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Changes in the activities of adenosine-metabolizing enzymes in six regions of the rat brain on chemical induction of hypothyroidism

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1. Rats (4 weeks old) were made hypothyroid by treatment with propylthiouracil and a low-iodine diet for a further period of 4 weeks. Synaptosomal membranes, myelin and 105000 *g* soluble fractions were obtained from six regions of the brain. 2. Hypothyroidism resulted in 2–5-fold increases in membrane-bound 5′-nucleotidase activity in synaptosomal fractions obtained from cerebellum, cortex, striatum and hippocampus. By contrast, myelin 5′-nucleotidase activity was slightly increased only in the medulla oblongata. 3. Hypothyroidism did not change adenosine deaminase activity, but decreased adenosine kinase activity by approx. 40% in soluble fractions obtained from cerebellum, hippocampus, striatum and hypothalamus. 4. It is suggested that these changes in hypothyroidism, in particular the increases in 5′-nucleotidase activity, could enhance the neuromodulatory effect of adenosine to decrease neurotransmitter release.

INTRODUCTION

It is now well documented that adenosine plays an important regulatory role in the functioning of the central nervous system. It acts as a neuromodulator primarily through inhibition of excitatory neurotransmitter release (Phillis & Wu, 1981; Williams, 1984; Snyder, 1985), producing effects such as sedation, decreased motor activity, inhibition of breathing and anticonvulsant actions (Katims *et al.*, 1983; Williams, 1984; Eldridge & Millhorn, 1987). These effects of adenosine appear to be presynaptic, are mediated by adenosine receptors of the A_1 subtype (Fredholm & Dunwiddie, 1988), which are widely distributed throughout the central nervous system (Lewis *et al.*, 1981; Goodman & Snyder, 1982; Daly, 1985; Bruns *et al.*, 1987; Weber *et al.*, 1988), and involve inhibition of release of acetylcholine, noradrenaline, dopamine or 5-hydroxytryptamine (Fredholm & Dunwiddie, 1988). The occupied A_1 receptor, coupled to G-proteins, may achieve this modulation through inhibition of adenylate cyclase, by inhibition of voltage-sensitive Ca^{2+} currents or by an increase in K^+ conductance (Proctor & Dunwiddie, 1983; Dolphin *et al.*, 1986; Scott & Dolphin, 1987; Trussel & Jackson, 1985, 1987; Fredholm & Dunwiddie, 1988). Adenosine also plays an important neuroprotective role in that its increased concentration within the central nervous system after metabolic insults such as ischaemia or hypoxia results in vasodilation and increased blood flow (Berne *et al.*, 1974, 1987; Winn *et al.*, 1979, 1981; McIlwain & Poll, 1986; Van Wylen *et al.*, 1986). It is envisaged that all these effects are mediated by adenosine that is formed within or released into extracellular spaces. Two routes of extracellular provision of adenosine appear likely. First, adenosine may be produced as the end product of an extracellular purine nucleotide phosphohydrolase pathway. ATP is packaged and released as a co-transmitter with 'classical' neurotransmitters such as acetylcholine (Nagy *et al.*, 1976), noradrenaline (Geffen & Livett, 1971; Lagercrantz, 1971) or 5-hydroxytryptamine (Da Prada & Pletscher, 1968). Extracellular hydrolysis of ATP has been described in synaptosomes from peripheral nerves (Zimmermann *et al.*, 1986) or the central nervous system (Nagy, 1986; Richardson *et al.*, 1987). In this pathway adenosine is formed from extracellular AMP via a membrane-bound 5′-nucleotidase ectoenzyme, and can be removed by uptake into cells by a widely distributed adenosine-transport system (Bender *et al.*, 1980, 1981; Bisserbe *et al.*, 1985). Second, in response to metabolic insults, adenosine may be produced intracellularly after a decrease in the energy charge of the cell and a resulting rise in AMP content (Winn *et al.*, 1979; Fredholm & Hedqvist, 1980; Wojcik & Neff, 1982; Jonzon & Fredholm, 1985; McIlwain & Poll, 1986). By analogy with heart muscle (Itoh *et al.*, 1986; Truong *et al.*, 1988; Newby, 1988), it would seem most likely that this route of adenosine formation should involve an intracellular, soluble, 5′-nucleotidase activity that has been noted in brain (Montero & Fes, 1982; Mallol & Bozal, 1983; Newby *et al.*, 1987).

