Intracerebroventricular infusion of the peptide melittin increases immunoreactive kinins in the cerebrospinal fluid of anesthetized dogs, probably secondary to activation of brain or cerebrospinal fluid kininogenases. Intracerebroventricular melittin also increases blood pressure and heart rate, possibly mediated by brain kinins, since intracerebroventricular bradykinin also increases blood pressure and heart rate. We tested whether the effects of centrally administered melittin on blood pressure and heart rate could be blocked by simultaneous infusion of a kinin receptor antagonist, [DArg°]Hyp³-Thi⁵[DPhe⁷]bradykinin, in normotensive awake rats. In the controls, intracerebroventricular infusion of kinin receptor antagonist given for 1 hour at a rate of 10 µg/hr blocked bradykinin-induced increases in blood pressure and heart rate by 80%. Basal blood pressure and heart rate were not affected by the kinin receptor antagonist alone. After a 30-minute infusion of melittin (8 µg/30 min), cerebrospinal fluid kininogenase activity (n=17) rose from 0.13±0.05 to 0.43±0.1 ng/ml/min (p<0.02). Although cerebrospinal fluid kinins increased from below sensitivity (0.02 ng/ml, n=12) to 0.19±0.1 ng/ml (n=17), this change was due to drastic increases in three rats, whereas in 12 of them kinins were below sensitivity. Incubation of bradykinin (10 ng) with 0.1 ml rat cerebrospinal fluid for 5 minutes destroyed 70% of kinins, suggesting that rapid destruction may have made detection of increased CSF kinins difficult. In normotensive awake rats (n=8), intracerebroventricular melittin increased blood pressure from 110.8±1.8 to 131.5±1.4 mm Hg (p<0.01) and heart rate from 378.8±5.8 to 430.0±8 beats/min (p<0.01). Simultaneous intracerebroventricular infusion of kinin receptor antagonist (10 µg/hr) partially but significantly blunted the blood pressure response to melittin; blood pressure increased to 122.5±2.5 mm Hg (p<0.001, melittin vs. melittin+kinin receptor antagonist) and heart rate decreased to 343.8±11.2 beats/min (p<0.01 vs. basal and p<0.001 vs. melittin). Pretreatment with intracerebroventricular meclofenamate (a prostaglandin synthesis inhibitor) abolished the blood pressure and heart rate response to melittin. The long-lasting hypertensive effect of melittin may be due to prostanoids, since a bolus intracerebroventricular injection of prostaglandin E₂ (500 ng) induced long-lasting hypertension. In summary, intracerebroventricular melittin increased the cerebrospinal fluid concentration of kallikrein without a concomitant increase in kinins. Kinin receptor antagonist diminished the hypertensive response induced by melittin and converted melittin-induced tachycardia to bradycardia. Meclofenamate also blocked the cardiovascular response to melittin. These results suggest that the cardiovascular response to intracerebroventricular melittin is mediated by activation of the endogenous kallikrein-kinin system in the brain, and ultimately by prostanoids. We conclude that the brain kallikrein-kinin system, once activated, can markedly influence regulation of blood pressure and heart rate. (Hypertension 1989;14:629-635)

The components of the kallikrein-kinin system have been found in the central nervous system.¹-³ A messenger RNA (mRNA) that codes for a kallikrein-like enzyme⁴ has been isolated from brain homogenates; kininogen and free kinins are found in the cerebrospinal fluid (CSF) in dogs and humans;⁵,⁶ and several enzymes capable of inactivating kinins are found in the central nervous system as well.⁷,⁸ Intracerebroventricular (ICV) infusion of kinins increases blood pressure and
heart rate\textsuperscript{9,10} as well as the release of antidiuretic hormones.\textsuperscript{11} These findings suggest that kinins generated within the central nervous system may participate in the regulation of cardiovascular function; however, evidence of this is indirect at best. Thomas et al\textsuperscript{18} have shown that infusion of melittin (a bee venom polypeptide) into the cerebral ventricles of dogs increases not only blood pressure and heart rate but also CSF concentrations of immunoreactive kinins. Melittin has also been found to accelerate kallikrein release from cellular membranes.\textsuperscript{12} Thus the cardiovascular changes induced by melittin may be the result of kinin generation secondary to activation of brain kallikrein. Thomas et al\textsuperscript{13} have also reported that hypertension induced by electric stimulation of the vagus nerve was accompanied by increased kinins in ventriculocisternal perfusates. The parallel changes in CSF kinins and blood pressure suggest that endogenous brain kinins may participate in cardiovascular regulation, though as yet proof is lacking.

