Antiinflammatory and Analgesic Activity of an Inhibitor of Neuropeptide Amidation

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ABSTRACT

4-Phenyl-3-butenoic acid (PBA) has been shown in vitro to be a turnover-dependent inactivator of peptidylglycine α-monoxygenase (PAM), the rate-limiting enzyme involved in the formation of amidated neuropeptides from their glycine-extended precursors. In the studies reported herein, we have shown that PBA produces a dose-dependent (50–500 mg/kg s.c.) inhibition of serum PAM activity in normal rats without affecting peptidylamidoglycolate lyase activity. Because amidated neuropeptides such as substance P and calcitonin gene-related peptide are involved in acute inflammation, we evaluated the effects of PBA on carrageenan-induced inflammation in rats. The acute administration of PBA (s.c. or i.p.) produced a dose-related inhibition of edema with maximum inhibition (67%) observed at 2 hr postphlogistic agent. In addition, the continuous administration of PBA to animals over a 7-day period using osmotic pumps not only inhibited hind paw swelling induced by carrageenan but also inhibited serum PAM activity and reduced tissue levels of substance P in hind paws. These results demonstrate for the first time a correlation between the antiinflammatory activity produced by an inhibitor of peptide amidation with its ability to inhibit serum PAM activity and lower endogenous tissue levels of substance P. Moreover, these results confirm our contention that PAM is an excellent pharmacological target for controlling the acute inflammatory response. We also demonstrate the ability of PBA to inhibit phenyl-p-quinone and acetylcholine-induced writhing in mice without affecting the spinally mediated tail immersion assay in rats. Because this analgesic effect was extremely rapid (within 15 min), PBA may be producing this effect by a mechanism other than peptide amidation.

Neuropeptides such as SP and CGRP are released from peripheral terminals of primary afferent sensory nerves and contribute significantly to the inflammatory response of a variety of diseases including rheumatoid arthritis (Garrett et al., 1992). These neuropeptides have been shown to be capable of producing vasodilatation, increasing vascular permeability, attracting and activating phagocytic white blood cells, releasing cytokines, lysosomal enzymes and prostaglandins from these cells, increasing the expression of adhesion molecules as well as causing the activation of synoviocytes (Matucci-Cernic and Partsch, 1992). In addition, the direct injection of these substances into inflamed joints of animals has been shown to directly increase the severity of the process (Levine et al., 1984). In several animal models of inflammation, the involvement of SP in the pathogenic process is further suggested by the observation that capsaicin, the active principle from chili peppers known to deplete SP from sensory nerve endings, significantly inhibits the inflammatory response (Colpaert et al., 1983; Lam and Ferrell, 1991).

It has become very clear that the synthesis, axonal transport and release of SP and CGRP increase during both acute and chronic inflammation. More importantly, the extent of these changes in neuropeptide dynamics in sensory afferents appears to depend on the time-course and severity of the inflammatory response. In acute inflammation induced by carrageenan, levels of SP in inflamed hind paws increased within 15 min after induction, reached peak levels in 30 min and remained elevated during the first 2 hr of inflammation (Gilligan et al., 1994). Similar increases in SP levels have also been reported in inflammatory pleural exudate produced in rats by injection of carrageenan (Tissot et al., 1988). The spontaneous as well as capsaicin-evoked release of SP and CGRP from spinal dorsal horn slices obtained from rats administered carrageenan has also been shown to be enhanced (Garry and Hargreaves, 1992). As expected, the content of these two neuropeptides in the dorsal horn was reduced during this response. In response to the inflammation produced by carrageenan and subsequent release of SP and CGRP from sensory nerve endings, cells from dorsal root ganglia have been shown to produce a rapid increase (within 2 hr) in mRNA coding for the preprotachykinin and pre-

ABBREVIATIONS: SP, substance P; CGRP, calcitonin gene-related peptide; PAM, peptidylglycine α-monoxygenase; PGL, peptidylamidoglycolate lyase; PBA, 4-phenyl-3-butenolic acid; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay.
proCGRP proteins indicating an up-regulation of neuropeptide synthesis (Iadarola and Draisci, 1988).

