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Interaction of hypothalamic $GABA_A$ and excitatory amino acid receptors controlling heart rate in rats

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ABSTRACT

We have previously shown that microinjection of drugs that impair γ-aminobutyric acid (GABA)-mediated synaptic inhibition into the dorsomedial hypothalamus (DMH) of rats generates cardiovascular and behavioral changes that mimic the response to stress. The purpose of this study was to examine the role of excitatory amino acid (EAA) receptors in the DMH in generating the cardiovascular changes caused by withdrawal of local GABAergic inhibition in urethan-anesthetized rats. Local treatment of the DMH with the nonselective EAA antagonist kynurenic acid blocked or reversed the increases in heart rate and blood pressure caused by microinjection of the $GABA_A$ antagonists bicuculline methiodide (BMI) or picrotoxin into the same region. Conversely, similar injection of xanthurenic acid, a structural analogue of kynurenic acid without significant effects on EAA receptors, did not significantly alter the cardiovascular changes produced by either $GABA_A$ antagonist. The tachycardic effects of BMI were also attenuated by injection of either the *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-Sphosphonopentanoic acid or the non-NMDA EAA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione. When the two EAA receptor antagonists were combined, their effects to suppress the BMI-induced tachycardia were additive. These findings suggest that the cardiovascular effects caused by blockade of GABAergic inhibition in the DMH of the rat are dependent on activation of local NMDA and non-NMDA EAA receptors.

Previous studies in our laboratory have provided evidence that the inhibitory neurotransmitter "(γ-amino-butyric acid (GABA) plays an important role in the dorsomedial hypothalamus (DMH) in regulating the cardiovascular and behavioral responses to stress. Microinjection of drugs that impair GABA-mediated neurotransmission into the DMH produces sympathetically mediated increases in heart rate and blood pressure in anesthetized (7) and conscious (24) rats. Injection of GABA antagonists at this site also produces behavioral changes in rats that include increases in locomotor activity (16), selective enhancement of shock avoidance (17), and experimental "anxiety" in a conflict paradigm (18). Therefore, blockade of GABAergic inhibition in the DMH causes cardiovascular and behavioral changes that mimic the response to stress in rats. Conversely, stimulation of $GABA_A$ receptors in the DMH produces anti-conflict or "anxiolytic" effects (18) and prevents the tachycardia and hypertension elicited by an air stress paradigm (1, 14). Taken together, these findings suggest that neurons whose activation is responsible for generating the cardiovascular and behavioral response to stress are found in the DMH and that the activity of these neurons is regulated, in part, by $GABA_A$ receptors.

As yet, the excitatory input to neurons in the DMH that generate these cardiovascular and behavioral changes has not been defined. Excitatory amino acid (EAA) receptors mediate the majority of fast, excitatory, synaptic transmission in the mammalian central nervous system (9). Stimulation of EAA receptors in the DMH causes increases in heart rate and blood pressure similar to those produced by $GABA_A$ antagonists injected at this site in urethan-anesthetized rats (19). Furthermore, recent studies have provided evidence that GABA and EAA receptors interact to modulate neuronal activity in several areas of the central nervous system (4, 8, 13, 20), including the hypothalamus (22). If a similar relationship between GABA and EAA receptors exists in the DMH, then the changes produced by withdrawal of local GABAergic inhibition may depend on activation of local EAA receptors. To test this hypothesis, the postsynaptic $GABA_A$ antagonists bicuculline methiodide (BMI) and picrotoxin were microinjected alone and in the presence of the nonselective EAA antagonist kynurenic acid into the DMH of urethan-anesthetized rats while heart rate and blood pressure were monitored. In addition, the role of specific subtypes of EAA receptors in generating the cardiovascular response to BMI was examined using the *N*-methyl-D-aspartate

(NMDA) receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) and the non-NMDA EAA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX).

METHODS

Male Sprague-Dawley rats (250-350 g, Taconic Farms, Germantown, NY, and Harlan Industries, Indianapolis, IN) were anesthetized with urethan (1.35 g/kg ip). The left femoral artery and vein were cannulated with PE-50 tubing filled with heparinized saline. The arterial line was connected to a pressure transducer for the direct measurement of arterial pressure with the pulse pressure signal triggering a cardiotachometer for the measurement of heart rate. Arterial pressure and heart rate were continuously recorded on a Beckman 511A strip-chart recorder while rectal temperature was monitored and maintained at 36-37˚C.

Microinjections were made with glass micropipettes as previously described (19). Briefly, glass capillary tubing was pulled, broken back, and beveled to a smooth tip with an outer diameter of 40-60 μm. The micropipette was attached to a length of PE-10 tubing, and the system was filled with mineral oil and water. The tubing was connected to a 10-μ1 Hamilton syringe mounted in a manual micrometer drive. The drug solution was drawn up into the tip of the pipette and subsequently delivered by slowly advancing the plunger of the syringe with the manual micrometer drive. All injections were unilateral (right side) and consisted of a total volume of 50 nl infused over 15-20 s. The micropipette remained in place for 1 min after ending the infusion.

