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Hypoxia and N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate, but not nerve growth factor, induce Na^+ channels and hypertrophy in chromaffin-like arterial chemoreceptors

(glomus cells/carotid body/O_2 sensors)

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ABSTRACT **Chronic hypoxia sensitizes the ventilatory reflex in mammals and causes enlargement of the carotid body, a peripheral arterial chemosensory organ. To investigate possible underlying mechanisms, in the absence of circulatory changes, we exposed cultures of dissociated rat carotid body containing the oxygen sensors (i.e., chromaffin-like glomus cells) to chronic hypoxia (6% O_2) over a period of 2 weeks. After a delay of a few days, the Na^+ current density in hypoxia-treated glomus cells increased significantly, reaching values up to 6 times that seen in normoxic (20% O_2) controls. In addition the whole-cell capacitance, an indicator of cell size, was also significantly larger (3–4 times control) in glomus cells exposed to chronic hypoxia. Both effects were mimicked qualitatively by chronic treatment of normoxic cultures with N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate, but not nerve growth factor, which is known to induce similar changes in the chromaffin cell line PC12. Thus, the physiological and morphological effects of chronic hypoxia on the carotid body *in vivo* may be due in part to a cAMP-mediated stimulation of Na^+ channel expression and hypertrophy in the chemosensory glomus cells.**

Conditions of chronic hypoxia, as occur in humans and animals living at high altitude (1) or in patients with hypoxic lung disease (2), cause enlargement or hypertrophy of the carotid body, an organ that senses blood P_{O_2} and controls ventilation. In addition, there is a time-dependent sensitization of this chemosensory pathway (3, 4), resulting in an increased respiratory drive that is thought to be important during acclimatization to hypoxia. The above structural and functional changes may be mediated via a direct action of low arterial P_{O_2} on the carotid-body oxygen sensors (i.e., the chromaffin-like glomus cells), which contain a unique class of O_2-sensitive K^+ channels (5–7), or indirectly, by way of blood-borne factors arising elsewhere in the circulation. Though there is evidence that the hypertrophic response of the carotid body during chronic hypoxia is accompanied in part by an increased size of the glomus cells (8, 9), the underlying mechanisms associated with the morphological and physiological adaptations of the carotid body to chronic hypoxia are largely unknown. Moreover, these mechanisms are especially difficult to investigate *in vivo*, where circulatory factors cannot be readily controlled or eliminated.

This laboratory has been investigating (7, 10, 11) chemotransduction mechanisms by using dissociated cell cultures of the rat carotid body in which the chemosensory glomus cells survive for several weeks and are accessible for electrophysiological patch-clamp/whole-cell recording. The ability to control the cellular, fluid, and gaseous environments in these cultures allowed us to investigate whether direct exposure of carotid body cells alone to chronic hypoxia could alter the physiological and morphological properties of the glomus cells, in the absence of extraneous circulatory factors. In this study, we compare the properties of glomus cells cultured in normoxic (20% O_2) and hypoxic (6% O_2) environments and provide direct evidence that prolonged hypoxia may alter both ion-channel expression and morphology in these cells. In addition, our studies suggest a role for cyclic nucleotides in the mediation of these responses.

METHODS AND MATERIALS

The procedures for the culture of glomus cells by combined enzymatic and mechanical dissociation of the rat carotid body were identical to those previously described (7, 10, 11). Briefly, carotid bodies were obtained from rat pups 5–12 days old and then incubated for 45 min in an enzyme solution containing 0.1% collagenase, 0.1% trypsin (GIBCO), and 0.01% deoxyribonuclease (Millipore). The tissues were then rinsed and mechanically teased in growth medium containing F-12 nutrient medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO), insulin (80 units/liter; Sigma), 0.6% glucose, 2 mM L-glutamine, and 1% penicillin/streptomycin. For the first 2 days, all cultures were grown in a control normoxic environment at 37°C in a humidified atmosphere. Thereafter, they were exposed to one of the following four conditions for up to 14 days: (*i*) a control normoxic environment (20% O_2/75% N_2/5% CO_2), (*ii*) a hypoxic environment (6% O_2/89% N_2/5% CO_2), (*iii*) a normoxic environment with 1 mM N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP) added to the medium, and (*iv*) a normoxic environment with nerve growth factor (NGF; 1 μg/ml) added to the medium. In several control experiments, this same NGF-containing medium supported long-term growth and survival (>2 weeks) of dissociated sympathetic neurons from rat superior cervical ganglia; the majority of these neurons died within 2 days when the NGF was omitted. All normoxic cultures were grown in a Forma Scientific (Marietta, OH) automatic CO_2 incubator, whereas hypoxic cultures were grown in a Forma Scientific O_2/CO_2 incubator in which the gas tensions could be independently controlled.