These two proinflammatory neuropeptides are synthesized as biologically inactive glycine-extended precursors that require a carboxyl-terminal posttranslational amidation for biological activity. The formation of the amide involves a two-step process (fig. 1) resulting from the sequential action of two enzymes. The first enzyme, PAM (EC 1.14.17.3), catalyzes the formation of a peptidyl-α-hydroxyglycine intermediate that is then converted by the second enzyme, PGL (EC 4.3.2.5), into the α-amidated product and glyoxylate (Bradbury et al., 1982; Katopodis et al., 1990; Li et al., 1994; Ping et al., 1995).

Several pharmacological approaches to reduce the neurogenic component of inflammation have been evaluated including interference with the release of neuropeptides from afferent terminals, enhancing the degradation of these peptides or blocking neuropeptide receptors (Barnes et al., 1990). Because α-amidation appears to be the rate-limiting step in the biological activation of these proinflammatory neuropeptides (Eipper et al., 1992), the enzymes involved in this process should be very attractive pharmacological targets for reducing neuropeptide formation and inhibiting the inflammatory response. Recently, we have demonstrated that PBA is a potent turnover-dependent inactivator of PAM with inactivation exhibiting the characteristics expected for mechanism-based inhibition (Katopodis and May, 1990). The k_m/aV value for this inhibitor was found to be approximately 6700 mM⁻¹ min⁻¹ indicating that this compound is an extremely potent PAM inactivator. Studies in vitro using cultured bovine endothelial cells (Oldham et al., 1992) as well as rat thyroid carcinoma cells (Bradbury et al., 1990) have demonstrated the ability of PBA to inhibit α-amidation and PAM activity without affecting PGL, resulting in decreased formation of the amide.

In our study, we have examined the effects of systemic administration of PBA on the activity of serum PAM in normal rats. We have found that PBA inhibits PAM activity in vivo without affecting PGL. Furthermore, this inhibitor of peptide amidation inhibited the acute inflammatory response produced by injection of carrageenan into rat hind paws. The antiinflammatory effect produced by PBA correlated with its ability to inhibit serum PAM activity and reduce levels of SP in rat hind paws. These results suggest that drugs inhibiting peptide amidation might represent a new and unique pharmacological class of antiinflammatory agents.

**Methods**

**Experimental animals.** Adult male, Sprague Dawley rats (175–225 g) and male ND4 Swiss Webster mice (18–20 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), housed in appropriate cages facilities and allowed food and water ad libitum. All experiments using animals were approved by the Institutional Animal Care and Use Committee of Mercer University (Macon, GA).

**Drugs and reagents.** PBA used in all experiments was purchased from Aldrich Chemical Co. (Milwaukee, WI) and recrystallized from hot ethyl acetate. For all injections, PBA was dissolved in saline, the pH adjusted with sodium hydroxide to 7.5 and administered s.c. (between the scapulae) using a constant volume (2 ml/kg). Type IV, λ-carrageenan, bradykinin acetate, serotonin hydrochloride, phenyl-p-quinone and acetylcholine chloride were all purchased from Sigma Chemical Co (St. Louis, MO). Bovine liver catalase (65,000 U/mg) was purchased from Boehringer Mannheim (Indianapolis, IN). α-Hydroxyhippuric acid was purchased from Aldrich and recrystallized before use. TNP-D-Tyr-Val-Gly was synthesized as reported previously (Katopodis and May, 1990). All other reagents, solvents and chemicals were of analytical grade.