Recently a series of bradykinin analogues capable of inhibiting the vasodilatory and musculotropic activity of kinins has been synthesized.\textsuperscript{14-16} These kinin receptor antagonists could be useful in clarifying whether brain kinins are involved in cardiovascular changes. Our purpose therefore was two-fold: 1) to determine whether the effects of centrally infused melittin on blood pressure and heart rate could be blocked by simultaneous administration of the kinin receptor antagonist [DArg\textsuperscript{9}]Hyp\textsuperscript{1}-Thr\textsuperscript{2,3} [D-Phe\textsuperscript{7}]bradykinin and 2) to ascertain whether pretreatment with meclofenamate, a prostaglandin synthesis inhibitor, could modify these changes. Our reasoning was that both kinins and melittin activate prostaglandin synthesis, and some prostaglandins are known to cause hypertension and tachycardia\textsuperscript{17,18} when injected centrally.

**Materials and Methods**

Bradykinin and melittin were purchased from Peninsula Labs., Inc. (Belmont, California); kinin receptor antagonist was generously provided by John Stewart and Raymond Vavrek (University of Colorado, Denver, Colorado) and meclofenamate by Martin Black (Warner-Lambert/Parke Davis, Ann Arbor, Michigan). All compounds were dissolved in artificial cerebrospinal fluid (aCSF). Male Sprague-Dawley rats weighing 250–300 g (Charles River Labs., Wilmington, Massachusetts) were housed individually in an air-conditioned room with a 12-hour light/dark cycle and fed a normal sodium diet (0.4% NaCl) with tap water ad libitum. After 2 or 3 days of adjustment to the new environment, the rats were anesthetized (sodium pentobarbital, 50 mg/kg i.p.), and a 22 gauge stainless steel cannula (15 mm long and bent 90° at the midpoint) was implanted into each lateral cerebral ventricle (1.5 mm from the midline, 1.0 mm posterior to the bregma, and 4.5 mm deep) and anchored to the skull with screws and dental cement. The free end was attached to a Silastic tube that was filled with aCSF, tunneled under the skin, and brought out between the scapulae, and the Silastic tube was occluded with a metal pin. Dead space of the cannula was 5–7 \( \mu \)l. After 3–5 days, placement was verified by the dipsogenic response to angiotensin II\textsuperscript{19}; the rats were then anesthetized with ether and a polyethylene tube (PE10 fused to PE50) filled with heparinized saline was implanted in the abdominal aorta via the femoral artery for measurement of blood pressure and heart rate. The catheter was tunneled under the skin and brought out at the back of the neck.

Experiments were performed the following morning. Awake rats were placed in transparent plastic restrainers and the canulae connected to a Hamilton syringe (50–100 \( \mu \)l). For ICV infusion, the syringe was driven by a variable-speed pump (model 990, Harvard Apparatus, Dover, Massachusetts); bolus ICV injections (10 \( \mu \)l followed by another 10 \( \mu \)l) were delivered by hand. There was no discernible effect on blood pressure or heart rate when this volume of aCSF was injected into the lateral ventricle. ICV infusions were performed at 20 \( \mu \)l/hr. Blood pressure and heart rate were measured via the arterial catheter with a transducer connected in series with a tachometer, and recorded with a Gould Brush 220 (Cleveland, Ohio). None of the rats were used in more than one experiment. After a stabilization period of 30 minutes, one of the following protocols was performed.