The likely importance of the membrane-bound 5′-nucleotidase ectoenzyme in the regulation of purine salvage and of synaptic transmission has been noted by several workers (Phillis *et al.*, 1979; Zimmermann *et al.*, 1979; Heymann *et al.*, 1984; Kreutzberg *et al.*, 1986; Richardson *et al.*, 1987). Previous work from our and other laboratories has shown that the membrane-bound 5′-nucleotidase activity in adipose tissue and muscle is altered by insulin treatment, diabetes or hypothyroidism (Jamal & Saggerson, 1987; Karnieli *et al.*, 1987; Klip *et al.*, 1988). In view of known behavioural changes in hypothyroidism (Gold *et al.*, 1981; Reus, 1986), we have measured membrane-bound 5′-nucleotidase activity in six regions of the rat brain after chemical induction of hypothyroidism. We report some region-specific increases in this activity in synaptosomal fractions.

The reactions catalysed by adenosine deaminase and adenosine kinase represent the likely major routes of adenosine disposal after its re-uptake or its intracellular formation (Phillips & Newsholme, 1979; Reddington & Pusch, 1983). In addition, inhibition of adenosine deaminase influences brain adenosine content and

potentiates presynaptic effects of adenosine (Muller & Patton, 1979; Zetterstrom *et al.*, 1982). These two soluble enzyme activities were therefore also measured in the present study.

MATERIALS AND METHODS

Chemicals

Enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.). Radiochemicals were from Amersham International (Little Chalfont, Bucks., U.K.).

Animals

These were male Sprague–Dawley rats bred at University College London. All animals had constant access to drinking water and to Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.), which contained (w/w) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 h/11 h, with light from 06:00 to 19:00 h. Rats to be made hypothroid were selected at age 4 weeks (80–90 g body wt.) and then fed on an iodine-deficient version of the No. 3 Breeding Diet and drank water containing 0.01% (w/v) 6-n-propyl-2-thiouracil (Chohan *et al.*, 1984; Saggerson & Carpenter, 1986). These animals were killed 4 weeks after commencement of this treatment when they weighed 140–170 g. Euthyroid controls fed on a normal diet were also 8 weeks old at time of death, when they weighed 260–280 g.

Preparation of subcellular fractions

Rats were stunned and then killed by decapitation. Brains were rapidly removed and placed on an ice-cold Petri dish covered with filter paper wetted with 0.32 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4). Six regions of the brain were separated by the dissection procedure outlined by Glowinski & Iverson (1966). The regions were: cerebellum, medulla oblongata (consisting of medulla oblongata and pons), striatum (consisting of the putamen nucleus, caudate nucleus and globus pallidus nucleus), cortex (corresponding to the telencephalon without the 'striatum' and including white and grey matter of the cerebral cortex), hippocampus and the hypothalamus. Pooled regions from four to six brains, or in some instances whole forebrains, were collected in ice-cold 0.32 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4), weighed, cut into small pieces and washed with the same medium to remove blood. Homogenates were prepared in a Potter–Elvehjem glass homogenizer with a Teflon pestle (six up-and-down strokes at 500 rev./min with a radial clearance of 0.2 mm). The homogenates were diluted with the ice-cold sucrose medium to give final concentrations of 11–15% (w/v), and then fractionated essentially as described by Booth & Clark (1978) with some minor modifications. After an initial centrifugation at 1500 $g_{av.}$ for 3 min, the resulting supernatants were centrifuged at 18000 $g_{av.}$ for 10 min to produce crude mitochondrial/synaptosomal pellets. The 18000 g supernatants were re-centrifuged at 105000 $g_{av.}$ for 45 min, and the resulting supernatants were stored at −80 °C. The pellets were resuspended in 5 ml of the sucrose medium and then mixed with 25 ml of 12% Ficoll medium [12% (w/w) Ficoll, 0.32 M-sucrose, 10 mM-Tris/HCl buffer (pH 7.4). Portions (5 ml) of these suspensions were pipetted into 14 ml centrifuge tubes, followed by a layer of 2.5 ml of 7% Ficoll medium [7% Ficoll, 0.32 M-sucrose, 10 mM-Tris/HCl buffer (pH 7.4)] and then a layer of 2.5 ml of 0.32 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4). After centrifugation at 110000 $g_{av.}$ for 1 h, the myelin and synaptosomal fractions banded at the upper and lower interfaces respectively, with mitochondria pelleting at the bottom of the tubes. The myelin fractions were suspended in 50 mM-Tris/HCl buffer (pH 7.4) and re-centrifuged for 45 min at 105000 $g_{av.}$. The resulting washed myelin fractions were finally resuspended in 2–3 ml of the same buffer and stored at −80 °C. The synaptosomal layers were removed from the lower interface and resuspended in 5 mM-Tris/HCl buffer (pH 8.0). After sonication for 30 s, the suspensions were left on ice for 30–45 min to achieve lysis of the synaptosomes, followed by centrifugation for 45 min at 105000 $g_{av.}$. The resulting synaptic-membrane fractions were resuspended in 2–3 ml of 50 mM-Tris/HCl buffer (pH 7.4) and stored at −80 °C.