**Evaluation of PAM and PGL activity.** Male rats were fasted overnight and anesthetized with ether to obtain blood samples for measuring PAM and PGL activity in serum. Blood (0.5 ml) was collected from the tail vein at different times and spun at 14,000 × g for 5 min. Serum was collected and stored at -70°C until assayed. PAM activity was determined as described previously (Katopodis et al., 1991). Briefly, 50 μl of serum sample (enzyme source) were added to 200 μl of the assay mixture containing tripeptide TNP-D-Tyr-Val-Gly as the enzyme substrate (40 μM), copper sulfate (15 μM), L-ascorbate (4 mM) and catalase (1 mg/ml) in [2-(N-morpholino)-ethanesulfuric acid] buffer (100 mM, pH 6.5). After a 30-min incubation period at 37°C, an aliquot (90 μl) was quenched with 10 μl of HClO₄ (3 M) and centrifuged at 14,000 × g for 5 min. A 20-μl aliquot was removed and used to assay for product using reverse phase HPLC at 344 nm on a C8 column with a mobile phase of 56% water/0.1% trifluoroacetic acid/44% acetonitrile at a flow rate of 1.5 ml/min. In this manner, both TNP-D-Tyr-Val-NH₂ as well as the TNP-D-Tyr-Val-α-hydroxyGly were quantitated simultaneously. PGL activity was assayed by measuring the conversion of α-hydroxyhippuric acid to benzamide (Katopodis and May, 1990). Briefly, 50 μl of the serum sample were added to 200 μl of a 100 μM MES buffer solution (pH 6.5) containing 2 mM of the enzyme substrate. After incubation for 30 min at 37°C, an aliquot of the assay mixture was quenched with 3 M HClO₄ centrifuged at 14,000 × g and 20 μl used to analyze for benzamide product by HPLC using a C8 reverse phase column. Product detection was performed at 225 nm using a mobile phase of 80% water/0.1% trifluoroacetic acid/20% acetonitrile at a flow rate of 1.5 ml/min. Enzyme activity was expressed as mU/ml which is the amount of enzyme required to produce one nanomole of product.
**Carrageenan edema, bradykinin and serotonin edemas.**
Carrageenan, bradykinin and serotonin edemas were induced in anesthetized, male Sprague-Dawley rats (150–175 g) by injecting 0.5 mg (0.05 ml) of carrageenan, 50 μg (0.1 ml) of bradykinin and 20 μg (0.1 ml) of serotonin, respectively, into the subplantar region of the left hind paws; the contralateral hind paws received saline only. Hind paw volumes (edema) were measured plethysmographically by displacement of mercury at 0, 1, 2, 3, 4 and 6 hr postadministration of carrageenan, 30 min after bradykinin and 1 hr after serotonin. Swelling was determined by subtracting the volume (ml) of the right hind paw from that of the left. PBA was administered s.c. 30 min before the administration of carrageenan and serotonin and 1 hr before bradykinin. In those experiments in which PBA was delivered continuously to rats over a 7-day period before the administration of carrageenan, ALZET osmotic pumps (Palo Alto, CA) were filled with drug and implanted s.c. between the scapulae into anesthetized rats.

**Extraction and quantitation of SP levels.** The effects of PBA on levels of SP were evaluated using a modification of a method previously described (Ahmed et al., 1994). Briefly, at different times after the administration of carrageenan, hind paw ankle joints were removed, immediately frozen on dry ice and stored at -80°C until assayed for SP. After the frozen sample was weighed, it was boiled for 7 min as a 10% w/v solution of 2 M acetic acid in 4% EDTA, pH 3.5, cut into small pieces and boiled for an additional 7 min. Samples were then homogenized for 60 sec in a Brinkmann Polytron (Westbury, NY), sonicated for 30 sec and centrifuged at 3000 × g for 20 min. Supernatants were lyophilized and then diluted in RIA buffer before analysis. SP levels were determined from these samples by using a commercially available RIA kit (INCSTAR, Stillwater, MN). Initial experiments conducted to insure the specificity of the substance P antibody for SP found the cross-reactivity for the glycine-extended precursor to be 1.7% at concentrations up to 100 ng/ml.

