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An Inhibitor of Opioid Peptide Degradation Produces Analgesia in Mice

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SIMMONS, W. H. AND R. F. RITZMANN. *An inhibitor of opioid peptide degradation produces analgesia in mice.* PHARMAC. BIOCHEM. BEHAV. 13(5) 715–718, 1980.—Bacitracin, a known inhibitor of brain peptidases which degrade the enkephalins and endorphins, produced dose-dependent analgesia in mice when injected into the lateral ventricle of the brain as determined by the tail-flick assay. The analgesic response peaked at 15 min post-injection but was reduced to control levels by 3.5 hours. Naloxone reversed the analgesic response to bacitracin, suggesting that opiate receptors may be involved. It is proposed that bacitracin produces analgesia by preventing the enzymatic destruction of endogenously released opioid peptides.

Bacitracin Analgesia Naloxone Enkephalin Enkephalinase Endorphin

THE endogenous opioid pentapeptides, (Met^5)enkephalin (Tyr-Gly-Gly-Phe-Met) and (Leu^5)enkephalin (Tyr-Gly-Gly-Phe-Leu), are subject to rapid enzymatic inactivation in brain tissue [4, 9, 14, 21] which has been proposed to account for their short-lived analgesic effect [2, 5, 6]. The larger opioid peptides, β, γ, and α-endorphin, are also degraded by brain peptidases although more slowly than the enkephalins [4, 14, 18, 28]. In addition, the N-terminal fragment [1–13] of the newly discovered potent endogenous morphinomimetic peptide, dynorphin, has been shown to be rapidly degraded by brain peptidases both *in vitro* and *in vivo* [11].

We report here that bacitracin, which inhibits opioid peptide degradation *in vitro* [1, 13, 17, 19, 20, 22, 25, 29, 30], can produce analgesia in mice when injected intracerebroventricularly (ICV). It is proposed that bacitracin acts by blocking the enzymatic inactivation of endogenously released antinociceptive peptides.

METHOD

The ability of bacitracin to produce analgesia was determined in male Swiss Webster mice, using the tail-flick procedure of D'Amour and Smith [8]. A high intensity light beam was focused on an area of the tail blackened with a felt-tipped pen and the tail-flick latency, i.e., the time required for the mouse to remove its tail from the radiant heat source, was measured with an IITC Inc. MOD 33 analgesia meter. Only mice which had pretest latencies in the range of 1.1 ± 0.2 sec were used in these experiments. Injections of bacitracin in saline, or saline alone (control), were given ICV in a volume of 10 μl according to the method of Ritzmann *et al.*, [27]. Tail flick latencies were determined 15 min (for dose-response studies) or at various times (for time course studies) after ICV injection. If animals failed to respond within 2 sec, the light beam was turned off. When repeated latency measurements were determined in a group of animals for time course studies, each successive measurement was made at a slightly different position on the tail (in both test and control animals) since a 2 sec exposure to the light beam caused peripheral damage resulting in subsequent artifactual analgesia. The number of animals showing a statistically significant ($p<0.05$) increase in latency compared to the mean pretest latency was determined. The results are expressed as percent mice showing analgesia and are corrected for analgesic responses in the ICV saline control group.

The effect of naloxone on bacitracin-induced analgesia was determined by comparing two groups of bacitracin-treated mice which were injected intraperitoneally (IP) with either 1 mg/kg naloxone in saline, or saline alone (control) in equal volumes. Statistical analysis was performed on the raw data using the Fisher exact probability test between the two groups. Differences were assumed significant at $p<0.05$.

RESULTS AND DISCUSSION

Figure 1 shows that ICV injection of bacitracin produced a dose-dependent analgesia in mice. The ED_{50} value was approximately 200 μg. Under identical conditions, morphine had an ED_{50} value of 200 ng, being 200 times more potent on a molar basis. The time course of analgesia following injection of 500 μg bacitracin is shown in Fig. 2 (A). After a peak effect at 15 min post-injection (100 % of the mice showing analgesia), the response decreased to about 50% after 75 min and disappeared completely by 3.5 hours. At no time period after injection did mice appear to be debilitated or sedated. No stress-induced analgesia was observed following ICV injection of saline in control mice. Intraperitoneal (IP) or intramuscular (IM) injection of bacitracin in doses as high as 500 mg/kg failed to produce analgesia.