**Protocol 1: Effects of Kinin Receptor Antagonist on Blood Pressure and Heart Rate Response to Intracerebroventricular Bradykinin**

Either aCSF alone (\( n=5 \)) or kinin receptor antagonist (5 \( \mu \)g/hr, \( n=6 \) and 10 \( \mu \)g/hr, \( n=6 \)) was infused into one lateral ventricle. A bolus of 0.5 \( \mu \)g bradykinin was injected into the opposite ventricle before and at the end of the infusion and maximum changes in blood pressure and heart rate determined.

In a separate group of rats, we studied the effects of kinin receptor antagonist on basal blood pressure and heart rate. After the equilibration period, kinin receptor antagonist (\( n=8 \)) was infused intracerebroventricularly at 10 \( \mu \)g/hr for 1 hour, while control rats (\( n=8 \)) received aCSF alone. Blood pressure and heart rate were recorded continuously throughout the experiment.

**Protocol 2: Effects of Kinin Receptor Antagonist on Blood Pressure and Heart Rate Response to Intracerebroventricular Melittin**

One group (control) was infused with aCSF and the other (experimental) with kinin receptor antagonist at 10 \( \mu \)g/hr for 1 hour. Thirty minutes after the beginning of the infusions, melittin was simultaneously infused into the contralateral ventricle for another 30 minutes (8 \( \mu \)g/30 minutes). The dose of melittin was selected in pilot experiments by determination of the infusion rate capable of inducing a
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20–30 mm Hg increase in blood pressure. Blood pressure and heart rate were continuously monitored during the infusions and for 2 hours thereafter.

**Protocol 3: Effect of Intracerebroventricular Meclomenamate on Blood Pressure and Heart Rate Response to Intracerebroventricular Melittin**

This was similar to protocol 2, except that instead of kinin receptor antagonist the experimental group received 200 µg/hr meclomenamate, a dose shown to be effective in inhibiting prostaglandin synthesis within the central nervous system.17,20

**Protocol 4: Effect of Intracerebroventricular Melittin on Kininogenase Activity and Cerebrospinal Fluid Kinin Concentration**

A separate group of rats (n=17) was anesthetized with sodium pentobarbital (Nembutal, Abbott Labs., North Chicago, Illinois) (50 mg/kg i.p.) and either meclomenamate or aCSF infused into the lateral ventricle for 30 minutes. Just before the end of the infusion, the arachnoid membrane was exposed; a glass cannula with a sharpened tip was inserted into the cisterna magna and 100–150 µl CSF withdrawn. Kininogenase activity was measured with and without soybean trypsin inhibitor (SBTI) (100 µg/ml) after 50 µl CSF was incubated with semipurified dog kininogen for 6 hours at 37°C.21 Free kinins were measured in different rats at the end of the aCSF or melittin infusion. A total of 100–150 µl CSF was rapidly mixed with 500 µl ethanol in a siliconized glass tube. The mixture was centrifuged at 3,000g for 5 minutes, and the supernatant was evaporated under a N2 stream at 80°C. The dried residue was reconstituted with 200 µl 0.1 M Tris HCl buffer, 0.1% neomycin sulfate, and 0.2% gelatin (pH 7.4). To measure kininase activity, CSF was drawn from normal rats (n=4), and 0.1 ml aliquots of CSF or saline were placed in siliconized glass tubes and then maintained at 37°C in a water bath. After a 15-minute equilibration period, 10 ng bradykinin was added to each tube and the mixture incubated for 5 minutes. The enzymatic reaction was stopped by immersing the tubes in boiling water. The samples were dried, reconstituted to 0.2 ml with the kinin radioimmunoassay buffer22 and assayed. The data obtained with saline were used to determine recovery. Kinins were measured by a radioimmunoassay that could detect 1 pg bradykinin/tube.22

**Protocol 5: Effects of Intracerebroventricular Prostaglandin E2 on Blood Pressure and Heart Rate**

Prostaglandin E2 (PGE2) (500 ng in 10 µl aCSF) was injected intracerebroventricularly as a bolus. Blood pressure and heart rate were monitored continuously for 60 minutes. For statistical analysis, the data were grouped into four periods: basal, 15, 30, and 60 minutes.