**Analgesic assays.** The effects of PBA on the perception of pain was evaluated using several different animal models. The spinally mediated tail-flick response to heat in fasted rats (250–290 g) was used by measuring the withdrawal latency following immersion of the rodent’s tail (2 inches) into hot water (50°C). PBA at several different dose levels was administered s.c. and the time to tail withdrawal was measured at 0, 15, 30 and 60 min post-drug administration. The analgesic effects of PBA were also evaluated in fasted mice after the injection of either phenyl-p-quinone or acetylcholine. Male, ND4 Swiss mice (15–20 g) were dosed s.c. with PBA or saline followed immediately by the injection of phenyl-p-quinone (2 mg/kg i.p.). After 5 min, the number of writhes (abdominal constrictions along with contortion of the trunk and extension of the hindlimbs) was counted over the next 10 min. When acetylcholine (6 mg/kg i.p.) was used as the noxious agent, PBA was administered s.c. 15 min before the administration of the algic agent. The number of writhes produced was counted over the last 5 min after the algic agent.

**Statistical analysis.** Data are presented as mean responses ± S.E.M. Two-way analysis of variance for repeated measures was used to test for significance. Comparison of means was performed by using Tukey’s post hoc tests. A probability of P < .05 was considered statistically significant.

**Results**

Our initial experiments were conducted to determine if the effects of PBA on PAM and PGL activity in vitro were similar to those observed in vivo. As shown in figure 2, PBA (500 mg/kg s.c.) significantly inhibited (>90%) the activity of PAM in serum within 1 hr after the administration of a single dose to conscious rats. This magnitude of inhibition remained during the first 3 hr after administration, decreased to 43% inhibition by 6 hr, and was not significantly different from control values after 24 hr. This same dose of PBA had no effect on serum PGL activity during the 24-hr observation period demonstrating the ability of PBA to selectively inhibit PAM activity. This inhibitory effect of PBA on serum PAM activity was also found to be dose-related (fig. 3) with the 50 and 150 mg/kg doses having similar time-courses of inhibition but of lesser magnitude than the effects caused by the 500 mg/kg dose.

These initial results suggested that PBA might be capable of producing an antiinflammatory effect in vivo since inhibition of PAM activity would result presumably in lowering of

**Fig. 2.** The effect of PBA in vivo on the activity of serum PAM and PGL. Normal, male rats were fasted overnight, administered PBA (500 mg/kg s.c.) and blood samples taken from the tail at different times over a 24-hr period. The activity of PAM and PGL in these samples was measured as described in “Methods.” Solid line, saline-treated PAM controls; dashed line, PBA effects on PAM; dotted line, saline-treated PGL controls; dot, dashed line, PBA effects on PGL. Data are presented as mean ± S.E.M. for each group (n = 6). * Statistically significant (P < .06) compared to time 0 (Tukey’s test).

**Fig. 3.** Dose-response effect of PBA on serum PAM activity in normal rats. Different doses of PBA were administered subcutaneously to fasted rats. Blood samples were taken from the tail and assayed for PAM activity as described in “Methods.” Solid line, saline-treated controls; dashed line, 50 mg/kg; dot, dashed line, 150 mg/kg; dotted line, 500 mg/kg. Data are presented as mean ± S.E.M. for each group (n = 6). *Statistically significant (P < .05) compared to time 0 (Tukey’s test).
endogenous levels of amidated neuropeptides such as SP and CGRP. Therefore, experiments were conducted to determine if PBA had any effects on inflammation induced by the subplantar injection of carrageenan. As seen in figure 4, the s.c. administration of PBA 30 min before the phlogistic agent produced a dose-related inhibition of hind paw edema at 2 and 3 hr postadministration of the phlogistic agent. This inhibitory effect was greatest at 2 hr with the 250 and 500 mg/kg doses producing 43 and 67% inhibition, respectively. A similar time-course of inhibition of carrageenan edema was observed in these animals after i.p administration of PBA (data not shown).