Analytical methods

Protein contents of subcellular fractions were measured by the method of Lowry *et al.* (1951), with bovine albumin as a standard. Lactate dehydrogenase (EC 1.1.1.27) was assayed spectrophotometrically at 25 °C (Saggerson, 1974). Succinate dehydrogenase (EC 1.3.99.1) was assayed spectrophotometrically at 37 °C with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride as electron acceptor (Prospero, 1974). 2′,3′-Cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37) was assayed spectrophotometrically at 25 °C with 2′,3′-cyclic $NADP^+$ as substrate (Sogin, 1976). Acetylcholinesterase (EC 3.1.1.7) was assayed spectrophotometrically at 25 °C by using acetylthiocholine as substrate and coupling the reaction to 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman *et al.*, 1961). Adenosine deaminase (EC 3.5.4.4) and adenosine kinase (EC 2.7.1.20) were assayed radiochemically at 37 °C with [2-^{3}H]adenosine as substrate. The procedures for these assays and the separation of reaction products by t.l.c. were essentially as described by Arch & Newsholme (1978), with minor modifications (Jamal & Saggerson, 1987). Both adenosine deaminase and adenosine kinase assays were initiated by addition of 0.01 ml (5–20 μg of protein) of brain 105000 g supernatant. 5′-Nucleotidase (EC 3.1.3.5) was assayed at 37 °C by monitoring the conversion of [2-^{3}H]AMP into [^{3}H]adenosine (Newby *et al.*, 1975). The reaction was initiated by the addition of 20–50 μg of myelin or synaptic-membrane protein. All three radiochemical assays were linear with respect to time in both the euthyroid and the hypothyroid states. Adenosine deaminase and 5′-nucleotidase assays were also linear with respect to protein concentration, whereas the assay for adenosine kinase was non-linear in this respect (see the Results and discussion section).

Statistical methods

Statistical significance was evaluated by Student's t test for unpaired samples.

RESULTS AND DISCUSSION

Distributions of marker enzymes

Preliminary experiments (Table 1), using fractionated homogenates from whole forebrain, established that marker enzymes were distributed in the appropriate fractions in the euthyroid and hypothyroid states. It was noted that hypothyroidism decreased the specific activity of 2′,3′-cyclic nucleotide phosphodiesterase in the myelin fraction by 30% ($P < 0.01$) and also resulted in a striking 68% decrease in the specific activity of lactate dehydrogenase in the soluble fraction ($P < 0.001$). It is noteworthy that hypothyroidism also results in a major decrease in lactate dehydrogenase activity in white-adipose-tissue cells (Baht & Saggerson, 1988). The reasons for these changes are unclear.

5′-Nucleotidase

Membrane-bound 5′-nucleotidase was observed in purified myelin and synaposomal-fraction membranes from all six brain regions tested. Association of a significant production of brain 5′-nucleotidase with myelin has been noted by Cammer *et al.* (1980), Heymann *et al.* (1984) and Casado *et al.* (1988). In addition, subcellular-fractionation studies have repeatedly shown the presence of the enzyme on membranes derived from synaptosomal fractions (see, e.g., Phillips & Newsholme, 1979). However, cytochemical evidence suggests that most of this 'synaptosomal' activity is not derived from neurons, but from glial-cell membranes (Kreutzberg & Barron, 1978; Kreutzberg *et al.*, 1978, 1986; Heymann *et al.*, 1984), and in a complement-fixation assay using antiserum to 5′-nucleotidase Richardson (1983) has provided clear evidence that the enzyme is absent from cholinergic neuron terminals.