Most whole-cell recordings were performed using the perforated-patch technique as described in our recent studies on these glomus cells (7, 11). Typical external recording solutions contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4. The pipette solutions for perforated-patch recording contained either 25 mM KCl plus 110 mM potassium gluconate or 135 mM KCl in 5 mM NaCl/0.1 mM $CaCl_2$/10 mM Hepes, pH 7.2/nystatin (500 μg/ml). Whole-cell currents recorded with the two pipette

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Abbreviations: Bt_2cAMP, $N^6O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; NGF, nerve growth factor; TTX, tetrodotoxin.
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solutions were generally comparable. Data were analyzed using PCLAMP Version 5.5 software (Axon Instruments, Burlingame, CA) and voltage-clamp traces were plotted on a Hewlett–Packard LaserJet III printer after subtraction of the capacity and leakage currents and filtering at 2000 Hz. For estimates of whole-cell capacitance, a hyperpolarizing voltage step, typically from a holding potential of −60 mV to a step potential of −120 mV, was applied to the cell and the resulting capacitative transient was recorded on the current trace. The whole-cell capacitance was then calculated by dividing the charge (obtained from integration of the capacitative transient) by the magnitude of the voltage step. Series resistance was typically <15 MΩ for perforated-patch recordings and was compensated in all experiments.

The drugs Bt_2cAMP, nystatin, and tetrodotoxin (TTX) were obtained from Sigma.

RESULTS

In the first part of this study, we used patch-clamp/whole-cell recording to compare the active and passive properties of glomus cells grown under normoxic and hypoxic conditions. Rat glomus cells grown in culture for 2–15 days under normoxic conditions contained small transient inward currents (Fig. 1 *A* and *B*), as we have reported (7, 11). This transient current was substantially reduced by TTX (see ref. 7), indicating that it was carried mainly via voltage-gated Na^+ channels. Typically, for cells grown in normoxic conditions, the peak inward current was <50 pA (holding potential, −60 mV) and remained relatively constant with time in culture (Fig. 1*A*). In contrast, when the cultures were grown under hypoxic conditions, the magnitude of the peak inward current increased progressively, reaching average values ≈6 times control after a 13-day exposure (Fig. 1*A*). These larger inward currents were substantially (and reversibly) reduced by 500–1000 nM TTX (Fig. 1*C Left*), indicating that they too were carried mainly via voltage-gated Na^+ channels. The effect of hypoxia may be due to a specific induction of Na^+ channels since the augmented Na^+ current developed with a lag period of 2–5 days (Fig. 1*A*), and it occurred in cells where