Bradykinin and serotonin are two mediators released early during acute inflammation, and have been suggested to play an important role during the early phase of carrageenan edema (Di Rosa et al., 1971). Because PBA appeared to be more effective during the early phase of carrageenan edema, experiments were conducted to determine if PBA was capable of inhibiting hind paw swelling produced by these two mediators. It is clear from the data in Table 1 that PBA lacked significant inhibitory activity on edema produced by either bradykinin or serotonin at a dose that produced significant inhibition of carrageenan edema.

The reduced effectiveness observed with PBA during the late phase of carrageenan edema (at 3 hr) could be due to the drug having a short duration of action rather than a lack of activity. To evaluate this possibility, PBA was administered to animals 2 hr after the induction of inflammation. As seen in figure 5, PBA inhibited the continuous increase in hind paw swelling observed in control animals at 3, 4 and 6 hr postadministration of the phlogistic agent. The 100 mg/kg dose was only effective at hr 3 although the 250 and 500 mg/kg doses produced significant inhibition of hind paw swelling for up to 6 hr postphlogistic agent.

Because these results suggested that the pharmacokinetics of PBA were responsible for its short duration of action in carrageenan edema, experiments were conducted in which PBA was administered to rats via osmotic pumps to prolong its duration of action. Our initial experiments using this method of drug delivery were designed to determine if continuous release of PBA (50–100 mg/kg/hr s.c.) over a 7-day period would produce a sustained reduction in serum PAM activity. Serum samples were taken every other day for analysis of PAM activity. Results from this study demonstrated that this method of dosing with PBA produced a sustained reduction (>75%) in PAM activity throughout the 7 day dosing period (data not shown).

Based on these results, this same dosing protocol was used to evaluate the effects of PBA on carrageenan edema in animals. Animals were treated with PBA (75 mg/kg/hr) for 1

<table>
<thead>
<tr>
<th>Phlogistic Agent</th>
<th>Hind Paw Volume (ml ± S.E.M.)</th>
<th>Inhibition (%)</th>
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<tr>
<td>Carrageenan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93 ± 0.12</td>
<td>66.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bradykinin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.39 ± 0.05</td>
<td>25.6</td>
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<tr>
<td>Serotonin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.81 ± 0.07</td>
<td>18.5</td>
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<sup>a</sup> Carrageenan (0.05 ml of 1% solution) was injected into left hind paw. Right hind paw received saline. Paws were measured 2 hr after phlogistic agent.

<sup>b</sup> Statistical significance (P < .05) compared to controls; n = 8 for each assay.

<sup>c</sup> Bradykinin (50 μg) was injected into left hind paw. Paws were measured 30 min after phlogistic agent.

<sup>d</sup> Serotonin (20 μg) was injected into the left hind paw. Paws were measured 1 hr after phlogistic agent.
wk, and then carrageenan was administered into the sub-plantar region of the hind paw. The degree of inflammation as well as serum PAM activity and SP levels in hind paw tissue were measured at different time periods. Results of these experiments are shown in figure 6. As expected, carrageenan produced a time-dependent increase in hind paw volume in control animals that reached a peak 3 hr postadministration of the phlogistic agent (fig. 6a). Although serum PAM activity did not change significantly in these animals (fig. 6b), there was a significant increase in levels of SP in the inflamed hind paws during the first 2 hr after carrageenan administration (fig. 6c). Interestingly, in animals treated for 7 days with PBA, both serum PAM activity and SP levels in hind paw tissue were significantly reduced compared to controls when measured before the administration of carrageenan (fig. 6b and c, time 0). In those animals treated with PBA, there was a significant inhibition of carrageenan edema at each time period (fig. 6a). This inhibition of hind paw swelling by PBA correlated with its ability to inhibit serum PAM activity and reduce SP levels in hind paw tissue of animals administered carrageenan (fig. 6b and c).

