral vanilloid receptors (Ferreira et al., 2004). Also, it has been demonstrated that both PKA and PKC stimulation in spinal cord are involved in capsaicin-induced hyperalgesia (Sluka et al., 1997). On top of this, the nociception caused by carrageenan, formalin and acetic acid is also sensitive to PKA or PKC inhibitors or gene deletion (Khasar et al., 1999; Malmberg et al., 1997).

Interestingly, systemic treatment with α,β-amyrin almost abolishes the mechanical hyperalgesia or the overt nociception produced by intraplantar injection of direct activators of PKA (8-Br-cAMP) or direct activators of PKC (TPA) into the rat or mouse paw. Of note, in vitro studies have shown that α,β-amyrin is capable of blocking the activity of both PKC and, in a special manner, PKA-dependent mechanisms (Hasmeda et al, 1999). Therefore, the ability of α,β-amyrin to interact with kinase pathways might explain its ability to inhibit PGE_2, capsaicin and bradykinin-induced nociception without directly interfering with their respective receptor binding sites. Apart from its nociceptive-producing effects, PKC pathway seems to be also involved in the tolerance to the analgesic effect of morphine (Granados-Soto et al., 2000). However, studies must be carried out in order to verify whether or not α,β-amyrin treatment could be also effective in reducing morphine tolerance.

Hyperalgesia is often observed during painful pathological processes. This sensitization results from activation of different intracellular kinase cascades leading to the phosphorylation of key membrane receptors and channels (Ji and Woolf, 2001). Thus, the interaction of kinases could also explain why α,β-amyrin is capable of reducing the thermal hyperalgesia produced by glutamate, which is dependent on retrograde sensitization of primary afferents (Ferreira e Lorenzetti, 1994), without changing the response to acute thermal stimulation. This seems to be unassociated with the transmission of acute thermal stimuli (Khasar et al., 1999; Malmberg et al., 1997).

In addition, the antinociceptive effects of α,β-amyrin herein reported extend the antiarthritic activity of α,β-amyrin triterpenes (Kweifio-Okai et al., 1994a; Kweifio-Okai et al., 1994b) and the anti-edematogenic of α,β-amyrin effect in carrageenan-induced mouse paw edema (Recio et al., 1995). They also confirm and extend the antinociceptive activity of the triterpene mixture (α-amyrin, β-amyrin and baurenol) in the writhes induced by acetic acid (Villasenor et al., 2004). Moreover, these results demonstrate that, at least in part, α,β-amyrin is responsible for the antinociceptive effect of *P. kleinii*.

In summary, we have demonstrated that the mixture of α-amyrin and β-amyrin triterpenes, isolated from resins of *Protium kleinii*, exhibit dose-related antinociception when assessed in chemical, but not thermal, models of nociception in mice, as well as producing anti-hyperalgesic effects in models of painful mechanical hypersensitivity in rats. Currently, the precise mechanism involved in its action is not completely understood, but the inhibition of both PKA and PKC pathways stimulated by different algogen mediators appears to account for α,β-amyrin's antinociceptive effect.

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Legends to Figures

Fig. 1- Molecular structure of α-amyrin (A) and β -amyrin (B).

Fig. 2 - Effects of α,β-amyrin given intraperitoneally (-■-),and orally (-□-), against the acetic acid-induced abdominal constriction. The total time (mean ± S.E.M.). Each point represents the mean ± S.E.M. for 6-10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, **P < 0.01. In some cases, the S.E.M. are hidden within the symbols.

Fig. 3 - Effects of α,β-amyrin given intraperitoneally (-■-, 0.1-10.0 mg/kg), orally (-□-, 5.0-100 mg/kg), intracerebroventricularly (-○-, 1.0-10.0 μg/site) and intrathecally (-●-,1.0-30.0 μg/site) against the early (panel A and B) and the second phase (panel C and D) of formalin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0-5 min) and the second phase (15-30 min) after subplantar injection of formalin into the hindpaw. Each point represents the mean ± S.E.M. for 6-10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, **P < 0.01. In some cases, the S.E.M. are hidden within the symbols.

Fig. 4 - Effects of α,β-amyrin given orally (-■-, 5-100 mg/kg), intraperitoneally (-□-, 0.3-30 μmol/paw) (panel A), intrathecally (-○-, 0.3-3.0 μg/site) and

intracerebroventricularly (-●-, 0.3-3.0 μg/site) (panel B) against capsaicin-induced licking in mice. The total time spent licking the hindpaw was measured in the 0-5 min after subplantar injection of capsaicin into the hindpaw. Effects of α,β-amyrin given intraperitoneally (10.0 mg/kg). The total time spent licking the hindpaw was measured after subplantar injection of TPA (C) and 8-Br-cAMP (D) into the hindpaw. Each point represents the mean ± S.E.M. for 8-10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, *P< 0.05 and **P < 0.01.

