Antinociceptive Properties of Mixture of α-Amyrin and β-Amyrin Triterpenes: Evidence for Participation of Protein Kinase C and Protein Kinase A Pathways

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ABSTRACT

The mixture of the two pentacyclic triterpenes α-amyrin and β-amyrin, isolated from the resin of Protium kleinii and given by intraperitoneal (i.p.) or oral (p.o.) 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 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 inhibits the neurogenic nociception induced by capsaicin. Moreover, i.p. treatment with α,β-amyrin was able to reduce the nociception produced by 8-bromo-cAMP (8-Br-cAMP) and by 12-O-tetradecanoylphorbol-13-acetate (TPA) or the hyperalgesia caused by glutamate. On the other hand, in contrast to morphine, tetradecanoylphorbol-13-acetate (TPA) or the hyperalgesia induced by glutamate; RTX, resiniferatoxin, or [3H]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 protein kinase A- and protein kinase C-sensitive pathways.

Natural products, since ancient times, have contributed to the development of modern therapeutic drugs, especially those derived from higher plants. Furthermore, they have contributed to our current understanding about the mechanisms involved in neurotransmitter action, especially regarding the process of pain transmission and treatment (for review, see Calixto et al., 2000). Species of the genus Protium (Burseraceae) are known for the production of oleoresin exudates that occur as a result of insect stings, broken branches, or other acts injurious to 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 agents (Corrêa, 1984). In early pharmacological studies, immunostimulant (Delaveau et al., 1980) and anti-inflammatory activities where demonstrated 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

ABBREVIATIONS: PCPA, dl-p-chlorophenylalanine methyl ester; L-NOARG, N³-nitro-L-arginine; BK, bradykinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; 8-Br-cAMP, 8-bromo-cAMP; i.pl., intraplantar; PGE₂, prostaglandin E₂; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; RTX, resiniferatoxin; PKC, protein kinase C; PKA, protein kinase A; NO, nitric oxide.
kleinii revealed the presence of a mixture of two triterpenes belonging to the ursane (α-amyrin) and oleane (β-amyrin) series. These triterpenes have been reported to exert anti-inflammatory effects when assessed in animal models of inflammation (Kweifio-Okai et al., 1994a,b; Recio et al., 1995; Akihisa et al., 1996). Furthermore, some in vitro studies have shown that α-amyrin is a relatively specific inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase (Hasmeda et al., 1999). In addition, α-amyrin and its palmitate and linoleate esters are effective in inhibiting collagenase (Kweifio-Okai et al., 1994a,b), whereas β-amyrin palmitate inhibits α1-adrenoceptors (Subarnas et al., 1993).

In our earlier study, we showed that the ether extract and the isolated pentacyclic triterpene brein obtained from P. 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.

**Materials and 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 being P. kleinii, a plant of the family Burseraceae. A voucher of this plant (excisata no. 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 diethyl ether in the proportion of 1:1 (w/v), being stirred and macerated at room temperature (21 ± 3°C) for approximately 2 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 natural compound present in this plant. This mixture was obtained in a 1:1 proportion by gas chromatography spectra. The chemical identification was obtained by using the 1H and 13C 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 2 h 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 pretreated 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% NaCl (10 ml/kg). After challenge, pairs of mice were placed in separate boxes, and the number of abdominal constrictions 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% formaldehyde), made up in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer), was 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 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 pretreated with naloxone (a nonselective 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 explore the possible contribution of serotonin to the antinociceptive effect of α,β-amyrin, animals were pretreated with dl-p-chlorophenylalanine methyl ester (PCPA) (an inhibitor of seroto-

![A](image1.png)  
![B](image2.png)

Fig. 1. Molecular structure of α-amyrin (A) and β-amyrin (B).
The response to the thermal stimuli was measured on the hot-plate test. The animals were pretreated i.p. with the angiotensin-converting enzyme inhibitor captopril (30 mg/kg i.p.) or with a,β-amyrin (10 mg/kg s.c.) or with vehicle (5 ml/kg i.p.). Control animals (vehicle, 10 ml/kg i.p.) or mice pretreated with a,β-amyrin (30 mg/kg i.p.) were injected 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 analgesimeter 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 (vehicle, 10 ml/kg i.p.) and for animals pretreated with a,β-amyrin (30 mg/kg i.p.) or with morphine (10 mg/kg s.c.). An automatic 20-s cut-off was used to minimize 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. To evaluate possible nonspecific muscle relaxant or sedative effects of a,β-amyrin, mice were tested on the rotarod (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 (model 7600; Ugo Basile). The bar rotated at a constant speed of 22 revolutions per minute. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 60 s. Animals were treated with a,β-amyrin (30 mg/kg i.p.) or with the same volume of vehicle (10 ml/kg i.p.) 30 min before being tested. The results are expressed as the time (seconds) for which animals remained on the rotarod. The cut-off time used was 60 s.

