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A subsidiary fever center in the medullary raphé?

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FEVER IS A COORDINATED THERMOREGULATORY RESPONSE against inflammatory stimuli and is organized by the central nervous system. PGE₂ is considered to be the final humoral mediator to influence the thermoregulatory effector mechanisms to raise body temperature. PGE receptors are divided into four subtypes (EP₁–EP₄) (22). Studies on mice lacking specific EP receptors have shown that the EP3 receptor is essential for PGE-mediated fever (42), although the EP1 receptor may also participate (25). According to studies that surveyed the brain for sites where microinjection of PGE₁, a similar prostaglandin that drives fever, the PGE-sensitive sites are located in the preoptic area (38, 43). Moreover, microinjection of small doses of a cyclooxygenase inhibitor into this region can attenuate fever (36). It is well established that the preoptic area contains intrinsically thermosensitive neurons and regulates body temperature (11, 19). It is also known that EP3 receptors are abundantly expressed in the preoptic area (8, 20, 27).

In febrile responses, body temperature is raised by both increased heat production and decreased heat loss and is kept at higher levels than normal (12). In rats, the interscapular brown adipose tissue (BAT) and the tail circulation are major effector organs of heat production and heat loss, respectively. Both are controlled by sympathetic nerves, which are activated during cold exposure (18, 31) or after central administration of PGE₂ or PGE₁ (6, 16). Tachycardia has also been observed with PGE₂-induced fever (16, 30) and during cold exposure (29). Recently, several investigations have indicated that sympathetic premotor neurons controlling tail vasoconstriction, BAT thermogenesis, and cardiac sympathetic activity are all located in the medullary raphé. Neurons there have been shown to project polysynaptically to tail vessels, interscapular BAT, and the stellate ganglion (which includes the main sympathetic supply to the heart) (2, 4, 10, 37). Second, chemical activation of medullary raphé neurons drives the sympathetic nerve supplies to tail vessels, BAT, and heart (5, 18, 33). Furthermore, several lines of evidence indicate that medullary raphé neurons are an essential synaptic relay mediating signals from the preoptic area to tail vessels and BAT during cold exposure and fever: 1) cold exposure and PGE₂ injection into the preoptic area both cause Fos expression in the medullary raphé (3, 4, 18, 21); 2) the tail vasodilation and the inhibition of BAT thermogenesis caused by preoptic warming can be suppressed by bicuculline injections into the medullary raphé (40, 41); 3) activation of tail and BAT sympathetic nerves and tachycardia induced either by cold exposure or by central administration of PGE may be suppressed by inhibition of medullary raphé neurons (13, 16, 28).

EP3 receptors are strongly expressed in the medullary raphé (8, 20), and it was recently demonstrated that more than half of the medullary raphé neurons projecting polysynaptically to BAT express the EP3 receptor (44). In fever, PGE₂ is readily detected in cerebrospinal fluid and may be distributed widely in the brain (7). The possibility thus arises that the medullary raphé neurons that mediate autonomic cold defense and fever responses might themselves respond directly to PGE₂ during fever, and this might reinforce the autonomic responses driven from the preoptic area. We therefore tested the hypothesis that the medullary raphé might be another brain site where PGE₂ could act to cause fever by driving the tail, BAT, and cardiac sympathetic nerve supplies.

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MATERIALS AND METHODS

Thirteen adult male Sprague-Dawley rats (250–420 g) were used in this study. All experiments were carried out in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