Hypothalamic sites where microinjection of $GABA_A$ antagonists or EAA agonists elicited short-latency tachycardia were determined as previously (19). The rat was placed in a stereotaxic instrument (David Kopf) with the upper incisor bar adjusted 5.0 mm above the interaural plane. With bregma serving as the reference point, the micropipette was lowered to the target coordinates at a 10˚ angle with respect to the sagittal plane to avoid damaging the midsagittal sinus. The stereotaxic coordinates for the target were 1.2 mm posterior to and 8.7 mm below bregma and 0.5

mm from the midline. Proper placement of the pipette was verified by injecting either 20 pmol BMI or 6.8 pmol NMDA. Only those sites at which BMI produced an increase in heart rate of ≥90 beats/min or NMDA produced an increase in heart rate of ≥50 beats/min with onsets of 30 s or less from the beginning of the infusion were examined further. If the change in heart rate failed to meet these criteria, the anterior-posterior, right-left, or height-depth coordinate was altered by 0.3 mm and the new placement tested until an active site was located. Once an active site was located, the experiment proceeded with typically three or four subsequent injections spaced in time such that any effects on heart rate caused by the previous injection had dissipated before the next injection. In some experiments two or three drugs were combined in solution and coinjected. The order of treatments within a series of experiments was staggered.

At the end of each experiment, the injection site was marked by injecting 50-100 nl of a suspension of india ink diluted 1:1 with saline. The pipette remained in place for 10 min after injection of the marker at which time the pipette was slowly removed. The animal was killed and perfused with 50 ml of saline followed by 300-500 ml of fixative (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) over 20-40 min. The brain was removed and stored in the fixative for 1-7 days. Coronal sections (40 μm) were cut on a freezing microtome, mounted on gelatin-coated slides, and stained with a 1% neutral red solution. The locations of the injection sites were determined according to the atlas of Paxinos and Watson (15).

In those experiments in which BMI was injected at various sites in the hypothalamus, a single site was marked in each animal, and the location of the remaining sites (numbering from 2 to 4) was determined based on their coordinates relative to the marked site. The angle of the pipette and the pitch of the rat's head were taken into account when plotting these sites.

Drugs used in these experiments included urethan, BMI, picrotoxin, NMDA, kynurenic acid, xanthurenic acid, *dl*-2-amino-5-phosphonopentanoic acid (all purchased from Sigma Chemical, St. Louis, MO) and 6-cyano-7-nitroquinoxaline-2,3-dione (Tocris Neuramin, UK). All drugs were dissolved in a saline solution with the final pH adjusted to 6-7.5.

Results are expressed as means ± SE. The data were analyzed by linear regression, paired *t* tests, and analysis of variance (ANOVA) with Newman-Keuls. The 5% limits of probability were accepted as significant.

RESULTS

Coinjection of the nonselective EAA antagonist kynurenic acid (0.5-5.0 nmol) attenuated, in a dose-related manner ($P < 0.05$ by linear regression), the increase in heart rate produced by 20 pmol BMI (Fig. 1). Kynurenic acid also blocked the increase in blood pressure caused by BMI, however, not in a dose-dependent manner at the doses of kynurenic acid tested. Coinjection of 20 pmol BMI and 5 nmol xanthurenic acid, a compound chemically similar to kynurenic acid with no activity at EAA receptors (10), produced increases in heart rate (+102 ± 17 beats/min) and blood pressure (+10 ± 2 mmHg) similar to those produced by 20 pmol BMI alone (+100 ± 13 beats/min and 14 ± 2 mmHg, $P > 0.05$ by paired *t* tests, $n = 3$). Injection of 5 nmol kynurenic acid alone at sites reactive to BMI produced no significant changes in baseline heart rate (-3 ± 4 beats/min) or blood pressure (+1 ± 2 mmHg, $n = 3$). In a series of control experiments, 20 pmol BMI caused reproducible elevations in heart rate and blood pressure over the course of five successive injections ($P > 0.05$, repeated ANOVA, $n = 4$).

Microinjection of 25 pmol picrotoxin, a noncompetitive postsynaptic $GABA_A$ antagonist, produced cardiovascular changes similar to those caused by BMI, i.e., marked increases in heart rate accompanied by modest elevations in blood pressure. Microinjection of 5 nmol kynurenic acid at the same site 5 min after injection of picrotoxin reversed the picrotoxin-induced tachycardia and hypertension (Figs. 2 and 3). Injection of 5 nmol xanthurenic acid did not significantly alter the pattern or the time course of cardiovascular changes produced by picrotoxin.

The ability of kynurenic acid to block or reverse the cardiovascular effects of the two $GABA_A$ antagonists BMI and picrotoxin suggests that local EAA receptors play a role in this response. To examine the role of specific subtypes of EAA receptors in these changes, the NMDA receptor antagonist AP5 and the non-NMDA EAA receptor antagonist CNQX were used. We have previously demonstrated (19) that microinjection of the EAA agonists NMDA or kainic acid into the DMH of urethan-anesthetized rats produces increases in heart rate and blood pressure similar to those caused by $GABA_A$ antagonists injected at the same site. We have also shown that coinjection of 50 pmol AP5 selectively blocks the effects caused by NMDA (Fig. 4), whereas 25 pmol CNQX selectively block the effects caused by kainic acid (Fig. 5). Therefore, this previous work has defined doses of AP5 and CNQX that are selective for their respective EAA receptor subtype.