Fig. 5 - Effects of i.p. injection of the α,β-amyrin (30 mg/kg) on bradykinin (A), capsaicin (B), carrageenan (C), substance P (D), 8-Br-cAMP (E) and TPA (F)-induced hyperalgesia in the rat paw. The closed column indicates the control values (C, phosphate-buffer solution-injection paws) and the diagonally-hatched column indicates the bradykinin (BK), capsaicin (CAP), carrageenan (CAR), substance P (SP), 8-Br-cAMP (E) or TPA-injected paws, in the absence of the α,β-amyrin. Each column represents the mean ± S.E.M. for 8-10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, *P< 0.05 and **P < 0.01.

Fig. 6 - Effects of α,β-amyrin given orally against glutamate-induced hyperalgesia in mice. The latency (mean ± S.E.M.) in the hot plate was measured at 5 after intrathecal injection of glutamate. Each point represents the mean ± S.E.M. for 6-8 animals. The asterisks denote the significance levels when compared with control

groups. Significantly different from controls, *P< 0.05, **P < 0.01. In some cases, the S.E.M. are hidden within the symbols.

Tables

Table 1- Summary of the effects of the various drugs on the antinociception caused by α,β-amyrin assessed in the formalin test

Drugs	Dose (mg/kg)	Formalin test	
		First Phase	Second Phase
Control	0	49 ± 2	155 ± 11
Naloxone	5	49 ± 4	165 ± 14
Morphine	5	9 ± 2**	22 ± 10**
Morphine + Naloxone	5 + 5	41 ± 4+	171 ± 14+
α,β-amyrin	10	25 ± 2**	46 ± 8**
α,β-amyrin + Naloxone	10 + 5	28 ± 5 NS	51 ± 7 NS
Control	0	56 ± 3	152 ± 11
PCPA	100	48 ± 4	171 ± 13
α,β-amyrin	10	29 ± 3**	51 ± 13**
α,β-amyrin + Naloxone	10 + 100	35 ± 7 NS	29 ± 17 NS
Control	0	55 ± 4	153 ± 16
L-arginine	600	60 ± 5	144 ± 21
L-NOARG	75	47 ± 5 NS	31 ± 11**
L-NOARG + L-arginine	75 + 100	58 ± 2 NS	155 ± 16+
α,β-amyrin	10	38 ± 3**	63 ± 16**
α,β-amyrin + L-arginine	10 + 100	39 ± 2 NS	43 ± 31 NS
Control	0	55 ± 2	140 ± 12
Prazosin	0.15	51 ± 4	125 ± 10
Yohimbine	0.15	50 ± 3	113 ± 12
Phenylephrine	10	12 ± 5**	2 ± 0.5**
Phenylephrine + Prazosin	10 + 0.15	48 ± 5+	109 ± 14+
Clonidine	0.1	15 ± 3**	6 ± 2**
Clonidine + Yohinbine	0.1 + 0.15	52 ± 4+	138 ± 24+
α,β-amyrin	10	18 ± 3**	20 ± 8**
α,β-amyrin + Prazosin	10 + 0.15	27 ± 5 NS	23 ± 10 NS
α,β-amyrin + Yohinbine	10 + 0.15	30 ± 4 NS	65 ± 5 NS

Each group represents means ± S.E.M. for 6 to10 animals. [a]P < 0.01 when compared to control value.

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Table 2 - Analgesic effect of morphine (s.c.) and α,β-amyrin (i.p.) in the hot plate and tail flick tests in mice.

Drugs	Dose (mg/kg)	Latency (s)	
		Tail-flick	Hot-plate
Control	0	7.42 ± 1.0	6.85 ± 0.7
Morphine	10	19.4 ± 0.6[a]	23.9 ± 1.5[a]
α,β-amyrin	30	8.4 ± 1.0	6.48 ± 0.8

Each group represents means ± S.E.M. for 6 to10 animals. [a]P < 0.01 when compared to control value.