Animals were anesthetized initially with pentobarbital sodium (30 mg/kg ip), and the hair over the trunk was shaved. The trachea was cannulated, and animals were then artificially ventilated with 2.0% isoflurane (Forthane; Abbott Laboratories, Chicago, IL) in pure oxygen. Respiratory pressure was monitored via a pressure transducer attached to a side tube, and expired CO₂ concentration was monitored by CO₂ analyzer (ADC). Ventilation was adjusted to keep expired CO₂ between 3.5 and 4.5%. The right femoral artery and vein were cannulated for monitoring blood pressure and for intravenous administration of drugs, respectively. The animal was then mounted prone in a stereotaxic apparatus, according to the coordinate system of Paxinos and Watson (32). A water-perfused silastic jacket was positioned around the animal’s shaved trunk, and the temperature of the perfusion water was used to manipulate skin and core temperature. Skin temperature was measured as the average of three thermocouples placed across the trunk between the skin and water jacket. Core temperature was measured by a thermocouple inserted 5 cm into the rectum and was maintained between ~37 and 38°C by perfusion of the jacket at 150–180 ml/min with water from a reservoir maintained at 43–45°C.

Two burr holes were made in the skull over the rostral hypothalamus and the medial medulla. Either the tail or the back was dissected to expose sympathetic nerves to the tail or interscapular BAT, as detailed below. When surgery was complete, isoflurane was gradually withdrawn and replaced by urethane (1.0–1.2 g/kg iv). The depth of anesthesia was frequently assessed throughout the experiment by testing withdrawal and corneal reflexes, and small additional doses of urethane (25–50 mg iv) were administered if necessary to abolish those reflexes.

Sympathetic nerve fiber recording. In eight rats, postganglionic sympathetic nerve activity was recorded from the rat tail lateral collector nerve (tail SNA), as described elsewhere (31). The lateral collector nerve was carefully exposed by dissection of the skin overlying the lateral vein. A pool filled with liquid paraffin was constructed around the tail. Connective tissue was removed from the nerve trunk, which was then desheathed. Nerve filaments were placed over a silver wire hook electrode, and their activity was recorded differentially with respect to a nearby thread of connective tissue, amplified (10,000-fold), and filtered (60–500 Hz). We monitored the activity continuously using an oscilloscope, and we detected the spikes that passed across a selected threshold voltage with a time-window discriminator; these spikes were counted as spikes per 15 s either online or offline on a computer-based analysis system (CED POWER1401 and Spike 2 software; CED, Cambridge, UK).

In five rats, postganglionic sympathetic nerve activity was recorded from a small nerve branch supplying the interscapular BAT (BAT SNA) on the animal’s right side. A lower thoracic dorsal spine was clamped, and the BAT nerve was exposed by separation and ligation of the tissue in the midline, as described elsewhere (18). A fine nerve branch entering the BAT was cut and dissected free. The nerve was placed over two fine silver wires, and its activity was recorded differentially under a pool of paraffin oil. BAT SNA was amplified (5,000–10,000-fold), filtered (1–500 Hz), and displayed as the raw signal but was rectified and integrated (time constant of 1 s) offline for quantitative measurements (using routines in Spike2). Heart rate was also calculated offline from signals triggered by the systolic peak of the blood pressure wave.

At the end of experiment, hexamethonium chloride (50 mg/kg in saline; Sigma) was given intravenously to confirm whether the recorded nerve activity was postganglionic sympathetic.

Drug preparation. PGE₂ (Sigma) was initially dissolved in pure ethanol and then diluted with artificial cerebrospinal fluid (aCSF), so the final concentrations of PGE₂ and ethanol were 50 ng/100 nl and 0.1%, respectively. aCSF consisted of (in mM) 128 NaCl, 2.6 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 20 NaHCO₃, and 1.3 Na₂HPO₄. Vehicle (0.1% ethanol in aCSF) was also made for control injections. To identify injection sites, PGE₂ and vehicle were mixed with 1% red and 2% yellow-green fluorescent microspheres (FluoSpheres, Molecular Probes, respectively).

Experimental procedure. Tail SNA and BAT SNA recordings were first identified functionally by their excitatory response to passing cold water through the water jacket for 1–3 min. This stimulus was repeated at intervals through the experiment. All subsequent tests were made from baseline conditions where BAT or tail SNA was low and stable. This was achieved by maintaining resting core temperature at a sufficiently high level (see RESULTS). In the case of BAT nerve recordings, low activity of presumed vasomotor origin (17) was always present.