[^1]Glutamate Binding Assay. Cerebral cortices obtained from rats (killed by decapitation) were dissected and homogenized in 20 volumes of ice-cold 0.32 M sucrose containing 10 mM Tris-HCl buffer, pH 7.4, and 1 mM MgCl₂. The homogenate was centrifuged at 1000 g, and the pellet was rehomogenized and centrifuged again. The second pellet was discarded, and both supernatants were pooled and centrifuged at 27,000 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 27,000 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 27,000 g for 15 min. The final pellet was resuspended in 10 mM Tris-HCl, and the suspensions were assayed for [^1]H-glutamate binding. Assays of [^1]H-glutamate binding were carried out in triplicate in a total volume of 0.5 ml containing 0.1 ml of membrane fraction (0.2–0.3 mg of protein), 50 mM Tris-HCl, pH 7.4, and 40 nM radioactive ligand ([^1]H-glutamate, 53 Ci/mmol), in the presence or absence of a,β-amyrin (concentration in the range 1–100 μM). Nonspecific binding was assayed similarly, except that 40 nM nonradioactive glutamate (displacer) was added to the incubation medium. Centrifugation at 12,000 g for 25 min was used to separate [^1]H-glutamate not bound to membranes. The supernatant was discarded, and 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 a,β-amyrin.
existence of the displacer. The results represent the means of three
independent experiments.

**[3H]Resiniferatoxin Binding Assay.** Binding assays were car-
ried out as described previously (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 ice-cold buffer
A, pH 7.4, which contained 5 mM KCl, 5.8 mM NaCl, 2 mM MgCl2,
0.75 mM CaCl2, 12 mM glucose, 137 mM sucrose, and 10 mM
HEPES. The homogenate was first centrifuged for 10 min at 1000
rpm at 4°C. The low-speed pellets were discarded, and the supernatants
were homogenized in ice-cold buffer (50 mM TES and 1 mM 1,10-
phenanthroline, pH 6.8) with a Polytron. The homogenate was cen-
trifuged to remove cellular debris (1000
rpm) and then stored at
−70°C 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 [3H]RTX.
For the measurement of the nonspecific binding, 100 nM nonradio-
active 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 37°C water bath.
After a 60-min incubation period, cooling the mixtures on ice termi-
nated the binding reaction, and then 100 µg of bovine α,γ-diglycer-
protein was added to each tube (to reduce nonspecific binding).

Finally, the bound and free membranes [3H]RTX were separated by
centrifugation for 15 min at 20,000g in 4°C. The pellet was quanti-
yzed by scintillation counting. Specific binding was calculated as the
difference of the total and nonspecific binding.

**[3H]Bradykinin Binding Assay.** The specific binding of [3H]bra-
dykinin (a high-affinity bradykinin B2 receptor ligand) was assayed
according to the method described previously (Manning et al., 1986)
with minor modifications. The ilea from guinea pigs were removed
according to the method described previously (Manning et al., 1986)
with minor modifications. The ilea from guinea pigs were removed
which are reported as geometric means accompanied by their respec-
tive 95% confidence limits. In the glutamate-induced hyperalgesia, the ID50
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 by i.p. (0.1–10
mg/kg) or by p.o. (25–100 mg/kg) route, dose-dependently
inhibited acetic acid-induced abdominal contractions. The calculated mean ID50
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 (0–5 min) and
the inflammatory phase (15–30 min) of the formalin-induced
licking. The calculated mean ID50 values for these effects
were >10 and 0.16 (0.07–0.37) mg/kg, respectively. Intra-
peritoneal administration with α,β-amyrin was significantly
more active to inhibit the inflammatory phase than the neu-ogenic 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 ID50 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 ID50
values for these effects (micrograms per 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-