At these selective doses, either AP5 or CNQX attenuated the cardiovascular response to BMI. The tracings shown in Fig. 6 are taken from a single experiment in which 5 pmol BMI produced a marked increase in heart rate that was reduced in the presence of either AP5 or CNQX and virtually eliminated when coinjected with both EAA antagonists in combination. Figure 7 summarizes the data from three groups of animals ($n = 5$ each group) in which three doses of BMI were injected alone or together with AP5 and/or CNQX. In the range of 2-20 pmol BMI produced dose-related increases in heart rate. In the presence of either 50 pmol AP5 or 25 pmol CNQX, the tachycardic response to each dose of BMI was reduced significantly ($P < 0.05$, repeated ANOVA with Newman-Keuls). In the presence of both AP5 and CNQX, the change in heart rate produced by each dose of BMI was further reduced compared with the same dose of BMI in the presence of either antagonist alone, suggesting that the effects of the two EAA antagonists were additive. The 5- and 20-pmol doses of BMI also produced significant elevations in blood pressure (+9 ± 1 and +11 ± 1 mmHg, respectively) that were attenuated by coinjection of the combination of AP5 and CNQX. Coinjection of either AP5 or CNQX significantly attenuated the increase in blood pressure caused by 5 pmol BMI but not that caused by the 20-pmol dose.

In all of the experiments described above, subsequent histology indicated that the sites of injection were in or immediately adjacent to the DMH according to the atlas of Paxinos and Watson

(15). To verify that the tachycardic effects of BMI were mediated by neurons in the DMH, 5 pmol BMI were injected at sites within and outside this area. When the injection site was within or <0.5 mm away from the DMH, BMI produced increases in heart rate of ≥50 beats/min (Fig. 8). At sites 0.5-1.0 mm lateral, dorsal, ventral, or posterior to the DMH, increases in heart rate ranged from 25 to 49 beats/min. At sites >1.0 mm away, the tachycardic response was, in most cases (6 of 9 injections), ≤10 beats/min.

DISCUSSION

Data presented in this study provide evidence that the cardiovascular effects resulting from blockade of GABAergic inhibition in the DMH of the rat are dependent on activation of local EAA receptors. Microinjection of either of the $GABA_A$ antagonists BMI or picrotoxin produced marked increases in heart rate accompanied by modest elevations in blood pressure. These cardiovascular changes could be either blocked or reversed by injection of the nonselective EAA antagonist kynurenic acid at the same site in the DMH. When coinjected with BMI, kynurenic acid attenuated the BMI-induced tachycardia and hypertension. Although its effects on heart rate were dose-dependent, kynurenic acid did not produce doserelated effects on blood pressure at the doses tested. One possible explanation is that the changes in blood pressure are more sensitive to EAA receptor blockade than the changes in heart rate, and therefore lower doses of kynurenic acid may be needed to demonstrate dose-related effects on blood pressure. However, it is also possible that the modest and variable changes in blood pressure produced by BMI prevented a clear demonstration of dose dependency. Kynurenic acid injected 5 min after injection of picrotoxin reversed the increases in heart rate and blood pressure caused by this agent. Conversely, xanthurenic acid, a structural analogue of kynurenic acid without significant effects on EAA receptors (10), produced no significant differences in the maximal changes in heart rate or blood pressure caused by either $GABA_A$ antagonist. The reproducible effects on heart rate and blood pressure caused by successive injections of BMI, the staggered order of the treatments within a series of experiments, and the negative controls using xanthurenic acid indicate that the reduced responses seen in the presence of kynurenic acid can be attributed to EAA receptor blockade and not to loss of responsiveness of the preparation or to nonspecific actions of kynurenic acid.

Therefore, these experiments suggest a role for activation of EAA receptors in the DMH in generating the cardiovascular effects caused by local injection of $GABA_A$ antagonists.

To determine the role of specific subtypes of EAA receptors in this response, AP5 and CNQX were used. AP5 has been identified as a competitive antagonist for the NMDA receptor (6, 23). Although CNQX has been shown to be a competitive antagonist at non-NMDA EAA receptors (3, 12, 23), doses of CNQX that are selective for this effect must be established because CNQX can also antagonize NMDA receptor-mediated responses by blocking the strychnine-insensitive glycine site associated with this receptor complex (2, 23). In a previous study involving injections of EAA agonists into the DMH of urethan-anesthetized rats, we demonstrated that coinjection of AP5 50 pmol/50 nl blocks the increases in heart rate and blood pressure caused by NMDA but not kainic acid and that, conversely, CNQX 25 pmol/50 nl blocks a similar cardiovascular response caused by kainic acid without affecting NMDA-induced tachycardia (19). Thus selectivity was previously demonstrated for these same doses (and concentrations) of AP5 and CNQX that reduced the tachycardic response caused by intrahypothalamic injection of BMI. When either AP5 or CNQX was coinjected with BMI, the tachycardiac response was reduced by a similar amount. When the two EAA antagonists were combined and coinjected with BMI, the effect on heart rate was additive. Therefore, this series of experiments suggests that there are two distinct components of the tachycardic response to injection of $GABA_A$ antagonists. One component appears to rely on activity at NMDA receptors, whereas the other is mediated through non-NMDA EAA receptors. The effects of AP5 and CNQX on BMI-induced changes in blood pressure, however, were less clear. Either AP5 or CNQX alone attenuated the hypertension caused by 5 pmol BMI, but their effects, when combined, were not additive. Conversely, only the combination of AP5 and CNQX produced a significant reduction in the blood pressure response to 20 pmol BMI. The inability of AP5 and CNQX to produce consistent reductions or additive effects on the increases in blood pressure caused by the $GABA_A$ antagonists may be, again, a consequence of the modest and variable nature of these changes. Nonetheless, the changes in blood pressure are sensitive to EAA receptor blockade as indicated by experiments in which kynurenic acid was used.

