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Microdialysis of excitatory amino acids in the periaqueductal gray of the rat after unilateral peripheral inflammation

W. M. Renno

Department of Anatomy, King Saud University, College of Medicine, Abha, Saudi Arabia, and Formerly at Department of Pathobiology, University of Minnesota, St. Paul, Minnesota, U.S.A.

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Summary. This study measured the release of glutamate (Glu) and aspartate (Asp) amino acid transmitters in the ventrocaudal compartment of the rat periaqueductal gray (PAG) following exposure to unilateral peripheral inflammation. The release of endogenous Glu and Asp from the rat ventrocaudal PAG was monitored with the microdialysis technique in unanesthetized, unrestrained rats. There was significant increase (1,300%) in the basal concentrations of Glu release in the 7 days Complete Freund's Adjuvant (CFA) treated group compared to 24 h mineral oil control group. Amino acid release was induced by infusing veratridine (75 μM, a sodium channel activator) directly through the 1 mm long dialysis probe. Perfusion of veratridine into the ventrocaudal PAG resulted in significant elevation of Glu and Asp amino acids. In the 24 h and 7 days CFA treated rats, veratridine-evoked release of Glu was significantly decreased in the lateral ventrocaudal PAG compared to control rats injected with mineral oil (CFA vehicle). The peak minus baseline concentrations of Glu in 24 h and 7 days CFA treated groups decreased 55.7% and 43.9%, respectively. In contrast, The basal and the peak minus baseline concentrations of Asp showed no significant change between control group and 24 h and 7 days CFA treated animals.The results provide direct evidence that Glu excitatory amino acid may be involved in nociception/nociception modulation pathway in the ventrocaudal PAG.

Keywords: Amino acids – Analgesia – Aspartate – Glutamate – Nociception – Pain

Introduction

The midbrain periaqueductal gray (PAG) is one of the major sites participating in pain modulation process via descending multisynaptic pathways that project to spinal cord (Basbaum and Field, 1984). The most sensitive portion

of this midbrain region to antinociception is the ventrocaudal PAG (Fang et al., 1989). Electrical stimulation or morphine microinjection into this portion of the PAG have been shown to produce analgesia accompanied by a strong inhibition of dorsal horn neurons which respond to noxious stimuli (Reynolds, 1969; Bennet and Mayer, 1979). The role played by the PAG in the above function is dependent on the interconnections of this midbrain area with the other regions of the central nervous system, particularly, the direct projection from the PAG to the spinal cord (Holstege and Kuypers, 1982), and the PAG-nucleus raphe magnus (NRM)-spinal cord projection pathway which is the major descending modulation pathway of nociception (Fields and Anderson, 1978). Inputs from laminae I and II and the lateral cervical nucleus of the spinal cord project directly to the lateral portion of the caudal PAG (Keay and Bandler, 1993).

Excitatory amino acid (EAA) glutamate (Glu) appears to play a significant role in the midbrain PAG. Microinjections of Glu into the rat PAG produced a potent analgesia and increased the threshold of a flexion reflex elicited by thermal stimuli to the hindpaw. It also caused excitation of neurons in the NRM (BehBehani and Fields, 1979). Furthermore, Jacquet (1988) has shown that EAA injection into the PAG produced a potent analgesia that is antagonized by prior injection of the N-methyl-D-aspartate (NMDA) antagonist suggesting that Glu may produce analgesia by activating directly the PAG projection neurons which influence the descending antinociception pathways. This analgesia is thought to be produced as a result of Glu acting on excitatory amino acid receptors.