**Statistical Methodology**

All results were expressed as mean±SEM. Profile analysis was used to compare controls and the two experimental groups over time in protocols 2 and 3. Since significant interactions between the groups were detected over time for both protocols, analysis of variance (ANOVA) was used to compare groups at each time point. If the results were statistically significant, Tukey’s least significant difference method was used to compare pairs of groups at each time point. This technique adjusts the critical level of each pairwise test to obtain an overall level of 0.05 for the experiments. Paired t tests were used to examine any change occurring between the control period and subsequent individual time points within each group. Differences in recovery time between kinins incubated with aCSF and melittin-infused rats were analyzed by Student’s t test. The overall effect of ICV PGE2 was tested by Hotelling’s t square analysis, and a paired t test for changes between control and posttreatment time points. Significance of all paired t tests was calculated with Bonferroni’s multiple comparison adjustment. For example, if three t tests were performed at each variable setting, Bonferroni’s adjustment was used to reduce the rejection level from 0.05 to 0.017. In protocol 1, paired t tests were used to compare the response before and after infusion. Probability values <0.05 were considered significant.

In protocol 4, profile analysis was used to compare control and melittin-infused rats across time. Probability values <0.05 were considered significant. No group-time interaction was detected; time effects were tested directly by pooling data across groups or time as appropriate.

**Results**

ICV infusion of 5 µg kinin receptor antagonist did not alter basal blood pressure and heart rate, but failed to inhibit the tachycardic action of a 0.5 µg ICV bolus injection of bradykinin (ΔDBP=36±2.0 vs. 29.5±2.2 mm Hg, p<0.05; ΔHR=68.3±6.5 vs. 50.0±9.3 beats/min, p>0.05; where ΔDBP is change in blood pressure and ΔHR is change in heart rate). Infusion of kinin receptor antagonist at 10 µg/hr significantly blocked the vasopressor and tachycardic effects of ICV bradykinin (ΔDBP=34.7±4.1 vs. 5.7±0.8 mm Hg, p<0.01; ΔHR=55.0±4.3 vs. 5.0±7.6 beats/min, p<0.001) (Figure 1) but produced no significant changes in basal blood pressure or heart rate by itself (Figure 2). On initiation of the kinin receptor antagonist infusion, there was a tendency for heart rate to increase, but this was not significant.

Central infusion of melittin resulted in a long-lasting increase in mean blood pressure, rising from 110.8±1.8 mm Hg (basal value) to 131.5±2.4 at the end of the infusion and 137.0±2.8 by 2 hours afterward (p<0.01). Simultaneous kinin receptor antagonist partially blocked these effects, with blood pressure reaching 122.5±2.1 at the end of the infu-
FIGURE 1. Line graphs showing effect of intracerebroventricular (ICV) infusion of kinin receptor antagonist (K-ant) on mean blood pressure (ΔMBP) and heart rate (ΔHR) response to ICV injection of bradykinin (BK). Responses to BK were determined before and after an infusion of artificial cerebrospinal fluid (aCSF) or K-ant. *p<0.05; **p<0.01; ***p<0.001 before versus after treatment.

Discussion

Kinin analogues, which are specific receptor antagonists of kinin-induced musculotropic and vasodilator activity, can be used to test whether kinins generated within the central nervous system help regulate cardiovascular function. The analogue we used ([DArg°]Hyp³-Thi⁵-⁸[DPhe⁷]bradykinin) is an effective kinin receptor antagonist that has only weak kinin-like agonistic activity.14-16 We found that central infusion of 10 μg/hr kinin receptor antagonist was effective in blocking the cardiovascular response to ICV bradykinin but had no significant effect on basal blood pressure or heart rate, suggesting that kinins in CSF or circumventricular structures are not involved in the control of resting blood pressure and heart rate. Doses 25 μg/hr or higher could not be used as they induced hypertension and tachycardia, possibly because at these

was not inhibited by SBTI (Figure 6). CSF kinins were below sensitivity (0.02 ng/ml) in all control rats (n=12), whereas they were 0.19±0.1 ng/ml in the melittin-infused rats (Figure 7). However, this change was due to a drastic increase in three of the 17 rats treated with melittin. A fourth rat had 0.025 ng/ml, whereas in the other 12 rats CSF kinin levels remained below sensitivity. To determine whether high kininase activity may explain the low kinin concentrations in CSF, kinins were added to CSF obtained from normal rats. Only 30.5±2.6% of added kinins were recovered after 5 minutes incubation.