The area responsive to the $GABA_A$ antagonists appears to be localized to the DMH. All sites reactive to BMI and picrotoxin were found to be within, or immediately adjacent to, an area defined as the DMH according to the atlas of Paxinos and Watson (15). Injection of BMI 0.5-1.0 mm away from the DMH resulted in lesser maximal increases in heart rate and longer latencies to onset. At sites >1 mm away, BMI produced an increase in heart rate of <25 beats/min or, in most cases, no significant change in heart rate. The distribution of hypothalamic sites reactive to BMI was similar to that seen with the EAA agonist NMDA in a previous study (19). The area of greatest reactivity to NMDA was centered in the DMH with unreactive sites lying 0.5-1.0 mm away from the nucleus. Therefore, neurons in the DMH can be activated to generate cardiovascular changes by either stimulating EAA receptors or blocking $GABA_A$ receptors.

The notion that neuronal excitability can be modulated by a balance between synaptic inhibition mediated by GABA receptors and synaptic excitation mediated by EAA receptors has been investigated in other areas of the central nervous system. Studies using hippocampal (4, 5, 8, 11, 20, 21) and cortical (13) slice preparations antagonist have demonstrated that, in the presence of a GABA antagonist (e.g., picrotoxin, BMI, penicillin), epileptiform burst discharge occurs in postsynaptic neurons in response to low-frequency stimulation of an afferent pathway. This bursting could be blocked with EAA antagonists, suggesting that endogenous EAAs are involved in mediating these effects. In a recent study using hypothalamic slices, van den Pol and colleagues (22) concluded that GABAergic and glutamatergic (EAA) neurons account for the majority of all presynaptic axons in the hypothalamus and may play a primary role in regulating neuronal output from this area. In the present study we have provided pharmacological evidence of a role for endogenous GABA and EAAs in regulating the activity of a single population of hypothalamic neurons capable of generating significant changes in cardiovascular function.

In summary, this study provides evidence that the cardiovascular response caused by removal of GABAergic inhibition in the DMH of the rat is dependent on activation of local NMDA and non-NMDA EAA receptors. Therefore, the activity of this hypothalamic mechanism may be regulated by a balance of GABA receptor-mediated synaptic inhibition and EAA receptor-mediated synaptic excitation.

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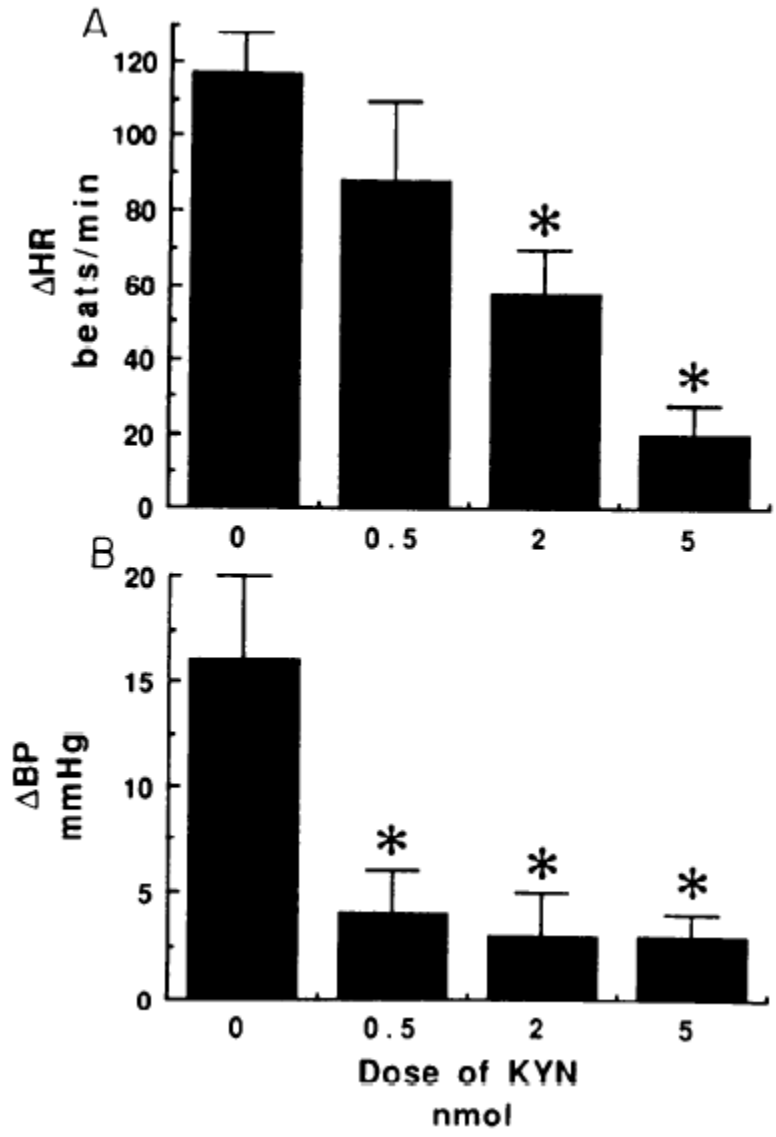