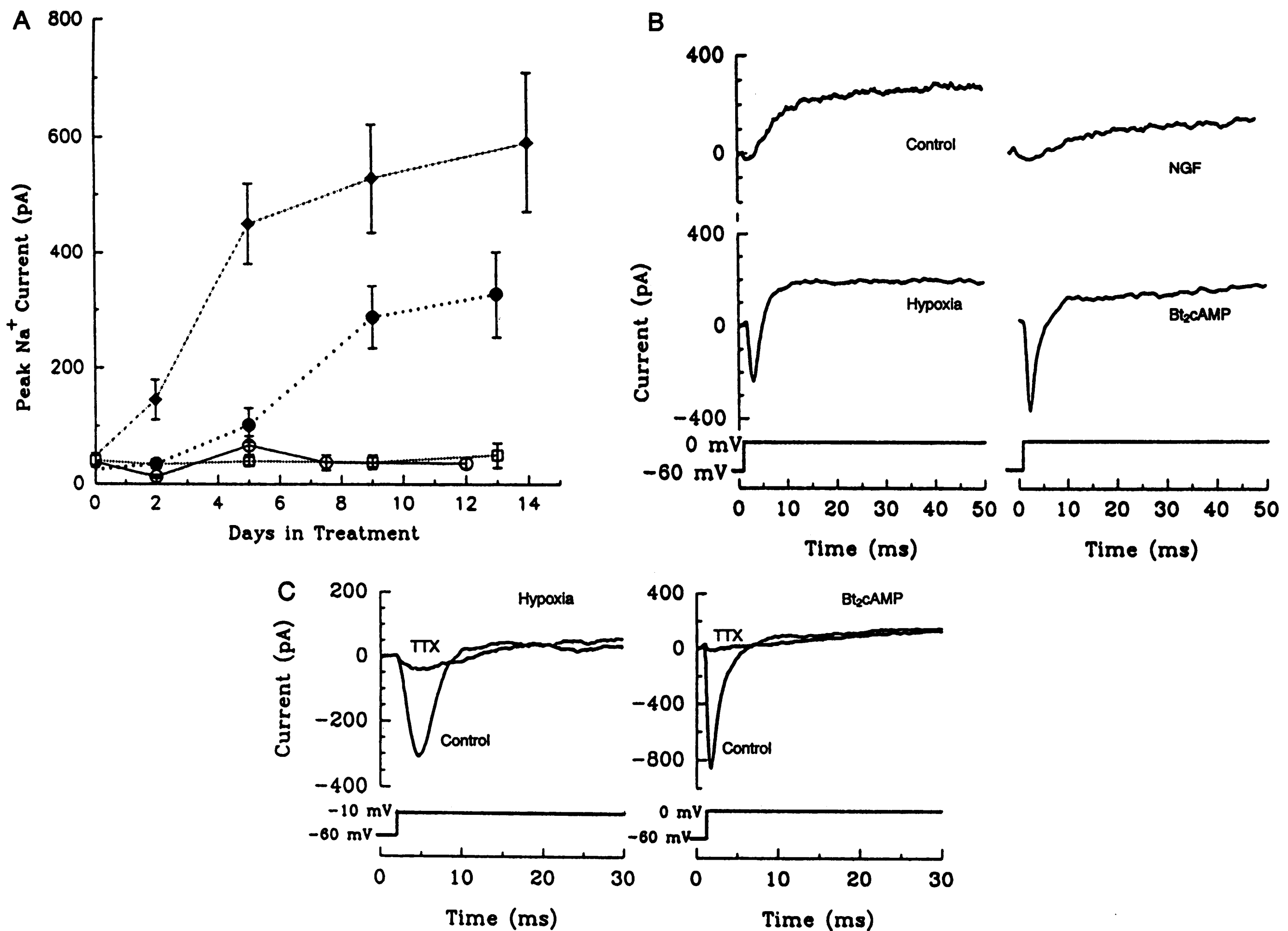

FIG. 1. Comparison of the effects of prolonged hypoxia, Bt_2cAMP, and NGF on the development of inward Na^+ currents in cultured chemosensory glomus cells. (*A*) Time course of the effect of various treatments on the peak inward Na^+ current elicited by a voltage step from −60 to 0 mV. □, Control (normoxia); ●, hypoxia; ◆, 1 mM Bt_2cAMP; ○, NGF (1 μg/ml). The progressive increase in peak Na^+ current is already significant ($P < 0.01$) at 5 days in hypoxic conditions (6% O_2) and at 2 days in Bt_2cAMP; at no time was there a significant change during NGF treatment. As in our previous studies (7, 11), control (normoxic) glomus cells had a mean peak Na^+ current of ≈50 pA that remained relatively constant over time in culture (□). Each point represents the mean ± SEM of a minimum of 9 cells from at least two separate platings; the graph is based on recordings from >250 glomus cells. All treatments were compared to controls using Student's *t* test. Significance levels were set at $P < 0.01$. (*B*) Whole-cell current traces recorded from glomus cells grown under various conditions. The control glomus cell, grown in a normoxic environment, had a typical small transient inward current followed by prolonged outward K^+ current; voltage step (lower traces) was from −60 mV to 0 mV. The NGF-treated cell showed no obvious difference in inward current from the control. In contrast, glomus cells exposed to hypoxia (6% O_2) and to Bt_2cAMP (1 mM) for 9 days showed a pronounced increase in the inward current, but there was no apparent change in the outward K^+ current. (*C*) Effect of TTX on the inward current in glomus cells exposed to hypoxia or Bt_2cAMP. The inward current recorded from glomus cells exposed to hypoxia (6% O_2) or 1 mM Bt_2cAMP for 9–10 days was almost completely blocked by 1 μM TTX. The residual inward current is likely due to the small Ca^{2+} current known to be present in these cells (7).

the magnitude of the outward K^+ current was similar to normoxic controls (Fig. 1*B*).

Since hypoxia causes elevation of cAMP in the carotid body (12–14), specifically in glomus cells (15), and since cAMP analogs induce Na^+ channel expression in PC12 (16) and skeletal muscle (17) cells *in vitro*, we tested whether the increase in inward current in glomus cells is mimicked by growing normoxic carotid body cultures in the continuous presence of the membrane-permeable cAMP analog Bt_2cAMP. As shown in Fig. 1*A*, the effect of 1 mM Bt_2cAMP was even more dramatic than chronic hypoxia, causing a greater increase in inward current throughout the treatment period and with a more rapid onset (see also Fig. 1*B*). This augmented inward current was again largely due to voltage-gated Na^+ channels since it was substantially reduced or eliminated by TTX (Fig. 1*C Right*), and there was no accompanying change in steady-state inactivation (data not shown). Thus, it is plausible that the signal transduction pathway leading to the enhanced inward Na^+ current in glomus cells exposed to chronic hypoxia, involves the elevation of intracellular cAMP produced by the hypoxic stimulus.

