A neurochemical mechanism for hypoxia-induced anapyrexia

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A neurochemical mechanism for hypoxia-induced anapyrexia. Am J Physiol Regul Integr Comp Physiol 283: R1412–R1422, 2002. First published August 15, 2002; 10.1152/ajpregu.00328.2002. —Hypoxia evokes a regulated decrease in body temperature, a response that has been termed anapyrexia, but the mechanisms involved are poorly understood. Therefore, the present study was undertaken to test the hypothesis that hypoxia-induced anapyrexia results from the activation of cAMP- and cGMP-dependent pathways in the preoptic region (PO). Adult male Wistar rats weighing 230–260 g were used. Body temperature was monitored by brielemetry, and the levels of cAMP and cGMP were determined in the anteroventral third ventricular region (AV3V), where the PO is located. Using immunohistochemistry, we observed that the PO contains a high density of cAMP- and cGMP-containing cells. Interestingly, hypoxia exposure raised the levels of cAMP and cGMP in the AV3V. Intra-PO microinjection of Rp-cAMPS, an inhibitor of cAMP-dependent protein kinase, attenuated hypoxia-induced anapyrexia. Similarly, intra-PO microinjection of the mixed β-adrenoceptor/serotonin (5-HT1A) receptor antagonist propranolol also impaired the drop in body temperature in response to hypoxia. The reduction in body temperature evoked by intra-PO serotonin, but not epinephrine, was blocked by Rp-cAMPS, indicating the involvement of a preoptic serotonin-cAMP pathway in the development of anapyrexia. Moreover, microinjection of Nω-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthesis, or Rp-cGMPs, an inhibitor of cGMP-dependent protein kinase, into the PO also attenuated hypoxia-induced anapyrexia. In conclusion, the present study supports that hypoxia-induced anapyrexia results from the activation of the serotonin-cAMP and NO-cGMP pathways in the PO.

IN AEROBIC ORGANISMS, OXYGEN is the final acceptor of the electrons from oxidative metabolism, being essential to keep electron flow through the respiratory chain, ATP synthesis, and, consequently, cell function. Therefore, a lack of oxygen represents a life-threatening situation to all aerobic species. A way of reducing hypoxia-induced cell damage is the reduction of temperature, which protects tissues from hypoxia by decreasing oxygen consumption and by slowing the rate of cellular damage that occurs from the formation of free radicals, chemical metabolites, and tissue edema (14, 36, 40). In fact, several animal species, including ectotherms and endotherms, reduce their temperature when exposed to hypoxia, a response that has been reported to be protective (36, 40). Actually, evidence has accumulated that the decrease in body core temperature (Tc) evoked by hypoxia is indeed a consequence of a downward resetting of the thermoregulatory set point, a response named anapyrexia, rather than the result of a direct effect of hypoxia on specific thermoeffectors (36). This notion is supported by the following observations: 1) hypoxia produces a downward shift in the thermoneutral zone of rats, cats, and squirrels (for a review, see Ref. 36); 2) endothermic (rats, hamsters, and mice) and ectothermic (lizards, alligators, toads, and fishes) species select a lower preferred ambient temperature after they are subjected to hypoxia (for a review, see Ref. 36); and 3) changes in the thermoneutral zone and behavioral thermoregulation have been shown to be directly related to changes in the thermoregulatory set point (9). Although anapyrexia seems to be crucial for survival during hypoxia, the neurochemical mechanisms involved in this response are unknown, even though a few mediators have been suggested (for a review, see Refs. 36, 40).

The cyclic nucleotides cAMP and cGMP are second messengers and produce most of their effects by activating cAMP-dependent protein kinase (protein kinase A) and cGMP-dependent protein kinase (protein kinase G), respectively. The first studies (27, 39) suggesting a participation of cyclic nucleotides in the regulation of Tc were reported in the 1970s. More specifically, these studies suggested that administration of cAMP analogs that mimic cAMP-like effects into the preoptic region (PO), which is the presumed brain Tc controlling site (5), increased Tc. However, these observations started to be contested in 1984, when it was reported that intra-PO administration of cAMP and cGMP analogs to rabbits produces a rapid decrease in Tc followed by a feverlike response (12). Interestingly, the fever, but not the decrease in Tc, was abolished by
treatment with paracetamol, indicating that cAMP and cGMP reduce $T_c$ by acting on the PO and that the pyretic effect of intra-PO cAMP observed in previous studies is likely to result from a local inflammatory response produced by the injection procedure (12).