Fig. 1. Maximal changes in heart rate (HR, A) and blood pressure (BP, B) after injection of 20 pmol bicuculline methiodide (BMI) alone or conjection with kynurenic acid (KYN). Baseline HR (350 ± 9 beats/ min) and BP (96 ± 2 mmHg) were not different between injections.

* Significant differences from effects of BMI alone ($P < 0.05$ by repeated ANOVA and Newman-Keuls, $n = 3$).

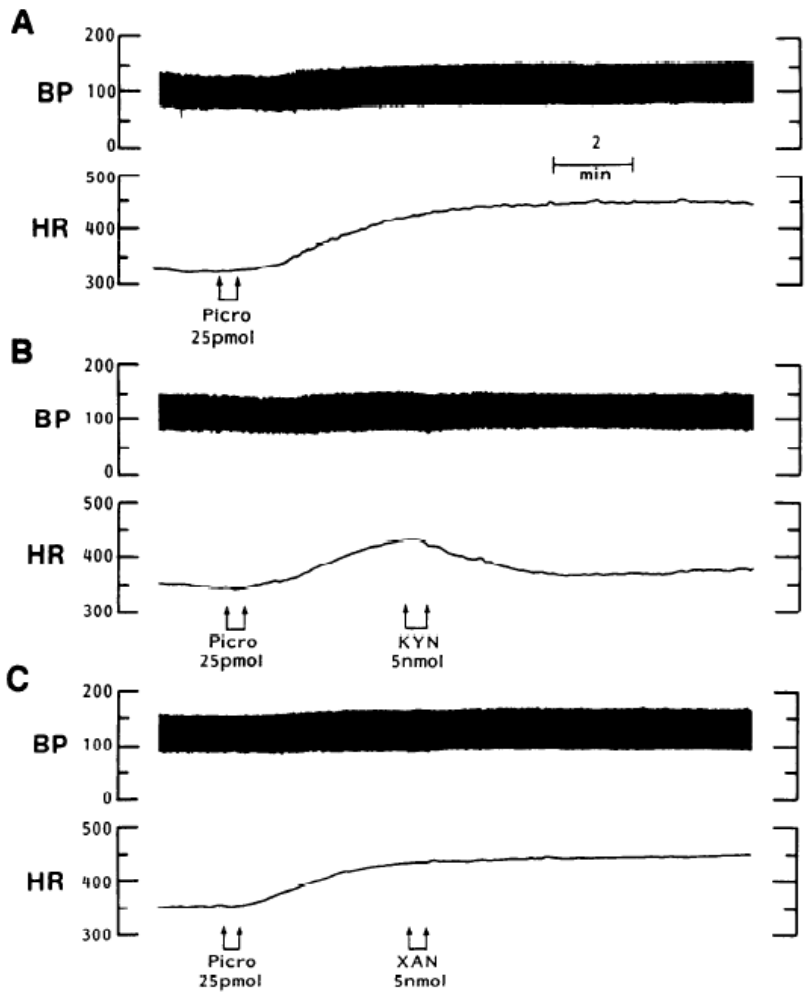

Fig. 2. Tracing of BP (mmHg) and HR (beats/min) depicting effects of microinjection of picrotoxin (Picro, A-C) and subsequent injection of KYN (B) or xanthurenic acid (XAN, C) at same site in dorsomedial hypothalamus in single urethan-anesthetized rat. Injections were made in 50 nl of saline and spaced 60-90 min apart.