Although many studies (Aimone and Gebhart, 1986; Wiklund et al., 1988; Beitz, 1990; Beitz and Williams, 1991) show that PAG neurons and axon terminals contain Glu and Asp, and suggest that EAA may be important neurotransmitters in the PAG-NMR pain modulation projection pathway, it is not clear whether or not these two amino acids exhibit differential release in the PAG in response to nociceptive stimuli. In the present study, an *in vivo* microdialysis procedure was utilized, which represents an important tool for monitoring extracellular levels of amino acids within specific regions of the brain in awake, freely moving animals (Renno et al., 1992). It allows analysis of extracellular levels of amino acids before, during and after stimulation of the PAG without the complicating effect of anesthetics (Benveniste and Huttemeier, 1990; DiChiara, 1990). Thus, using the microdialysis technique, this study was designed to determine the Glu and Asp basal concentrations and veratridine induced release concentrations in the ventrocaudal PAG in response to unilateral peripheral inflammation as a model for acute nociception in rats.

Materials and methods

Animals and treatment regiment

All animals used in this study were adult (225–350 g), male Sprague-Dawley rats. All surgical procedures were performed under ketamine/xylazine anesthesia (1 ml ketamine:

0.15 ml xylazine; 0.1 ml/100 g, i.m.; ketamine-HCl obtained from Fort Dodge Laboratories and xylazine obtained from Mobay Corp.). Animals were maintained at 22°C with 12 h light/dark cycles and were provided with rat chow and water ad libitum. Unilateral injection of Complete Freund's Adjuvant (CFA) into the hind paw causes a unilateral lesion that produces hypersensitivity to noxious pressure and heat and an increased paw volumes and hyperalgesia beginning several hours after injection. The hyperalgesia reportedly lasts for over one month (Stein et al., 1988). Furthermore, the nociceptive lesion is localized to a single peripheral site (Iadarola et al., 1988).

Three groups were employed in this study. In the first two groups, unilateral peripheral neuropathy was induced by subcutaneous injection of 120 μl of CFA (0.5 mg/ml heat killed *Mycobacterium butyricum* suspended in 85% oil/15% emulsified - Difco, Detroit, MI) 24 h and 7 days before dialysis. The third group of rats (control) received an equal volume of mineral oil (vehicle) 24 h before dialysis (Butler et al., 1992; Williams and Beitz, 1993). All rats were injected following the 6–14 days recovery period from chronic cannula implantation surgery. A fourth control group was injected with 120 μl of vehicle 7 days before dialysis. This group showed no significant difference when compared to 24 h control (mineral oil) rats (data not shown). The procedures on rats were approved by the University of Minnesota Medical Branch Animal Care and Use Committee and were in accord with the NIH guide for the *Care and Use of Laboratory Animals*.

Dialysis tube implantation and design

Rats were anesthetized with ketamine/xylazine and incised along the dorsal midline of the skull. A chronic microdialysis guide cannula (constructed from 20 gauge stainless tubing) was stereotaxically implanted into the PAG at a depth of 4.5 mm above the target site (A/P axis = 7.9 mm caudal to bregma; V/D axis = 6.8 mm from the skull surface; and M/L axis = 0.73 mm from midline according to the Rat Brain Atlas of Paxinos and Watson 1986). The guide cannula was fastened to the cranium with skull screws and dental cement, and the skin incision was closed with wound clips. Six to 14 days after implantation, a microdialysis probe was lowered into the PAG and secured into place. The microdialysis probe was constructed from a piece of 25 gauge tubing with a 1 mm hollowed opening located 0.5 mm from the distal end. The epoxied end (0.5 mm) of a piece of dialysis fiber (200 μm diameter; 5,000 mol. wt. cutoff; Spectrum Medical Industries) was inserted into the tubing and cemented into place. The opposite end of the microdialysis probe was attached to a fluid swivel via a piece of microline tubing. A piece of silica capillary tubing (145 μm o.d., Cole Parmer, Chicago, IL) was threaded into the dialysis fiber via a small slit cut in the microline tubing. The silica tubing was fastened to a piece of PE-10 tubing which served as the outflow route from the probe. The dialysis system was attached to a peristaltic pump (Rainin Rabbit-Plus) and 60 μl samples were collected via a Gilson microfraction collector. All microdialysis experiments were performed in awake freely moving animals (Renno et al., 1992).