CSF kininogenase activity was also significantly increased by melittin infusion compared with aCSF (0.43±0.09 vs. 0.13±0.05 ng/ml/min; p<0.02) and...
concentrations the kinin analogue acts as a partial agonist on brain kinin receptors.

Thomas et al suggested that the cardiovascular changes induced by melittin are accompanied by an increased concentration of kinins in the CSF. We have shown that central infusion of melittin induces a long-lasting hypertensive and tachycardic response associated with increased kininogenase activity. Pretreatment with kinin receptor antagonist significantly attenuated the vasopressor response to melittin, indicating that kinins generated in the CSF or circumventricular structures partially mediate its vasopressor activity. Higher doses may be needed to determine whether all of the hypertensive activity of centrally administered melittin is due to kinins. However, such experiments cannot be done with the kinin antagonist we used, because as stated above, at a high dose (just two to three times higher than the dose used here) it spontaneously increases blood pressure and heart rate.

It is not clear why simultaneous ICV kinin receptor antagonist and melittin induced bradycardia. Slowing of the heart rate is the normal reflex response to an acute increase in blood pressure. It is possible that increased kinins in brain tissue masked the reflex response, which was then restored by blockade of kinins. Bradycardia was not due to failure to block the effect of CSF kinins, because kinin receptor antagonist inhibited the hypertensive and tachycardic effects of exogenous kinins injected into the lateral ventricle but did not convert tachycardia to bradycardia. Another possibility is that one of the pharmacological actions of ICV melittin is bradycardia, which was masked by kinin-induced tachycardia but became apparent after inhibition of kinins by kinin receptor antagonist.

Central infusion of meclofenamate, a cyclooxygenase inhibitor, blocked the vasopressor and tachycardic effects of ICV melittin. The cardiovascular changes induced by melittin were long-lasting (Figures 3 and 4). We found that the hypertensive response to a bolus ICV injection of PGE_2 was long-lasting as well. Long-lasting overall changes in heart rate were also detected ($p<0.05$), although differences between individual time points and baseline levels were not significant because of the high individual variability. The cardiovascular effects of ICV PGE_2 were not altered by the kinin receptor antagonist (data not shown). These results suggest that kinins initiate the melittin-induced changes in blood pressure and heart rate, but brain prostaglandins are the final mediators. Other workers have also found a close relation between the central cardiovascular actions of kinins and prostaglandins. Thomas et al assumed that the melittin-induced increase in CSF

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**FIGURE 3.** Line graphs showing effect of intracerebroventricular (ICV) infusion of melittin (10 μg/hr) on mean blood pressure (MBP) and heart rate (HR) response to ICV infusion of meclofenamate. Control, control period; aCSF, artificial cerebrospinal fluid. ***$p<0.001$ changes from control period for melittin versus melittin plus K-ant; ++$p<0.01$ compared with control period.

