Exercise Training Differentially Affects Intrinsic Excitability of Autonomic and Neuroendocrine Neurons in the Hypothalamic Paraventricular Nucleus

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INTRODUCTION

During exercise training (ET), dynamic cardiovascular adjustments take place to maintain proper blood pressure and to efficiently adjust blood supply to different vascular beds according to regional metabolic demands. Cardiovascular responses to acute dynamic exercise include moderate increase in blood pressure accompanied by a marked tachycardia (Ludbrook 1983; Michelin and Morris 1999; Rowell 1992), whereas ET leads to unchanged pressure levels with reduced baseline heart rate (Clausen 1977; Moore and Palmer 1999). Although these changes are known to be mediated in large by adjustments in autonomic control of the heart, including parasympathetic withdrawal and sympathetic activation (Negrao et al. 1992; Robinson et al. 1966), the precise central mechanisms underlying exercise-induced changes in cardiovascular control have yet to be fully characterized.

The primary source of OT and VP neuropeptides in the NTS is the paraventricular nucleus of the hypothalamus (PVN) (Buijs et al. 1978; Sawchenko and Swanson 1982; Sofroniew 1980), a key center for the integration of autonomic and neuroendocrine responses (Swanson and Sawchenko 1980). A growing body of evidence supports an important role for the PVN in the control of cardiovascular function under various physiological as well as pathological conditions (Akine et al. 2003; Allen 2002; Deering and Coote 2000; Porter and Brody 1985, 1986; Veerasingham et al. 2005; Zhang et al. 2002; Zhang and Ciriello 1985). Furthermore, work from our laboratory indicates that projections from the PVN to the dorsal brain stem are activated during exercise training (Braga et al. 2000; Dufloth et al. 1997).

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METHODS

Male Wistar rats (n = 16, 120-150 g) were purchased from Harlan Laboratories, Indianapolis, IN. Rats were housed in a 12/12-h light/dark schedule and allowed free access to food and water. All the procedures used in these studies adhere to the policy of Wright State University regarding the use and care of animals.

Exercise training protocol

Rats displaying ability to walk on a treadmill (4–6 sessions at 0.2 ≤ 0.4 mph, 0% grade, ~10 min/day), were randomly assigned to exercise training (ET, n = 8) or sedentary (S, n = 8) groups. Their treadmill performance and body weight at the beginning of the experiments was similar between groups. (see Fig. 1, A and C). Low-intensity ET protocol was performed twice/day, 5 days/wk over 6 wk. Exercise intensity was increased progressively by a combination of time and velocity, attaining the maximal duration (45 min/session) by week 2. Increments of treadmill speed were also progressive to attain 50–60% of maximal exercise capacity as determined by means of progressive exercise tests on weeks 0, 2, and 4. The test consisted of graded exercise on the treadmill, starting at 0.2 mph, with increments of 0.2 mph every 3 min up to the maximal running speed attained by each rat. Tests were also repeated for both groups by week 6 just to compare the efficacy of the training protocol. Rats allocated to S protocol were handled every day. During S and ET protocols, body weight was measured weekly.

Retrograde labeling of PVN-NTS projecting neurons

At the end of the sixth week of the ET protocol, both S and ET rats were anesthetized by intraperitoneal injection of ketamine/xylazine mixture (90 mg/kg and 5 mg/kg, respectively), placed in a stereotaxic frame and injected with a fluorescent retrograde tracer in the area of the NTS as previously described (Li et al. 2003). The dorsal medulla was exposed after retraction of overlying muscles and occipital membranes. A small part of the occipital bone was removed to expose the medulla. Rhodamine-labeled microspheres (Lumaflor, Naples FL) were pressure-injected unilaterally (200 nl) into the NTS area at the level of the obex. The injection point was 1.0 mm lateral to the midline, and 0.8 mm below the dorsal surface. After the injection, muscles were sutured together, and the wound was closed. The location and extension of the injection sites were confirmed histologically. Injections in the NTS area were mostly restricted to caudal aspects of the nucleus of the solitary tract, although they also extended ventrally into the dorsal motor nucleus of the vagus (see also Stern 2001).

Slice preparation

Three days after the injection of the retrograde tracer, coronal hypothalamic slices (150–300 μm) containing the SON and PVN from S and ET rats in the preceding text, were obtained for electrophysiological recordings using a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding, CA) as previously described (Stern et al. 1999). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused through the heart with cold artificial cerebrospinal solution (ACSF) in which NaCl was replaced by an equimolar amount of sucrose, a procedure known to improve the viability of neurons in adult brain slices (Aghajanian and Rasmussen 1989). The standard ACSF solution contained (in mM) 120 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, 26 NaHCO3, 20 glucose, and 0.4 ascorbic acid, pH 7.4 (297–300 mosM). After the slicing procedure, hypothalamic slices were placed in a holding chamber containing standard ACSF and stored at room temperature (22–24°C) until used.