Baseline collection and veratridine depolarization

Microdialysis experiments were begun after the microdialysis probe has been implanted for a period of 18–20 h. The microdialysis probe was perfused with a modified Ringer's solution (147 mM NaCl, 4.0 mM KCl and 2.2 mM $CaCl_2$; pH 7.4; osmolarity = 300 mOs) at a flow rate of 3–5 μL/min for 45–60 min to establish a steady state concentration difference between the perfusate and the sample medium (the extracellular fluid). Following this steady state period, samples were collected at 12 min intervals in polypropylene tubes and maintained at 5°C until analyzed for amino acids by high pressure liquid chromatography as described below.

Five to 8 samples per rat were used to determine the baseline concentration of each amino acids. Depolarization by veratridine (Sigma) was achieved by changing the

perfusion to a solution containing 75 μM veratridine in modified Ringer's solution for 24 min and then back to modified Ringer's solution for an additional 120 min. Following each experiment, the animals were killed and perfused with 4% paraformaldehyde in 0.1 M Sorensen's buffer. The brain was then removed for histological confirmation of probe placement. Only animals with probes located in the lateral ventrocaudal PAG were included in this study.

High pressure liquid chromatography and amino acid content analysis

Amino acid concentrations in the dialysate samples were determined using a precolumn o-phthaldialdehyde derivatization method (Lindroth and Mopper, 1974) which was modified as previously described (Beitz, 1990). Amino acid concentrations were measured using a Gilson dual pump high pressure liquid chromatography apparatus with Spectro Vision fluorometer. Twelve microliters of o-phthaldialdehyde were mixed with 49 μl of perfusate sample and allowed to react for 2 min. Forty-one μl of the mixture was then injected into the column. A nonlinear gradient of 5–60% methanol in sodium acetate buffer was used on a C18 reverse phase column (Phenomenex; 250 mm $\times$ 4.6 mm) to separate amino acids over 25 min period. Derivatives were detected fluorometrically with a Spectro Vision FD-100 fluorometer and quantified based on linear calibration of known amino acid standards. The baseline concentrations of amino acids are expressed as picomoles and the concentrations measured in each sample collected after treatment with veratridine are expressed as the mean peak minus basal concentration for each amino acid studied.

Technical considerations

In vivo experiments that use invasive techniques, such as microdialysis, require a cautious interpretation of the results. This is partially due to the inherent tissue destruction that occurs with the invasive procedure. In addition, the brain environment around the probe may have been altered (Ungrerstedt, 1984; Benveniste and Huttemeier, 1990). These factors need to be taken into account when interpreting data resulting from any microdialysis study including the present investigation. In this context it is important to note that our experiments were conducted seven to ten days after chronic cannula implantation surgery to insure complete recovery. Furthermore, the dialysis procedure was performed approximately eighteen hours after the probe was inserted through the guide cannula into the PAG. This time frame was used to allow the environment around the probe to normalize following probe insertion. Many microdialysis studies (Ungrerstedt, 1984; Benveniste and Huttemeier, 1990; Di Chiara, 1990) indicate that the integrity of the blood brain barrier in the region around the probe is restored within 30 min to 2 hr following probe insertion, our timeframe (18 hr) should allow more than adequate time for the tissue to adjust to the presence of the probe. Finally, it should be pointed out that differences observed in the present study were between 24 h and 7 days CFA treated rats and mineral oil-treated controls. All groups had chronic cannulas implanted 7–10 days prior to experimentation and all groups had 1 mm dialysis probes implanted approximately 18 h before dialysis. Thus because all groups received identical treatment except for CFA injections it is unlikey that the damage due to dialysis implantation or probe size is responsible for the differences observed in Glu release.

Data analysis

Only the rats in which the probes were placed correctly in the lateral ventrocaudal PAG were used for data analysis. For each rat the first 6 baseline samples were averaged for each amino acid investigated, and compared to the average of the highest two fraction

peaks observed following veratridine treatment. The mean baseline value was subtracted from the mean peak value to obtain peak-minus-baseline (P-B) values for each amino acid for each rat. This method of calculating the P-B values was used to normalize individual baseline variations from rat to rat (Renno et al., 1992; Skilling et al., 1992). The data obtained from the veratridine-induced release above baseline was analyzed using paired student's t-test. Statistical analysis among experimental and control groups [24 h CFA (n = 9), 7-days CFA (n = 7) and 24 h (control) mineral oil (n = 7)] was accomplished using ANOVA. P-values were determined using the Scheffe F-test.

