Microinjections of α-melanocyte stimulating hormone into the nucleus ambiguus of the rat elicit vagally mediated bradycardia

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Chitravanshi VC, Bhatt S, Sapru HN. Microinjections of α-melanocyte stimulating hormone into the nucleus ambiguus of the rat elicit vagally mediated bradycardia. Am J Physiol Regul Integr Comp Physiol 296: R1402–R1411, 2009. First published March 18, 2009; doi:10.1152/ajpregu.90978.2008.—Neurons that immunostain for α-melanocyte stimulating hormone (α-MSH) have been identified in the nucleus ambiguus (nAmb). The presence of mRNA for melanocortin type 4 receptors (MC4Rs) has also been reported in this nucleus. On the basis of this information, it was hypothesized that activation of MC4Rs in the nAmb may play a role in the regulation of cardiac function. This hypothesis was tested in urethane-anesthetized, artificially ventilated, adult male Wistar rats. Microinjections (30 nl) of α-MSH (0.1, 0.2, 0.4, 0.8, and 1.2 mM) into the nAmb of anesthetized rats elicited decreases in heart rate (HR; 1.3 ± 0.6, 3 ± 1, 11 ± 2, 46.3 ± 3, and 43.3 ± 7 bpm, respectively) and no changes in mean arterial pressure (MAP). Maximum decreases in HR were elicited by 0.8 mM concentration of α-MSH. Bradycardic responses to α-MSH were similar in unaesthetized midcollular decerebrate rats. Microinjections of artificial cerebrospinal fluid (30 nl) into the nAmb did not elicit a HR response. Bilateral vagotomy completely abolished α-MSH-induced bradycardia. The decreases in HR elicited by α-MSH (0.8 mM) were completely blocked by a selective MC4R antagonist. Direct application of α-MSH on the nAmb neurons increased their firing, which was blocked by prior applications of the MC4R antagonist. Microinjections of the MC4R antagonist into the nAmb did not alter reflex bradycardic responses elicited by intravenous infusions of phenylephrine, suggesting that MC4Rs did not play a role in mediating the parasympathetic component of baroreflex-mediated cardiac responses to α-MSH.

Bradycardic responses to α-MSH may activate the parasympathetic preganglionic nAmb neurons via MC4Rs, leading to bradycardic responses.

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(2–3% in 100% oxygen), the trachea was cannulated with polyethylene (PE) tubing, and the rats were artificially ventilated using a rodent ventilator (model 683; Harvard Instruments, Holliston, MA). Both femoral veins were cannulated. One of the venous cannulas was used for injections of urethane (1.2–1.4 g/kg), which was administered in 12–15 aliquots at 2-min intervals; the total volume of the anesthetic solution was 0.55–0.75 ml injected over a period of about 25–30 min. Urethane and perfused transcardially with 10% sucrose solution followed by 4% paraformaldehyde at 4°C overnight, and then placed in 30–40 m). The sections were then incubated with a biotinylated secondary antibody (rabbit polyclonal anti-MC4R; Abcam, Boston, MA) diluted in blocking buffer (1:1,000), followed by 3 washes in TPBS for 5 min each. The sections were then incubated overnight at 4°C with primary antibody (rabbit anti-MC4R: Abcam, Boston, MA) diluted in blocking buffer containing 0.3% Triton (TPBS). The sections were then washed with PBS containing 0.3% Triton (TPBS). The sections were then washed and treated with 0.02% hydrogen peroxide for 2 min and washed, followed by 1 h incubation in blocking buffer consisting of TPBS and goat serum (5%, Vector Laboratories, Burlingame, CA). The sections were then incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-MC4R; Abcam, Boston, MA) diluted in blocking buffer (1:1,000), followed by 3 washes in TPBS for 5 min each. The sections were then washed with PBS containing 0.3% Triton (TPBS). The sections were then washed and treated with a stabilized solution of active diaminobenzidine (“Stable DAB”, Vector Laboratories) until completion of the reaction (at least 20 min of ejection solution (2 nl) was visually confirmed under a modified binocular horizontal microscope. Controls consisted of 2 nl of aCSF (pH 7.4). The involvement of recorded nAmb neurons in cardiovascular regulation was ascertained by their excitation in response to bolus injections of PE (2–4 µg/kg iv). Immunohistochemical staining for MC4Rs. For immunohistochemical identification of MC4Rs, the rats were deeply anesthetized with urethane and perfused transcardially with 10% sucrose solution followed by 4% paraformaldehyde in PBS. The brain was removed and stored in 4% paraformaldehyde at 4°C overnight, and then placed in a 30% sucrose solution until it sank to the bottom of the container (usually 2–4 days). The sections of the brain were cut (30–40 µm) at −13 to −20°C, using a cryostat (model CM1900, Leica Microsystems, Bannockburn, IL), placed on subbed slides and stored at −20°C. Subsequently, the sections were brought to room temperature and washed with PBS containing 0.3% Triton (TPBS). The sections were then treated with 0.02% hydrogen peroxide for 2 min and washed, followed by 1 h incubation in blocking buffer consisting of TPBS and goat serum (5%, Vector Laboratories, Burlingame, CA). The sections were then incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-MC4R). Absorbance was measured continuously in the expired gas collected via a side-arm (PE-50 tubing) inserted into the tracheal cannula using an infrared CO2 analyzer for small animals (Micro-Capnometer; Columbus Instruments, Columbus, OH). Rectal temperature was maintained at 36.5 ± 0.5°C using a temperature controller (model TCAT-2AC; Physitemp Instruments, Clifton, NJ). One of the femoral arteries was cannulated, and blood pressure (BP) was recorded using a polygraph (model 7D; Grass Instruments, West Warwick, RI). The BP signals were digitized and mean arterial pressure (MAP) and heart rate (HR) signals were derived from them using 1401 A/D converter and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All of the tracings were stored on a computer hard drive.