Figure 2 (A) also shows that if mice were pretreated with naloxone (1 mg/kg, IP) 15 minutes prior to the ICV injection of bacitracin, the subsequent analgesic response to bacitracin was significantly reduced compared to saline-pretreated

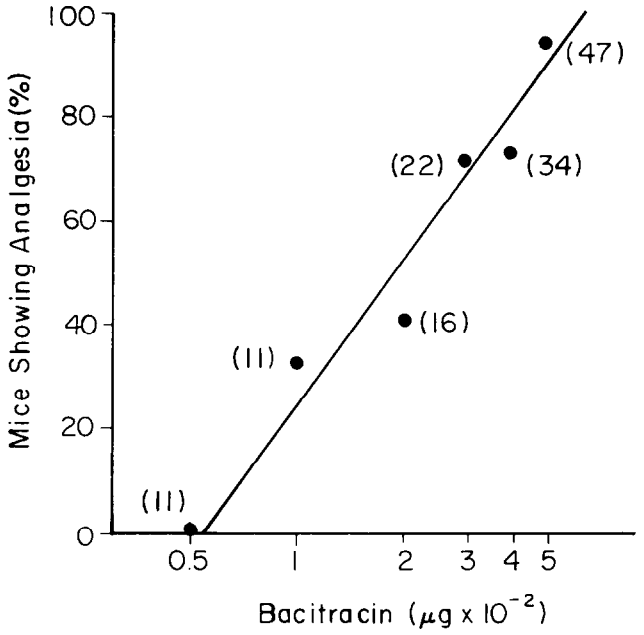

FIG. 1. The analgesic effect of bacitracin 15 min after intracerebroventricular (ICV) injection using the tail-flick test in mice. After determining pretest tail-flick latencies, mice were injected ICV with 10 μl of saline (control) or 10 μl of saline containing 50–500 μg bacitracin. Fifteen minutes after injection, tail-flick latencies were again determined and the number of animals showing a statistically significant ($p<0.05$) increase in latency compared to the mean pretest latency was determined. The results are expressed as percent mice showing analgesia. Numbers in parentheses indicate number of animals tested at each dose.

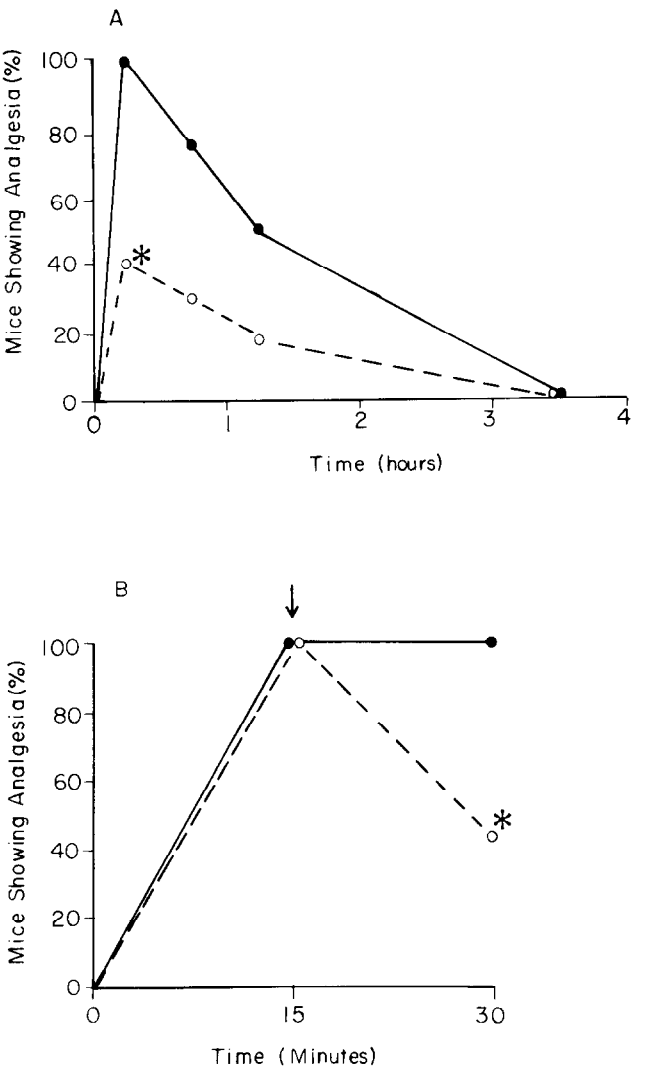

FIG. 2. Effect of naloxone on bacitracin-induced analgesia. (A) After determining pretest tail-flick latencies, mice (2 groups, n=12) were injected intraperitoneally with 1 mg/kg naloxone in saline (○) or saline (●). Fifteen minutes later, both groups were injected intracerebroventricularly (ICV) with 10 μl of saline containing 500 μg bacitracin. Tail-flick latencies were then determined at 15, 45, 75 and 210 minutes after bacitracin injection. The percent mice showing analgesia in each group was calculated at each time period, taking into account analgesic responses in respective control groups which received either naloxone or saline intraperitoneally, but saline without bacitracin ICV. (B) After determining pretest tail-flick latencies, mice (2 groups, n=7) were injected with 10 μl saline containing 500 μg bacitracin. Fifteen minutes later, the mice were tested for tail-flick latency and then immediately injected intraperitoneally (indicated by arrow) with either 1 mg/kg naloxone in saline (○) or saline (●) in equal volumes. The tail-flick latency was again determined 15 minutes after the intraperitoneal injection. Results are expressed as percent mice showing analgesia at each time period. *Significant at $p<0.05$.