Results

The basal Glu concentration showed a slight non-significant tendency to increase (using Scheffe F-test) in rats treated with CFA injections 24 h prior to dialysis (Fig. 1A). This increase was approximately 300 ± 115% above the basal concentrations of the control group treated with mineral oil 24 h before dialysis experiments. The basal Glu concentration of the 7 days CFA treated group showed a significant increase ($P < 0.02$). This increase was 1,300 ± 680% above the basal Glu concentration of control group (Fig. 1A).

The concentration of Glu and Asp increased significantly following 75 μM veratridine perfusion into the lateral ventrocaudal PAG in control rats. The peak concentrations of Glu and Asp increased approximately 8,000% and 1,000% above the baseline, respectively. However, after veratridine depolarization into the lateral ventrocaudal PAG of CFA treated animals the concentration of Glu in the dialysate was found to decrease significantly ($P < 0.006$) compared to control rats (Fig. 1B). The peak minus baseline (P-B) concentration of Glu decreased from 44.16 ± 5.69 (S.E.M.) picomoles in control (24 h mineral oil) group to 19.56 ± 2.03 picomoles in 24 h CFA treated group. The P-B concentration of Glu in this group decreased approximately 55.7% when compared to control animals. Similarly, the 7-days CFA treated group showed a significant decrease in P-B of Glu release following veratridine stimulation (Fig. 1B). The P-B of Glu concentration decreased to 24.77 ± 3.50 picomoles in 7 days CFA treated group. This decrease was nearly 43.9% compared to control group.

Figure 2 shows the mean basal Asp concentration and the mean peak Asp concentration in dialysate samples obtained from lateral ventrocaudal PAG. The basal Asp concentration showed no significant change between control group and 24 h and 7 days CFA treated groups (Fig. 2A). Likewise, the P-B of Asp concentration showed no significant change between the 24 h mineral oil control group and the two experimental groups treated with CFA 24 h and 7 days before the dialysis experiments (Fig. 2B).

Discussion

The present study was designed to test the hypothesis that Glu and/or Asp are neurotransmitters associated with the PAG during peripheral nociception in