We next investigated whether NGF might act as an intermediary signal, since NGF causes development of action potentials and induces Na^+ channel expression in PC12 cells (16, 18), whose parental chromaffin cells are closely related embryologically to glomus cells (19, 20); in addition, during embryonic development at least some glomus cells appear sensitive to NGF (21). As shown in Fig. 1 *A* and *B*, chronic treatment of carotid body cultures with NGF (1 μg/ml) had no effect on the inward Na^+ current. Thus, it is unlikely that hypoxia increases the number of Na^+ channels in glomus cells by locally elevating the levels of NGF.

Chronic hypoxia also causes enlargement of the carotid body *in vivo* and this is accompanied in part by glomus cell hypertrophy (1, 8, 9). To test whether such structural changes might be due to a direct effect of hypoxia on carotid body cells, rather than the production of ectopic humoral growth factors, we compared the input or whole-cell capacitance (which is proportional to cell surface area) in glomus cells grown under normoxic and hypoxic conditions. As shown in Fig. 2, chronic hypoxia caused hypertrophy of glomus cells as reflected by a 3- to 4-fold increase in whole-cell capacitance after a 14-day exposure; this is roughly equivalent to a doubling of the cell diameter if a spherical shape is assumed. Since glomus cells tend to flatten out with age in culture, and their true shape is more ovoid than spherical, this estimate of diameter is somewhat arbitrary. However, direct measurements of tyrosine hydroxylase-positive glomus cells in two dimensions, after immunofluorescent identification under the fluorescent microscope (10), confirmed that, in both hypoxia- and Bt_2cAMP-treated cultures, the average diameter of glomus cells was significantly larger ($P < 0.01$) than in untreated normoxic controls after 9–14 days (data not shown); this estimate was obtained from the mean of the short and long axes, oriented perpendicular to each other and parallel to the plane of the coverslip. The extent of the hypertrophy increased progressively with exposure time and was already significant by day 5 of hypoxic treatment (Fig. 2). As with the increase in number of Na^+ channels (see above), the hypertrophic effect of hypoxia could be mimicked qualitatively by growing the cells under normoxia in the continuous presence of 1 mM Bt_2cAMP, but not NGF (Fig. 2); however, hypoxia (6% O_2) was more effective than 1 mM Bt_2cAMP in promoting hypertrophy in glomus cells, whereas the converse was true for the increase in number of Na^+ channels (cf. Figs. 1*A* and 2).