Currently, some studies using small volume microinjections have confirmed that intra-PO administration of cAMP and cGMP analogs that activate protein kinases A and G, respectively, produces a decrease in the $T_c$ of rats (34, 35). Consistent with this notion, cAMP increases the thermosensitivity of warm-sensi-
tive preoptic neurons, an effect that seems to be asso-
ciated with increased heat loss mechanisms and a decrease in $T_c$ (4). Moreover, the use of small volume microinjections also permitted the identification of the anteroventral PO (AVPO) as the preoptic site most sensitive to the thermoregulatory effects of cyclic nu-
cleotides (34, 35). Although cAMP and cGMP seem to act in the AVPO by reducing $T_c$, no attempt has ever been made to verify the involvement of these cyclic nucleotides in anapyrexia.

We then hypothesized that the activation of cAMP- and cGMP-dependent pathways in the PO mediates hypoxia-induced anapyrexia. Inasmuch as the rise in cAMP during anoxia seems to be under the control of the monoaminergic system (41, 42), whereas rises in cGMP may be driven by nitric oxide (NO; for a review, see Ref. 25), the present study aimed to test the hy-
pothesis that hypoxia-induced anapyrexia results from the activation of the monoamine-cAMP and NO-cGMP pathways in the PO.

METHODS

Animals and Drugs

Male Wistar rats (230–260 g) exposed to a daily 12:12-h light-dark cycle (lights on at 6:00 AM) were used. The ani-

mals were housed at 24–25°C, and the experiments were performed at the same temperature. The protocols used are in accordance with the guidelines for animal care and hand-

ling of the Local Institutional Committee (University of Sao Paulo) and of the American Physiological Society (1).

All drugs were purchased from Sigma-Aldrich (St. Louis, MO), Rp-cAMPS, Rp-cGMPs, (+/-)propranolol hydrochlo-

ride, (+/-)epinephrine hydrochloride, serotonin hydrochlo-

ride, $N^\omega$-monomethyl-L-arginine (L-NMMA), and sodium ni-

troprusside (SNP) were all dissolved in pyrogen-free sterile saline. The doses of Rp-cAMPS, Rp-cGMPs, serotonin, and L-NMMA were chosen on the basis of previous studies that evaluated the thermoregulatory effects of these drugs (16, 34, 35). The doses of propranolol, epinephrine, and SNP were chosen on the basis of pilot experiments and of previous reports (22, 28, 31, 35). All doses used were threshold, i.e., they presented a thermoregulatory effect when microinjected into the AVPO, but not when microinjected peri-AVPO.

As to the pharmacology of these agents, Rp-cAMPS and Rp-cGMPs are specific membrane-permeable inhibitors of protein kinases A and G, respectively, resistant toward cyclic nucleotide phosphodiesterases (3, 8). As to propranolol, it is a mixed $\beta$-adrenoceptor/5-HT1A receptor antagonist (10, 24). Last, L-NMMA is a nonsselective NO synthase inhibitor (25), whereas sodium nitroprusside is an NO donor, i.e., it has the property of releasing NO in biological systems (25).

Surgical Preparation and Microinjection

Animals were anesthetized with 2,2,2-tribromoethanol (Aldrich) at the dose of 250 mg/kg ip and fixed in a stereotaxic frame. A stainless steel guide cannula (22 gauge, thin wall, Small Parts) was introduced 2 mm above the right AVPO (relative to bregma: anteroposterior +0.4 mm, lateralateral -0.1 to -0.5 mm, dorsoventral 7.0 mm from the skull sur-
face). These coordinates were adapted from Paxinos and Watson (26) and based on previous studies (34, 35). The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion and infection. Immedi-
ately after, each animal was removed from the stereotaxic frame and a biotelemetry probe capsule (model PDT4000 temperature/activity; Mini-Mitter, Sunriver, OR) was im-
planted into the peritoneal cavity. After surgery, animals were treated with 100,000 units of benzyl-penicillin (intra-
muscularly) and allowed to recover for 1 wk.

A 5-μl Hamilton syringe and a dental injection needle (Mizzy, 200 μm OD) connected to a PE-10 tube were used for the microinjections. The injection needle was 2-mm longer than the guide cannula so that the AVPO was reached by the needle only at the time of injection. Injection was performed in a volume of 100 nl over a period of 1 min and 30 s more were allowed to elapse before the injection needle was re-

moved from the guide cannula to avoid reflux. All injections were performed using a microinjector machine (model 310, Stoelting).

At the end of each experiment, 2% Evans blue solution was microinjected to mark the injected sites for later histological analysis. The animals were then euthanized, and their brains were excised and postfixed in a 10% formalin solution for at least 2 days, after which they were transferred to a 30% sucrose solution, where they stayed for 2 more days. The brains were then cryosectioned in 30-μm slices and mounted on chrome-gelatin-coated glass slides, after which they were stained by the cresyl-Nissl method.