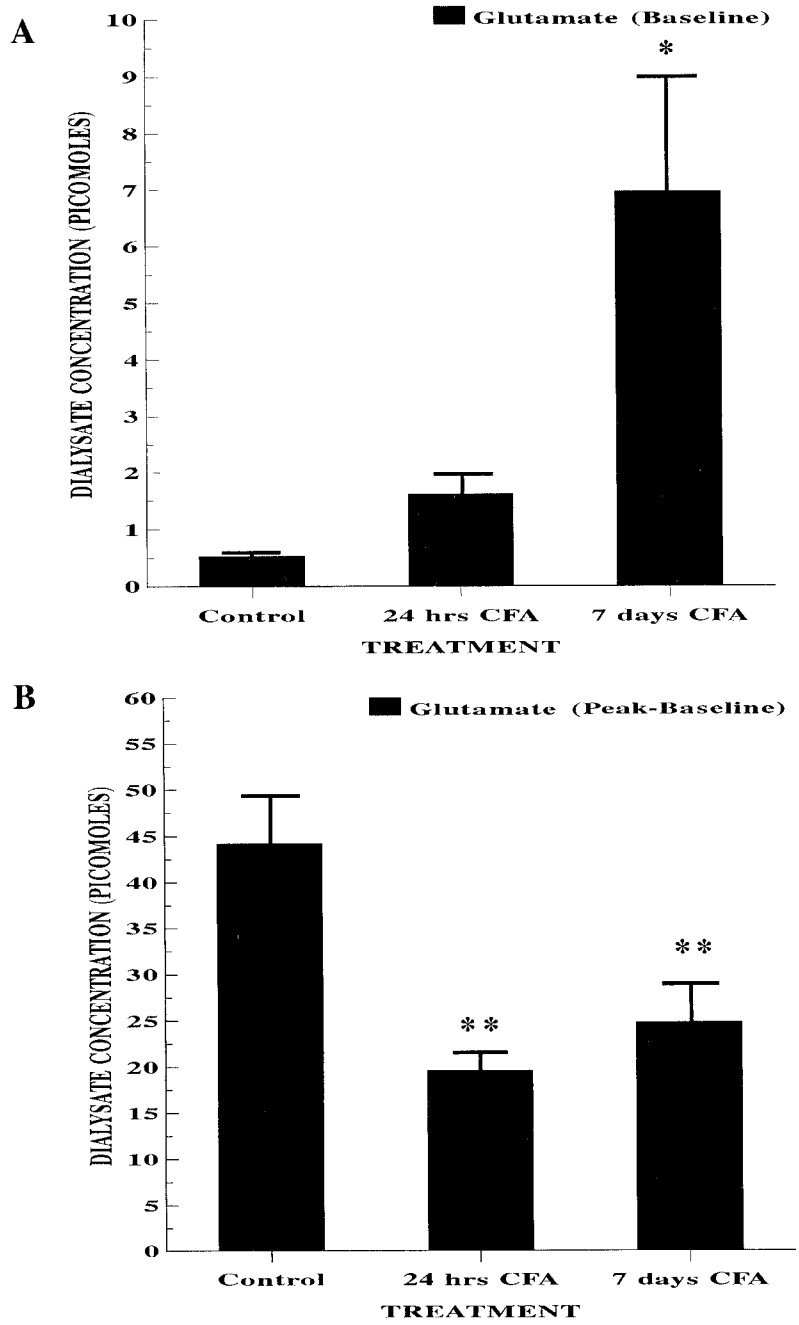

Fig. 1. Effect of unilateral peripheral inflammation on the baseline concentration and on the veratridine-induced release of glutamate in the lateral ventrocaudal PAG. **A** Basal concentrations were determined as mean ± S.E.M. values from 6 consecutive fractions per rat. The 7 days CFA injected group showed a significant increase in Glu base line when compared to control group. The asterisk indicates $P < 0.02$ by ANOVA and Schéffe F-test between different groups. **B** Effect of unilateral peripheral inflammation after 24 h and 7 days of CFA administration on the veratridine-induced release of Glu concentration in the lateral ventrocaudal PAG in vivo. The rats were perfused with 75μM veratridine for 24 min. The results represent the average of 2 fractions containing the highest concentrations of Glu minus baseline within 36–48 min following veratridine depolarization. Note the selective effect of CFA treatment on Glu release from the lateral ventrocaudal PAG as compared to mineral oil control groups. The double asterisk indicates $P < 0.006$ by ANOVA and Schéffe F-test between different groups