Although other workers have previously measured 5′-nucleotidase activity in various regions of the rat brain (Sun *et al.*, 1976; Phillips & Newsholme, 1979; Nagata *et al.*, 1984), we are unaware of any regional study in which activity measurements were made in both purified myelin and synaptosomal-membrane fractions. Table 2 shows that 5′-nucleotidase specific activities in the myelin fraction were relatively uniform between regions, except for a slightly lower activity in the medulla oblongata in the euthyroid state. In euthyroidism, cerebellum, hippocampus, hypothalamus and striatum showed similar activities in the membranes derived from synaptosomes, whereas medulla oblongata showed a relatively high activity (Sun *et al.*, 1976) and cortex showed a relatively low activity (Sun *et al.*, 1976; Phillips & Newsholme, 1979).

Previous studies using homogenates or membrane preparations from rat or mouse brain have shown a decrease in brain 5′-nucleotidase activity in hypothyroidism (Smith *et al.*, 1980; King *et al.*, 1983; Shanker *et al.*, 1984). However, these studies were deliberately conducted during the 'critical period' of myelination, which is highly sensitive to thyroid-hormone status (Balazs *et al.*, 1969; Rosman *et al.*, 1972; Walters & Morell, 1981). With regard to the present study, it should be stressed that induction of hypothyroidism was not commenced until this process was essentially complete.

Table 2 shows that hypothyroidism caused significant increases in 5′-nucleotidase activity in cerebellum (5.5-fold), cortex (4.8-fold), striatum (3.9-fold) and hippocampus (2.3-fold). There were no significant changes in the medulla oblongata or the hypothalamus. By contrast, the 5′-nucleotidase activity in the myelin fraction was unchanged, except for a modest increase in the medulla oblongata. Hypothyroidism therefore brings about a region-specific increase in membrane-bound 5′-nucleotidase activity that is almost entirely confined to structures at, or near to, nerve terminals.

Adenosine deaminase and adenosine kinase

These activities were assayed in the 105000 *g* supernatants. Adenosine deaminase activities in cortex and cerebellum were very similar to those reported by Phillips & Newsholme (1979). Hypothyroidism had no significant effect on adenosine deaminase activity. Preliminary experiments revealed an anomaly in the assay of

Table 1. Distribution of marker enzymes in subcellular fractions from rat whole forebrain

Homogenates were prepared and fractions isolated as described in the Materials and methods section. The values are means ± S.E.M. for four separate preparations, except where it is indicated that $n = 3$.

Fraction	Condition	Enzyme activity (nmol/min per mg of protein)			
		Acetylcholin-esterase	2′,3′-Cyclic nucleotide phosphodiesterase	Lactate dehydrogenase	Succinate dehydrogenase
Synaptic membranes	Control	172 ± 8	2050 ± 128	1667 ± 49	4.3 ± 0.3
	Hypothyroid	148 ± 28	2250 ± 128	227 ± 15	4.5 ± 0.3
Myelin	Control	28 ± 2	14583 ± 850	463 ± 71	1.5 ± 0.1
	Hypothyroid	21 ± 2	10000 ± 517 ($n = 3$)	240 ± 36	0.7 ± 0.1 ($n = 3$)
105000 *g* supernatant	Control	32 ± 2	275 ± 20	7550 ± 567	1.2 ± 0.1
	Hypothyroid	24 ± 3 ($n = 3$)	427 ± 40	2567 ± 195	1.0 ± 0.1
Mitochondria	Control	50 ± 3	829 ± 62	983 ± 168	23 ± 2
	Hypothyroid	39 ± 5	727 ± 66	435 ± 23	21 ± 2

Table 2. Effects of hypothyroidism on activities of adenosine-metobolizing enzymes in regions of rat brain

The values are expressed as nmol/min per mg of protein and are means ± S.E.M. for the numbers of measurements shown in parentheses. Statistical significance of changes relative to the control is indicated by a, b, c, d, which represent $P < 0.05$, < 0.02, < 0.01, < 0.001 respectively.

