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EFFECTS OF HYPOXIA ON ATRIAL MUSCARINIC CHOLINERGIC RECEPTORS AND CARDIAC PARASYMPATHETIC RESPONSIVENESS*

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Abstract—Chronic exposure of rats to hypoxia resulted in a lower resting heart rate and a supranormal increase in heart rate in response to parasympathetic blockade by atropine. The density of muscarinic cholinergic receptors labeled by the antagonist [^{3}H]quinuclidinyl benzilate was elevated significantly in the atria of animals kept hypoxic for 2–4 weeks. Chronic hypoxia did not change the affinity of the receptor for [^{3}H]quinuclidinyl benzilate, the weight of the atria, or the amount of protein per atrial pair. Thus, the decrease in resting heart rate may be explained by the increase in the density of atrial muscarinic cholinergic receptors.

During the first day or two following exposure to hypoxia, heart rate and cardiac output increase, probably as a result of increased catecholamine output [1–3]. Following the initial increase, cardiac output decreases to below control values and resting heart rate returns to normal, or decreased, values [2–4], even though circulating levels of catecholamines are still elevated [1, 2]. Two hypotheses have been put forward to explain this adaptation of heart rate in the presence of increased catecholamine levels. Maher *et al.* [2] have shown that the activity of catechol-*O*-methyltransferase is increased in hypoxic animals, thus causing a more rapid degradation of released catecholamines. Alternatively, Voelkel *et al.* [4] have shown that the adrenergic responsiveness of the heart is decreased in hypoxic rats and that this decreased responsiveness is manifested by a diminished capacity of isoproterenol to stimulate cardiac adenylate cyclase activity *in vitro*. This effect appears to result from both a decrease in the density of beta-adrenergic receptors and a decrease in the maximum catalytic activity of cardiac adenylate cyclase. The present study was conducted to examine a possible role of cardiac muscarinic cholinergic receptors in the phenomenon of a lowered resting heart rate since the influence of the parasympathetic nervous system on resting heart rate is typically great [5]. Thus, the density and properties of atrial muscarinic receptors were determined using the specific binding of the radiolabeled antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB). Additionally, the effect of various doses of atropine on resting heart rate was examined to determine the influence of these receptors and the parasympathetic input.

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METHODS

Animals. Male Sprague–Dawley rats weighing 250–300 g were used in these experiments. Some animals were kept in a hypobaric pressure chamber at a simulated altitude of 4250 m (P_B = 450 torr) for 2–4 weeks. Control animals were maintained at a laboratory altitude (Denver) of 1600 m (P_B = 650 torr) in the same room and were exposed to the same 12 hr light–dark cycle as were the experimental animals. The animals were removed from the chamber in the morning, and the heart rates were measured or the animals were killed 1–2 hr following removal.

Heart rate measurements. Basal and atropine-stimulated heart rates were measured using an electrosphygmomanometer as previously described by Voelkel *et al.* [4]. Atropine was administered via a cannula in the tail vein.

Tissue preparation. After decapitation of an animal, the heart was quickly removed, and the atria, left ventricle and right ventricle were dissected and weighed. The atrial pair from each animal was homogenized (Polytron, speed 6 for 5 sec) in 100 vol. of cold 20 mM Tris–HCl (pH 7.5) containing 9 g/l NaCl (Tris–saline). The homogenate was centrifuged at 20,000 *g* for 10 min, and the pellet was resuspended (Polytron, speed 6 for 5 sec) in 900 vol. of Tris–saline buffer; and the resulting mixture was used in the binding assays.

Muscarinic receptor assay. This assay, which was originally described by Fields *et al.* [6], was modified so that an aliquot (0.9 ml) of the tissue mixture was incubated with 400–4500 cpm (0.013 to 0.14 nM) of [^{3}H]-(−)-quinuclidinyl benzilate ([^{3}H]QNB) (40.2 Ci/mmole, New England Nuclear Corp., Boston, MA), in 0.1 ml of distilled water containing 1 μg bovine serum albumin, 1.4 mM ascorbic acid and, in half of the tubes, atropine (final concentration of

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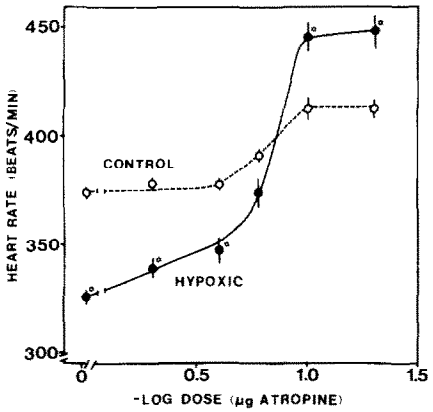

Fig. 1. Heart rate responses to atropine in control and hypoxic rats. Rats were kept at simulated high altitude for 2 weeks at which time their heart rates were measured following i.v. injections of various doses of atropine. Results are expressed as the mean ± S.E.M. obtained after each dose of atropine (N = 7). Key: (*) $P < 0.01$ relative to the control value at the same dose of atropine using a Newman–Keuls test [9] for multiple comparisons.