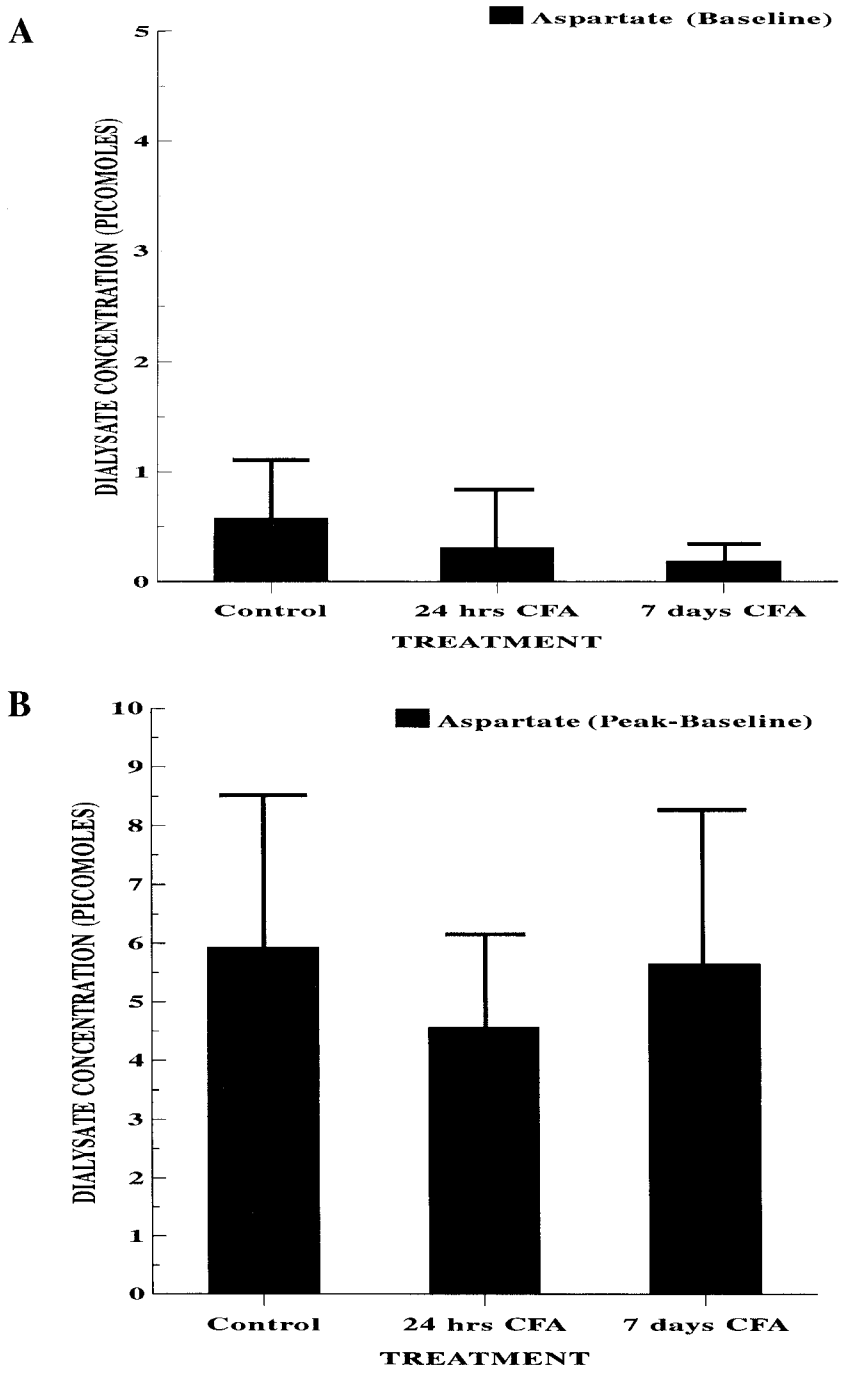

Fig. 2. Effect of unilateral peripheral nociception on the baseline concentration and on the veratridine-induced release of aspartate in lateral ventrocaudal PAG. **A** Basal concentrations were determined as mean ± S.E.M. No significant differences were observed between the control and CFA injected groups (P = 0.49). **B** Effect of peripheral nociception after 24 h and 7 days of CFA administration on veratridine-induced release of Asp concentration in the lateral ventrocaudal PAG in vivo. There were no significant differences among the groups tested (P = 0.27)

the rat. The results indicate that nociceptive stimuli in the rat hind paw causes a significant increase in Glu basal concentration release in the PAG which is in agreement with available biochemical and physiological evidence that favors Glu rather than Asp (Evans, 1989). Glutamate appears to play an important functional role in the midbrain PAG. Behbehani and Fields (1979) originally demonstrated that microinjections of Glu into the rodent PAG produced a potent analgesia by activating PAG neurons involved in the descending pain modulation system. A few years later, Jacket (1988) has shown that injection into the PAG of EAAs produces a strong analgesia that is antagonized by prior injection of the NMDA antagonist, 2-amino-7-phosphonoheptanoate. Moreover, receptor binding studies have demonstrated high levels of Glu receptors in this midbrain region (Albin et al., 1990; Cotman et al., 1987). Thus, these studies suggest that Glu acts on EAA receptors within the PAG to activate components of the endogenous analgesic system. Indeed, immunocytochemical investigations have shown PAG to exhibit one of the highest densities of Glu immunoreactive neuropil staining among all midbrain structures (Ottersen and Storm-Mathisen, 1984). In addition, many fibers and terminals in the PAG have been shown to contain Glu-like immunoreactivity (Clemente et al., 1987). Together, these studies implicate EAAs in general and Glu in particular in nociception/nociception modulation system.