Enzyme	Condition	Brain region					
		Cerebellum	Cortex	Hippocampus	Hypothalamus	Striatum	Medulla oblongata
Adenosine kinase	Control (5)	0.69±0.8	0.41±0.05	0.37±0.05	0.27±0.03	0.38±0.06	0.34±0.05
	Hypothyroid (4)	0.39±0.04[b]	0.25±0.05	0.23±0.02[a]	0.15±0.02[c]	0.22±0.02[a]	0.25±0.03
Adenosine deaminase	Control (4–5)	1.78±0.13	1.87±0.15	1.27±0.40	3.62±0.95	1.34±0.34	2.37±0.46
	Hypothyroid (5–6)	1.80±0.25	1.98±0.30	1.42±0.20	2.66±0.16	2.03±0.33	3.12±0.54
5′-Nucleotidase (myelin)	Control (4–5)	9.03±2.41	14.2±2.8	13.9±3.2	14.4±2.9	10.6±2.2	6.78±1.89
	Hypothyroid (4–5)	7.25±0.36	14.0±1.6	16.8±2.7	15.0±2.2	15.8±2.7	12.9±0.7[a]
5′-Nucleotidase (synaptic)	Control (4–5)	8.99±2.27	3.31±0.26	8.28±2.81	8.27±1.59	9.36±2.08	16.4±3.7
	Hypothyroid (4–5)	49.3±4.4[d]	16.0±1.2[d]	18.8±1.4[d]	9.68±1.37	36.2±4.8[c]	17.6±1.4

adenosine kinase in that, although time courses were linear at various protein concentrations, the specific activity increased considerably with protein concentration (Fig. 1). Since thyroid status did not appreciably alter the nature of this dependence, comparison of adenosine kinase activities in the two states was still a valid exercise. As a precaution all assays were performed with 5.0 μg of soluble protein (125 μg/ml in the assays). The reason for this phenomenon is not known. Possibly it reflects enzyme dissociation/disaggregation or loss of some necessary activator on dilution of the soluble extract. Although not commented on by Phillips & Newsholme (1979), it is clear from Tables 2 and 3 of that study that the specific activity of adenosine kinase is enriched to a substantially greater extent than that of adenosine deaminase during isolation of high-speed soluble fractions from homogenates of rat brain. It remains to be established whether this is a related phenomenon. Table 2 shows that hypothyroidism decreased adenosine kinase specific activity by approx. 40% in cerebellum, hippocampus, hypothalamus and striatum. The cell types responsible for these changes are unknown; nor is it known whether they are expressed at sites where they may readily influence the re-utilization of adenosine.

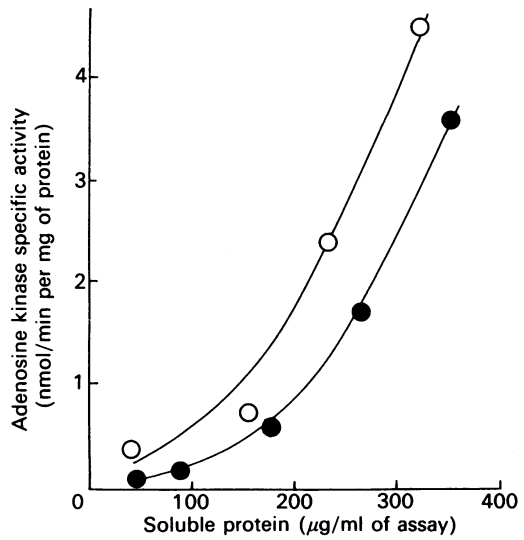

Fig. 1. Dependence of adenosine kinase specific activity on protein concentration

Adenosine kinase was assayed as described in the Materials and methods section. This was in a final volume of 0.04 ml, with the indicated concentrations of soluble protein obtained from 105000 *g* supernatants of whole forebrain. The values are taken from a single representative experiment. ○, Euthyroid; ●, hypothyroid.

General considerations

The brain regions showing the largest changes in synaptosomal-membrane 5′-nucleotidase activity were cerebellum, cortex, striatum and, to a lesser extent, hippocampus. A consequent possible increase in extracellular adenosine formation could be accentuated by a decrease in adenosine kinase activity in the cerebellum, striatum and hippocampus as a result of diminished re-utilization of the nucleoside after re-uptake. The striatum has a high content of both A_1 and A_2 adenosine receptors, whereas A_1 receptors are abundant in the cerebellum, cortex and hippocampus (Bruns *et al.*, 1987). Furthermore, it is in the cortex, the striatum and the hippocampus that presynaptic adenosine receptors are known to inhibit neurotransmitter release (Fredholm & Dunwiddie, 1988). Richardson *et al.* (1987) have suggested that an increase in the synthesis or expression of postsynaptic 5′-nucleotidase would enhance neuromodulation through an increase in adenosine formation. The present study demonstrates examples of such an increase in activity. It is speculated that this may contribute to the co-existence of depression with thyroid dysfunction (Gold *et al.*, 1981; Reus, 1986).

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