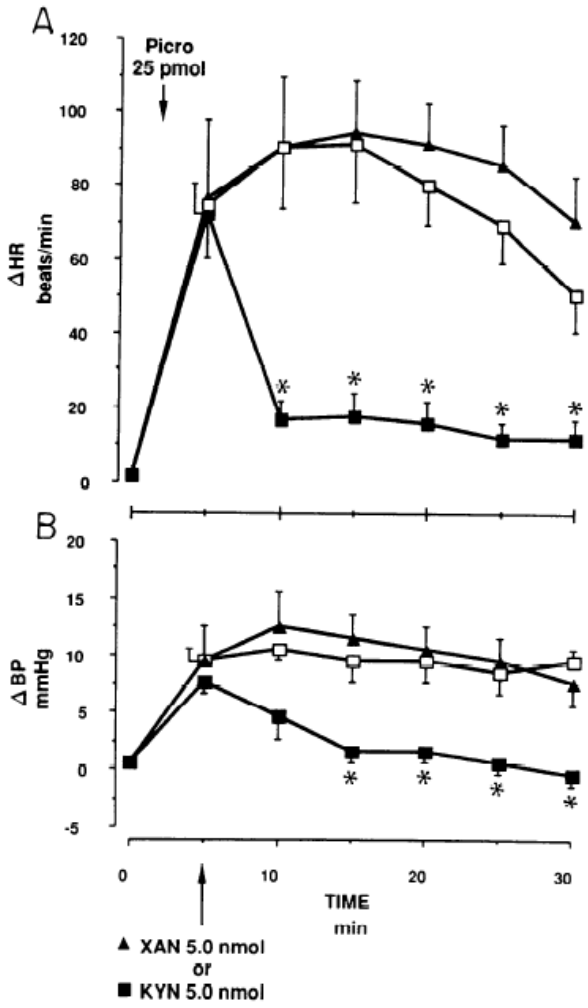

Fig. 3. Time course of changes in HR (A) and BP (B) after injection of Picro without further treatment (open squares) and Picro followed by injection of KYN (filled squares) or XAN (filled triangles) at same site. Baseline HR (364 ± 20 beats/min) and BP (104 ± 3 mmHg) were not different between injections. * Significant difference from Picro alone (open squares) at corresponding time points ($P < 0.05$ by repeated ANOVA and Newman-Keuls, $n = 4$).

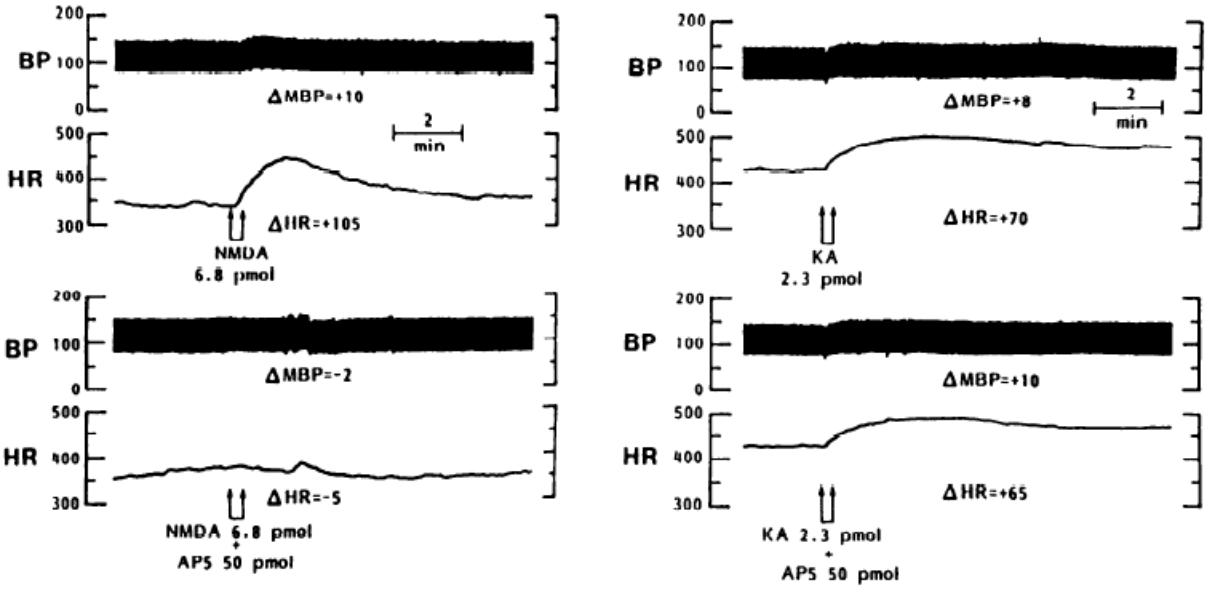

FIG. 4. Tracing of BP (mmHg) and HR (beats/ min) depicting effects of microinjection of *N*-methyl-D-aspartate (NMDA, *left*) and kainic acid (KA, *right*) alone (top panels) or in presence of 50 pmol 2-amino-Sphosphonopentanoic acid (AP5, I bottom panels). Injections were made in 50 nl of saline. AP5 was delivered in same solution as NMDA and KA. Left and right tracings are taken 1 from different experiments. Maximal changes in mean blood pressure (MBP) and HR are indicated.