Because most antiinflammatory drugs also have analgesic activity, experiments were conducted to determine if PBA possessed the ability to increase the threshold to pain. Initial experiments were performed using the rodent tail-flick assay to determine if PBA possessed any central analgesic activity after acute administration. After obtaining baseline values, PBA was administered s.c. to rats followed by immersion of the rodent tail into 50°C water and measuring the time to tail withdrawal at 15, 30 and 60 min post-drug administration. In these experiments, PBA did not have any effect on time to tail withdrawal at any time period with doses up to 750 mg/kg (data not shown).

The phenyl-p-quinone and acetylcholine-induced writhing assays in mice are known to be inhibited by both central as well as peripheral-acting analgesic agents (Gyires and Torma, 1984). The i.p. injection of these two substances produces painful responses (writhes) as manifested by a series of abdominal constrictions with contortions of the trunk and extension of the hindlimbs. Because PBA lacked central analgesic activity, these two assays were used to evaluate the peripheral analgesic activity of PBA. As seen in figure 7, PBA produced a significant dose-related inhibition of phenyl-p-quinone-induced writhing with more than 85% inhibition of writhing occurring at 250 mg/kg. In the acetylcholine-induced writhing assay, because the number of writhes produced in each control animal was small (1 to 8), the effects of PBA were expressed as the percentage of animals per group that writhed (all or none response) at each dose level (fig. 8). Again, PBA showed significant inhibitory activity with an ED_{50} of 105 mg/kg that is similar to the dose of PBA that reduced phenyl-p-quinone-induced writhing by 50%. It is noteworthy that the 100 mg/kg dose that did not show any activity on carrageenan edema produced a significant analgesic effect in both writhing assays.

**Discussion**

Approximately 50% of the known neuropeptides are synthesized as biologically inactive glycine-extended precursors that require a carboxyl-terminal posttranslational amidation for biological activity. Formation of the active neuropeptide requires two enzymes, PAM that catalyzes the formation of the α-hydroxyglycine derivative and PGL that rapidly converts the stable intermediate to the amidated peptide and glyoxylate (Katopodis et al., 1990; Katopodis et al., 1991). Data from previous studies suggest that PAM is the rate-limiting enzyme in this biochemical process and, as a result, is subject to regulation by pharmacological agents (Mueller et al., 1993). However, very few compounds have been synthesized and evaluated either in vitro or in vivo as specific inhibitors of PAM activity. More importantly, none of these compounds have been extensively studied for their potential therapeutic benefit in disease states involving α-amidated neuropeptides.

We have previously reported that the olefinic substrate analog PBA is a potent turnover-dependent inactivator of PAM with inactivation exhibiting the characteristics of enzymatic inhibition and irreversible inactivation (Gonon and Ogonowski, 1988). Since PBA is a noncompetitive inhibitor of PAM, it is possible that PBA could be evaluated in future studies to provide insights on the role of PAM in regulating the synthesis of active neuropeptides.

**Fig. 6.** Effect of continuous administration of PBA on carrageenan edema, serum PAM activity and SP levels in rat hind paws. Osmotic pumps containing PBA were implanted s.c. into normal rats. Seven days later, carrageenan edema was induced in both control and PBA-treated animals. Hind paw volumes, serum PAM activity and SP levels in hind paw tissue were evaluated as described in “Methods.” Open bars, control animals; solid bars, PBA-treated animals (75 mg/kg/hr). Data are presented as mean ± S.E.M. for each group (n = 5 or 6 animals). *Statistically significant (P < .05) compared to controls for that time period (Student’s t test); **Statistically significant (P < .05) compared to controls at time 0.
Effect of PBA on phenyl-p-quinone-induced writhing in mice. Different doses of PBA were administered subcutaneously to fasted, male ND4 Swiss mice followed immediately by the injection of phenyl-p-quinone (2 mg/kg i.p.). After 5 min, the number of writhes were counted over the next 10 min. Approximate ID$_{50}$ = 100 mg/kg.

Fig. 7.