Vagotomy. Vagotomy was necessary in experiments designed to investigate the role of parasympathetic innervation to the heart in mediating the α-MSH-induced HR responses. In these experiments, silk sutures were placed loosely around the vagus nerves bilaterally connected via PE tubing to one of the channels on a picospritzer 2–2.4 mm deep from the dorsal medullary surface. The sites eliciting induced responses, experiments were performed on decerebrate rats. Decerebration. To exclude the effects of urethane on α-MSH-induced responses, experiments were performed on decerebrate rats. The procedure for decerebration was similar to that reported earlier (3, 16). Briefly, anesthesia was induced and maintained by isoflurane. To minimize bleeding, the external and internal carotid, as well as the pterygopalatine arteries were ligated bilaterally under an operating microscope (OPMI-1; Carl Zeiss, Thornwood, NY), while all other branches in the carotid sinus region remained patent for continued blood flow in this region. The rats were placed in a prone position in a stereotaxic instrument (David Kopf Instruments). The parietal bones were removed, and the sagittal sinus was ligated rostrally and caudally and sectioned between the ligatures. A transection was made at midcerebellar level, and the rostral portion of the brain was removed by suction. The cranial cavity was then packed with cotton balls. Tracheal inhalation of isoflurane was discontinued, and a stabilization period of 60 min was allowed.

Baroreflex function test. To measure the parasympathetic component of the baroreflex, phenylephrine (PE) was infused intravenously, and baroreflex function curves were obtained by plotting reflex decreases in HR in response to increases in MAP. Approximately linear increase in MAP was induced by infusing PE (125 µg/ml) with a 5-ml syringe connected to the femoral venous cannula and mounted on an infusion pump (model 341, Sage Instruments, Cambridge, MA). PE was administered continuously every 30 s by ramped infusion at a rate of 0.12, 0.17, and 0.25 ml/min until the MAP increased by 60 mmHg. The protocol was repeated after bilateral microinjections of aCSF and MC4R antagonist (0.2 mM) into the nAmb (for selection of the MC4R antagonist concentration, see Blockade of α-MSH responses by MC4R antagonist in the RESULTS). In each experiment, PE infusions were made before any microinjections into the nAmb (control), after microinjection of aCSF, and after microinjection of MC4R antagonist; the interval between PE infusions was at least 20 min. Extracellular neuronal recording. The procedure for extracellular neuronal recording has been described elsewhere (17). Briefly, five-barreled glass micropipettes (Medical Systems, Greensvile, NY) were used. The tip size was adjusted, so that the resistance of the barrels was 4–8 MOhm. In this configuration, the recording barrel was in the center of the micropipette tip (∼5–10 µm), with ejection barrels surrounding it. The central barrel used for recording was filled with 4 M NaCl, and the other barrels contained aCSF, L-Glu, α-MSH, and MC4R antagonist. The micropipette was inserted into the nAmb using the coordinates mentioned earlier. The extracellularly recorded neuronal activity was filtered (100–10,000 Hz) and amplified (∗10,000–20,000) (using model DAM-80 amplifier with low-noise headstage probe; World Precision Instruments) and window discriminator (Fredrick Haer, Brunswick, ME). The amplified signals were digitized and analyzed using 1401 A/D converter and Spike 2 software (Cambridge Electronic Design) and stored on a computer hard drive. Ejection of the contents in different barrels on neurons was accomplished by application of pressure pulses (15-ms duration, 20 psig), and the volume of ejected solution (2 nl) was visually confirmed under a modified binocular horizontal microscope. Controls consisted of 2 nl of aCSF (pH 7.4). The involvement of recorded nAmb neurons in cardiovascular regulation was ascertained by their excitation in response to bolus injections of PE (2–4 µg/kg iv).