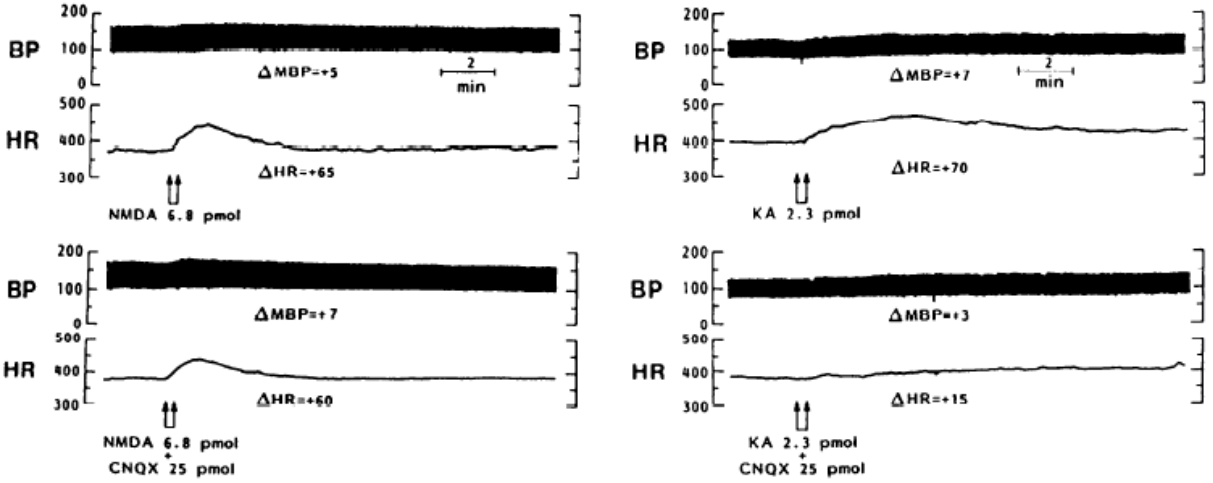

Fig. 5. Tracing of BP (mmHg) and HR (beats/min) depicting effects of microinjection of NMDA (left) and KA (right) alone (top panels) or in presence of 25 pmol 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, bottom panels). Injections were made in 50 nl of saline. CNQX was delivered in same solution as NMDA and KA. Left and right panels are taken from different experiments. Maximal changes in MBP and HR are indicated.

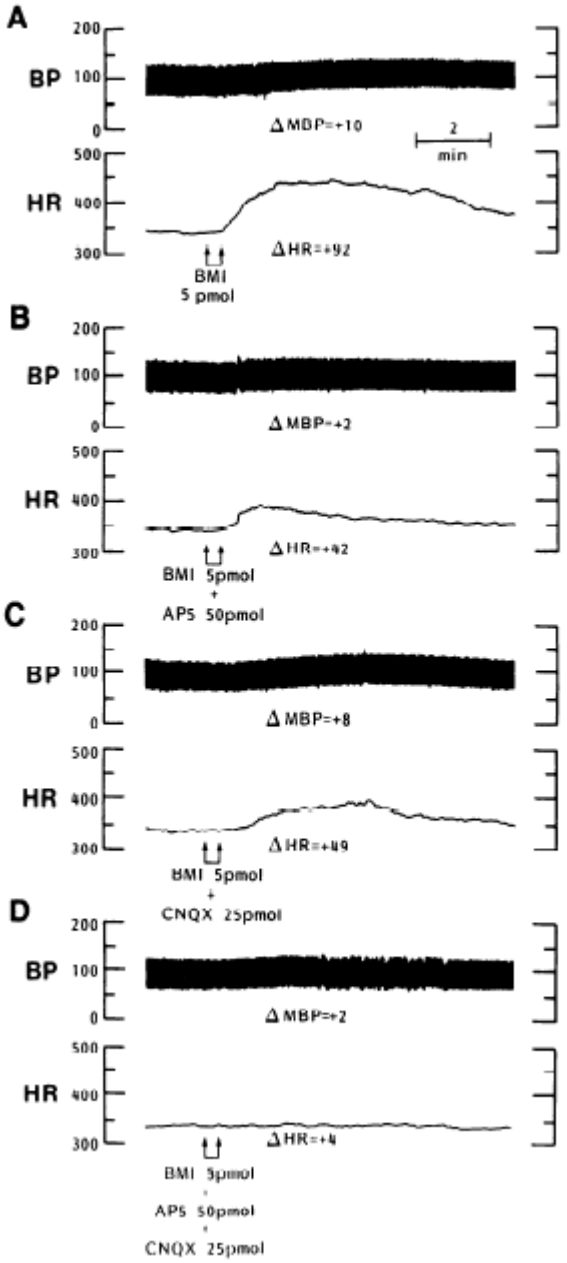

Fig. 6. Tracing of BP (mmHg) and HR (beats/min) depicting effects of microinjection of BMI alone (*A*) and in presence of AP5 (*B*), CNQX (*C*) or AP5 + CNQX (*D*) in single urethan-anesthetized rat. BMI was delivered in same solution as excitatory amino acid (EAA) antagonists. Injections were made in 50 nl of saline and spaced 30-45 min apart. Maximal changes in MBP and HR are indicated. Analysis of these experiments indicated that effects of AP5 and CNQX were not different as function of time.