The results in this study are in agreement with the proposed hypothesis for the glutamatergic analgesic action in the PAG suggested by Jacquet (1988). According to the hypothesis, Glu activates a group of interneurons in the PAG. These neurons are apparently the first of a three-stepped descending

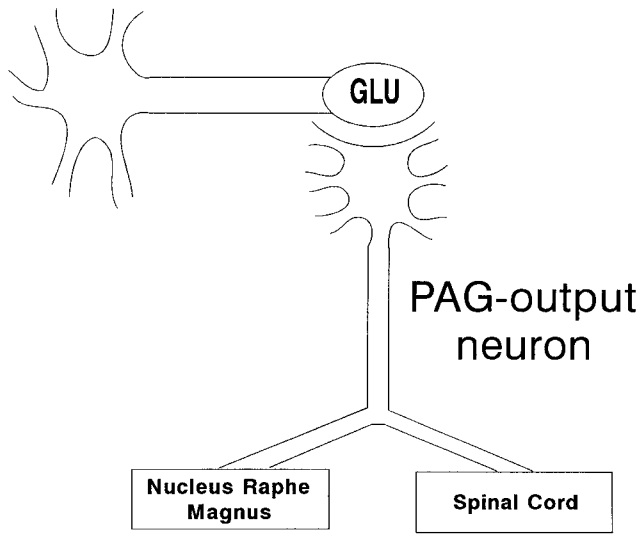

Fig. 3. A diagram of hypothesized glutamate role within the PAG

inhibition of ascending nociceptive information on the level of the spinal cord (Basbaum and Fields, 1984). The present experiments showing a significant increase in basal release of Glu in the ventrocaudal PAG following peripheral nociceptive inflammation clearly support the above hypothesis proposed by Jacquet. Furthermore, the decrease in veratridine-induced release in CFA treated groups points out to the continuous increase in basal release of Glu which in turn makes less Glu available in the PAG output neurons upon further depolarization with veratridine. In other words, the peripheral nociception stimuli causes net increase in basal Glu release from ventrocaudal PAG which excite the output PAG neurons. This in turn depletes the Glu concentration available for depolarization. Because of Glu direct excitation of cell bodies (Goodchild et al., 1982), it is possible that an EAA-receptor-linked system in the PAG serves to turn on or maintain descending pain inhibition. In this regard, EAA receptor antagonists when microinjected into the rostroventral medulla block the tail flick inhibition produced by Glu at the same site and electrical stimulation in the PAG (Aimone and Gebhart, 1986; Jacquet, 1988; VanPraag and Frenk, 1990). In addition, Gold and his colleagues (1990) have shown that microinjections of kainate (a glutamate receptor agonist) into the PAG increases tail flick latency in a dose dependent fashion. These findings suggest that neuronal activity in certain PAG neurons that control descending antinociception system is under the influence of Glu (Jacquet, 1988). Together with the present data, it seems that peripheral nociceptive stimuli turn on the endogenous descending analgesia system by releasing EAA Glu.