Did the progressive increase in inward Na^+ current, produced by chronic hypoxia and Bt_2cAMP (Fig. 1*A*), result simply from the accompanying increase in growth or hyper-

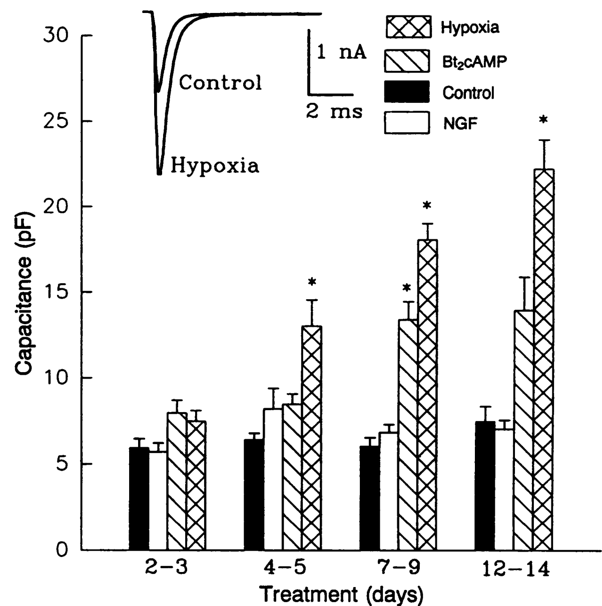

FIG. 2. Hypertrophy of chemosensory glomus cells induced by hypoxia and Bt_2cAMP but not NGF. After 5 days in a hypoxic (6% O_2) environment, the input capacitance (which is proportional to cell surface area) is significantly (*, $P < 0.01$) increased relative to age-matched controls; a progressive increase was observed with longer exposure times up to 14 days. The continuous presence of 1 mM Bt_2cAMP in the medium for 9–14 days also caused a significant, though less pronounced, increase in input capacitance in glomus cells grown under normoxic conditions. Control and NGF-treated (1 μg/ml) cells showed no significant changes in capacitance over 14 days in culture. (*Inset*) Superimposed capacity current transients from a control glomus cell and a glomus cell exposed to 13 days of hypoxia, in response to a voltage step from −60 to −120 mV. Note that input capacitance was determined by integration of the capacity current transient and dividing the resulting charge by the voltage step.

trophy of glomus cells (Fig. 2) or from a specific inductive effect? To address this, we compared the peak inward and outward current densities (elicited from holding potentials of −60 mV) by dividing the peak currents by membrane capacitance (pA/pF) at various treatment intervals. As shown in Fig. 3*A*, chronic hypoxia resulted in a significant increase in inward Na^+ current density by 7–9 days, though the even more pronounced effect of Bt_2cAMP was already quite substantial by 2–3 days of treatment. Once induced, the increased Na^+ current density persisted for at least 2 weeks with both treatments. In contrast, the outward current density, due mainly to K^+ channel activity (7, 11), remained relatively constant and indeed the trend was toward a decrease rather than an increase after 1 week of treatment with hypoxia or Bt_2cAMP (Fig. 3*B*). In addition, the normalized density of leakage channels (obtained on dividing the leakage current by membrane capacitance) was not significantly altered by either treatment (data not shown). These data suggest that both hypoxia and Bt_2cAMP promote a specific induction of Na^+ channels in glomus cells. Whether the density of calcium channels, also known to be present in these cells (5, 7, 22), is similarly augmented requires further study.