 ### Drugs.** Formalin, morphine hydrochloride, and acetic
acid were from Merck (Darmstadt, Germany); naloxone hydrochloride
was from DuPont (Garden City, NY); capsaicin was from Calbiochem
(San Diego, CA); and PCPA, glutamate, L-arginine, L-NOARG,
yohimbine, clonidine, L-phenylephrine, prazosin, TPA, 8-Br-cAMP,
PGE2, substance P, and carrageenan were from Sigma-Aldrich (St.
Louis, MO). [3H]BK and [3H]glutamate were from Amershams Bio-
sciences, Inc. (Piscataway, NJ), and [3H]RTX was from PerkinElmer
Life and Analytical Sciences (Boston, MA). Morphine and naloxone
were dissolved in 0.9% NaCl solution just before use, whereas capsai-
cin 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 ID50 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 respec-
tive 95% confidence limits. Data were analyzed by analysis of vari-
ance or t test and complemented by Dunnett's or Newman-Keuls post
hoc test. P values less than 0.05 (P < 0.05) were considered as
indicative of significance. The ID50 values were determined by linear
regression analysis from individual experiments using GraphPad
software (GraphPad Software, Inc., San Diego, CA), and are reported
as geometric means accompanied by their respective 95% confidence
limits. In the glutamate-induced hyperalgesia, the ID50 value was calculated on the peak (5 min) of glutamate response.

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 to 10
animals. The asterisks denote the significance levels compared with
control groups. Significantly different from controls, **, P < 0.01. In some
cases, the S.E.M.s are hidden within the symbols.
dependent inhibition of both phases of the formalin-induced licking (Fig. 3, B and D). The calculated mean ID$_{50}$ values for these effects (micrograms per 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 $\alpha,\beta$-Amyrin. The pretreatment of animals with naloxone given 15 min before injection of morphine largely reversed the antinociception caused by morphine when analyzed for both phases of the formalin-induced licking, leaving the antinociception caused by morphine when analyzed for both phases of the formalin-induced licking when tested for both phases of formalin-induced nociception (Table 1). The pretreatment of animals with $\alpha,\beta$-amyrrin unaffected (Table 1). The pretreatment of animals with $\alpha,\beta$-amyrrin unaffected by $\alpha,\beta$-amyrrin, given 15 min prior, significantly reversed the antinociception effect caused by N$\mathrm{N^\prime}$-nitro-$\alpha$-arginine, but it did not reverse the antinociception effect caused by $\alpha,\beta$-amyrrin against either phases of the formalin test (Table 1). Treatment of the animals with prazosin or yohimbine, 10 min prior, significantly reversed the antinociception caused by phenylephrine and clonidine, respectively, but it failed to interfere significantly with the antinociception caused by $\alpha,\beta$-amyrrin against both phases of formalin-induced nociception (Table 1).

Algogen-Induced Overt Nociception. The results of Fig. 4, A and B, show that $\alpha,\beta$-amyrrin, given orally (5–100 mg/kg), by i.p. (0.3–30 mg/kg), by i.t. (0.3–3.0 $\mu$g/site), or by i.c.v. (0.3–3.0 $\mu$g/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) $\mu$g/site, and 0.89 (0.69–1.15) $\mu$g/site and 89 ± 2, 82 ± 4, 84 ± 2, and 88 ± 2%, respectively.

Hyperalgesia in the Rat Paw. When assessed in the Randall-Selitto model of nociception, $\alpha,\beta$-amyrrin (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), carrageenan (300 $\mu$g/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 $\alpha,\beta$-amyrrin, 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, $\alpha,\beta$-amyrrin, over the same range of doses where it was effective in inhibiting glutamate-induced hyperalgesia, had no effect in the hot-plate assay (data not shown).

Hot-Plate and Tail-Flick Tests. $\alpha,\beta$-Amyrrin (30 mg/kg i.p.), given 30 min prior, did not cause any significant increase 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).

**Rotarod Test.** α,β-Amyrin (30 mg/kg), given i.p. 30 min prior, did not significantly affect the motor response of the animals. The control response in the rotarod test was 60 s versus 60 s in the presence of α,β-amyrin (n = 8) (data not shown).

**[3H]BK, [3H]RTX, and [3H]Glutamate Binding Studies.** High concentrations of α,β-amyrin (up to 100 μg/ml) were not able to alter either [3H]bradykinin, [3H]RTX, [3H]glutamate specific binding to guinea pig ilium, rat spinal cord membranes, or cerebral cortex in vitro (data not shown). In the same conditions, Hoe140 (100 nM), capsaicin (30 μM), or nonradioactive glutamate (30 μM) blocked the specific binding of [3H]bradykinin, [3H]RTX, and [3H]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 seems to be independent of the activation of important endogenous analgesic systems, namely, opioidergic, serotonergic, 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 nonselective 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 amyрин was just as efficacious as systemically administered α,β-amyrin in producing antinociception.