$T_c$ Measurements

$T_c$ was measured continuously by biotelemetry (model ER-

4000; Mini-Mitter). Data were acquired at 5-min intervals.

Experimental Protocols

Experiment 1: Immunolocalization of cAMP- and cGMP-containing cells in the PO. Rats were anesthetized with 2,2,2-tribromoethanol (Aldrich) and subjected to transcordial perfusion with PBS (0.1 M, pH 7.4) followed by 4% parafor-
maldehyde in PBS, after which their brains were rapidly removed and postfixed in 4% paraformaldehyde for 4 h at 4°C. The brains were then transferred to a 30% sucrose solution and kept in it for 2 days at 4°C, after which they were frozen under isopentane at -50°C and cryosectioned (12 μm, Leica, CM1850). The cryosections containing the PO were then mounted on chrome-gelatin-coated glass slides. After being washed with 0.1 M glycine and PBS, the sections were incubated at ambient temperature with 0.5% Triton X-100 on April 15, 2017 http://ajpregu.physiology.org/ Downloaded from http://ajpregu.physiology.org/ by 10.220.32.246 on April 15, 2017
used (1:10), the cross-reactivity of the cAMP antiserum against cGMP is <0.001% (32). The cGMP antiserum also presents no cross-reactivity against cAMP (33). Moreover, Fandsen and Krishna (13) reported that cAMP has to be present at 10^6- to 10^6-times higher concentration than cGMP to show comparable competition for binding to cGMP antiserum. Furthermore, a recent study using rabbit anti-formaldehyde-fixed cGMP antiserum demonstrated that the immunostaining of cGMP in the hippocampus can be selectively increased by sodium nitroprusside (38), a result that is in line with the high specificity of this sort of antiserum. Taken together, these data demonstrate that the antibodies used in the present study are highly selective to cAMP or cGMP.

The primary antibody was visualized by the incubation of sections with biotinylated goat anti-rabbit IgG antibody (dilution 1:100 for 2 h), which complexed with avidin biotinylated horseradish peroxidase. These reagents were obtained from a commercially available kit (Vector Laboratories, code PK-6101) and the complex was visualized by the addition of the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride according to manufacturer’s instructions (Sigma Chemical). Cells that stained for cAMP and cGMP developed a dark brown color.

The number of cAMP- and cGMP-immunopositive cells was quantified at a magnification of ×400 in a Leica microscope (model DML), and a grid was used to delineate the area of quantification. The image was acquired into a Super-Macintosh computer with the use of an MTI converter and the number of immunopositive cells per square millimeter was determined in software provided by the National Institutes of Health. The cell density (number of cells/mm^2) was quantified in the AVPO, in the medial PO (MPO), in the lateral PO (LPO), and in the median preoptic nucleus (MnPO). The delimitation of each area was made on the basis of the atlas of Paxinos and Watson (26) and is shown in Fig. 2.

Experiment 2: Effect of hypoxia on the content of cAMP and cGMP in the anteroventral third ventricular region. Rats were housed in plastic chambers (5 liters) continuously ventilated throughout the chamber for 30 min. Immediately after, the animals were decapitated and their brains excised. The anteroventral third ventricular region (AV3V) region after, the animals were decapitated and their brains excised. The anteroventral third ventricular region (AV3V) region was then dissected under a stereomicroscope based on the anatomical landmarks previously described (19, 21), frozen under liquid nitrogen, and stored at −70°C until assay. This procedure has already been used in previous studies (34, 35).

Samples were homogenized on ice in 150 μl of a 6% (wt/vol) TCA solution by sonication using a Digital 600-W microprocessor cell disrupter (The Vir Tris). Sonication was chosen for tissue homogenization because it disrupts cells, releasing their intracellular content and permitting the determination of the intracellular content of cAMP and cGMP. The resulting homogenates were then centrifuged at 10,000 g for 10 min at 2°C, and the pellets were processed for protein determination. TCA was extracted with water-saturated diethyl ether and the samples were lyophilized. This methodology was based on previous studies (34, 35, 41, 42). For cAMP determination, the samples were then reconstituted in 8 ml of the assay buffer provided in the kit (Amersham Pharmacia Biotech, code RPN225) and processed by enzyme immunoassay according to manufacturer’s instructions. For cGMP determination, the samples were reconstituted in 2 ml of the assay buffer provided in the kit (Amersham Pharmacia Biotech, code RPN226) and processed by enzyme immunoassay according to manufacturer’s instructions.