Intracerebroventricular administration of MC4Rs. For intracerebroventricular administration of MC4Rs, the rats were deeply anesthetized with urethane and perfused transcardially with 10% sucrose solution followed by 4% paraformaldehyde in PBS. The brain was removed and stored in 4% paraformaldehyde at 4°C overnight, and then placed in a 30% sucrose solution until it sank to the bottom of the container (usually 2–4 days). The sections of the brain were cut (30–40 µm) at −13 to −20°C, using a cryostat (model CM1900, Leica Microsystems, Bannockburn, IL), placed on subbed slides and stored at −20°C. Subsequently, the sections were brought to room temperature and washed with PBS containing 0.3% Triton (TPBS). The sections were then treated with 0.02% hydrogen peroxide for 2 min and washed, followed by 1 h incubation in blocking buffer consisting of TPBS and goat serum (5%, Vector Laboratories, Burlingame, CA). The sections were then incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-MC4R). Absorbance was measured continuously in the expired gas collected via a side-arm (PE-50 tubing) inserted into the tracheal cannula using an infrared CO2 analyzer for small animals (Micro-Capnometer; Columbus Instruments, Columbus, OH). Rectal temperature was maintained at 36.5 ± 0.5°C using a temperature controller (model TCAT-2AC; Physitemp Instruments, Clifton, NJ). One of the femoral arteries was cannulated, and blood pressure (BP) was recorded using a polygraph (model 7D; Grass Instruments, West Warwick, RI). The BP signals were digitized and mean arterial pressure (MAP) and heart rate (HR) signals were derived from them using 1401 A/D converter and Spike 2 software (Cambridge Electronic Design) and stored on a computer hard drive. Ejection of the contents in different barrels on neurons was accomplished by application of pressure pulses (15-ms duration, 20 psig), and the volume of ejected solution (2 nl) was visually confirmed under a modified binocular horizontal microscope. Controls consisted of 2 nl of aCSF (pH 7.4). The involvement of recorded nAmb neurons in cardiovascular regulation was ascertained by their excitation in response to bolus injections of PE (2–4 µg/kg iv).
RESULTS

Baseline values for MAP and HR in urethane-anesthetized rats were 99.7 ± 2 mmHg and 405 ± 9 bpm, respectively (n = 64). The values for baseline MAP and HR in the decerebrate rats (n = 9) were 83 ± 3 mmHg and 428 ± 10 bpm, respectively. There were no significant differences (P > 0.05) between the baseline values of MAP and HR in urethane-anesthetized and decerebrate rats.

Concentration-response of α-MSH. Five different concentrations (0.1, 0.2, 0.4, 0.8, and 1.2 mM) of α-MSH, 30 nl each, were used for concentration-response experiments. Fifteen rats were used for concentration-response studies. In one group (n = 5), 2 concentrations (0.1 and 0.2 mM) were filled in two separate barrels of the 4-barreled micropipette, while the other two barrels contained l-Glu (5 mM) and aCSF. First, the nAmb was identified by microinjections of l-Glu, which was followed by testing lack of any responses to microinjections of aCSF. Then, the responses to the two concentrations of α-MSH were studied. The interval between the microinjections of the α-MSH was at least 40 min to avoid tachyphylaxis. The sequence of microinjections of the two concentrations of α-MSH was changed in different rats. In the second group of rats (n = 5), the same protocol was used with two different concentrations (0.4 and 0.8 mM) of α-MSH. In the third group of rats (n = 5), the procedure was similar except that only one concentration (1.2 mM) was used. Unilateral microinjections of l-Glu (5 mM) were used to identify the nAmb; bradycardia (56 ± 6, bpm), unaccompanied by any change in MAP, was observed. The interval between the microinjections of l-Glu and other agents was at least 5 min. Microinjections of different concentrations (0.1, 0.2, 0.4, 0.8, and 1.2 mM) of α-MSH into the nAmb elicited decreases in HR (1.3 ± 0.6, 3 ± 1, 11 ± 2, 46.3 ± 3, and 43.3 ± 7, bpm; Fig. 1A) (n = 15). In this and other series of experiments,
microinjections (30 nl) of aCSF into the nAmb elicited no responses. The maximal decrease in HR was elicited by 0.8 mM concentration; the bradycardic response to a higher concentration of α-MSH (1.2 mM) was not significantly different from that of 0.8 mM concentration. Therefore, 0.8 mM concentration of α-MSH was selected for further studies in other groups of rats. The onset, peak, and duration of the responses elicited by 0.8 mM concentration of α-MSH were 5–15 s, 40–90 s, and 10–15 min, respectively.