0.3 μM) to define nonspecific binding. Specific binding represented 50–85% of the total binding with much (50–80%) of the nonspecific binding being accounted for by the filter blanks and counter background, especially at low ligand concentrations. The concentration of tissue was such that less than 20% of the radioligand was bound. Following incubation in new polypropylene tubes (Sarstedt 538) for 45 min at 37°, the reaction was terminated by the addition of 10 ml of cold 10 mM Tris–HCl (pH 7.5) containing 9 g/l NaCl, and the mixture was poured over glass fiber filters (Schleicher & Schuell, No. 30) which were washed with an additional 10 ml of cold buffer. The filter was dried by vacuum suction, and the radioactivity trapped on the filter was determined in 3 ml of Triton–toluene (1:2) containing 3.53 mg/ml 2a70 (RPI) using a Searle Delta 300 scintillation counter (35% counting efficiency). The binding measured with this method appeared to be to muscarinic cholinergic receptors since the affinity constants for various drugs such as QNB (20–60 pM), atropine and scopolamine (1–5 nM), oxotremorine (10 nM; no GTP), and carbachol (1–3 μM; no GTP) appeared to be in the proper range. B_{max} values for [^{3}H]QNB binding were linear with protein up to at least twice the normal (110 μg) amount of protein used per assay tube.

Protein assay. The concentration of protein was determined in each tissue preparation by the method of Lowry *et al.* [7], using bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) as a standard.

Calculations and statistics. The [^{3}H]QNB saturation curves were transformed by the method of Scatchard [8], and the affinity of the receptors for [^{3}H]QNB (K_d) and the density of binding sites (B_{max}) were determined by linear least squares analysis. The statistical comparisons in Table 1 and Fig. 2 were made using Student's two-tailed *t*-test, and those in Fig. 1 were made using the Newman–Keuls multiple comparison test following a one-way analysis of variance [9]. The data were examined for statistical significant differences between control and hypoxic animals at each dose of atropine.

RESULTS

Heart rate responses to atropine in control and hypoxic rats. Rats kept 2–4 weeks at simulated high altitude (4250 m) had a significantly lower mean resting heart rate than did control animals kept at 1600 m (Fig. 1). This is in agreement with previously published data [4]. The effect of an intravenous injection of atropine on heart rate was also examined (Fig. 1). The maximum heart rate caused by atropine was significantly greater in the chronically hypoxic rats than in the control rats. The ED_{50} for atropine (8.9 μg/rat) was the same in the hypoxic and control rats.

Effect of hypoxia on atrial weight and protein content. Chronic hypoxia led to right ventricular hypertrophy in the animals used in this study. The ratio of right ventricular weight to total ventricular weight in a typical experiment was 0.242 ± 0.010 in controls

Table 1. Effect of hypoxia on atrial weight, protein content, density and properties of muscarinic receptors*

	Atrial wt	Protein/Atrial pair	B_{max}	K_d
	Relative values			
Control (N = 31)	100.0 ± 3.59	100.0 ± 4.66	100.0 ± 2.73	100.0 ± 4.35
Hypoxic (N = 30)	92.6 ± 3.65†	94.2 ± 4.33†	122.1 ± 4.78‡	102.5 ± 5.33†

* Animals were kept at simulated high altitude for 2–4 weeks. The data from four separate experiments were combined; each value is the mean ± S.E.M. (N = number of animals). The values for each animal have been normalized to the average of the control values for that set of four experiments and are expressed as percent control. The mean control B_{max} was 528 fmoles/mg protein. The mean control K_d value for [^{3}H]QNB was 0.048 nM.

† Not significant.

‡ $P < 0.001$.

