Maintenance of adenosine A\textsubscript{1} receptor function during long-term anoxia in the turtle brain

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Lutz, Peter L., and Liscia Manuel. Maintenance of adenosine A\textsubscript{1} receptor function during long-term anoxia in the turtle brain. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R633–R636, 1999.—It has been established that adenosine has a critical role in the extraordinary ability of the turtle brain to survive anoxia. To further investigate this phenomenon we compared rat and turtle brain adenosine A\textsubscript{1} receptors using cyclopentyl-1,3-dipropylxanthine,8-[dipropyl-2,3-\textsuperscript{3}H(N)] ([\textsuperscript{3}H]DPCPX) saturation binding analyses and determined the effects of prolonged anoxia (6, 12, and 24 h) on the adenosine A\textsubscript{1} receptor of the turtle brain. The rat brain had a 10-fold greater density of A\textsubscript{1} receptors compared with the turtle [rat cortex receptor density (B\textsubscript{max}) = 1,400 \pm 134.6 fmol/mg protein, turtle forebrain B\textsubscript{max} = 103.2 \pm 4.60 fmol/mg protein] and a higher affinity [dissociation constant (K\textsubscript{d}) rat cortex = 0.328 \pm 0.035 nM, K\textsubscript{d} turtle forebrain = 1.16 \pm 0.06 nM]. However, the turtle K\textsubscript{d} is within the reported mammalian range, and the B\textsubscript{max} is similar to that reported for other poikilotherms. Unlike the mammal, in which A\textsubscript{1} receptor function is rapidly compromised in anoxia, in the turtle forebrain no significant changes in the A\textsubscript{1} receptor population were seen during 24-h anoxia. However, in the hindbrain, whereas the B\textsubscript{max} remained unchanged, the K\textsubscript{d} significantly decreased from 2.1 to 0.5 nM after 6 h anoxia and this higher affinity was maintained at 12- and 24-h anoxia. These findings indicate that, unlike the GABA\textsubscript{A} receptor, the protective effectiveness of adenosine in the anoxic turtle brain is not related to an enhanced receptor number. Protection from a hypoxia-induced compromise in A\textsubscript{1} receptor function and an increased A\textsubscript{1} sensitivity in the hindbrain may be important factors for maintaining the adenosine-mediated downregulation of energy demand during long-term anoxia.

cyclopentyl-1,3-dipropylxanthine,8-[dipropyl-2,3-\textsuperscript{3}H(N)]; ischemia

IT IS WELL ESTABLISHED that adenosine plays a critically protective role in the brain during the initial period of energy deficit caused by hypoxia or ischemia (31). Intracellular adenosine, derived from a breakdown of ATP, increases during anoxia and is released from the cell by a nucleoside transporter (26). The extracellular adenosine acts to reduce electrical activity through an adenosine A\textsubscript{1} receptor-mediated activation of K\textsuperscript{+} channels, a blockage of Ca\textsuperscript{2+} channels, and suppression of excitatory neurotransmitter release (8, 28). However, the protection is only temporary, and after 2–3 min of anoxia the mammalian brain starts to fail. ATP depletion causes a halt in ion pump activity with a consequent loss of ion gradients, an uncontrolled increase in intracellular Ca\textsuperscript{2+}, and a massive loss of excitatory neurotransmitters, such as glutamate, aspartate, and dopamine, into the extracellular space (30). The latter event produces a wave of destruction outside the immediately affected region (30).

There is also evidence that adenosine receptor function is interfered with by hypoxia/ischemia. Ischemia produces a rapid depletion in adenosine A\textsubscript{1} receptors in the gerbil brain (23) and rat brain (13, 20), and as little as 2 min of anoxia results in a persistent downregulation of hippocampal receptors in the gerbil (1). Even relatively mild hypoxic exposure (7.7\% O\textsubscript{2}) produces a rapid and substantial decrease in hippocampal A\textsubscript{1} receptor density in the neonate rat (1). Any permanent or long-lasting impairment of adenosine receptor function would be an important contributing cause of brain damage induced by hypoxia/ischemia (1). For example, because the density of A\textsubscript{1} receptors has been shown to be a critical factor in determining the regional strength of adenosine’s inhibitory action, a reduction in receptors could compromise adenosine’s retaliatory effect (13).