**FIGURE 4.** Line graphs showing effect of intracerebroventricular (ICV) infusion of meclofenamate (200 μg/hr) on mean blood pressure (MBP) and heart rate (HR) response to ICV infusion of melittin. Control, control period; aCSF, artificial cerebrospinal fluid. ***$p<0.001$ changes from control period for melittin versus melittin plus meclofenamate; ++$p<0.01$ compared with control period. MBP and HR responses to melittin are redrawn from Figure 3.
kinins was due to activation of tissue kallikrein. We found that melittin increased kininogenase activity in CSF from the cisterna magna; this increase was resistant to SBTI, suggesting that the enzyme involved may be similar to glandular kallikrein anddiscounting enhanced permeability of plasma constituents as a source of increased CSF kininogenase. Immunocytochemical studies have suggested that tissue kallikrein is present inependymal structures and hypothalamic cell bodics. We found that human CSF contains small amounts of kininogen. Although the data clearly suggest that increases in brain kinins mediate the cardiovascular effects of melittin, we could not obtain evidence that CSF withdrawn from the cisterna magna at the end of the melittin infusion contains large amounts of kinins. These data contrast with those obtained by Thomas et al., who performed ventriculocisternal perfusions in dogs and found that melittin increased kinins several times in the perfusate. This apparent discrepancy may be explained by the high kininase activity of the rat brain and CSF. Kariya et al. injected 5 μg kinins into the rat ventricle and found that half were destroyed within 26 seconds. Our results indicate that 1 ml rat CSF can destroy 12–15% of added kinins per minute. We feel that any kinins still present in the CSF after melittin were destroyed by the time we drew the CSF from the cisterna magna. By perfusing saline at a relatively high rate, Thomas may have protected kinins overflowing into the CSF. These data are consistent with the hypothesis that kinins act as paracrine hormones, acting at or near the site where they are generated. It is also possible that melittin induced a transitory increase in brain kinins, which in turn caused formation of prostaglandins, the final mediators of the long-lasting cardiovascular changes.

Because we were unable to clearly demonstrate a melittin-induced increase in CSF kinins, participation of brain kinins would seem to depend on the kinin receptor antagonist data and on the increased CSF kininogenase activity after melittin infusion. Thus we cannot rule out the possibility that the suppressor effects of kinin receptor antagonist on melittin-induced cardiovascular changes are caused by some nonspecific effect. However, this is unlikely because we are not aware of any property of kinin receptor antagonist other than its capability to block kinins that could explain its blunting of the hypertensive and tachycardic response to melittin.

The neural pathways stimulated by kinins or melittin may originate in or after the third ventricle, as infusion of kinin receptor antagonist or meclofenamate into one lateral ventricle blocked melittin-induced cardiovascular effects on the opposite side. Lewis and Phillips demonstrated that the structures responsible for the blood pressure response to centrally injected kinins are located in the third ventricle, while Diz and Jacobowitz reported that
microinjections of kinins into the dorsomedial and posterior hypothalamic nuclei resulted in pressor responses and tachycardia. Immunoocytochemical studies of the brain have shown that kinins are contained in neuronal cell bodies located in the hypothalamus, with dense clusters overlying the periventricular and dorsomedial nuclei. These data are consistent with the theory that kinins formed in neural structures in or around the third ventricle may be involved in the regulation of cardiovascular function. Within this context, CSF kinins may be only a fraction of those generated locally because the half-life of kinins injected into the brain is very short. Studies involving artificial stimuli such as melittin are useful because they demonstrate that the endogenous brain kallikrein-kinin system can be activated and that kinins can reach sufficient concentrations within the central nervous system to stimulate the neuronal pathways involved in cardiovascular regulation. Although it has been suggested that CSF kinins accumulate during vagal stimulation and increases in central vasopressin or during activation of endogenous brain kallikrein-kinin system can be activated and that kinins can reach sufficient concentrations within the central nervous system to stimulate the neuronal pathways involved in cardiovascular regulation. Although it has been suggested that CSF kinins accumulate during vagal stimulation and increases in central vasopressin or during cerebral hemorrhage, it is still unclear what causes activation of the brain kallikrein-kinin system.

In conclusion, central infusion of melittin results in activation of endogenous brain kallikrein. The blunting effects of the kinin receptor antagonist and the prostaglandin synthesis inhibitor indicate that increased concentration of endogenous brain kinins leads to the formation of prostanooids in those structures of the brain involved with cardiovascular regulation. Thus kinins and prostaglandins mediate the cardiovascular response to ICV melittin. Although the CSF or circumventricular kallikrein-kinin system might not normally participate in regulation of basal blood pressure and heart rate, involvement of locally formed kinins (paracrine system) cannot be excluded. Once activated, the brain kallikrein-kinin system can induce hypertension and changes in heart rate.

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key words: kinin antagonist • melittin • kallikrein • blood pressure • heart rate
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