Effect of bilateral vagotomy. In these experiments (n = 7), nAmb was identified by microinjection of L-Glu (5 mM); a decrease in HR (81.4 ± 11 bpm) was observed. Five minutes later, microinjections of α-MSH (0.8 mM) at the same site elicited a bradycardia (38.6 ± 4 bpm). Forty minutes later, bilateral vagotomy was performed, and a 10-min period of stabilization was allowed. Bilateral vagotomy did not change the basal MAP; the MAP values before and after bilateral vagotomy were 103.3 ± 6 and 102.5 ± 6 mmHg, respectively (P > 0.05). However, bilateral vagotomy increased the basal HR significantly; the basal HR values before and after the vagotomy were 415 ± 8 and 438.3 ± 8 bpm, respectively (P < 0.01). Microinjections of L-Glu, as well as α-MSH into the same site in the nAmb failed to elicit bradycardic responses after bilateral vagotomy (Fig. 1B).

Reproducibility of α-MSH-induced responses. Absence of tachyphylaxis to repeated microinjections of α-MSH was tested as follows (n = 5). The concentration of α-MSH that elicited maximal bradycardic responses (0.8 mM) was microinjected into the nAmb at least 3 times, at 40-min intervals. The decreases in HR induced by three consecutive microinjections of α-MSH were 46.3 ± 3, 43.3 ± 7, and 47.6 ± 3 bpm, respectively (P > 0.05). Thus, no tachyphylaxis of responses was observed with repeated microinjections of α-MSH when the interval between injections was 40 min.

Site specificity of α-MSH responses. The concentrations of α-MSH that elicited maximal bradycardic responses when microinjected into the nAmb (n = 5) did not elicit a response when injected intravenously, indicating that leakage of the drug, if any, from the injection site to the peripheral circulation was not responsible for the observed responses. The bradycardic responses elicited from the nAmb were site specific because microinjections of α-MSH (0.8 mM) or L-Glu (5 mM) into the adjacent areas (e.g., ventral region of the dorsal medullary reticular nucleus; 0.6 mm rostral and 2.3 mm lateral to the CS, and 2.6 mm deep from the dorsal medullary surface) elicited no HR responses.

Blockade of α-MSH responses by MC4R antagonist. Prior to microinjections of α-MSH or MC4R antagonist, the nAmb was always identified by microinjections of L-Glu (5 mM), which elicited decreases in HR (76 ± 10 bpm) without concomitant changes in MAP. In one group of rats (n = 5), 5 min after the microinjection of L-Glu, microinjection of the maximally effective concentration of α-MSH (0.8 mM) into the nAmb elicited a decrease in HR (34 ± 4, bpm). Forty minutes later, the MC4R antagonist (0.1 mM) was microinjected at the same site, which was followed by another microinjection of α-MSH. The bradycardic responses to microinjections α-MSH (0.8 mM) were attenuated (15 ± 4 bpm) by prior microinjections of MC4R antagonist into the nAmb, but the reduction in response was not statistically significant (P > 0.05). Forty minutes later, α-MSH was microinjected again at the same site, and a bradycardia (20 ± 2 bpm) was observed, indicating that there was a recovery of responses (Fig. 2A). In another group of rats (n = 10), microinjection of α-MSH (0.8 mM) into the nAmb elicited a decrease in HR (43 ± 3 bpm), which was completely blocked (1.5 ± 1, bpm) by prior microinjections of a higher concentration (0.2 mM) of the MC4R antagonist (P < 0.05). Recovery of α-MSH-induced bradycardic responses (38 ± 3, bpm) was observed within 40 min (Fig. 2B). Microinjections of the MC4R antagonist (0.1 mM) did not alter the responses to L-Glu; the decreases in HR before and after the microinjection of the antagonist were 76 ± 10 and 81 ± 11 bpm, respectively (Fig. 2C). Even higher concentration of the antagonist (0.2 mM) did not alter the responses to L-Glu; the decreases in HR before and after the microinjection of the antagonist were 69 ± 8 and 90 ± 20 bpm, respectively (Fig. 2D). Microinjections of aCSF did not alter the responses to α-MSH. Microinjections of the MC4R antagonist alone into the nAmb did not elicit significant cardiovascular responses. A typical tracing of the blockade of α-MSH responses in the nAmb is shown in Fig. 3.