A glass micropipette (tip ~50 μm OD) was positioned stereotactically into the medullary raphe (3.0 mm posterior to lambda, 0.0–0.5 mm lateral to midline, and 9.5 mm deep to the dural surface). In most cases, the appropriate location of the pipette was confirmed functionally by injecting i-glutamic acid (glutamate, 50 mM in aCSF, 60–180 nl; Sigma) to activate tail or BAT sympathetic nerves. The pipette was then removed from the brain, refilled with PGE₂ or vehicle, and lowered into the same position. The pipette was washed with aCSF at least twice whenever the injectate was changed. PGE₂ or vehicle was injected in volumes of 300 nl in the first instance, followed by repeated injections of up to 1,000 nl. Injections were made at a rate of 300 nl/min. At least 5 min elapsed between injections. In 10 of the 13 rats, the pipette was subsequently repositioned into the right preoptic area (0.3–1.0 mm from the midline, between 0.1 mm anterior and 0.3 mm posterior to the bregma, and 8.0–9.0 mm below the dural surface), where 300 nl of PGE₂ were injected.

At the end of experiment, animals were deeply anesthetized with pentobarbital sodium (325 mg iv) and perfused transcardially with saline followed by 4% paraformaldehyde. The brain was removed and placed in the same fixative at least overnight. After cryoprotection with 20% sucrose in PBS, 40-μm frozen coronal sections were made of the preoptic area and medulla. The locations of microinjection sites were identified by detecting the fluorescent microspheres, using fluorescence microscopy (Leitz DMRB, Leica). Relevant sections were photographed under both light and fluorescence optics using a digital camera (SPOT camera Real time, Diagnostic Instruments). In each case, the center of the injection was identified and mapped onto standard sections of the atlas of Paxinos and Watson (32).

Statistical analysis. The resting core temperature, skin temperature, blood pressure, heart rate, tail SNA (spikes/15 s), and integrated amplitudes of BAT SNA were measured as the average values of these variables over 3 min just before each brain injection or cooling period (prestimulus control). The prestimulus integrated BAT SNA was normalized as 100%. Prestimulus control values for three injection groups (vehicle-raphé, PGE₂-raphé, and PGE₂-preoptic area) were compared, using one-way ANOVA for repeated measures. In the case of glutamate injections and cooling episodes, which caused short-lasting activations, the peak response of each variable was averaged over a 30-s period. In the case of PGE₂ or vehicle injections, the onset of tail and BAT sympathetic activation was defined as the time when tail or BAT SNA reached 20% of its peak response. When there was no identifiable response, values were averaged over the 5 min immediately after the injection. In all cases, the response value was compared with the prestimulus control, and Student’s paired t-test was used to assess the significance of changes. To assess differences in response between vehicle-raphé and PGE₂-raphé injections and between PGE₂-raphé and PGE₂-preoptic area injections, Student’s paired t-test was used in all experiments where BAT SNA was recorded. The same compari-
sons were made with the unpaired t-test in experiments where tail SNA was recorded because paired observations in the same animal were not always available. All values are shown as means ± SE, and P < 0.05 was considered significant.

RESULTS

Tail and BAT SNA responses to cooling. Figures 1 and 2 show representative examples of tail and BAT SNA recordings in different rats. In the top traces, tail fiber SNA is displayed as spike counts of a few-fiber preparation (Fig. 1), whereas BAT SNA is displayed as raw traces (Fig. 2). Both signals were abolished by hexamethonium (50 mg/kg) given at the end of the experiment, demonstrating that they were of postganglionic sympathetic activity.

In the prestimulus control condition, thermal and cardiovascular variables were maintained in a stable state, where both tail and BAT SNA were minimal. When cold water was perfused through the water jacket for 1–3 min, this caused an increase in both tail (Fig. 1) and BAT SNA (Fig. 2), as expected on the basis of published work (18, 31). The threshold skin temperatures for activation of tail and BAT SNA were 37.9 ± 0.5°C (n = 8) and 35.7 ± 0.7°C (n = 5), respectively. In both cases, there were components of activation attributable both to the immediate fall in skin temperature and to the delayed (by ~5 min) fall in core temperature (Figs. 1 and 2).