Electrophysiology and data analysis

For electrophysiological recordings, a slice was transferred to a submersion-type recording chamber, continuously perfused (~2 ml/min) with a standard solution bubbled with a gas mixture of 95% O2-5% CO2. All recordings were performed at 30–32°C. Patch pipettes (3–5 MΩ) were pulled from thin-wall (1.5 mm OD, 1.17 mm ID) borosilicate glass (GC150T-7.5, Clark, Reading, UK) on a horizontal electrode puller (P-97, Sutter Instruments, Novato, CA). The pipette internal solution contained (in mM) 135 K-gluconate, 20 KCl, 10 HEPES (pH 7.4), 4 MgATP, 20 phosphocreatine (Na*), 0.3 NaGTP, and 0.2 EGTA, pH 7.3 (295 mosM). For intracellular labeling, biocytin (0.2%) was added to the internal solution. Whole cell recordings of SON/PVN neurons were made under visual control using infrared differential interference contrast (IR-DIC) video microscopy in combination with epifluorescence illumination (in the case of fluorescently labeled, PVN-NTS projecting neurons) as previously described (Stern 2001). Recordings were obtained with a Multiclamp 700B (Axon Instruments, Foster City, CA) amplifier. No correction was made for pipette liquid junction potential (measured to be ~10 mV). The series resistance was monitored throughout the experiment, and data were discarded if series resistance during recordings doubled from the one obtained at the beginning of the recording. The voltage output was digitized at 16-bit resolution in conjunction with pClamp 8 software (Digidata 1320, Axon Instruments). Data were digitized at 10 kHz and transferred to a disk. All neurons included in the analysis had membrane potentials of ~40 mV or more negative, and action potentials of at least +55 mV. Cell input resistance and cell capacitance was calculated in voltage clamp using a 5-mV pulse while holding the cells at ~70 mV. To measure action potential properties, neurons were current-clamped close to threshold and a 5-mA, 0.1-nA depolarizing pulse was applied. Spike height was measured from the beginning of the fastest rising phase to the peak of the action potential, whereas spike width was measured at 50% of the peak, from threshold.

To study the input-output relationship of SON/PVN neurons, repetitive firing was evoked by injecting depolarizing current pulses of varying amplitudes. Frequency-current (F-I) plots were generated by plotting the instantaneous firing frequency (i.e., the inverse of the interspike interval) for various interspike intervals (ISIs) during the

FIG. 1. Efficacy of the exercise training protocol. Plot of maximal speed attained on treadmill (A) and body weight changes (C) during the 6-week exercise training protocol. Comparison of the effects of ET on treadmill performance at the 6th week (B) and body weight gain during the 6-wk period (D). *P < 0.05 vs. sedentary, Student’s t-test.

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depolarizing steps. Similarly, frequency-time (F-T) plots were constructed by plotting the time course of the instantaneous firing frequency of spikes evoked during pulses of varying amplitudes. F-I and F-T plots were fitted by either a linear or exponential functions, respectively, and the slope and decay time constants of these functions calculated and compared between S and ET groups.

To quantitatively assess changes in HAP amplitude during repetitive firing, the HAP amplitudes of individual spikes evoked by a 120-pA depolarizing pulse were determined. In addition, the percent-change in amplitude of each progressive HAP (with respect to the 1st spike HAP) was plotted as a function of the spike number within the train. This plot was best fitted by a linear regression function, and the slope of this function was used to determine and compare the rate of progressive decrease in HAP amplitude between experimental groups. The afterhyperpolarizing potential (AHP) area was measured of progressive decrease in HAP amplitude between experimental groups. The afterhyperpolarizing potential (AHP) area was measured of progressive decrease in HAP amplitude between experimental groups. The afterhyperpolarizing potential (AHP) area was measured of progressive decrease in HAP amplitude between experimental groups.

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Retrograde tracing and oxytocin/vasopressin immunohistochemistry

In a separate set of experiments (n = 4 control rats), immunohistochemistry was performed to study co-localization of a retrograde tracer and oxytocin (OT) or vasopressin (VP) immunoreactivity in PVN-NTS neurons. For these studies, we used Fluorogold (4% in PBS) as a fluorescent retrograde tracer (Ju et al. 1989). In our experience, this is the fluorescent tracer that is best preserved after immunohistochemical procedures. The injection procedure was the same as described in the preceding text. Three days after the injections, rats were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and perfused transcardially with 0.1M phosphate buffered saline (PBS, 150 ml) followed by 4% paraformaldehyde (500 ml). Fixed brains were cryoprotected at 4°C with a 0.1M phosphate buffer with 30% sucrose for a minimum of 48 h. Sections (30 μm) were then cut using a cryostat, transferred into 0.01M PBS, and incubated in a solution of 0.01M PBS with 0.01% Triton X-100 and 10% normal goat serum for 1 h. Sections were then incubated 24 h in a cocktail of primary antibodies containing a rabbit polyclonal anti-Fluorogold antibody (Chemicon International, Temecula, CA, n = 1:10,000) and a polyclonal guinea pig anti-OT or anti-VP (Bachem, Torrence, CA, n = 1:100,000 dilution). The reaction was followed by a 4-h incubation in the presence of fluorescently labeled secondary antibodies (donkey anti-rabbit FITC-labeled and donkey anti-guinea pig Cy3-labeled antibody; 1:400). All antibodies were diluted with PBS containing 0.1% Triton X-100. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Control experiments were performed by omitting primary or secondary antibodies.