Effect of α-MSH in decerebrate rats. The nAmb was identified by microinjection of L-Glu (5 mM) in a group of decerebrate rats (n = 9); a decrease in HR (168.9 ± 18 bpm) was observed. Five minutes later, microinjections of α-MSH (0.8 mM) at the same site elicited bradycardia (43.9 ± 6 bpm) with no changes in MAP.

Effect of MC4R antagonist on baroreflex. In a group of rats (n = 5), the nAmb was identified bilaterally by microinjections of L-Glu (5 mM); the decrease in HR was 95.8 ± 17.2 bpm. The following three protocols were used to test the baroreceptor function (Fig. 4). First, control baroreflex responses were obtained before any microinjections into the nAmb; reflex decreases in HR (% change from basal value) were 1.06 ± 0.4, 5.16 ± 1.3, 8.44 ± 1.8, 12.22 ± 1.8, 14.55 ± 1.7, and 19.6 ± 3.5 in response to 10, 20, 30, 40, 50, and 60 mmHg increases in aCSF. Microinjections (30 nl) of L-Glu; the decreases in HR before and after the microinjection of L-Glu (5 mM) were 5–15 s, 40–90 s, and 10–15 min, respectively. The onset, peak, and duration of the responses elicited by 0.8 mM concentration of α-MSH were 5–15 s, 40–90 s, and 10–15 min, respectively. The following three protocols were used to test the baroreceptor function (Fig. 4). First, control baroreflex responses were obtained before any microinjections into the nAmb; reflex decreases in HR (% change from basal value) were 1.06 ± 0.4, 5.16 ± 1.3, 8.44 ± 1.8, 12.22 ± 1.8, 14.55 ± 1.7, and 19.6 ± 3.5 in response to 10, 20, 30, 40, 50, and 60 mmHg increases in aCSF. Microinjections (30 nl) of L-Glu; the decreases in HR before and after the microinjection of L-Glu (5 mM) were 5–15 s, 40–90 s, and 10–15 min, respectively. The onset, peak, and duration of the responses elicited by 0.8 mM concentration of α-MSH were 5–15 s, 40–90 s, and 10–15 min, respectively. The following three protocols were used to test the baroreceptor function (Fig. 4). First, control baroreflex responses were obtained before any microinjections into the nAmb; reflex decreases in HR (% change from basal value) were 1.06 ± 0.4, 5.16 ± 1.3, 8.44 ± 1.8, 12.22 ± 1.8, 14.55 ± 1.7, and 19.6 ± 3.5 in response to 10, 20, 30, 40, 50, and 60 mmHg increases in aCSF.
of MC4R antagonist into the nAmb (open triangles and dashed line). The
the nAmb (open circles and dotted line), and after bilateral microinjections
control baroreflex responses before any microinjections into the nAmb (solid
circles and straight line), after bilateral microinjections of aCSF (30 nl) into
MAP. Twenty minutes later, the same protocol was repeated
in MAP. Twenty minutes later, the same protocol was repeated after bilateral microinjections of aCSF into the nAmb; reflex decreases in HR were 0.84 ± 0.5, 4.28 ± 1.7, 7 ± 2.4, 11.6 ± 2.2, 14.4 ± 2.1, and 17.75 ± 3%. After an interval of 20 min,
the same protocol was repeated after bilateral microinjections of MC4R antagonist (0.2 mM) into the nAmb; reflex decreases in HR were 0.8 ± 0.4, 4.82 ± 1.4, 8.11 ± 1.9, 9.88 ± 2, 11.66 ± 2.1, and 17.55 ± 2.5%. No significant differences
(P > 0.05) in the reflex HR decreases in response to different increases in MAP were observed after the microinjections of either aCSF or MC4R antagonist into the nAmb. The concentration of MC4R antagonist (0.2 mM) used in these experiments was previously shown to completely block the effects of the maximally effective concentration of α-MSH (0.8 mM) in the nAmb (see Blockade of α-MSH responses by MC4R antagonist).