Effect of PBA on acetylcholine-induced writhing in mice. Different doses of PBA were administered subcutaneously to fasted, male ND4 Swiss mice followed immediately by the injection of acetylcholine (6 mg/kg i.p.). The number of writhes were counted over the next 5 min. Due to the small number of writhes per animal, the percentage of animals that writhed at each dose level (all or none response) was determined. Numbers in parenthesis represent the number of animals that writhed over the total number of animals tested at that dose.

Fig. 8.

Expected for a mechanism-based inhibitor (Katopodis and May, 1990). This compound has been shown in vitro to have an apparent $K_i$ near 1 $\mu$M and to be capable of inhibiting the activity of PAM isolated from endothelial cells (Oldham et al., 1992) as well as penetrating the membranes of rat CA77 cells and decreasing intracellular amidating activity and levels of thyrotropin releasing factor (Bradbury et al., 1990). We now report the ability of PBA to inhibit serum PAM activity following subcutaneous administration to rats without affecting PGL activity (fig. 2). This inhibitory effect was observed within 1 hr after drug administration and was dose-related (fig. 3) with the 500 and 150 mg/kg doses producing greater than 90 and 65% inhibition, respectively. The inhibitory effect produced by the single injection of PBA was maintained during the first 3 hr after drug administration but began to wane by 6 hr and approach control values after 24 hr. A similar change in the time-course of PAM activity was observed after incubation of CA77 cells with PBA (Bradbury et al., 1990). Because PBA acts as a mechanism-based inactivator and produces irreversible inhibition of the enzyme, our results suggest that the return of serum PAM activity was likely due to a compensatory increase in synthesis of the enzyme in response to an inactivation of PAM and the subsequent decrease in formation of amidated peptides.

Although the exact source of serum PAM is not clear, the highest concentration of this enzyme has been measured in the central nervous system with the next highest levels found in the submandibular glands and serum (Eipper et al., 1985). PAM has been shown to be primarily located within secretory granules and its presence in serum may represent accumulation due to exocytosis from a variety of neuronal and endocrine cells along with its peptide products (Schafer et al., 1990). We now report the ability of PBA to inhibit serum PAM activity suggests that this compound should be effective in disease states where amidated peptides have been shown to play important roles.

The results of our studies with PBA in carrageenan edema clearly demonstrate that PBA is capable of inhibiting an acute inflammatory response (fig. 4). These results illustrate, for the first time, that the enzyme PAM may be an attractive target for the pharmacological control of acute inflammation. Several other studies have shown that interference with the activity of the peripheral sensory nervous system can reduce inflammation associated with carrageenan edema. The administration of capsaicin, a compound known to produce chronic depletion of type C afferent sensory nerves, was found capable of inhibiting carrageenan-induced hind paw edema in rats by 36% (Lam and Ferrell, 1991) and carrageenan-induced pleurisy by 40% (Raychaudhuri et al., 1991). A similar degree of inhibition of carrageenan edema was also observed following chronic denervation of the hind paw and dorsal rhizotomy (Lam and Ferrell, 1989; Sluka et al., 1994). The percent inhibition produced in our studies by PBA are consistent with these findings (fig. 4).

The early phase of carrageenan edema (hr 2) appeared to be more susceptible to the inhibitory effects of PBA (fig. 4). During this time period, SP has been shown to increase very rapidly in hind paw tissue and synovial fluid of rats after injection of carrageenan (Gilligan et al., 1994). Levels of this amidated peptide more than doubled within 15 min after carrageenan administration, reached their peak within 30 min and slowly decreased with time but remained elevated for up to 24 hr (Bileviciute et al., 1993). We have also found a similar increase in levels of SP in inflamed hind paws of rats administered carrageenan (fig. 6c). This increase in neuropeptide levels may reflect not only the initial release from sensory nerve endings but also an increased conversion of the glycine-extended precursor to the active peptide by PAM. The glycine-extended precursors of neuropeptides have been found in plasma (Eipper and Mains, 1988) suggesting that some of the precursor is available within the nerve ending for
Taken together, these results strongly support our premise that PBA could be producing its antiinflammatory effect during this early phase of carrageenan edema by inhibiting PAM activity and reducing tissue levels of inflammatory neuropeptides. Other mediators of inflammation, such as serotonin and bradykinin, have also been identified and shown to play important roles during the early phase of carrageenan edema (Vinegar et al., 1987; Di Rosa et al., 1971). Therefore, PBA could also be exerting some of its antiinflammatory effects by interfering with the activity of these two mediators. However, we found that the dose of PBA that produced greater than 65% inhibition of carrageenan-induced hind paw swelling was ineffective in reducing hind paw edema induced by either serotonin or bradykinin (Table 1).