In contrast to the mammal (and most other vertebrates) the brain of the freshwater turtle Trachemys scripta is able to withstand anoxia for >24 h at room temperature (2, 18). The mechanisms behind this remarkable ability have been the subject of intense research (2, 9, 18). Adenosine, which is released during anoxia (17, 21), appears to play a critical role. Superfusing the anoxic isolated turtle cerebellum with adenosine receptor blockers theophylline or 8-cyclopentyltheophylline (CPT) causes rapid depolarization (25), and superfusion of the brain with the adenosine receptor blocker aminophylline prevents the (normal) anoxia-induced increase in brain blood flow (10). Adenosine causes a decrease in turtle brain N-methyl-o-aspartate receptor activity and an associated decrease in Ca\textsuperscript{2+} permeability (4), and adenosine appears to mediate anoxia-induced “channel arrest” (24), but we know very little about the mechanisms involved. For example, we have no information on how the adenosine receptor responds to anoxia in the turtle brain. This is particularly interesting considering the high vulnerability of the mammalian adenosine receptor to hypoxia/anoxia. It is also of interest to determine if the extraordinary efficiency of adenosine in facilitating complete anoxia tolerance in the turtle brain is related to it possessing a comparatively high density of adenosine receptors. An earlier study showed that turtle and mammalian brains have similar densities of the inhibitory acting GABA\textsubscript{A} receptor (16) while the energy-consuming systems of the turtle are 10–30% that of the mammal (18).

The purpose of this study, therefore, was to compare the A\textsubscript{1} receptor densities of turtle and rat brains and to
determine the effects of prolonged anoxic exposure (6–24 h) on the A1 adenosine receptors in the turtle brain.

MATERIALS AND METHODS

Materials. All studies were approved by the Institutional Animal Care and Use Committee. Freshwater turtles (Trachemys scripta, 0.75–1.3 kg) were purchased from William A. Lemberger Animals (Oshkosh, WI). Cyclopentyl-1,3-dipropylxanthine, 8-[dipropyl-2,3-3H(N)] ([3H]DPCPX) (specific activity 95.5–105 Ci/mmol) was purchased from DuPont-New England Nuclear. Theophylline and all other reagents were purchased from Sigma (St. Louis, MO) or Boehringer Mannheim.

Brain preparation. Turtles were placed in sealed plastic chambers (36 × 25 cm) at room temperature (25°C). The chambers were initially flushed with 100% N2 at a high rate of flow for 1 min, and the flow was then reduced to a constant rate of 5 ml/min. We have found that this results in the chamber air being completely replaced by nitrogen within 10 min (7). Turtles were held in the chambers for periods of 6, 12, and 24 h, after which they were killed by decapitation. Control (air) turtles were immediately killed on removal from living quarters. The cerebral hemispheres (forebrain) and hindbrain (remainder of brain minus brain stem) were dissected on ice and immediately frozen in liquid N2 and stored at −80°C.

Binding assays. Tissue was processed by standard methods (3) with some modifications. Briefly, the tissue was homogenized in 10 ml cold 50 mM Tris-HCl buffer (pH 7.4) with a Biospec tissue tearor at setting 5 for 30–45 s. The homogenates were centrifuged at 48,000 g at 0°C for 10 min. The pellet was resuspended in the same volume of Tris and centrifuged at 48,000 g for 20 min. The pellets were then frozen at −20°C until use, up to 4 days. The pellet was suspended in enough volume of Tris buffer to bring the protein concentration to −0.30–0.60 mg/ml, and the suspensions were incubated with 2 IU/ml of adenosine deaminase (ADA) for 30 min at 25°C. These suspensions were used in the final saturation binding experiments.

For the adult rat brain, the cerebral hemispheres (forebrain) and cerebellum were processed similarly, except the tissue was homogenized in 20 ml cold buffer. Also, per the observations of Bruns et al. (3), the final tissue suspensions were incubated with 1 IU ADA/ml.

Binding was carried out in polypropylene tubes (Sarstedt) containing 200 µl of homogenate (40–80 µg protein), 25 µl Tris buffer containing 10 mg/ml BSA ± 1 mM theophylline and 25 µl [3H]DPCPX diluted in Tris buffer and 0.1% BSA. [3H]DPCPX concentrations were 8.2 nM–16 µM.

The incubation lasted for 1.5 h at 25°C and was terminated by the addition of 4 ml ice-cold Tris buffer and filtration under reduced pressure through Whatman GF/B glass fiber filters. Filters were washed quickly two times with ice-cold Tris, partially dried, and allowed to stand in 3 ml Ecolume scintillation fluid overnight. The filters were counted at an efficiency of 42%.

Specific binding was calculated as the difference between binding in the presence and absence of 1 mM theophylline. Protein concentrations were determined by the modified method of Lowry et al. (15).

Data analysis. Data are presented as means ± SE. All experiments were analyzed for dissociation constant (Kd) and receptor density (Bmax) using the Lundon software program ReceptorFit Saturation Two-Site. A nonlinear least-squares regression analysis was used for computing Kd and Bmax.