Confocal imaging acquisition and analysis

Sequential 30-μm sections through the hypothalamus, between Bregma –1.80 and –2.12 ( Paxinos et al. 1985) were immunostained as described in the preceding text. Sections were examined with a Zeiss LSM 510 confocal microscope system. An argon-krypton laser was used to excite the FITC and Cy3 fluorochromes, at 488 and 543 nm, respectively. Fluorescent signal cross-talk among the channels was avoided by setting image-acquisition parameters with individually labeled sections. Each optical section was averaged three times. Six consecutive optical focal planes were obtained, and a projection image of the focal planes was displayed. To determine co-localization of the two fluorescent markers, confocal images obtained from the different fluorophores were merged. A positive co-localization was considered by the appearance of yellow (red + green) profiles in the merged image. Furthermore, an identifiable structure needed to be clearly discernible in each image prior to merging. The number of retrogradely labeled and/or OT/VP immunoreactive cells was counted in the ipsilateral PVN (with respect to the brain stem injection). A total number of 587 retrogradely labeled neurons located throughout the rostrocaudal extension of the PVN were analyzed for co-expression with OT or VP. The number of double-labeled neurons was obtained and expressed as percent co-localization. Figures were composed using Adobe Photoshop (Adobe Systems).

Statistics

Numerical data are presented as means ± SE. Unpaired Student’s t-test was used for comparing electrophysiological properties between neurons recorded from S and ET rats. However, when necessary, and as indicated in the text, a two-way ANOVA was used. Because no differences were observed between SON and PVN MNCs for any of the variables studied (results not shown), data from these neuronal populations were pooled together. Differences in the incidence of functional properties across experimental groups (e.g., low-threshold spikes, spontaneous firing activity), were analyzed using a χ² test was used. Differences were considered statistically significant at P < 0.05.

RESULTS

Efficacy of the exercise training protocol

As depicted in Fig. 1A, the low-intensity training protocol efficiently increased the maximal running speed in the ET group, whereas no change was observed in S rats (from 0.78 ± 0.04 to 2.01 ± 0.05 mph, P < 0.05 for ET and from 0.80 ± 0.04 to 0.76 ± 0.04 mph for S rats, week 0 to week 6, respectively). After 6 wk of training, effective running distance attained during maximal exercise test was markedly increased in the ET group when compared with S controls (919 ± 74 vs. 145 ± 12 m, a 6.3-fold increment). During this period of time, body weight increased in both groups (from 178 ± 3 to 342 ± 11 g in ET and 183 ± 4 to 400 ± 8 g in S rats, P < 0.05, Fig. 1B), but weight gain was significantly reduced in the trained group when compared with the S group (+164 ± 8 vs. +215 ± 6 g, ET vs. S, respectively, P < 0.05).

Exercise-induced changes in basic intrinsic membrane properties PVN-NTS neurons and MNCs

Electrophysiological recordings from these S and ET rats were obtained from MNCs located in the SON (n = 17), MNCs located in the lateral magnocellular subdivision of the PVN (Swanson and Kuypers 1980), n = 14, and retrogradely labeled, PVN-NTS projecting neurons (n = 34). All of the later were located within the ventral parvocellular (PaV) subnucleus of the PVN, and based on their general appearance, 30/34 were classified as type A, as previously described (Stern 2001). Due to incomplete intracellular labeling, we were unable to determine the type of the remaining four neurons.

A typical example of an identified PVN-NTS neuron in the hypothalamic slice preparation is shown in Fig. 2A. Using retrograde tract tracing in combination with immunohistochemistry in fixed brain tissue (see METHODS), a large proportion of PVN-NTS neurons was found to be immunoreactive for OT (41.0%, 80/195) or VP (21.2%, 83/372; see Fig. 2B). As summarized in Table 1, resting membrane potential (V_m) and input resistance did not differ between S- and ET-PVN-
NTS neurons (n = 17 on each group). On the other hand, cell capacitance was significantly larger in ET-PVN-NTS neurons (+21%, P < 0.05). No differences in the incidence of spontaneous firing activity was observed between S- and ET-PVN-NTS neurons (12/14 and 11/14, respectively, P > 0.05, χ² test).

Electrophysiological recordings were also obtained from SON/PVN MNCs (n = 13 and 18 in S and ET rats, respectively). As summarized in Table 1, no changes in resting V̄, input resistance or cell capacitance were observed in MNCs after ET (P > 0.05 in all cases).

Exercise-induced changes in action potential properties of PVN-NTS neurons and MNCs

Individual Na⁺ action potentials were evoked in S- and ET-PVN-NTS neurons (n = 17 in each group). Na⁺ spike threshold values were similar between S and ET-PVN-NTS neurons (−32.8 ± 2.0 and −33.1 ± 1.1 mV, respectively, P > 0.05). Representative examples of Na⁺ spikes obtained from an S and ET rats are shown in Fig. 3A, and the mean values corresponding to various spike properties are shown in B. Spikes recorded from ET-PVN-NTS neurons were larger in amplitude (−13%, P < 0.01) and displayed faster rise times (~27%, P < 0.05) when compared with S NTS-projecting neurons. On the other hand, no differences were observed in either spike width or decay rate (P > 0.05 in both cases).

Na⁺ spikes in PVN-NTS neurons were followed by a prominent hyperpolarizing afterpotential (HAP). The amplitude of the HAP was similar in S- and ET-PVN-NTS neurons (−16.4 ± 1.8 and −15.7 ± 1.9 mV, respectively, n = 17/group, P > 0.05).

Differently from PVN-NTS neurons, the action potential waveform of SON/PVN MNCs did not differ between S and ET animals (n = 10/each group, see Fig. 3B). Similarly, no differences were observed in the HAP amplitude between S and ET MNCs (S: −17.6 ± 3.9 mV; ET: −22.2 ± 2.6 mV, P > 0.05).