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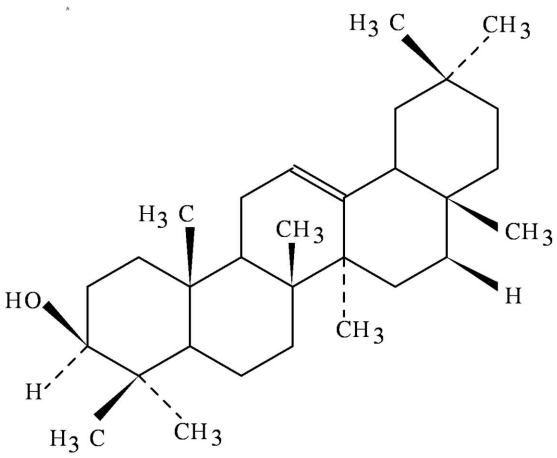
Fig. 1-

A



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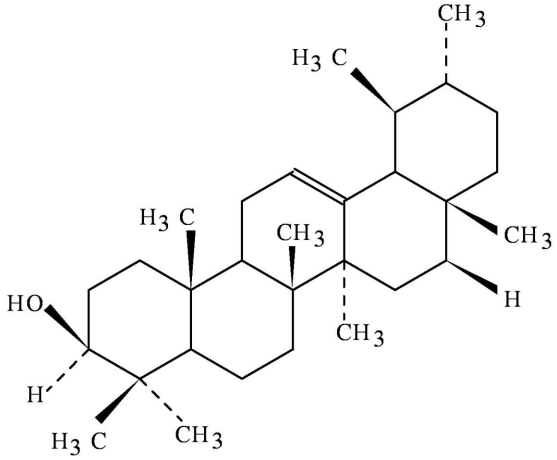