For protein determination, the pellets were diluted in 4 ml of sodium hydroxide (0.1 N) using a Digital 600-W microprocessor cell disrupter (The Vir Tris). The solution obtained was assayed for protein determination using the BioRad protein assay (BioRad Laboratories, code number 500–0002).

Experiment 3: Effect of Rp-cAMPS or propranolol on hypoxia-induced anapyrexia. After the animals habituated to the experimental conditions (~3 h), initial T_c (T_ci) was determined and the rats received intra-AVPO microinjections of Rp-cAMPS (1 μg in 100 nl), propranolol (1 μg in 100 nl), or pyrogen-free saline (100 nl). Thirty minutes later, a humidified hypoxic gas mixture containing 7% O_2 (AGA, Brazil) was ventilated throughout the chamber for 1 h, after which the chamber was ventilated again with room air. Another experimental group was treated intra-AVPO in the same way, but was kept under normoxia during the whole experiment. T_c was recorded throughout the experiments.

Experiment 4: Effect of Rp-cAMPS on the thermoregulatory response to epinephrine or serotonin. Rats were treated with an intra-AVPO microinjection of saline (100 nl) or epinephrine (12 μg in 100 nl) or with a comicroinjection of Rp-cAMPS (1 μg in 100 nl) and epinephrine (12 μg in 100 nl). In another set of experiments, the animals received intra-AVPO microinjections of saline (100 nl) or serotonin (20 μg in 100 nl) or with a comicroinjection of Rp-cAMPS (1 μg in 100 nl) and serotonin (20 μg in 100 nl). T_c was monitored throughout the experiments.

Experiment 5: Effect of Rp-cGMPS or l-NMMA on hypoxia-induced anapyrexia. Rats were microinjected intra-AVPO with Rp-cGMPS (1 μg in 100 nl), l-NMMA (12.5 μg in 100 nl), or pyrogen-free saline (100 nl). Thirty minutes later, hypoxia was applied for a 1-h period, after which the animals were returned to normoxic conditions. Another experimental group was treated intra-AVPO in the same way, but was kept under normoxia during the whole experiment. T_c was recorded throughout the experiments.

Experiment 6: Combined effect of Rp-cGMPS and sodium nitroprusside on T_c. Animals were treated with an intra-AVPO microinjection of saline (100 nl) or sodium nitroprusside (20 μg in 100 nl) or with a comicroinjection of Rp-cGMPS (1 μg in 100 nl) and sodium nitroprusside (20 μg in 100 nl). T_c was monitored.

Statistical Analyses

The results are reported as means ± SE. The values of T_c (°C) are the changes from initial T_c (T_ci; the T_c at 5-min intervals averaged over the last 30 min of the preceding 3-h stabilization period) plotted at 5-min intervals. Two-way ANOVA followed by the Tukey-Kramer multiple comparisons test was used for the analysis of T_c data. cAMP and cGMP data are expressed as femtomoles per microgram protein of AV3V. Unpaired Student’s t-test was used to assess differences between cyclic nucleotide levels. Ordinary ANOVA was used to analyze immunohistochemical data. Values of P < 0.05 were considered statistically significant.

RESULTS

In all experimental protocols, T_c ranged from 36.4 to 37.4°C and no significant difference in T_ci values was observed among experimental protocols. Intra-AVPO microinjection of saline evoked a rise in T_c, which reached maximum values of ~1°C above T_ci, 2.5 h after injection (see Figs. 5–8). Therefore, to eliminate the
effect of the intra-AVPO microinjection on $T_c$, the thermoregulatory effect of any drug was compared with the respective saline-treated group, an approach that has already been used in previous reports (34, 35). AV3V basal cAMP and cGMP levels in normoxic rats were $19.4 \pm 0.7$ ($n = 11$) and $1.4 \pm 0.3$ fmol/$\mu$g ($n = 9$), respectively.

**Experimental Protocols**

Experiment 1: Immunolocalization of cAMP- and cGMP-containing cells in the PO. By using immunohistochemistry, we observed that the PO of normoxic rats contains a strong immunoreactivity to both cAMP and cGMP, which was localized in the cell bodies. As to the distribution of cAMP and cGMP immunoreactivity within the PO, the AVPO presented the higher density of cAMP- and cGMP-positive cells compared with the MPO, LPO, and MnPO ($P < 0.05$, ordinary ANOVA). The immunoreactivity can be seen by the dark brown color staining. Slices processed as negative controls, i.e., which were not incubated with the primary antibody, presented no staining, even though a yellow background color developed. These data are depicted in Figs. 1 and 2.