Repeated, brief (1–2 min) cooling periods were sometimes used, and these were particularly effective at stimulating BAT SNA (Fig. 2). In 6 of 13 rats (e.g., in Figs. 1 and 2), cooling the skin caused transient increases in heart rate of 41 ± 11 beats/min, which were accompanied by rise in blood pressure of 9 ± 2 mmHg.

Effects of PGE2 injection into the medullary raphe on tail and BAT SNA. In 11 rats, injections of PGE2 were made into the medullary raphe while tail (n = 6) or BAT (n = 5) SNA were recorded. In two other rats, tail SNA was recorded but only vehicle injections were made into the medullary raphe. Before PGE2 or vehicle injections were made into the medullary raphe, the animal was maintained in a state where tail or BAT SNA was minimal but not far from its thermal threshold, and blood pressure and heart rate were stable (Figs. 1 and 2 and Table 1). In 8 of 13 rats, the appropriate location of the injection pipette was first confirmed by an excitatory response to microinjections of sodium glutamate (Figs. 1 and 2). Glutamate injection produced a transient increase in tail SNA (10 ± 5 to 65 ± 15 spikes/15 s; n = 3) or BAT SNA (485 ± 45% of control; P < 0.01, n = 5), accompanied by small increases in blood pressure (102 ± 2 to 109 ± 3 mmHg; P < 0.01, n = 8) and heart rate (381 ± 18 to 398 ± 18 beats/min; P < 0.01, n = 8).

Neither vehicle nor PGE2 injections (300–1,000 nl) into the medullary raphe caused any significant change in tail SNA or BAT SNA; blood pressure and heart rate also did not change.

Fig. 1. Effect of PGE2 injection into the medullary raphe and the preoptic area (POA) on tail sympathetic nerve activity (SNA), core (rectal) temperature, trunk skin temperature, heart rate, and blood pressure. Volumes (in nl) of solutions injected into the medullary raphe or the preoptic area are indicated above the trace. Horizontal bars under tail nerve activity show cooling periods, when cold water was pumped through the water jacket. bpm, Beats/min. Insets (a and b): expanded segments of raw tail nerve activity after injection of PGE2 into the medullary raphe (a) or into the preoptic area (b), at times indicated under the tail nerve activity trace. Hexamethonium (18 mg iv) was given during a cooling period at the end of the record, as indicated.
From those when PGE2 was injected into the medullary raphé (Table 1). In all of the rats tested, unilateral injection of 300 nl PGE2 into the preoptic area significantly excited tail (n = 5) and BAT (n = 5) SNA (Figs. 1–3). Tail SNA began to increase 2.8 ± 0.3 min after the PGE2 injection, reached its peak over the next 1.5–3 min, and lasted for ~20 min (15–23 min). BAT SNA started to increase after 1.3 ± 0.3 min, reached its peak 1–2 min later, and maintained this level for a further 6–23 min before declining. Accompanying the increased tail or BAT SNA were increases in blood pressure, heart rate, and core temperature (Figs. 1–3). Core temperature began to increase 2.4 ± 0.3 min (n = 10) after preoptic PGE2 injections, reaching 0.9–2.9°C (mean 1.8 ± 0.2°C; n = 10) above resting levels, and remained elevated for at least 1 h.

Postmortem reconstruction of the sites of PGE2 injections confirmed that all were positioned in the ventral medullary raphé, level with the caudal part of the facial nucleus (Fig. 4A).