ET enhances the input-output function in PVN-NTS neurons

Repetitive firing was evoked by injecting depolarizing current pulses of incremental amplitude (10–200 pA). Representative examples of repetitive firing evoked in S- and ET-PVN-NTS neurons are shown in Fig. 4.

To characterize the input-output (I/O) function, a plot of the number of evoked action potentials as a function of current was generated (see Fig. 4B). Qualitatively, the pattern of the I/O

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<th>TABLE 1. Basic membrane properties of PVN-NTS neurons and MNCs in the PVN and SON of S and ET rats</th>
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Summary of resting membrane potential (V̄), input resistance (IR) and cell capacitance (CC) values, of paraventricular nucleus of the hypothalamus (PVN)-nucleus of the solitarii tract (NTS) neurons and magnocellular cells (MNCs) obtained from sedentary (S) and exercise training (ET) rats. Cell capacitance was significantly higher in ET-PVN-NTS-projecting, when compared to S-PVN-NTS neurons (*P < 0.05). Numbers of neurons is in parentheses.
function differed between S- and ET-PVN-NTS neurons. In S-PVN-NTS neurons, the plot displayed a roughly parabolic relationship: the firing discharge increased progressively at low intensity stimulation, reached a peak at $94.6 \pm 10.4$ pA ($\sim 45\%$ of maximum stimulation), and decreased thereafter. The decrease in firing frequency at relatively stronger depolarization was observed to occur concomitantly with a large degree of adaptation and/or dampening of action potential amplitude, which sometimes resulted in failure to evoke an action potential (see Fig. 4A). On the other hand, the I/O plot obtained from ET-PVN-NTS neurons displayed a linear relationship throughout the stimulus intensity range. In these neurons, the firing discharge increased progressively with stimulation amplitude with little or no evidence of a high degree of adaptation and/or action potential dampening (see Fig. 4B). The peak firing frequency was observed on average at $180.3 \pm 59.6$ pA ($\sim 90\%$ of the maximum stimulation) a value significantly higher than that observed in S-PVN-NTS neurons ($P < 0.0001$). Overall, a two-way ANOVA indicated that the I/O function was significantly different between S- and ET-PVN-NTS projecting neurons, the latter generating a higher number of action potentials in response to depolarizing stimulation ($F = 33.0, P < 0.0001$).

F-I relationships for early and late ISIs were constructed to further characterize the I/O function in these neurons (Fig. 4C). In general, ISIs displayed a linear relationship between current and frequency. In S-PVN-NTS neurons, the mean slope for the first ISI was $0.19 \pm 0.01$ Hz/pA. As a reflection of adaptation, the slope of subsequent ISIs decreased progressively. For example, the slope of the fourth and sixth ISIs were $0.06 \pm 0.02$ and $0.05 \pm 0.02$ Hz/pA, respectively, representing a $68.7 \pm 9.8$ and a $80.2 \pm 8.2\%$ decrease with respect to the first ISI, respectively ($P < 0.0001$ for both 4th and 6th ISIs).

While F-I slopes in ET-PVN-NTS neurons also decreased as a function of the ISI (1st ISI: $0.15 \pm 0.01$ Hz/pA, 4th ISI: $0.10 \pm 0.01$ Hz/pA, and 6th ISI: $0.08 \pm 0.01$ Hz/pA, $P < 0.0001$), the decrease was less pronounced, with slopes of $0.10 \pm 0.01$ Hz/pA and $0.08 \pm 0.01$ Hz/pA, respectively ($P < 0.0001$ for both 4th and 6th ISIs).
0.001 for 4th and 6th when compared with 1st ISI), the degree of change in these neurons was significantly lower that that observed in S-PVN-NTS neurons (4th ISI: 36.2 ± 6.5%, P < 0.01; 6th ISI: 58.8 ± 7.5%, P < 0.05). In fact, the F-I slopes for ISIs fourth and sixth were significantly steeper than those observed in sedentary rats (P < 0.05).

Differences in adaptation during repetitive firing between the two experimental groups also become clear when plots of instantaneous firing rate versus time (F-T) were constructed (see Fig. 4D). In S-PVN-NTS neurons, and for relatively small current steps (e.g., 60 pA), the F-T plots showed frequency adaptation and were best fitted by a monoexponential function with a mean time constant (τ) of 111.8 ± 40.6 ms. At higher current steps though, half of the F-T plots in this group were best fitted either by a monoexponential function (with τ = 50.5 ± 10.5 and 66.9 ± 10.6 ms for 90- and 120-pA pulses, respectively) or a linear function (slope: −0.08 ± 0.03 and −0.13 ± 0.05 Hz/ms for 90- and 120-pA pulses, respectively).

In ET-PVN-NTS neurons, a lower degree of adaptation was also evident in the F-T plots. For example, at current steps of 60 pA, evidence of adaptation was observed only in 6/12 tested neurons. The F-T plots of those neurons showing adaptation decayed monoexponentially with a τ = 198.5 ± 42.4 ms (not significantly different from that observed in S-PVN-NTS neurons). The other six F-T plots displayed a mostly flat relationship (results not shown). At higher current steps, and differently from S-PVN-NTS neurons, all the F-T plots decayed monoexponentially with τs significantly slower than those observed in S-PVN-NTS neurons. In this case the τs for 90- and 120-pA current pulses were 176.0 ± 10.4 and 154.9 ± 20.8 ms, respectively (P < 0.03 and P < 0.05 when compared with S-PVN-NTS neurons, respectively).