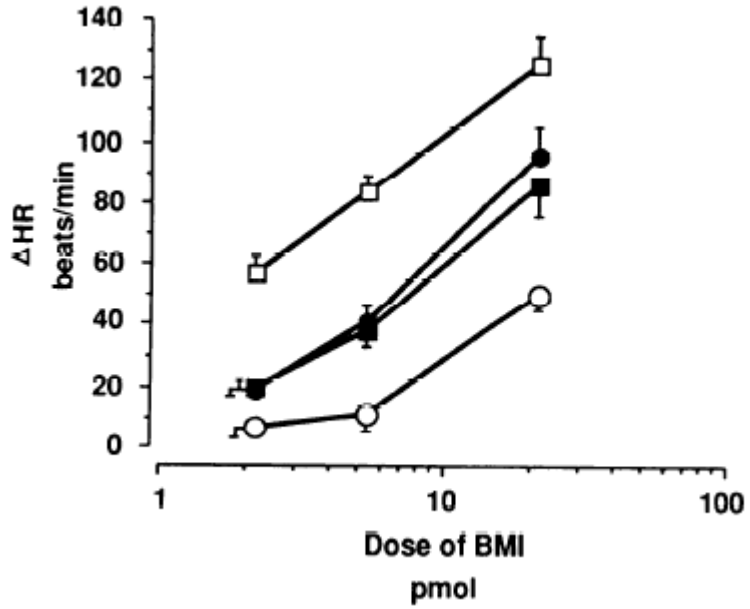

FIG. 7. Maximal changes in HR after injection of BMI alone (open squares) and in presence of 50 pmol AP5 (closed circles), 25 pmol CNQX (closed squares) or 50 pmol AP5 + 25 pmol CNQX (open circles). Graph summarizes data from 3 sets of experiments (n = 5 each set) in which 1 dose of BMI was injected alone and in presence of each EAA antagonist and combination in single rat as depicted in Fig. 6.

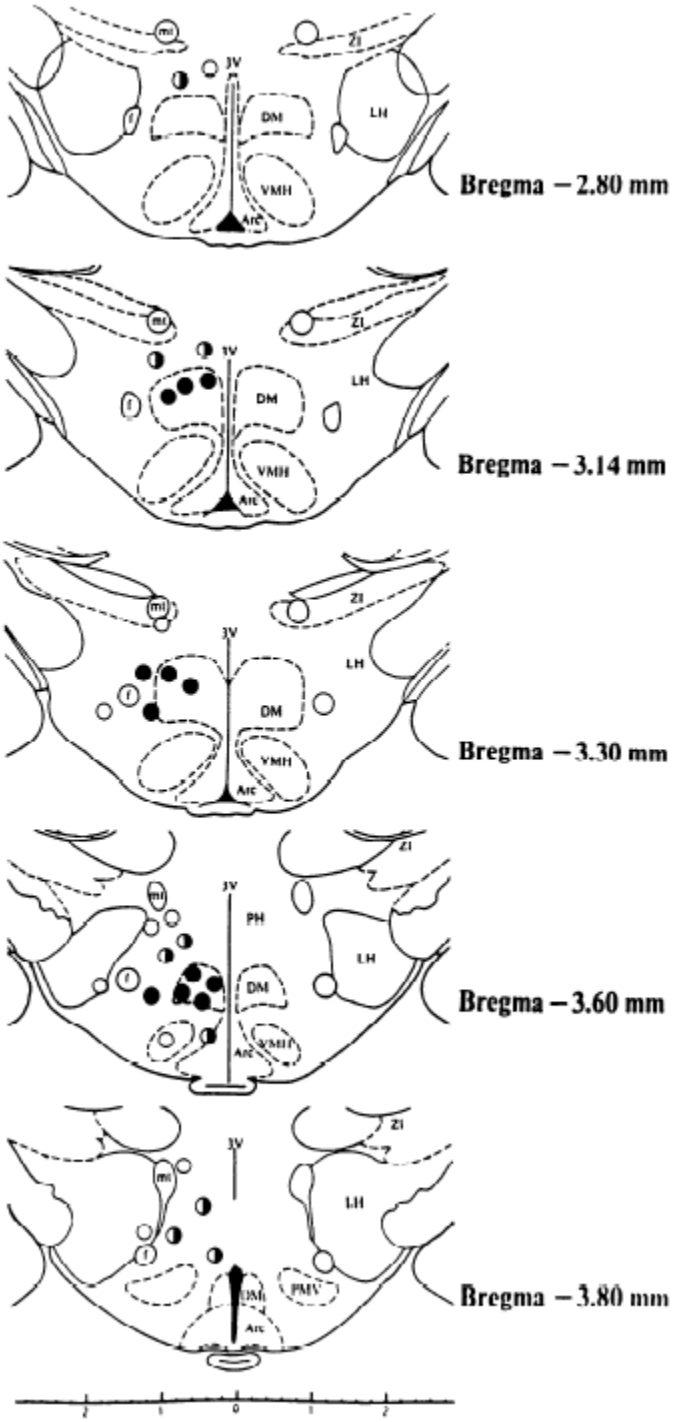

Fig. 8. Schematic coronal sections of rat brain adapted from Paxinos and Watson (15). Effects on HR after microinjection of 5 pmol BMI at each of 30 sites in 11 rats. Open circles, HR increased by <25 beats/min; half-filled circles, HR increased by 25-49 beats/min; filled circles, HR increased by ≥50 beats/min. Arc, arcuate nucleus; DM, dorsomedial hypothalamus; f, fornix; LH, lateral hypothalamic area; mt, mammillothalamic tract; PH, posterior hypothalamus; PMV, ventral premammillary nucleus; VMH, ventromedial hypothalamus; 21, zona incerta; 3V, third ventricle.