Fig. 2-

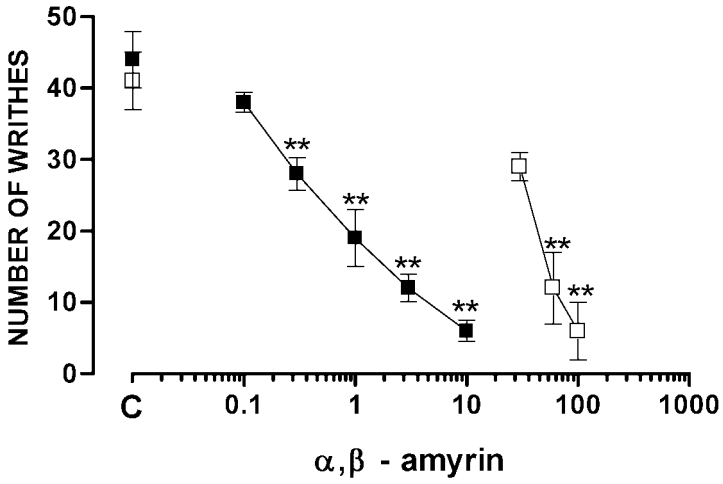

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Fig. 3-

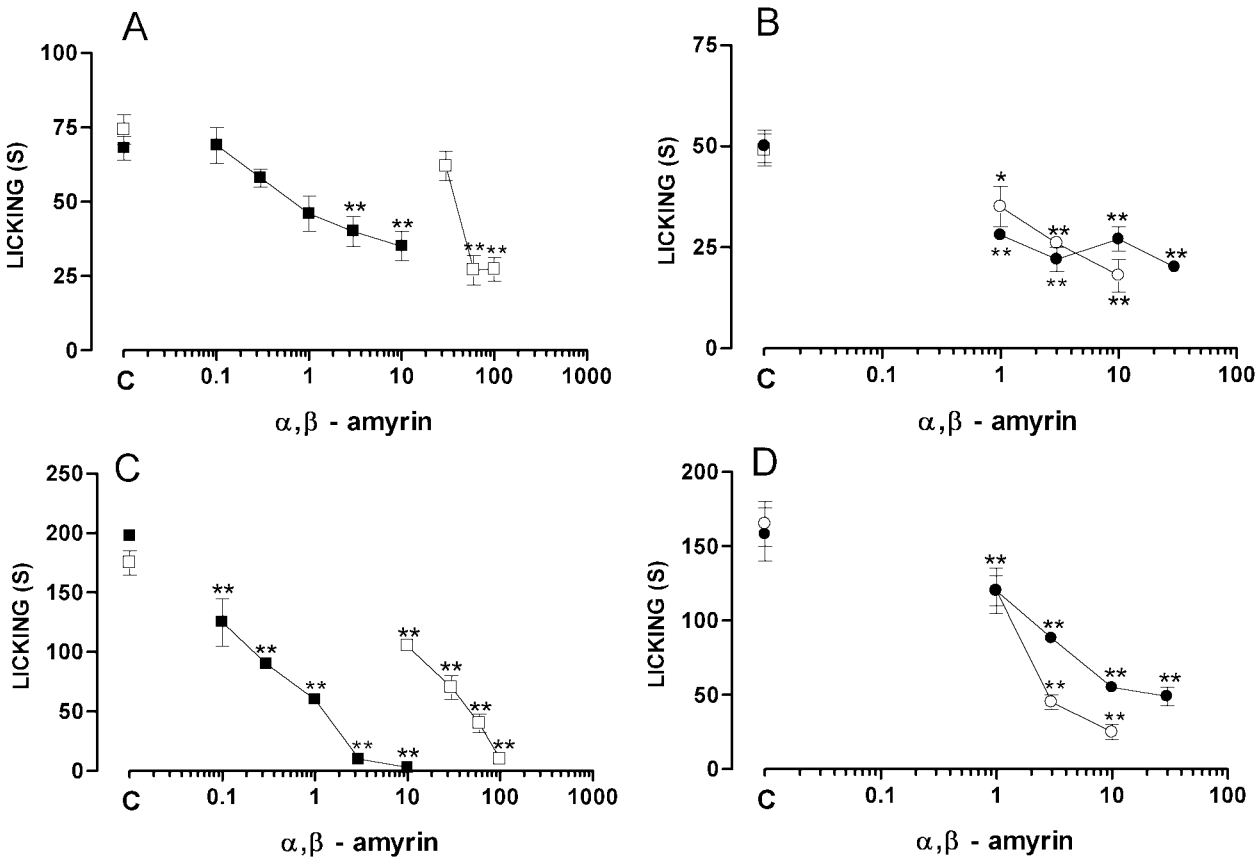

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Fig. 4-