Using the cresyl-Nissl method, which stains all cell types, it was observed that the AVPO, the MPO, the LPO and the MnPO present the same cell density (data not shown), a result that confirms that the AVPO contains a higher percentage of cAMP- and cGMP-containing cells among the preoptic sites studied.

Experiment 2: Effect of hypoxia on the content of cAMP and cGMP in the AV3V. As shown in Fig. 3A, exposition to hypoxia for 30 min caused a significant ($P < 0.05$, Student’s t-test) increase in the levels of cAMP in the AV3V from $19.4 \pm 0.7$ to $23.3 \pm 1.5$ fmol/$\mu$g protein. Similarly, the levels of cGMP in the AV3V were also raised ($P < 0.05$, Student’s t-test) during hypoxia from $1.4 \pm 0.3$ to $2.0 \pm 0.2$ fmol/$\mu$g protein (Fig. 3B).

Experiment 3: Effect of Rp-cAMPS or propranolol on hypoxia-induced anapyrexia. A typical site of microinjection into the AVPO is shown in Fig. 4. The animals in which the site of microinjection was located in the AVPO were considered to have been injected intra-AVPO, whereas microinjections located in nuclei surrounding the AVPO were considered to be peri-AVPO. Among the peri-AVPO regions are the organum vasculosum of the laminae terminalis (rostrally), the septum (rostrally), the anterior commissure (dorsally), the lateral PO (laterally), and hypothalamic nuclei located caudally to the PO, such as the paraventricular nucleus. In Figs. 5–8, it is shown that peri-AVPO micro-

![Fig. 1. Immunolocalization of cAMP (A; lower magnification; B, higher magnification) and cGMP (C; lower magnification; D, higher magnification) in the preoptic region of the rat brain. Staining is visualized as a dark brown color. E: a brain slice processed as negative control (without the primary antibody) is shown.](image-url)
injections produced no effect, whereas intra-AVPO microinjections evoked thermoregulatory effects, implying that the diffusion of the injected compounds was small and that the effects of the agents were restricted to the AVPO. In other words, when the drug was injected outside the AVPO (peri-AVPO), the amount of drug that reached the AVPO was not high enough to produce a thermoregulatory response.

The intra-AVPO microinjection of Rp-cAMPS at the dose of 1 μg produced no change in Tc compared with the groups that received intra-AVPO saline or peri-AVPO Rp-cAMPS. Moreover, exposition of rats to 7% of inspired oxygen evoked anapyrexia, a response similar to that observed in a previous study (37). In more detail, when inspired oxygen was reduced from 21% to 7%, Tc dropped quickly during the first 30 min, reaching Tc values of ~1.5°C below Tc, and continued to drop more slowly until the end of hypoxia exposure (1 h). A maximum decrease in Tc of ~2°C was observed after 1 h of hypoxia. When room air was applied again, Tc started to return toward baseline control values (normoxic values). Peri-AVPO administered Rp-cAMPS did not affect the thermoregulatory response to hypoxia. However, when microinjected intra-AVPO, Rp-cAMPS (1 μg) significantly (P < 0.05; 2-way ANOVA) attenuated hypoxia-induced anapyrexia. These data are plotted in Fig. 5.

Similar to Rp-cAMPS, intra-AVPO microinjection of propranolol (1 μg) did not affect the Tc of normoxic animals (Fig. 6A), but significantly (P < 0.05; 2-way ANOVA) attenuated hypoxia-induced anapyrexia (Fig. 6B). On the other hand, peri-AVPO propranolol had no effect on hypoxia-induced anapyrexia (Fig. 6B).

![Fig. 2. Number of cAMP- and cGMP-immunopositive cells per mm² of the preoptic region (PO) subdivisions (A and B). Values are means ± SE. Number of animals in each group is shown in parentheses. *P < 0.05 compared with the other subdivisions of the PO. C: boxed areas in which the number of immunopositive cells were counted are depicted. Diagram has been adapted from Paxinos and Watson (26). AC, anterior commissure; OX, optic chiasm; LPO, lateral PO; MPO, medial PO; MnPO, median preoptic nucleus; AVPO, anteroventral PO.](http://ajpregu.physiology.org/)

![Fig. 3. Effects of hypoxia (7% inspired oxygen) on cAMP (A) and cGMP (B) levels in the anterovenral third ventricular region (AV3V). Values are means ± SE. Number of animals in each group is shown in parentheses. *P < 0.05 compared with the normoxic control.](http://ajpregu.physiology.org/)
Experiment 4: Effects of Rp-cAMPS on the thermoregulatory response to epinephrine or serotonin. Intra-AVPO microinjection of epinephrine at the dose of 12 μg produced a significant decrease in $T_c$ ($P < 0.05$; 2-way ANOVA), which was evident 5 min after the injection and peaked $\sim 1^\circ C$ below $T_{ci}$. The overall response lasted 35 min. The comicroinjection of Rp-cAMPS with sodium nitroprusside completely abolished the drop in $T_c$ elicited by sodium nitroprusside intra-AVPO. These results are depicted in Fig. 6C.