Although PBA was less effective during the late phase of carrageenan edema (hr 3), we suspected that the reason for this short duration of action was the pharmacokinetics of the drug and not due to its selectivity for mediators associated only with this early phase, especially because we could not demonstrate any inhibitory effects of PBA on serotonin and bradykinin activity. Therefore, we administered PBA 2 hr after the induction of the inflammation to determine if it had the ability to inhibit the late phase of carrageenan edema. As seen in Figure 5, PBA not only prevented the increase in paw edema observed in the controls during this time period but also caused a reversal of hind paw swelling. These results support the conclusion that the reduced effectiveness of PBA observed at 3 hr postadministration of carrageenan was due to the kinetics of the drug and not because it lacked the ability to inhibit the late phase of the edema.

Because PBA was found to have a short duration of action, experiments were conducted to evaluate its antiinflammatory activity following continuous administration over a 7-day period using osmotic pumps. In these studies, we also determined the effects of PBA on serum PAM activity and hind paw tissue levels of SP during the inflammatory response. The results of these experiments (Fig. 6) clearly show the ability of PBA to inhibit carrageenan edema in animals dosed continuously for 7 days with the drug. More importantly, this inhibitory effect of PBA on carrageenan edema (Fig. 6a) correlated with its ability to inhibit serum PAM activity (Fig. 6b) and reduce levels of SP (Fig. 6c) in hind paw tissue of animals during this acute inflammatory response. Taken together, these results strongly support our premise that the in vivo administration of an inhibitor of α-amidation can interfere with the endogenous synthesis of inflammatory neuropeptides such as SP resulting in a significant lowering of levels of these peptides in inflamed joints and an antiinflammatory effect.

The analgesic effects produced by PBA are more difficult to explain based on a mechanism of inhibition of PAM activity. We have clearly shown that PBA was very effective in inhibiting pain associated with the injection of either phenyl-p-quinone or acetylcholine in mice (Figs. 7 and 8). This analgesic effect was observed within 15 min or less after its administration to these animals. The effectiveness of PBA as an analgesic agent appears to be greater than its antiinflammatory activity as demonstrated by its ED\(_{50}\) in both analgesic assays being approximately 100 mg/kg. The site of action for this analgesic effect seems to be the periphery since PBA was ineffective in preventing the spinally mediated tail immersion assay in rats (data not shown). At least three classes of mediators are known to be involved in producing peripheral pain: neuropeptides such as SP and CGRP (Gamse, 1982; Smith et al., 1994), bradykinin (Dray and Perkins, 1993) and prostaglandins (Doherty et al., 1987). It is noteworthy that prostaglandins increase very rapidly (within 10 min) after the administration of the algesic agent, potentiate the effects of other mediators of pain and are believed to be the major site of action of nonsteroidal antiinflammatory agents capable of inhibiting pain (Cronstein and Weissmann, 1995). In view of the rapid appearance of the analgesic effects produced by PBA as well as its greater potency as an analgesic agent compared to its antiinflammatory activity, it does not appear that the mechanism of action of PBA in these models of pain is related to its ability to inhibit peptide amidation. Because we have established that PBA does not interfere with the activity of bradykinin, it is more likely that PBA is producing its analgesic effects by inhibiting the synthesis of prostaglandins. In this regard, studies are in progress to determine if PBA is capable of inhibiting cyclooxygenase activity and the subsequent release of prostaglandins in addition to its effects on PAM.

References


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