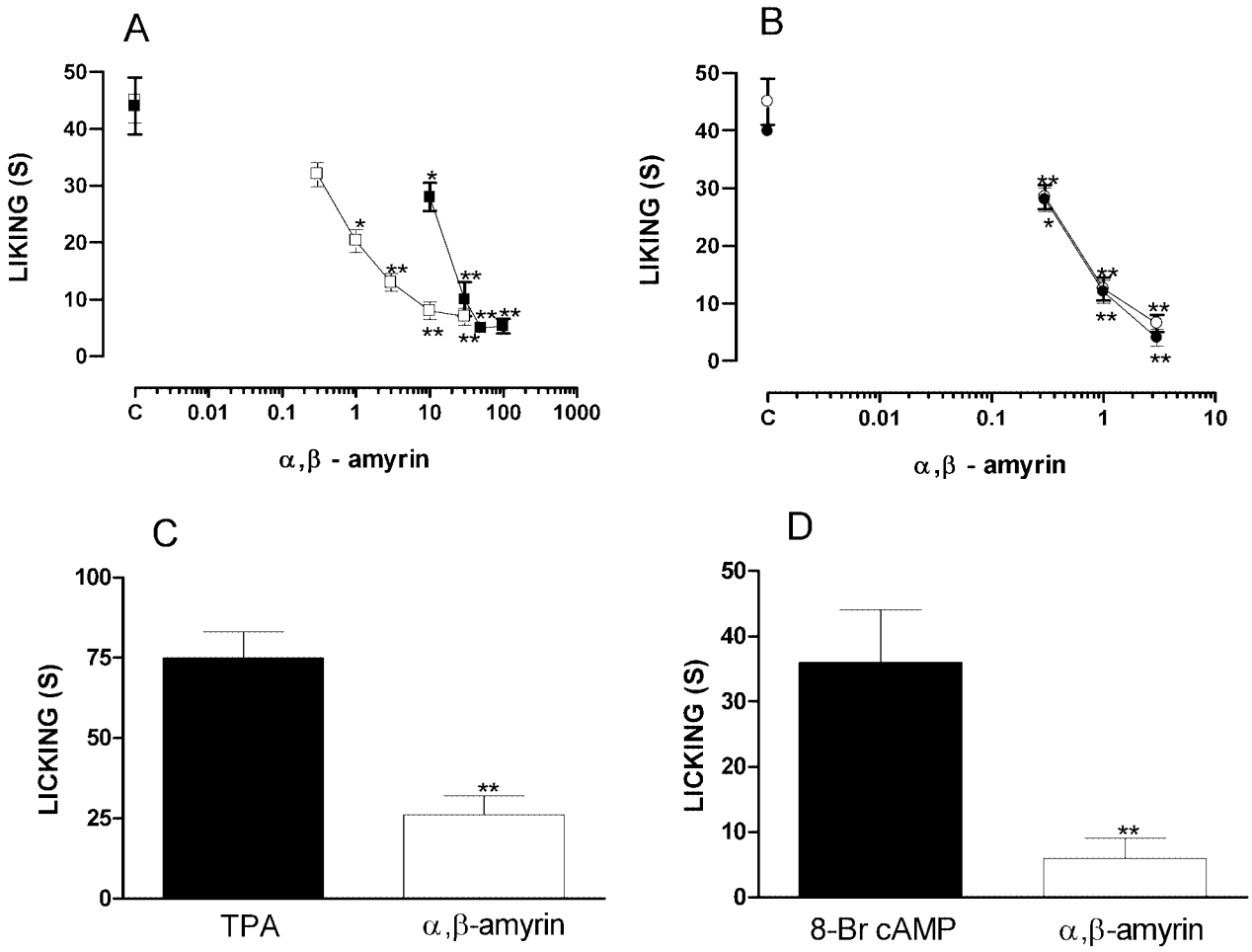

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Fig. 5-

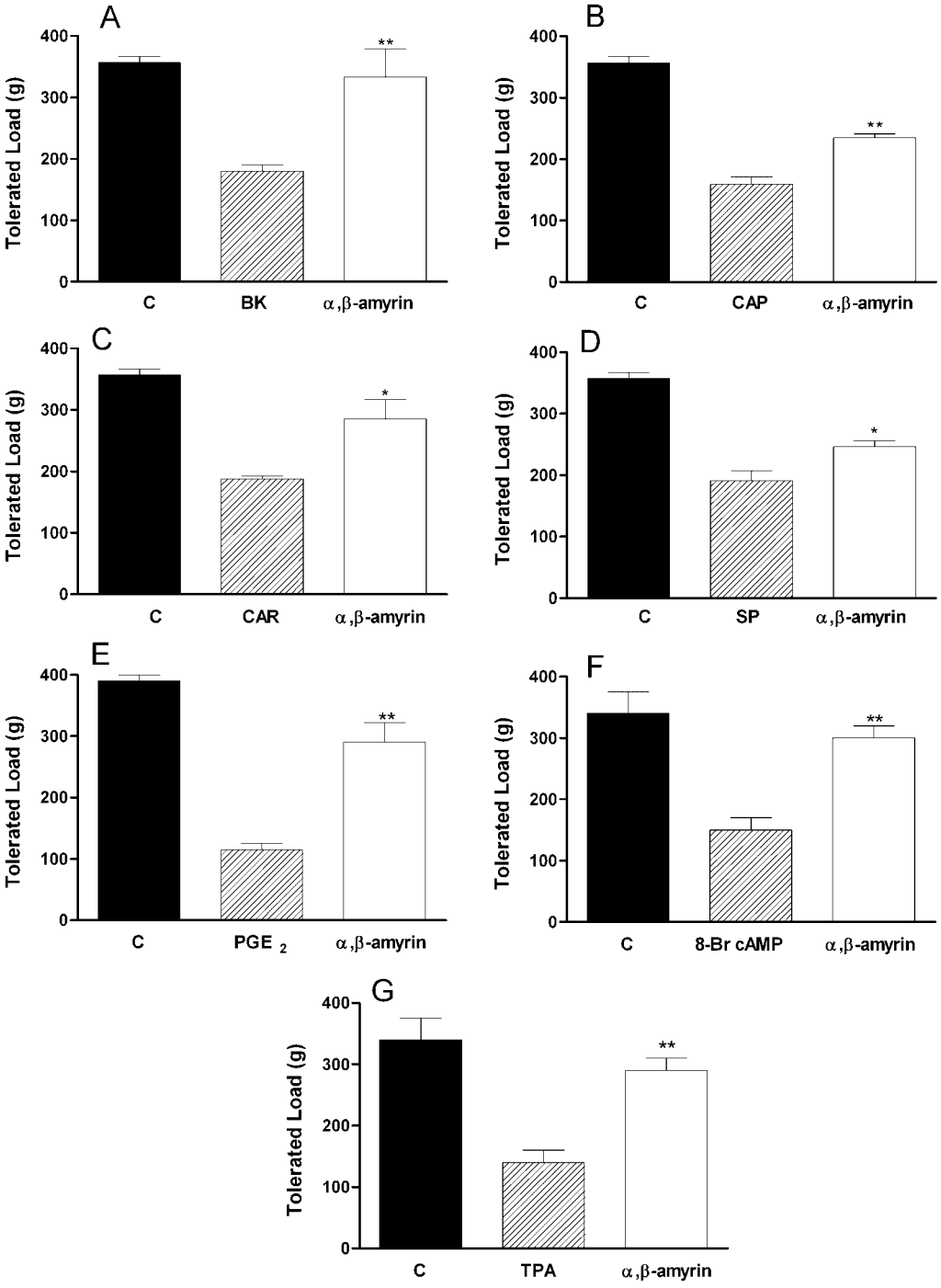

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Fig. 6-