Altogether, these results indicate a lower degree of adaptation during repetitive firing in PVN-NTS neurons during exercise training.

ET diminished the input-output function of MNCs

Interestingly the I/O function was differentially affected in MNCs. In this case, a two-way ANOVA indicated that ET-MNCs responded to input stimulation by generating a lower number of action potentials than S-MCNs (F = 33.8, P < 0.0001) (see Fig. 5, A and B).

Similarly to PVN-NTS neurons, F-I relationships in MNCs displayed a linear relationship between current and frequency and a progressive decrease in F-I slope at higher ISIs. Representative examples of F-I plots in MCNs are shown in Fig. 5C. No differences were observed in the F-I relationships between S- and ET-MNCs. For example, in S-MNCs, the mean slopes for the first, fourth, and sixth ISIs were 0.13 ± 0.01, 0.09 ± 0.01 (a 23.8 ± 6.3% decrease to 1st ISI), and 0.07 ± 0.01 Hz/pA (a 33.6 ± 9.2% decrease to 1st ISI), respectively. Similar results were observed in ET-MNCs: the mean slopes for the first, fourth, and sixth ISIs were 0.12 ± 0.01, 0.09 ± 0.01 (a 28.8 ± 2.4% decrease with respect to ISI1), and 0.07 ± 0.01 Hz/pA (a 33.5 ± 7.0% decrease with respect to ISI1), respectively (P > 0.05 in all cases).

FIG. 5. ET diminished the input-output function of MNCs. A: repetitive firing evoked in an S- and a ET-MNC (top and bottom, respectively) in response to increasing depolarizing steps. B: plot of the mean number of spikes as a function of current injection. ET-MNCs neurons responded to current stimulation by generating a lower number of action potentials than S-MCNs (F = 33.8, P < 0.0001, 2-way ANOVA, see text). C: frequency-current (F-I) plots for interspike intervals (ISIs) 1, 4, and 6, obtained from the same S- and ET-MNCs shown in A (left and right, respectively). D: F-T plots obtained during depolarizing current steps of 90, 120 and 170 pA, from the same S- and ET-MNCs neurons shown in A (left and right panels, respectively). No significant differences were observed in F/I nad F/T plots between S- and ET-MNCs.
EXERCISE-INDUCE CHANGES IN SON AND PVN NEURONS

The I/O function in MCNs was further studied using F-T plots. Representative examples are shown in Fig. 5D. At relatively low stimulation levels (e.g., 60 pA), evidence of adaptation was observed only in 50% of recorded S- and ET-MNCs (13/26). The F-T plots of MNCs showing adaptation were best fitted by a monoexponential function, with a $\tau = 297.8 \pm 45.6$ and 193.0 $\pm$ 49.3 ms in S- and ET-MNCs, respectively. Values were not significantly different between groups ($P > 0.05$).

On the other hand, a different pattern and degree of adaptation between S- and ET-MNCs was observed at higher stimulation levels. In S-MNCs, F-T plots were fitted by a monoexponential function with adaptation $\tau$ of 239.7 $\pm$ 44.5 and 203.8 $\pm$ 31.4 ms for stimulation amplitudes of 90- and 120-pA, respectively. On the other hand, the F-T plots in ET-MNCs were best fitted by either a monoexponential (12/31) or a linear function (16/31). For those neurons showing a monoexponential F-T relationship, the adaptation $\tau$s were 196.5 $\pm$ 46.3 and 259.9 $\pm$ 28.4 ms for stimulation amplitudes of 90- and 120-pA, respectively. These values were not significantly different from those observed in S-MNCs ($P > 0.05$ in all cases). For those F-T relationships best fitted by a linear function, the mean slopes were 8.4 $\pm$ 2.7 and 20.8 $\pm$ 6.5 Hz/s, for stimulation amplitudes of 90 and 120 pA, respectively.

Thus whereas a difference in the pattern of spiking adaptation (e.g., monoexponential vs. linear) appears to exist between S and ET rats, the overall degree of adaptation between these two experimental groups was not significantly different.

Exercise-induced changes in HAPs and AHPs during repetitive firing

During repetitive firing, action potentials in S-PVN-NTS neurons failed to completely repolarize, and a progressive decrease in their amplitude was evident. This phenomenon was much less pronounced in ET-PVN-NTS neurons (see examples in Fig. 4A, 1, and 2, respectively). Overall, the mean HAP amplitude in PVN-NTS neurons during exercise training was significantly larger (S-PVN-NTS: $-16.1 \pm 1.6$ mV; ET-PVN-NTS: $-24.1 \pm 1.5$ mV, $n = 13$, $P < 0.01$). Furthermore, the rate of progressive decrease in HAP amplitude during the train of spikes was significantly reduced during exercise training (S-PVN-NTS: $-9.6 \pm 2.3$%/spike, ET-PVN-NTS: $-6.6 \pm 0.4$%/spike, $n = 13$, $P < 0.001$).

No differences in HAP properties during repetitive firing were observed in MNCs (HAP mean amplitude: S-MNCs: $-21.5 \pm 1.9$, ET-MNCs: $-21.8 \pm 0.9$; HAP rate of progressive amplitude decrease: S-MNCs: $-1.5 \pm 0.4$, ET-MNCs: $-1.1 \pm 0.1$, $n = 8$, $P > 0.05$ for both parameters).