mice. Similarly, Fig. 2 (B) demonstrates that when naloxone was injected IP (1 mg/kg) 15 min after ICV injection of bacitracin, the analgesic effect of bacitracin was significantly reversed, decreasing to about 40% of that observed in mice not treated with naloxone. Thus, naloxone reversed the effect of bacitracin as well as prevented the full development of the bacitracin-induced analgesia. Naloxone, by itself, had no effect on tail-flick latencies at the time periods studied.

Bacitracin has been observed to enhance binding of enkephalins to receptors *in vitro* [19, 22, 29], potentiate the enkephalin-induced increase in cyclic GMP levels in rat striatal slices [23], and enhance and prolong the analgesic activity of centrally administered β-endorphin in the rat [25]. These effects have been attributed to the ability of bacitracin to inhibit the enzymatic degradation of the opioid peptides. Indeed, bacitracin has been shown to inhibit two enkephalin-degrading peptidases in brain particulate fractions: a low affinity aminopeptidase which cleaves the Tyr^1-Gly^2 bond [17, 20, 30] and a high affinity dipeptidyl carboxypeptidase which cleaves the Gly^3-Phe^4 bond and is distributed in brain in parallel with opiate receptors [13, 20, 30]. In addition, bacitracin has been shown to inhibit brain aminopeptidase degradation of α-endorphin, γ-endorphin, and endopeptidase fragments of β-endorphin *in vitro* [1,25].

It is therefore suggested that the bacitracin-induced analgesia which we observed is due to *in vivo* inhibition of peptidases which normally inactivate endogenous opioid peptides. This peptidase inhibition could result in increased levels of brain antinociceptive peptide(s) which, in turn, could be responsible for the observed increase in pain threshold. Bacitracin appears to exert its effect centrally since IP or IM injections of high doses failed to produce

analgesia. This is consistent with the known inability of bacitracin to pass the blood-brain barrier [12].

Experiments with naloxone support the proposed participation of endogenous opioid peptides in bacitracin-induced analgesia. Naloxone, an opiate antagonist which has been shown to block the analgesic effect of ICV-injected enkephalins [2, 5, 6], and β-endorphin [3, 16, 24], significantly reduced the analgesic response of subsequently administered bacitracin and reversed the analgesia in animals already injected with bacitracin. These results suggest that the effect of bacitracin involves opiate receptors and hence is due to increased levels of endogenous opiates. While inhibition of opiate-degrading peptidases is the favored explanation for bacitracin-induced analgesia, a direct effect of bacitracin at the opiate receptor cannot be eliminated.

Other attempts to produce analgesia by inhibiting enkephalin-degrading enzymes have been described. D-Phenylalanine, a weak inhibitor of carboxypeptidase A, has been observed to produce long lasting analgesia in mice [7,10] (also D-leucine) and man [10] following systemic administration. The mechanism of action of these D-amino acids is unclear, however, since (1) carboxypeptidase cleavage appears to be unimportant in the inactivation of enkephalins and endorphins [4, 14, 15], and (2) D-phenylalanine fails to inhibit known enkephalinases [30].

Corroborating our results in mice, Patthy *et al.* [25] reported that 30 μg bacitracin injected ICV in the rat produced a slight but statistically significant analgesia. Pinsky *et al.* [26] showed that ICV infusion of a combination of the proteinase inhibitor aprotinin and bacitracin at doses which were ineffective alone (50 kallikrein inhibiting units and 5 μg, respectively) caused naloxone reversible epileptiform spiking, analgesia to tail pinch, stiff tail and flaccid immobility in the rat. We have also observed that ICV injection of 300–500 μg of bacitracin alone, a dose which produces analgesia but otherwise normal behavior in mice, causes catatonia, catalepsy and wet dog shakes in rats. Behaviors of this type have been reported following central administration of opioid peptides [3, 6, 11, 16, 24] and further support the contention that bacitracin is acting by potentiating the effect of endogenous opioid peptides. The observed species differences in the response to bacitracin could be due to different rates of tonic release of the opioid peptides in brain regions responsible for these behaviors.

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