Effect of α-MSH on nAmb neurons. Activity was extracel-
lularly recorded from a total of 36 neurons in 7 rats. Twenty-
two neurons were spontaneously active, and the remaining 14 were initially silent but were activated by application of L-Glu (5 mM, 2 nl). The increases in neuronal firing mentioned below refer to maximum changes in firing compared with control firing just before the intravenous injection of PE or direct application of drugs. The volume of aCSF or drugs directly ejected onto the neurons was always 2 nl. Involvement of the recorded neurons in HR control was tested by their excitation in response to intravenous bolus injections of PE. Eight of these neurons did not respond to bolus injection of PE; the firing rate before and after PE was 2.5 ± 1 and 2.2 ± 1 spikes/s, respectively. These neurons were not studied any further. Direct application of aCSF to remaining neurons (28 neurons) elicited minimal changes in their firing (0.35 ± 0.2 spikes/s; P > 0.05) (Fig. 5A). After 3–4 s, an intravenous bolus injection of PE elicited a reflex increase in firing rate (13.6 ± 2 spikes/s; P < 0.001) (Fig. 5B). The increases in MAP were 15 ± 3 and 26 ± 5 mmHg in response to intravenous bolus injections of 2 and 4 μg/kg of PE, respectively. After an interval of 3–4 s, direct application of L-Glu (5 mM) elicited an increase in firing rate (19.6 ± 2 spikes/s; P < 0.001) (Fig. 5C). After the neuronal firing returned to basal rates, 3–4 s later, α-MSH (0.8 mM) was directly applied to these neurons; the neuronal firing rate was significantly increased (10.6 ± 1

Fig. 3. Blockade of α-MSH responses in the nAmb. Top: tracing shows pulsatile arterial pressure (PAP, mmHg); middle: tracing shows mean arterial pressure (MAP, mmHg), bottom: tracing shows heart rate (HR, beats/min).

A: microinjection of L-Glu (5 mM) into the nAmb elicited a decrease in HR but no change in MAP. B: microinjection of artificial cerebrospinal fluid (aCSF) at the same site elicited no responses. C: after 5 min, microinjection of alpha-MSH (0.8 mM) at the same site elicited bradycardic response. After an interval of 40 min, aCSF was microinjected again at the same site. Within 5 min, MC4R antagonist (0.2 mM) was microinjected at the same site (not shown). D: two minutes later, alpha-MSH was again microinjected at the same site; the responses to alpha-MSH were blocked. E: two minutes later, the responses to microinjections of L-Glu (5 mM) were tested; the MC4R antagonist did not alter L-Glu-induced responses. F: bradycardic responses to alpha-MSH recovered within 40 min.

Fig. 4. Baroreflex function curves. Linear regression curves showing the control baroreflex responses before any microinjections into the nAmb (solid circles and straight line), after bilateral microinjections of aCSF (30 nl) into the nAmb (open circles and dotted line), and after bilateral microinjections of MC4R antagonist into the nAmb (open triangles and dashed line). The r^2 values for the regression curves of the bradycardic reflex were 0.993, 0.996, and 0.966 for control, aCSF, and MC4R antagonist groups, respectively.

Fig. 5. Group data of the effect of alpha-MSH on nAmb neurons (n = 28, 7 rats). A: direct application of aCSF to the neuron elicited a small change (P > 0.05) in firing rate; the volume of all direct applications onto the neurons was 2 nl. B: intravenous bolus injection of phenylephrine (PE) elicited an increase (P < 0.001) in the neuronal firing. C: application of L-Glu (5 mM) increased (P < 0.001) the neuronal firing. D: application of alpha-MSH (0.08 mM) increased the neuronal firing (P < 0.01). E: prior application of the MC4R antagonist (0.2 mM) to the neuron blocked the excitatory effect of alpha-MSH on these neurons (P < 0.05). F: antagonist did not alter the responses (P < 0.001) to application of L-Glu. G: increase in firing (P < 0.01) in response to alpha-MSH application recovered within 10–15 s.