Since the PAG area has extensive EAA efferent projections to numerous sites in the forebrain (Mantyh, 1983; Beitz, 1989; Rezvi et al., 1991) which have been implicated in nociception modulation when they are stimulated (Hardy, 1985), it is also possible that Glu release in the PAG area is playing a role in the ascending pathways during peripheral inflammation. The fact that PAG neurons receive direct spinal cord connection from Lamina I and II (Keay and Bandler, 1993; Holstage and Kuypers, 1982) implies that the ascending pathways could be influenced by the PAG neurons. Glutamate release could influence the nociceptive information on their way to the higher centers. Although many studies have concentrated on the role of Glu on the descending pathways specially the PAG-NMR of the ventromedial medulla (Wiklund et al., 1988; Beitz and Williams, 1991; Fields et al., 1991; Jensen and Yaksh, 1992), this, however, does not eliminate the possible role of Glu in the ascending pathways. Indeed, the changes in Glu release during peripheral nociceptive stimuli could affect both ways. Further investigations are needed to reveal the specific role of Glu in the ascending analgesic pathway during peripheral inflammation.

In the present study, Asp showed a significant release upon veratridine stimulation but it did not show any change in the basal or veratridine-stimulated release concentrations among the control and experimental (CFA treated) groups suggesting that at least in the unilateral peripheral nociception Asp role in the ventrocaudal PAG may be limited. In fact, the PAG neurons also exhibit immunostaining for the enzyme aspartate

aminotransferase (an enzyme which interconverts Glu and Asp) (Clements, 1987). Therefore, the Asp release after KCI (Beitz, 1990) or veratridine stimulation (Renno et al., 1992) may participate in other functions that PAG is known to have other than the activation of descending analgesic pathway. On the other hand, it is very possible that Asp plays a metabolic rather than a neurotransmitting role in the ventrocaudal PAG. The other possibility is that Asp participates in the nociceptive process but the levels of this amino acid just do not change during peripheral inflammation.

Recent microdialysis data indicate that veratridine stimulation (Renno et al., 1992) or KCl depolarization (Beitz, 1990) of the PAG results in significant Glu and Asp release which indicate that both amino acids are released in the PAG following depolarization of this region but the mean baseline concentration of Asp in PAG dialysates is approximately one seventh (Renno et al., 1992) and one tenth (Beitz, 1990) the mean baseline concentration of Glu. In this study, Glu release was approximately eight times more than Asp upon depolarization by veratridine in control group. Thus, previous studies (Beitz, 1990; Renno et al., 1992) in addition to this investigation support the hypothesis that Glu may be more important of the two putative EAA transmitters in the PAG. In vitro receptor binding studies have also shown high levels of quisqualate-metabotropic receptor subtype in the PAG (Albin et al., 1990), while NMDA receptors were least dense. Based on these data one would predict that quisqualate (a glutamate receptor agonist) should have a greater effect on neurons in this region than NMDA. Electrophysiological data (Jensen and Yaksh, 1992) indicate that both quisqualate and glutamate are highly effective in activating PAG neurons, which is consistent with the pharmacological profile of a high density of quisqualate sensitive receptors in the PAG. Furthermore, the lower levels of aspartate-evoked release in this region (Renno et al., 1992; Beitz, 1990) parallels the low density of NMDA receptors (Albin et al., 1990). Since Asp appears to be a selective NMDA receptor agonist (Patneau and Mayer, 1990), it might be reasonable, based on the binding data, to assume that this amino acid plays a less important role in the PAG function than Glu. Further investigations are needed to determine the role of Asp in the ventrocaudal PAG and to elucidate its precise function during nociception process.

In conclusion, this study provides the first in vivo biochemical evidence indicating that putative unilateral peripheral nociception causes a significant increase in Glu basal concentration release in the midbrain PAG. This supports the hypothesis that glutamatergic neurons in the PAG are activated during nociception. Thus, endogenous glutamate may directly turn on the descending analgesic system that project either directly to the spinal cord or indirectly through the nucleus raphe magnus. It is also likely to play a role in ascending pathways to the forebrain. Further studies, however, are needed to establish the origin of this amino acid transmitter release and to dissect out the exact Asp amino acid role in the PAG.

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Author's address: Dr. Waleed M. Renno, Department of Anatomy, King Saud University, Abha Branch, P. O. Box 641, Abha, Saudi Arabia,
Fax #: 966 (7) 226-4918 or 966 (7) 224-7570; E-mail: A03A002@KSU.EDU.SA

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