Intra-AVPO microinjection of serotonin at the dose of 20 μg caused a significant ($P < 0.05$; 2-way ANOVA) decrease in $T_c$ of $\sim 1^\circ C$ (Fig. 6D). However, different from the thermoregulatory response to epinephrine, this response was completely abolished by the presence of Rp-cAMPS (Fig. 6D).

Experiment 5: Effect of Rp-cGMPS or L-NMMA on hypoxia-induced anapyrexia. Intra-AVPO microinjection of Rp-cGMPS at the dose of 1 μg caused no thermoregulatory effect in normoxic rats. However, intra-AVPO microinjection of Rp-cGMPS (1 μg) significantly ($P < 0.05$; 2-way ANOVA) attenuated hypoxia-induced anapyrexia. Peri-AVPO Rp-cGMPS did not affect the course of the anapyrexia elicited by hypoxia. Figure 7 shows these data.

Administration of L-NMMA into the AVPO did not affect the $T_c$ of normoxic rats compared with the group treated with intra-AVPO saline or peri-AVPO L-NMMA (Fig. 8A). Moreover, peri-AVPO L-NMMA did not alter the course of hypoxia-induced anapyrexia. On the other hand, the drop in $T_c$ in response to hypoxia was significantly ($P < 0.05$; 2-way ANOVA) attenuated by intra-AVPO microinjection of L-NMMA (Fig. 8B).

Experiment 6: Combined effect of Rp-cGMPS and sodium nitroprusside on $T_c$. Intra-AVPO microinjection of sodium nitroprusside at the dose of 20 μg elicited a significant decrease in $T_c$ ($P < 0.05$; 2-way ANOVA), which was evident 5 min after the injection and peaked $\sim 1^\circ C$ below $T_{ci}$. The overall response lasted 35 min. The comicroinjection of Rp-cGMP with sodium nitroprusside completely abolished the drop in $T_c$ elicited by sodium nitroprusside intra-AVPO. These results are depicted in Fig. 8C.

**DISCUSSION**

The present study provides evidence that an increased production of cAMP and cGMP in the PO mediates hypoxia-induced anapyrexia, a conclusion that is supported by the observations that 1) the PO contains a high density of cAMP- and cGMP-producing cells (Figs. 1 and 2); 2) the levels of cAMP and cGMP in the AV3V were raised under conditions of hypoxia (Fig. 3); 3) cAMP and cGMP seem to reduce $T_c$ by acting on the PO (12, 34, 35); and 4) intra-AVPO microinjection of Rp-cAMPS and Rp-cGMP, which are inhibitors of protein kinase A and G, attenuated hypoxia-induced anapyrexia (Figs. 5 and 7). Taken together with a
recent study (34) showing that a decrease in the intracellular content of cAMP and cGMP in the PO mediates fever, these data may provide a general neurochemical model for the control of the thermoregulatory set point. More specifically, we previously observed that intra-AVPO microinjection of Rp-cAMPS or Rp-cGMPS alone does not affect the Tc of rats, but a comicroinjection of these agents into the AVPO produces a feverlike response (34). Moreover, the AV3V levels of cAMP and cGMP seem to be reduced in the course of prostaglandin E2 and endotoxin fever, respectively (34, 35). It is then suggested that a raised cAMP and cGMP production in the PO decreases the thermoregulatory set point, i.e., anapyrexia (present study), whereas a reduced production of these nucleotides increases the thermoregulatory set point, i.e., fever (34).

By using immunohistochemistry, the present report showed that the AVPO is a brain region that contains a high density of cAMP- and cGMP-containing cells (Figs. 1 and 2), an observation that is in line with the finding that the AVPO is the most sensitive preoptic site to the thermoregulatory effects of cAMP and cGMP (34, 35). Since in vivo experiments (34, 35) and electrophysiological measurements (4) support that cAMP and cGMP seem to act on the PO by reducing Tc, we then verified the role of these nucleotides in anapyrexia.