DISCUSSION

In this study, *in vitro* simulation of chronic hypoxia (over a 2-week period) was found to induce a selective increase in Na^+ channel density and hypertrophy in glomus cells of the rat carotid body. In a previous study on cultures of dissociated carotid bodies from embryonic rabbit, there was a

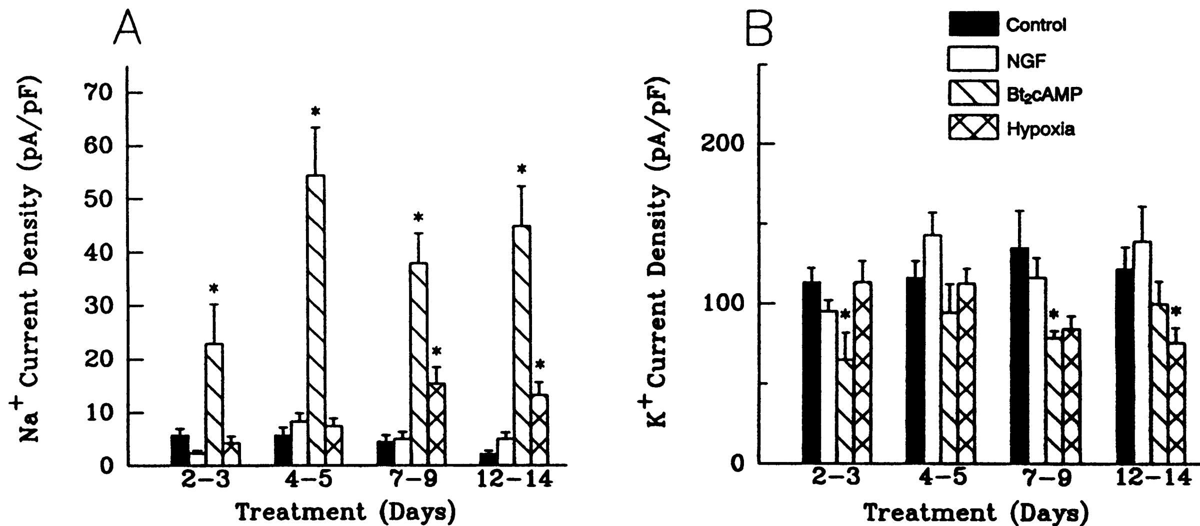

FIG. 3. Comparison of the effects of chronic hypoxia, Bt_2cAMP, and NGF on the density of Na^+ and K^+ currents in glomus cells. (*A*) Changes in Na^+ current density in glomus cells exposed to various treatments. Significant (*) increases in the peak Na^+ current density were recorded after 9 days in hypoxia (6% O_2). Bt_2cAMP (1 mM) caused significant increases as early as 2 days after exposure. (*B*) K^+ current density in glomus cells exposed to various treatments. K^+ current density in hypoxia and Bt_2cAMP-treated cells was equal to or less than that in control cells at all time points. In fact, significant (*) reductions in the K^+ current were seen after 7–14 days in both hypoxia- and Bt_2cAMP-treated glomus cells. Note that NGF-treated cells did not differ significantly from controls at any time point. (*A* and *B*) Significance levels were set at $P < 0.01$ (Student's *t* test). To calculate Na^+ current density, peak Na^+ currents elicited by voltage steps from −60 to 0 mV were divided by the input capacitance of the same cell. For the K^+ current density, steady-state K^+ currents elicited during a step of −60 to +50 mV (after ≈30 ms) were divided by the input capacitance.