**Effects of PGE2 injection into the preoptic area on tail and BAT SNA.** The effects of PGE2 injection into the preoptic area on tail and BAT SNA were also investigated in 10 of the 11 rats that had been given PGE2 injections into the medullary raphé. This was done when resting thermal and cardiovascular variables were stable, and these did not differ significantly from those when PGE2 was injected into the medullary raphé (Table 1). In all of the rats tested, unilateral injection of 300 nl PGE2 into the preoptic area significantly excited tail (n = 5) and BAT (n = 5) SNA (Figs. 1–3). Tail SNA began to increase 2.8 ± 0.3 min after the PGE2 injection, reached its peak over the next 1.5–3 min, and lasted for ~20 min (15–23 min). BAT SNA started to increase after 1.3 ± 0.3 min, reached its peak 1–2 min later, and maintained this level for a further 6–23 min before declining. Accompanying the increased tail or BAT SNA were increases in blood pressure, heart rate, and core temperature (Figs. 1–3). Core temperature began to increase 2.4 ± 0.3 min (n = 10) after preoptic PGE2 injections, reaching 0.9–2.9°C (mean 1.8 ± 0.2°C; n = 10) above resting levels, and remained elevated for at least 1 h.

Postmortem reconstruction of the sites of PGE2 injections confirmed that all were positioned in the preoptic area (Fig. 4B).

**DISCUSSION**

The present study was prompted by the finding that the EP3 receptor, the key prostaglandin receptor responsible for febrile responses (42), is abundantly expressed by cells in the medullary raphé, including neurons that are also connected polysynthetically to BAT (44). The density of EP3 receptors in the medullary raphé appears to be quite similar to that in the medial preoptic area (8, 20). Medullary raphé neurons have been implicated as a critical relay in the efferent pathways from the preoptic area to BAT, tail vessels, and heart during PGE1- or PGE2-induced fever (13, 16), as well as for the BAT and tail fiber responses to cold exposure (18, 28). It was therefore natural to suggest that these EP3 receptors on medullary raphé neurons might be directly involved in those sympathetic components of the febrile response. The present study has made the first direct investigation of PGE2 action in the medullary raphé and clearly showed the hypothesis was incorrect. Local injections of PGE2 had no measurable effect on BAT or tail SNA, core temperature, blood pressure, or heart rate. By contrast, injections of PGE2 into the preoptic area caused vigorous

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**Table 1. Baseline levels of tail SNA and thermal and cardiovascular variables before vehicle and PGE2 injections**

<table>
<thead>
<tr>
<th></th>
<th>Before Vehicle-Raphé</th>
<th>Before PGE2-Raphé</th>
<th>Before PGE2-POA</th>
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<tr>
<td><strong>Tail nerve recording</strong></td>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Tail SNA, spikes/15 s</td>
<td>9±4</td>
<td>14±9</td>
<td>7±4</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>38.4±0.4</td>
<td>38.4±0.4</td>
<td>38.1±0.4</td>
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<tr>
<td>Blood pressure, mmHg</td>
<td>105±2</td>
<td>102±3</td>
<td>100±4</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>395±24</td>
<td>392±26</td>
<td>355±19</td>
</tr>
<tr>
<td><strong>BAT nerve recording</strong></td>
<td></td>
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<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>37.2±0.3</td>
<td>36.9±0.3</td>
<td>37.4±0.3</td>
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<tr>
<td>Blood pressure, mmHg</td>
<td>96±3</td>
<td>94±3</td>
<td>93±2</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>367±24</td>
<td>366±30</td>
<td>355±24</td>
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Values are means ± SE; n = no. of animals. Values indicate the average value over 3 min before each injection. SNA, sympathetic nerve activity; POA, preoptic area; BAT, brown adipose tissue. There was no significant difference between columns for any variable.
activation of tail and BAT SNA, with accompanying increases in core temperature, blood pressure, and heart rate, as expected from previous work (14, 30, 35, 43).