Several reports have demonstrated that rats and mice under conditions of anoxia present increased levels of cAMP in several brain regions, including the PO (15, 41, 42). However, in these studies the animals were subjected to a drastic drop in inspired oxygen fraction from 21% to 0%, followed by ~2 min of complete anoxia. This experimental protocol is quite different from the models habitually used to study anapyrexia. In our laboratory, anapyrexia has been induced by subjecting animals to a 7% oxygen atmosphere for a period longer than 30 min (37). In agreement with previous studies, animals inspiring 7% of oxygen reduced their Tc by ~1.5°C after 1 h of hypoxia (Figs. 5–8). Whether cAMP levels are raised in the PO under the conditions used to induce anapyrexia had never been tested. First, we attempted to use immunohistochemistry to determine whether hypoxia increases the number of cAMP-positive cells or the intensity of cAMP staining, but the results obtained were not consistent, probably because of methodological limitations. Therefore, we measured the levels of cAMP by enzyme immunoassay in the AV3V, where the PO is located. As a result, we observed that cAMP levels were raised in the AV3V after 30 min of hypoxia exposure (7% inspired oxygen, Fig. 3). This period of time was chosen because in 30 min of hypoxia Tc dropped ~1.5°C and would have continued to decrease had the animals not been decapitated. Even though in the present study cAMP levels were determined only in the AV3V, where the PO is located. As a result, we observed that cAMP levels were raised in the AV3V after 30 min of hypoxia exposure (7% inspired oxygen, Fig. 3). This period of time was chosen because in 30 min of hypoxia Tc dropped ~1.5°C and would have continued to decrease had the animals not been decapitated. Even though in the present study cAMP levels were determined only in the AV3V, it should be emphasized that hypoxia seems to be a more global stimulus and as such may increase the levels of cAMP not only in the PO, but also in other brain regions, such as the cerebral cortex and the hippocampus (41). However, the levels of cAMP in these regions are unlikely to play thermoregulatory actions because the hypoxia-induced accumulation of cAMP in the cerebral cortex and hippocampus is not affected by exposition to low and high ambient temperatures, whereas that of the PO is (41).
To directly test the hypothesis that a raised intracellular content of cAMP in the PO mediates anapyrexia, the recently developed protein kinase A inhibitor Rp-cAMPS (3) was employed. As shown in Fig. 5, intra-AVPO Rp-cAMPS (1 μg/100 nl) did not affect the Tc of euthermic animals compared with saline-treated animals, but significantly attenuated hypoxia-induced anapyrexia, providing direct evidence that an increase in preoptic cAMP mediates anapyrexia. Moreover, it should be pointed out that the Tc of rats that received intra-AVPO Rp-cAMPS returned more rapidly to baseline values after hypoxia was withdrawn (Fig. 5). Nevertheless, it should be considered that at the end of hypoxia exposure, Rp-cAMPS-treated rats presented higher Tc values in relation to saline-treated rats, a fact that makes any conclusion on the role of cAMP in the return of Tc after hypoxia speculative.

Since the rise in cAMP under conditions of anoxia seems to be dependent on the monoaminergic system (41, 42) and as such is blocked by treatment with propranolol, we then tested whether a monoamine-cAMP pathway in the PO is involved in anapyrexia. Interestingly, we observed that intra-AVPO propranolol (1 μg/100 nl), similarly to Rp-cAMPS, attenuated the decrease in Tc evoked by hypoxia (Fig. 6B). Since propranolol is a mixed β-adrenoceptor/5-HT1A receptor antagonist (10, 24), this result would imply that a catecholamine-cAMP or a serotonin-cAMP pathway in the PO is involved in anapyrexia. However,
the observation that Rp-cAMPS failed to alter the thermoregulatory response to epinephrine (Fig. 6C) indicates that the effect of propranolol on anapyrexia and hypoxia-induced cAMP production is more likely to be due to inhibition of a 5-HT<sub>1A</sub> receptor-cAMP pathway in the PO. In support, the drop in T<sub>e</sub> evoked by intra-AVPO serotonin was abolished by the protein kinase A inhibitor Rp-cAMPS (Fig. 6D). Indeed, 5-HT<sub>1A</sub> receptor agonists have been shown to evoke dose-dependent decreases in the T<sub>e</sub> of several species, including humans (30). In addition, a recent study (23) reported that activation of the 5-HT<sub>1A</sub> receptor increases cAMP formation in rat hippocampal neurons, even though the transduction mechanism remains uncertain.