Also relevant are our findings showing that α,β-amyrin was able to 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 [3H]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 a modulator of the nociceptive processes, being able to produce hyperalgesia or antinociceptive effects, depending on the experimental model and the dose or site of administration tested (Aley et al., 1998). 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 NG-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 B₂ 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 sensitization through protein kinase activation, including PKC, PKA, and mitogen-activated kinases (Scholz and Woolf, 2002). Evidence now suggests that the mechanical hyperalgesia produced by PGE₂ could 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 and Willis, 1997). On top of this, the nociception caused by carrageenan, formalin, and acetic acid is sensitive to PKA or PKC inhibitors or gene deletion (Malmberg et al., 1997; Khasar et al., 1999). 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 PKA and, in a special manner, PKC (Hasmeda et al., 1999). Therefore, the ability of α,β-amyrin to interact with kinase pathways might explain its ability to inhibit PGE₂, capsaicin-, and bradykinin-induced nociception without directly interfering with their respective receptor binding sites. Apart from its nociceptive-producing effects, PKC pathway seems also to be involved in the tolerance to the analgesic effect of morphine (Granados-Soto et al.,...
However, studies must be carried out to verify whether $\alpha,\beta$-amyrin treatment also could be 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 also could explain why $\alpha,\beta$-amyrin is capable of reducing the thermal hyperalgesia produced by glutamate, which is dependent on retrograde sensitization of primary afferents (Ferreira and Lorenzetti, 1994), without changing the response to acute thermal stimulation. This seems to not be associated with the transmission of acute thermal stimuli (Malmberg et al., 1997; Khasar et al., 1999).

In addition, the antinociceptive effects of $\alpha,\beta$-amyrin herein reported extend the antiarthritic activity of $\alpha,\beta$-amyrin triterpenes (Kweifio-Okai et al., 1994a,b) and the antiedematogenic of $\alpha,\beta$-amyrin effect in carrageenan-in-

**Fig. 5.** Effects of i.p. injection of the $\alpha,\beta$-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-buffered solution, injection paws), and the diagonally hatched column indicates the BK- (BK), capsaicin- (CAP), carrageenan- (CAR), substance P- (SP), 8-Br-cAMP- (E), or TPA-injected paws, in the absence of the $\alpha,\beta$-amyrin. Each column represents the mean ± S.E.M. for 8 to 10 animals. The asterisks denote the significance levels compared with control groups. Significantly different from controls, *, $P < 0.05$ and **, $P < 0.01$.

**Fig. 6.** Effects of $\alpha,\beta$-amyrin given orally against glutamate-induced hyperalgesia in mice. The latency (mean ± S.E.M.) in the hot-plate was measured at 5 min after intrathecal injection of glutamate. Each point represents the mean ± S.E.M. for six to eight animals. The asterisks denote the significance levels compared with control groups. Significantly different from controls, **, $P < 0.01$. In some cases, the S.E.M.s are hidden within the symbols.
duced 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 P. kleinii, exhibit dose-related antinociception when assessed in chemical, but not thermal, models of nociception in mice, as well as producing antihyperalgesic 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 alogen mediators seems to account for α,β-amyrin’s antinociceptive effect.

Acknowledgments

We are indebted to Dr. Ademir Reis for botanical classification of P. kleinii.

References


Aley KO, McCarter G, and Levine JD (1999) Nitrergic nociceptive signaling pathway in rodent paw edema (Recio et al., 1995). They also confirmed in mice a triterpene isolated from resin of P. kleinii/H9251. They also confirmed in mice a triterpene isolated from resin of P. kleinii/H9252.

...α,β-amyrin’s antinociceptive effect.

FIGURE 2

TABLE 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Tail-Flick Latency</th>
<th>Hot-Plate Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>7.42 ± 1.0</td>
<td>6.85 ± 0.7</td>
</tr>
<tr>
<td>Morphinol</td>
<td>10</td>
<td>19.4 ± 0.6α</td>
<td>23.9 ± 1.5α</td>
</tr>
<tr>
<td>α,β-Amyrin</td>
<td>30</td>
<td>8.4 ± 1.0</td>
<td>6.48 ± 0.8</td>
</tr>
</tbody>
</table>

α P < 0.01 compared with control value.


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