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spikes/s; \( P < 0.01 \) (Fig. 5D). The duration of \( \alpha \)-MSH effect was 6.5 ± 1 s. Subsequently, MC4R antagonist was directly applied to the neuron; no significant change in firing was observed. After an interval of 3–4 s, \( \alpha \)-MSH was again applied onto the neuron; the change in neuronal firing (0.9 ± 0.2 spikes/s) was not statistically significant (\( P > 0.05 \)), indicating that the effect of \( \alpha \)-MSH was blocked by the antagonist (Fig. 5E). The MC4R antagonist did not alter the responses to L-Glu, which elicited an increase in neuronal firing rate (19.6 ± 1.9 spikes/s; \( P < 0.01 \)) (Fig. 5F). The neuronal response to \( \alpha \)-MSH application recovered within 10.5 ± 1 s; application of \( \alpha \)-MSH elicited a significant increase in neuronal firing (9.9 ± 1.1 spikes/s; \( P < 0.01 \)) (Fig. 5G). Typical tracings of a neuronal recording are shown in Fig. 6.

**Immunohistochemical staining of MC4Rs in the nAmb.** Immunostaining for MC4Rs was observed in the nAmb (\( n = 5 \)). Fig. 7A shows in low magnification the general location of the nAmb in an immunostained section. A cresyl violet-stained adjacent section confirmed the general location of nAmb (Fig. 7B). At a higher magnification of the circled area in Fig. 7A, MC4Rs were seen on the cells and fibers in the nAmb (Fig. 7C). When the primary antibody was omitted as a control procedure, no labeling of MC4Rs in the nAmb was detected (Fig. 7D).

**Histological identification of microinjection sites.** The nAmb sites, where microinjections of L-Glu and \( \alpha \)-MSH elicited bradycardia were marked with India ink in 22 rats. A typical nAmb site marked with India ink (30 nl) is shown in Fig. 8A. Composite diagrams of the microinjection sites in the nAmb are presented in Figs. 8, B–F.

**DISCUSSION**

The main observation in this study was that microinjections of \( \alpha \)-MSH into the nAmb elicited bradycardic responses. This is the first report of central cardiovascular effects of \( \alpha \)-MSH in
the nAmb. Local distortion of brain tissue or any nonspecific effects were not responsible for these observations because microinjections of aCSF into the nAmb did not elicit any response. Concentrations of α-MSH microinjections into the nAmb that elicited decreases in HR did not elicit a response when injected intravenously, indicating that leakage of α-MSH, if any, from the microinjection site into the peripheral circulation was not responsible for the observed responses. The α-MSH-induced bradycardic responses elicited from the nAmb were site specific because similar microinjections into the adjacent areas (e.g., ventral part of the dorsal medullary reticular nucleus; 0.6 mm rostral and 2.3 mm lateral to the CS, and 2.6 mm deep from the dorsal medullary surface) elicited no responses. In view of the proximity of caudal ventrolateral medulla (CVLM) to the nAmb, it is possible that α-MSH-induced bradycardic responses could be partly mediated via the CVLM. However, bilateral vagotomy abolished the α-MSH or L-Glu-induced responses, excluding this possibility. Moreover, microinjections of L-Glu, used for the identification of nAmb, did not elicit either a pressor or depressor response, suggesting that the RVL and CVLM did not participate in these responses, respectively. In this context, it may be noted that bradycardia with no concomitant arterial pressure responses to microinjections of L-Glu was consistently used as the criterion for the identification of the nAmb in all experiments.

Microinjections of α-MSH into the nAmb of midcollicular decerebrate rats also elicited bradycardic responses, which were not statistically different from those elicited in urethane-anesthetized rats. These experiments revealed that urethane anesthesia did not alter the responses to α-MSH qualitatively or quantitatively. Moreover, the presence of neural structures located rostral to the brain stem (e.g., hypothalamus) was not necessary for α-MSH-induced effects in the nAmb.