Trains of action potentials in PVN-NTS neurons were followed by a prominent AHP (see examples in Fig. 6). When the magnitude of the AHP was compared between S- and ET-PVN-NTS neurons, the AHP area per spike (see METHODS) was found to be significantly smaller in ET- as compared with S-PVN-NTS rats (S: $1.79 \pm 0.45$ mV $\cdot$ s$^{-1}$ $\cdot$ spike$^{-1}$, $n = 8$; ET: $0.79 \pm 0.15$ mV $\cdot$ s$^{-1}$ $\cdot$ spike$^{-1}$, $n = 13$, $P < 0.05$, Student’s t-test).

On the other hand, no significant differences in AHP area between S- and ET-MNCs were observed (S: $0.16 \pm 0.05$ mV $\cdot$ s$^{-1}$ $\cdot$ spike$^{-1}$, $n = 8$; ET: $0.17 \pm 0.04$ mV $\cdot$ s$^{-1}$ $\cdot$ spike$^{-1}$, $P > 0.5$, Student’s t-test; Fig. 6B).

FIG. 6. The afterhyperpolarizing potential (AHP) is diminished in PVN-NTS neurons during exercise training. A: examples of evoked AHPs (arrows) in S- and ET-PVN-NTS neurons (top and bottom, respectively). Both AHPs are shown aligned and expanded in the inset for better comparisons (arrowhead points to the AHP evoked in the ET neuron). Note the smaller AHP evoked in the ET-PVN-NTS neuron. B: on average, the AHP area in PVN-NTS neurons was significantly smaller in ET when compared with S rats ($*P < 0.05$, Student’s t-test). No differences in AHP magnitude were observed in MNCs between S and ET rats. Scale bar in A: 12 mV and 250 ms for the vertical and horizontal lines, respectively.

DISCUSSION

Enhanced I/O function in PVN-NTS projecting neurons during ET

A major finding of this work is that the I/O function of PVN-NTS projecting neurons is enhanced during exercise training. This is partly expressed as an increased number of evoked actions potentials, and steeper F-I slopes in response to depolarizing stimulation. Changes in the input-output function in PVN-NTS neurons during exercise training were more prominent at high stimulation levels. In this sense, while the firing rate of PVN-NTS neurons recorded from both S and ET rats increased progressively at low stimulation levels, S-PVN-NTS neurons were only able to sustain continuous firing discharge up to $\sim 45\%$ of the maximal stimulation used. At higher stimulation, their firing rate decreased progressively, and a high degree of adaptation, and dampening of action potential amplitude became evident (see Fig. 5A). On the other hand, ET-PVN-NTS neurons were able to sustain firing discharge throughout the stimulus range, showing little degree of adaptation/dampening.

To systematically study evoked responses using a wide range of depolarizing steps, neurons were initially hyperpolarized to approximately $-45$–$50$ mV, requiring a relatively large current injection for differences in spiking to become apparent between groups. It is important to consider, however, that most PVN-NTS neurons were shown to be spontaneously active (Stern 2001). Thus it is likely that under more physiological conditions, less current injection/depolarization would be needed to evoke similar responses. In addition, PVN and SON neurons have been shown to be capable of following high-frequency excitatory synaptic inputs (Cui et al. 2001; Gribkoff and Dudek 1990). Thus these differences in I/O function between experimental groups would be expected to occur under more physiological conditions as a consequence of temporal and spatial summation of EPSPs during high-frequency synaptic activation. It is worth noting that the enhanced cell capacitance observed in PVN-NTS neurons during ET in this study would further facilitate temporal summation of
excitatory postsynaptic potentials (EPSPs). Altogether, these results support an enhanced fidelity in the I/O function during exercise training, enabling PVN-NTS neurons to maintain activity and to more efficiently relay afferent information during high levels of activity in afferent pathways.

Despite the fact that the neurochemical identity of the recorded PVN-NTS neurons was not determined, our combined tract tracing and immunohistochemistry experiments indicate that the majority (~65%) of PVN-NTS neurons under basal conditions express either OT or VP peptides. In addition, preliminary results from our laboratory suggest that the degree of colocalization, at least with OT, is increased during exercise training (unpublished observations). Thus it is likely that the majority of our recordings were in fact obtained predominantly from OT and, to a lesser extent, VP neurons. Moreover, the homogeneous changes observed across PVN-NTS neurons during ET would suggest that despite being neurochemically heterogeneous, this neuronal population is similarly affected during ET. Thus even though VP and OT peptides have opposing effects on cardiovascular function within the NTS (Braga et al. 2000; Dufloth et al. 1997; Michelini 2001; Michelini and Morris 1999), our results are in agreement with the fact that the release of both peptides is enhanced during ET (Braga et al. 2000; Dufloth et al. 1997).

The resting membrane potential of PVN-NTS neurons in this study was found to be more depolarized than what we have previously reported (Stern 2001). Even though we can’t precisely explain at present these differences, potential factors underlying these differences include the use of different rat strains (Wistar vs. Sprague Dawleys), as well as difference in rat age/size (120–150 vs. 250–350 g).