suggestion that chronic hypoxia over a period of only 2 days resulted in higher resting potentials in glomus cells (23), but this point requires confirmation. These neuroendocrine cells are now generally considered to be the actual sensors of arterial P_{O_2} and respond directly to acute hypoxia by a decrease in K^+ conductance (5–7). Our findings suggest plausible mechanisms by which chronic hypoxia, during *in vivo* exposure, may produce the known altered physiological and morphological responses in the mammalian carotid body. One physiological alteration, thought to be involved in the ventilatory acclimatization to hypoxia, is the sensitization of the carotid body afferent response to chemosensory stimuli (3, 4). In light of the present findings, this sensitization may well be due to an increased Na^+ channel density in glomus cells, since Na^+ channels play a major role in controlling excitability in a number of neurons and neuroendocrine cells. Thus, a selective increase in Na^+ channel density would amplify the depolarization (and subsequent neurotransmitter release) in glomus cells resulting from a given chemosensory stimulus. In addition, it was found that the induction of Na^+ channels in glomus cells could be mimicked qualitatively in a normoxic environment by chronic treatment with the membrane-permeant analog of cAMP (i.e., Bt_2cAMP). Since acute hypoxia is known to elevate intracellular cAMP levels in the carotid body (12–14), and specifically in glomus cells (15), the possibility is raised that hypoxia may induce Na^+ channels in glomus cells via a cAMP-mediated pathway. It is noteworthy that elevated intracellular cAMP also causes Na^+ channel induction in skeletal muscle cells (17) and in the neuroendocrine PC12 cell line (16), whose parental chromaffin cells share several biochemical and ultrastructural similarities with glomus cells (10, 20, 21) and a common neural crest origin (19). However, although Na^+ channels could also be induced in PC12 cells by NGF treatment (16, 18), the latter was ineffective when applied to glomus cells in this study. Hence, it appears that NGF does not mediate the induction of Na^+ channels in glomus cells during chronic hypoxia.

Our studies also suggest that enlargement of the carotid body *in vivo*, after exposure of humans or animals to chronic hypoxia (1, 2, 8, 9), may be partly due to a direct effect of low P_{O_2} in promoting hypertrophy of the O_2-sensitive glomus cells, since in culture they almost doubled in size after 13 days in hypoxia (P_{O_2} ≈ 50 torr; 1 torr = 133.3 Pa). Since the cultures contained only carotid body cells, this finding suggests that circulatory growth factors (arising external to the carotid body) may not be involved in glomus cell hypertrophy observed after chronic exposure of animals to hypoxia (8, 9). However, we cannot exclude the possibility that chronic hypoxia may have increased the sensitivity of glomus cells to potential growth factors present in the serum used to grow the cells. This study also indicates that chronic treatment with Bt_2cAMP promotes hypertrophy in glomus cells, an effect likely mediated via elevation of intracellular cAMP. Interestingly, elevation of intracellular cAMP in related PC12 cells causes an increase in cell size, as does the addition of NGF (16, 18), but the latter was ineffective on glomus cells. The hypertrophy observed in glomus cells may also play a role in the sensitization of the carotid chemoreceptor response during chronic hypoxia if, as a consequence, more excitatory neurotransmitter is released for a given chemosensory stimulus.

Although both the induction of Na^+ channels and hypertrophy of glomus cells were mimicked by chronic treatment with Bt_2cAMP under normoxic conditions, chronic hypoxia was more effective in promoting hypertrophy but was less effective in inducing Na^+ channels. In fact, the effect of Bt_2cAMP in increasing Na^+ channels was nearly complete by day 5 of treatment—i.e., at a time when the effect of hypoxia on the same response had hardly begun. Thus these results suggest that cAMP may not be the sole mediator of the effects of chronic hypoxia. Indeed, in addition to cAMP elevation (15), hypoxia elicits other responses in glomus cells including the elevation of intracellular calcium (22) and a decrease in cGMP levels (15). Though in preliminary experiments, chronic treatment with Bt_2cGMP (200 μM) did not mimic the effects of Bt_2cAMP in augmenting the Na^+ currents in glomus cells (unpublished observations), the potential role of cGMP and other second messengers in carotid body adaptation to chronic hypoxia requires further investigation.

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