The mechanism of the bradycardic responses elicited by α-MSH microinjections into the nAmb can be explained as follows based on our current knowledge regarding the medullary control of cardiac function (24, 36, 41, 42). α-MSH may have excited parasympathetic preganglionic nAmb neurons, increased the vagal input to the heart, causing bradycardia. This contention was supported by the results of our extracellular neuronal recording in vivo, in which direct application of α-MSH to nAmb neurons resulted in excitation of these neurons. α-MSH-induced excitation of nAmb neurons was mediated via MC4Rs because prior applications of the MC4R antagonist prevented the excitatory effect of α-MSH on these neurons. Moreover, excitation of these neurons by direct applications of L-Glu indicated that activity was being recorded from neurons and not fibers of passage (23). Although the volume ejected by pressure application was very small (2 nl), activation of adjacent interneurons cannot be excluded entirely. Glutamate receptors are likely to be present on the nAmb neurons containing MC4Rs. This possibility is consistent with the ubiquitous distribution of glutamate receptors in the CNS. Reflex activation of nAmb neurons by intravenous bolus injections of PE indicated that the recorded neurons were involved in HR control (34). Lack of responses to aCSF applications indicated that neuronal excitation was not due to an artifact. In agreement with previous reports (23, 24, 25), many (38.8%) neurons in the nAmb were found to be initially silent. There are no reports regarding the effect of α-MSH on extracellular neuronal activity in vivo for comparison of our results. However, patch-clamp studies in vitro have shown that MC4R agonists depolarize some neurons (e.g., POMC neurons in the hypothalamic arcuate nucleus) by decreasing predominantly transient outward potassium (Iₖ) conductance (39), and this effect is likely to cause an increase in the action potential firing (6).
The bradycardic responses to microinjections of α-MSH into the nAmb were blocked by prior microinjections of a selective MC4R antagonist at the same site, suggesting that these responses were mediated via MC4Rs in this nodule. Cyclo[CO-(CH2)2-CO-D-Nal(2')-Arg-Trp-Lys]-NH2 has been reported to be a selective antagonist for MC4Rs (2). Furthermore, this antagonist did not alter responses to microinjections of an unrelated agonist, l-Glu, into the nAmb. Since microinjections of the antagonist did not elicit significant cardiovascular responses, it was concluded that under normal physiological conditions MC4Rs in the nAmb were not under tonic control of endogenous α-MSH.

The presence of MC4Rs in the nAmb was confirmed by our immunohistochemical studies. There are no immunohistochemical studies on the presence of MC4Rs in the nAmb for comparison of our results. However, in situ hybridization studies have shown the presence of MC4R mRNA in the nAmb (27, 40). The presence of α-MSH-containing fibers in the nAmb has been reported (30). These fibers arise in the hypothalamus and descend in lateral ventrolateral tegmental fiber pathway to the nAmb. The descending fibers decussate in the midbrain and thereafter project ipsilaterally to the nAmb (11, 15, 30). Activation of these descending pathways may release α-MSH in the nAmb, activate preganglionic parasympathetic neurons, which innervate the heart via the vagus nerves, and elicit bradycardia. Similar α-MSH projections from the hypothalamus to the dorsal vagal nucleus complex have been reported (20).

The majority of cardiac vagal neurons in the nAmb is normally silent, and their firing is controlled by synaptic inputs (45). Major synaptic inputs to cardiac vagal neurons in the nAmb include excitatory glutamatergic pathways from the NTS, excitatory cholinergic inputs that facilitate glutamatergic synaptic pathways innervating these neurons, and inhibitory GABA inputs (44) that may mediate the inhibition of these neurons during inspiration. In our study, microinjections of the MC4R antagonist into the nAmb did not alter reflex bradycardic responses elicited by intravenous infusions of PE, suggesting that α-MSH does not influence the parasympathetic component of baroreflex-induced bradycardia.

It is possible that MC4Rs in nAmb are activated in some pathological conditions. As mentioned in the introduction, α-MSH has been reported to reverse hemorrhagic shock conditions and provide strong protection against myocardial ischemia/reperfusion in experimental animals and humans (9, 10). These effects have been reported to be mediated via MC4Rs in the brain and involve activation of an effenter vagal cholinergic pathway (26). Although this effenter cholinergic pathway has not been characterized, it may involve the nAmb. It is possible that in the aforementioned pathological conditions, α-MSH is released from α-MSH-containing terminals in the nAmb, activating MC4Rs on parasympathetic preganglionic neurons providing innervations to the heart and causing bradycardia.

In summary, microinjections of α-MSH into the nAmb elicit bradycardic responses, which are mediated via MC4Rs. Direct application of α-MSH to nAmb neurons revealed that this peptide excites these neurons via MC4Rs. It was concluded that activation of nAmb neurons caused bradycardia via an increase in vagal activity because bilateral vagotomy abolished the bradycardic effect of α-MSH.

**Perspectives and Significance**

We hypothesize that in pathological situations such as stress, ischemic stroke and hemorrhagic shock, α-MSH may be released in the nAmb, causing stimulation of parasympathetic preganglionic neurons, efferent vagal activation and consequent bradycardia. Bradycardia can be beneficial in certain pathological conditions and may provide strong protection against myocardial ischemia/reperfusion injury. An alternative approach for the treatment of these conditions could be to activate pharmacologically a physiologically well-defined vagal cholinergic pathway controlling cardiac function using a relatively safe agent, such as α-MSH. These possibilities remain to be explored.

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REFERENCES