**Diminished I/O function in MNCs during ET**

Differently from PVN-NTS neurons, the I/O function in MNCs (both in S and ET rats) displayed a mostly linear relationship throughout the stimulation range, with no evidence of strong adaptation and/or action potential dampening. Furthermore, and in clear contrast to PVN-NTS projecting neurons, the I/O function in SON/PVN MNCs was diminished during exercise training. In this neuronal population, neurons from ET rats responded to input stimulation by generating a lower number of action potentials than in S rats. The decrease excitability in MNCs during ET is in agreement with a recent study showing reduced plasma levels of OT in exercise trained rats (Braga et al. 2000). Furthermore, preliminary studies by Mueller et al. (2002a,b), showed that ET inhibited SON neuronal activation and VP plasma levels in response to baroreceptor unloading. Altogether, these combined results support a decreased excitability of MNCs during exercise training.

**Potential mechanisms underlying changes in neuronal excitability during ET**

Repetitive firing properties in various neuronal types are known to be influenced by a variety of intrinsic membrane properties. For example, the I/O function can be readily affected by changes in input resistance (Powers and Binder 2001). The fact that input resistance in PVN-NTS as well as MNCs did not change during ET, suggests that other mechanisms underlie the changes in I/O function reported herein. For example, the characteristic action potential dampening/failure observed in S PVN-NTS neurons at high stimulation levels could result from the fast inactivation of voltage-gated Na⁺ channels during prolonged depolarization. This could be in part due to the robust and progressive decrease in spikes HAP amplitude during repetitive firing, resulting in failure to efficiently repolarize the membrane after an action potential, to remove Na⁺ channel inactivation. On the other hand, the larger HAPs, as well as the lower rate of progressive HAP amplitude decrement observed during exercise training, would enhance the capacity of PVN-NTS neurons to repolarize the membrane, resulting in turn in a more efficient removal of Na⁺ channel inactivation during repetitive firing. This is supported by the faster and larger action potentials, as well as the lower degree of action potential dampening observed during repetitive firing in PVN-NTS neurons during exercise training. Thus it is tempting to speculate that changes in Na⁺ channel availability and/or channel properties (e.g., shift in the voltage-dependent properties of inactivation) may contribute to the ability of PVN-NTS neurons to sustain firing in response to strong depolarizing inputs.

The precise ionic mechanisms underlying HAPs in PVN-NTS neurons is at present unknown. A likely candidate, known to contribute to spike repolarization and HAPs in MNCs (Greffrath et al. 2004) and other neuronal populations (see (Sah and Faber 2002) for review), is the large-conductance (BK) Ca²⁺-dependent K⁺ channel. Thus determining whether BK channels are expressed in PVN-NTS neurons, what their role is in controlling spike frequency adaptation, and whether changes in their properties occur during exercise training, are key questions to be addressed in future studies.

Another important membrane property known to influence repetitive firing is the slow AHP that typically follows a train of action potentials in SON and PVN neurons (Armstrong et al. 1994; Bourque and Brown 1987; Greffrath et al. 1998, 2004). The AHP results from progressive accumulation of intracellular Ca²⁺ and subsequent activation of Ca²⁺-dependent SK potassium channels (Greffrath et al. 1998). We have recently shown that similarly to MNCs, PVN-NTS neurons display a prominent AHP, which influences spike frequency adaptation during repetitive firing (Stern 2001). Furthermore, spike frequency adaptation was strongly reduced when the AHP was blocked by the SK channel blocker apamin (unpublished observations). Thus it is likely that the diminished AHP observed in these neurons during exercise training also contributes to the lower degree of adaptation observed in PVN-NTS neurons. A reduction in AHP magnitude during exercise training could result from one or more mechanisms combined, including a reduction in intracellular Ca²⁺ accumulation during spiking, a reduction in the Ca²⁺ sensitivity of the Ca²⁺-dependent SK channels and/or intrinsic changes in the SK channels per se.

Last, neuronal structural remodeling is another potential mechanism contributing to changes in intrinsic neuronal excitability during ET. In this sense, the increased cell capacitance found in this study in ET-PVN-NTS neurons could reflect changes in neuronal membrane surface area (Neher and Marty 1982), a fact supported by preliminary studies from our laboratory (Jackson et al. 2004). Thus it is possible that as a consequence of ET, PVN-NTS neurons undergo both functional and structural plasticity. This is supported by previous studies in other brain regions showing ET-induced central
neuronal plasticity (see Cotman and Berchtold 2002 for review), including neurogenesis (van Praag et al. 1999b), functional synaptic plasticity (van Praag et al. 1999a) as well as changes in GABA neurotransmitter markers (Kramer et al. 2002).

The different, and in some cases even opposing, changes in intrinsic excitability observed in MNCs during ET support the fact that functionally discrete neuronal populations within the PVN circuitry can be differentially affected by physiological challenges, such as exercise activity. Because changes in I/O function in MNCs cannot be explained by changes in basic intrinsic properties (e.g., input resistance, cell capacitance), action potential waveform, AHP properties or spike frequency adaptation, it is likely that mechanisms other than those altered in PVN-NTS neurons are involved.

Functional implications

As summarized in the preceding text, accumulating evidence indicates that enhanced activation of VP and OT projections within the NTS [which mostly originate from the PVN (Buijs et al. 1978; Sawchenko and Swanson 1982; Sofroniew 1980)] play key roles in mediating adaptive cardiovascular changes during exercise (Braga et al. 2000; Michelin and Bonagamba 1988). Thus our present results suggest that changes in intrinsic properties of PVN-NTS neurons might constitute a mechanism contributing to enhanced activation and hormonal secretion from these peptidergic terminals during ET, an effect that could be mediated, at least in part, through an improved stimulus-secretion coupling mechanism.