Limitations on present observations. Before the negative conclusions are accepted, two critical points must be established. Was sufficient PGE2 injected into the medullary raphe, and was it injected into the correct place? The doses and volumes of PGE2 injected into the medullary raphe and the preoptic area in the present study (≥150 ng in 300 nl) were high compared with doses used in previous studies (14, 35). For injections into the preoptic area, this need not concern us because, although it is possible that the injections could have spread to neighboring brain regions, it is established that cells in the preoptic area mediate the febrile response (9, 34, 35). For injections into the medullary raphe, moreover, larger injections are a positive advantage if we want to be sure of a negative result.

With regard to the appropriate location of injections in the medullary raphe, in most cases we tested the location of the pipette functionally by showing that glutamate injections stimulated tail or BAT SNA. In all cases, injection sites were localized histologically post mortem to the ventral medullary raphe region at the level of the caudal half of the facial nucleus. This is the region previously shown to contain neurons in the efferent pathways to tail vessels, BAT, and cardiac sympathetic fibers (5, 18, 33) and shown to have a major concentration of EP3 receptors (8, 20).

We conclude that PGE2 was injected in a sufficient dose and into the correct region of the medullary raphe.

Actions of EP3 receptors on medullary raphe neurons. It is known that some of the medullary raphe neurons that express EP3 receptors are connected polysynaptically to BAT (44), and we would therefore have expected these to respond to local PGE2. The simplest hypothesis was that the EP3 receptors on medullary raphe neurons were excitatory [as has been shown for dorsal raphe neurons (15)] and that activating them led directly to increased heat conservation, thermogenesis, and tachycardia via direct spinal projections to the cutaneous vasconstrictor, BAT, and cardiac pathways. This hypothesis was clearly disproved.

In most instances, however, EP3 receptors reduce intracellular cAMP levels, which is likely to have an inhibitory action on cellular excitability (22, 39). If EP3 receptors with an inhibitory action on medullary raphe neurons were to participate synergistically in the fever response, one would need to postulate that the cells with the EP3 receptor were inhibitory interneurons. The excitatory pathways from the medullary raphe to BAT, tail vessels, and heart would then be disinhibi-
That possibility is not excluded by the data of Yoshida et al. (44) because the time allowed for transynaptic retrograde transport of pseudorabies virus from BAT in those experiments (71 h) could have enabled it to cross three synapses. Our data, however, provide no support for this hypothesis either.

Finally, we could postulate that activation of EP3 receptors on medullary raphe neurons acts to inhibit the sympathetic premotor neurons of the tail, BAT, and cardiac pathways. Our data provide no support for this possibility, although they do not completely refute it: PGE2 injections into the medullary raphe caused no inhibition of the low level of ongoing activity in the tail sympathetic supply (Fig. 1), nor did they obviously diminish the subsequent responses of tail or BAT sympathetic activity to cooling (Figs. 1 and 2). Any such action would have opposed the febrile response, however, rather than contribute to it; i.e., it could not provide a basis for the “raphe fever hypothesis.”

EP3 receptors and the febrile response. One point that should be noted is that, although EP3 receptors have been believed to be critical for fever induction in mice (42), Oka et al. (25) recently indicated that both EP1 and EP3 receptors might be necessary for fever induction. In rats, there is other evidence that PGE2 induces fever primarily by EP1 rather than EP3 receptors (23, 24, 26), although it causes pressor responses and tachycardia predominantly via EP3 receptors (1). In our experiments, whatever receptors were responsible, the fact remains that preoptic PGE2 injections were effective at producing febrile responses, whereas raphe PGE2 injections were not. EP3 receptors are evidently present on medullary raphe neurons, including those connected polysynaptically to BAT (44), yet we could demonstrate no functional response to an agonist (PGE2) that should have activated those EP3 receptors.

In conclusion, despite the expression of EP3 receptor by neurons in the medullary raphe, we found that these neurons are incapable of driving febrile responses to PGE2 independently of excitation from the preoptic area. Together with previous studies, these findings indicate that in fever, the medullary raphe neurons appear merely to transmit signals for heat production, heat conservation, and tachycardia originating from the main fever center in the preoptic area.

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