Although the present results support an involvement of preoptic cAMP in anapyrexia, this molecule is unlikely to be the only second messenger acting on the PO to produce anapyrexia because treatments that only increase or decrease cAMP levels usually do not affect T<sub>e</sub> (34). Thus a synergistic agent should exist, with cGMP being a putative candidate. This suggestion is supported by our previous observation that cAMP and cGMP may act synergistically in the PO to reduce the T<sub>e</sub> of rats (34). In agreement with this hypothesis, we observed that hypoxia (7% O<sub>2</sub> for 30 min) increased the levels of cGMP in the AV3V, a response similar to that obtained to cAMP (Fig. 3). Moreover, intra-AVPO treatment with the protein kinase G inhibitor Rp-cGMPs (8) had no effect on basal T<sub>e</sub>, but significantly attenuated hypoxic anapyrexia (Fig. 7).

Since NO seems to be an important inducer of cGMP synthesis in vitro and in vivo (25), the role of preoptic NO in hypoxia-induced anapyrexia was investigated. Both the NO donor sodium nitroprusside and the cGMP analog 8-Br-cGMP have been reported to reduce the T<sub>e</sub> of unanesthetized rats when administered into the AVPO (35). Now, we add that the hypothermic effect of intra-AVPO sodium nitroprusside (20 μg/100 nl) is abolished by Rp-cGMPs (Fig. 8C). These data, taken together with the present observation that intra-AVPO L-NMMA (an inhibitor of NO synthesis) also impaired the drop in T<sub>e</sub> evoked by hypoxia (Fig. 8B), support the hypothesis that the preoptic NO-cGMP pathway plays a major role in hypoxia-induced anapyrexia. It has been reported that intracerebroventricular administration of an inhibitor of NO synthesis abolishes hypoxia-induced anapyrexia in rats (7), but the brain site in which NO acts to reduce T<sub>e</sub> had not been assessed. According to the present data, the PO is likely to be a site in which intracerebroventricularly administered inhibitors of NO synthesis may act to attenuate anapyrexia.

On the basis of the notion that the PO seems to play a key role in the determination of the thermoregulatory set point (5) and that cAMP and cGMP act as second messengers, it is suitable to suggest that an increased production in preoptic cAMP and cGMP represents the final step to produce anapyrexia. Previous studies using intracerebroventricular administration of drugs have suggested a few molecules, such as endogenous opioids, adenosine (for review, see Ref. 36), and dopamine (2), as putative mediators of anapyrexia. However, these studies did not attempt to identify the site of action of the proposed mediators, precluding the identification of a detailed neurochemical mechanism. On the basis of the present observations, we then suggest that raised levels of preoptic cAMP and cGMP represent the final common pathway to all the mediators of anapyrexia proposed to date. In support, endogenous opioid receptors are coupled to adenylate cyclase (17) and might affect T<sub>e</sub> by changing cAMP levels in the PO. Also, the interaction between opioids and NO has been proposed (6). As to adenosine, its receptors are G coupled (20) and as such adenosine might also produce anapyrexia by altering cAMP levels in the PO. Finally, intra-PO dopamine-induced hypothermia seems to be associated with raised cAMP levels (11).

In summary, the present study is the first to propose a neurochemical mechanism for hypoxia-induced anapyrexia, which is likely to result from the activation of the serotonin-cAMP and NO-cGMP pathways in the PO. The proposed model, which is schematically shown in Fig. 9, may represent the final common pathway for all the mediators of anapyrexia proposed to date.

**Perspectives**

Evidence has accumulated that a decreased T<sub>e</sub> is a very effective way of reducing hypoxia-induced cell damage. On the basis of this rationale, forced hypo-
thermia has been used in clinical and surgical procedures to protect the brain and the heart from the deleterious effects of hypoxia (18, 29). However, forced hypothermia, different from anapryrexia, occurs as a deviation of $T_c$ from the respective thermoregulatory set point and as such is accompanied by mechanisms of heat production and heat conservation to restore $T_c$, creating undue physiological and psychological stress (14, 36, 40). These disadvantages are not present during anapryrexia. We now propose a neurochemical mechanism for hypoxia-induced anapryrexia, which may lead to new and more effective strategies to modulate $T_c$ under oxygen-limiting situations. In this context, we propose the combined use of 5-HT$_{1A}$ agonists with NO donors, which are currently used in patients as anxiolytic and vasorelaxing drugs, respectively, to induce anapryrexia in humans. This pharmacological approach may be used in combination with the currently used cold blankets (18, 29) to reduce the $T_c$ of patients.

In addition to its clinical application, it should be emphasized that the present proposed model may be part of a more general neurochemical model for the control of the thermoregulatory set point. Actually, using situations in which the thermoregulatory set point is raised (fever; Ref. 34) or decreased (anapryrexia; present study), we have shown that a decrease in preoptic cAMP and cGMP raises the thermoregulatory set point, whereas increases in the preoptic levels of these nucleotides reduce the thermoregulatory set point.

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