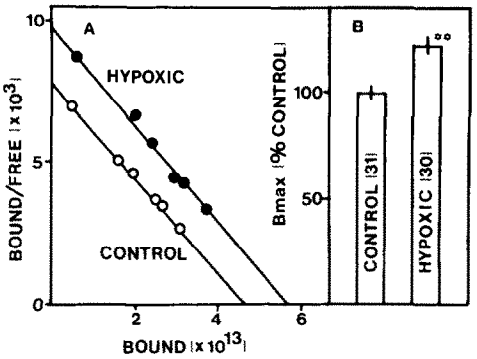

Fig. 2. Effect of hypoxia on the density of muscarinic cholinergic receptors. (A) Scatchard plot [8] of the binding of [^{3}H]QNB to atrial membranes from control and hypoxic animals. Data are combined from sixteen to seventeen animals that had been kept hypoxic for 2 weeks. The abscissa indicate specifically bound [^{3}H]QNB (moles/mg protein), and the ordinate indicates specifically bound divided by free [^{3}H]QNB (l/mg protein). The final concentrations of [^{3}H]QNB ranged from 0.013 to 0.14 nM. (B) The density of atrial [^{3}H]QNB binding sites was determined in thirty-one control and thirty hypoxic animals. Data were normalized as described in Table 1 and are the mean ± S.E.M. Values were from four separate experiments in which animals were kept hypoxic for 2–4 weeks. Key: (**) $P < 0.001$.

and 0.301 ± 0.003 ($N = 10$, $P < 0.001$) in hypoxic animals. In contrast, neither atrial weight nor the amount of protein per atrial pair was affected significantly in these animals (Table 1).

Effect of hypoxia on the density of muscarinic receptors. The specific binding of [^{3}H]QNB to muscarinic cholinergic receptors in homogenates of atria from control and hypoxic rats was determined at six concentrations of radioligand. The affinity of the receptors for [^{3}H]QNB was unchanged by the treatment (Table 1). On the other hand, the density of muscarinic receptors was 17–27% greater in atria from hypoxic rats compared to control rats (Fig. 2). This is in contrast with the finding that, overall, no consistent significant changes were found in either the right or left ventricle. All the values for the density of sites per mg protein, combined from three separate experiments ($N = 21$), were normalized to the mean control value found in each experiment. Values for the left ventricle were: control, 100 ± 5.6; hypoxic, 91 ± 4.8; and for the right ventricle: control, 100 ± 2.4; hypoxic, 107 ± 4.1.

DISCUSSION

The observation that hypoxic animals have a decreased cardiac output and a lower resting heart rate than control animals has previously been addressed in terms of the levels of circulating catecholamines and the function of the sympathetic nervous system [2, 4]. However, the normal resting heart rate of a young animal is strongly influenced by tonic parasympathetic activity to the sino-atrial node [5]. The present finding that the density of atrial muscarinic cholinergic receptors was increased following chronic hypoxia could provide the basis for the observed decrease in resting heart rate. Support for this hypothesis comes from the data in Fig. 1 which shows that the blockade by atropine of parasympathetic activity mediated by muscarinic receptors resulted in a supranormal increase in heart rate. This would require increased parasympathetic activity in the chronically hypoxic animals. Increased parasympathetic activity could result from either an increase in the amount of acetylcholine released or an increase in the responsiveness of the heart to acetylcholine. The present experiments do not bear on the first possibility but they do suggest that the second possibility, i.e. increased responsiveness of the heart, is responsible, at least in part, for the observed decrease in resting heart rate.

The increase in the density of atrial muscarinic cholinergic receptors following chronic hypoxia has been expressed as fmoles of receptor per mg of protein. A constant concern in studies such as these is that there may be alterations in the amount of protein per g wet weight or per atrial pair which could artifactually cause changes in the density of receptors when expressed per mg protein. As can be seen in Table 1, there were no significant changes in atrial weight or in the amount of protein per atrial pair. Thus, there was no change in the amount of protein per g wet weight, and the reported increase in muscarinic receptor density is significant whether expressed as fmoles/mg protein or fmoles/g wet weight.

Maher *et al.* [3] reported that goats kept hypoxic for 10 days had a higher resting heart rate than goats kept at sea level. Additionally, they found no difference in the heart rate increase caused by the administration of atropine methyl bromide in control or hypoxic goats. In our experiments, however, atropine had a greater effect in hypoxic rats than in control rats. The difference between the present results and those reported by Maher *et al.* [3] may be due to the facts that younger animals, a different species, and longer periods of exposure to simulated high altitude were used in the current study.

The supranormal increase in heart rate that followed an injection of atropine may have been a consequence of either increased sympathetic activity in hypoxic rats resulting in an elevated heart rate following parasympathetic blockade or an increased intrinsic (i.e. independent of autonomic control) heart rate. The former is less likely since Voelkel *et al.* [4] have shown that hypoxia induces a decrease in sympathetic responsiveness in rat heart. Thus, the increased heart rate in hypoxic rats with parasympathetic blockade was most likely due to an increased intrinsic rate. The mechanism for such an increase is not clear.

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