Statistical analysis of data was performed using the SAS Institute program MP ANOVA (1 way) was done to compare control and 6, 12, and 24 h anoxia affinities and maximal binding and all with the adult rat. Where significance of P < 0.05 was found, it was confirmed by Tukey-Kramer all-pairs analysis and Dunnett’s control comparison analysis.

RESULTS

A representative Scatchard plot comparing a control and 12-h anoxic turtle hindbrain is shown in Fig. 1. All the results were linear, consistent with a single high-affinity population of receptors. The fore- and hindbrain of control turtles had similar Bmax values, but the forebrain had a significantly higher affinity for the adenosine A1 receptor compared with the hindbrain (Kd forebrain = 1.16 ± 0.06 nmol, Kd hindbrain = 1.81 ± 0.21, P < 0.05) (Fig. 2). The rat forebrain and cerebellum had similar Kd and Bmax values (Fig. 2), but compared with the turtle, the rat brain had a >10-fold higher density of receptors (mean Bmax rat forebrain = 1,400.0 ± 134.60 fmol/mg protein, Bmax turtle forebrain = 103.2 ± 4.60 fmol/mg protein) and a somewhat higher affinity (Fig. 2).

Exposure to anoxia for up to 24 h produced no significant changes in either Kd or Bmax in the turtle forebrain (Fig. 3). However, in the turtle hindbrain a significant fall in Kd occurred within 6 h of anoxia, and this enhanced affinity was maintained over 24 h of anoxia (Fig. 4). Yet no change was seen in the Bmax of the turtle hindbrain throughout this period (Fig. 4).

DISCUSSION

Although the turtle brain adenosine A1 receptor had a lower affinity (Kd = 1.16–1.81 nmol) than the rat brain (Kd = 0.33–0.54 nmol), the turtle values are within the range found for other mammals (Kd = 0.17–2.1 nmol; Ref. 11), indicating that the turtle brain adenosine A1 receptor is activated at similar extracellular adenosine concentrations as the mammal.

By contrast, the density of the A1 receptors in the turtle brain is <10% that of the rat brain. The Bmax values that we report for the rat are similar to those
found for other mammals, for example, bovine brain (1,460 fmol/mg protein; Ref. 14) and fetal sheep medulla (1,222 fmol/mg protein; Ref. 12). This difference between turtles and mammals may be phylogenetically related, because a similar degree of difference in A1 receptors is also seen between fish and mammals. With the use of an A1 receptor agonist, [3H]cyclohexyladenosine ([3H]CHA), the Bmax for the gerbil hippocampus is 1,250 fmol/mg protein (32) and for human cerebral cortex it is 9,200 fmol/mg protein (19). By comparison, goldfish and eel whole brains have [3H]CHA Bmax values of 69.58 and 120 fmol/mg protein, respectively (27, 29). It is probable, therefore, that the brains of vertebrate poikilotherms have densities of adenosine A1 receptors about one-tenth that of mammals, matching the differences in brain metabolic intensity between the two groups (18), and the turtle is no exception in this respect. By contrast, the turtle and mammal have similar density in GABA<sub>A</sub> receptors (16). GABA, a major inhibitory neurotransmitter, is released during prolonged anoxia in the turtle brain (18).

The efficiency of adenosine in promoting turtle brain anoxia tolerance does not, therefore, lie in the turtle having a corresponding higher number of adenosine A1 receptors, although the density of the A1 receptor has an important influence on the intensity of adenosine-mediated depression (13). Other factors must be involved. Unlike in the mammal, where a rapid reduction in adenosine A1 binding sites occurs during even brief hypoxia/ischemia (1, 13, 20), no such deterioration is seen over 24 h of anoxia in the turtle brain. In the mammal, the vulnerability of the A1 binding sites to hypoxia/ischemia is thought to compromise adenosine function and has been linked to postischemic damage (1). In the turtle brain, the ability to maintain adenosine A1 receptor number during prolonged anoxia is likely to be one of the many key adaptations for brain anoxia survival (18). Adenosine continues to be released during prolonged anoxia in the turtle and probably continues to play a long-term protective role (17). Maintenance of receptor function would be essential for this purpose. The increased A1 sensitivity in the hindbrain might result in the receptor being activated at lower extracellular adenosine levels during anoxia, thereby enhancing its effectiveness. We have no knowledge of the mechanisms involved. However, a decrease in affinity, or desensitization, of the A1 receptor that is activated by the A2A receptor (5) or the A3 receptor (6) has recently been found in the rat striatum. The turtle brain may be protected against A2A or A3 receptor activation during anoxia.
REFERENCES