Extensive studies using the magnocellular neurosecretory system as a model provided important insights into mechanisms underlying stimulus-secretion coupling in peptidergic terminals. In this system, a close link between the electrical activity of OT and VP neurons, and hormone secretion from neurohypophysial terminals, has been established (see Poulain and Wakerley 1982; Renaud and Bourque 1991 for review). The amount of hormone release from axonal terminals has been shown to be dependent on both the number of action potentials as well as the firing pattern during a train of spikes. In this sense, the amount of hormone release per spike is increased during high-frequency firing (frequency-dependent secretion) (Bicknell 1988; Poulain and Wakerley 1982). This is particularly evident in OT neurons, which display little fatigue during repetitive firing, even up to frequencies of 50 Hz (Bicknell 1988). In addition, bursting activity, observed in VP and OT neurons under particular physiological conditions (dehydration, lactation), has been shown to maximize hormone release from these terminals (Cazalis et al. 1985; Dutton and Dyball 1979). Assuming that similar stimulus-secretion coupling mechanisms operate in OT/VP PVN-NTS neurons, it is likely that the enhanced input-output function observed in PVN-NTS neurons during ET, will be translated into an improved stimulus-secretion coupling at the brain stem terminals, resulting in an enhanced hormonal release, which in turn might contribute to cardiovascular fine-tuning during this physiological challenge. Finally our results indicate that modulation of intrinsic properties of autonomic-related PVN neurons may constitute a mechanism by which exercise training improves sympathetic outflow in hypertensive and congestive heart failure disorders (DiCarlo et al. 2002; Zheng et al. 2005; Zucker et al. 2004).

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References


Effects of excitatory amino acid antagonists on synaptic responses of supraoptic neurons in slices of rat hypothalamus. 


Localization of cardiac vagal preganglionic motoneurones in the rat: immunocytochemical evidence of synaptic inputs containing 5-hydroxytryptamine. 


Negrao CE, Moreira ED, Coleman A, Michelinic LC, and Stern JE. Plastic changes in the cellular properties of preautonomic neurons of the hypothalamic paraventricular nucleus (PVN) during exercise training. 


Ju G, Han ZS, and Fan LZ. Fluorogold as a retrograde tracer used in combination with immunohistochemistry. 


Kramer JM, Beatty JA, Plowey ED, and Waldrop TG. Exercise and hypertension: a model for central neural plasticity. 


Li Y, Zhang W, and Stern JE. Nitric oxide inhibits the firing activity of hypothalamic paraventricular neurons that innervate the medulla oblongata: role of GABA. 


Ludbrook J. Reflex control of blood pressure during exercise. 


Michelinic LC. Oxytocin in the NTS. A new modulator of cardiovascular control during exercise. 


Michelinic LC and Bonangamba LG. Baroreflex reflex modulation by vasopressin microinjected into the nucleus tractus solitarii of conscious rats. 


Michelinic LC and Morris M. Endogenous vasopressin modulates the cardiovascular responses to exercise. 


Miura M and Reis DJ. Termination and secondary projections of carotid sinus nerve in the cat brain stem. 


Moore RL and Palmer BM. Exercise training and cellular adaptations of normal and diseased hearts. 


Neher E and Marty A. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. 


Paxinos G, Watson C, Pennisi M, and Topple A. Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. 


Porter JP and Brody MJ. Neural projections from paraventricular nucleus that subserve vasomotor functions. 


Poullain DA and Wakerley JB. Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin. 


Powers RK and Binder MD. Input-output functions of mammalian motoneurones. 


Renaud JP and Bourque CW. Neurophysiology and neuropharmacology of hypothalamic magnocellular neurones secreting vasopressin and oxytocin. 


Robinson BF, Epstein SE, Beiser GD, and Braunwald E. Control of heart rate by the autonomic nervous system. Studies in man on the interrelation between baroreceptor mechanisms and exercise. 


Rowell LB. Reflex control of the circulation during exercise. 


Sah P and Faber ES. Channels underlying neuronal calcium-activated potassium currents. 


Sawchenko PE and Swanson LW. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. 


Sofroniew MV. Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human. 


Stern JE. Electrophysiological and morphological properties of pre-autonomic neurones in the rat hypothalamic paraventricular nucleus. 


Stern JE, Galarreta M, Foehring RC, Hestrin S, and Armstrong WE. Differences in the properties of ionotropic glutamate synaptic currents in oxytocin and vasopressin neuroendocrine neurons. 


Sullivan JM and Connors NA. An orthograde labelling study of axons of the dorsal motor nucleus of the vagus to rat cardiac ganglia as demonstrated by autoradiography. 


Swanson LW and Kuyper HG. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. 


Swanson LW and Sawchenko PE. Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. 


van Praag H, Christie BR, Sejnowski TJ, and Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. 


van Praag H, Kempermann G, and Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. 


Veerasingham SJ, Yamazato M, Berecek KH, Wyss JM, and Raizada MK. Increased PI3-kinase in presynaptic brain areas of the spontaneously hypertensive rat. 


Zhang K, Li YF, and Patel KP. Reduced endogenous GABA-mediated inhibition in the PVN on renal nerve discharge in rats with heart failure. 


Zhang K, Li YF, Cornish KG, Zucker IH, and Patel KP. Reduced endogenous GABA-mediated inhibition in the paraventricular nucleus in rats with heart failure. 

