Inhibition of adenylate cyclase in rat brain synaptosomal membranes by GTP and phenylisopropyladenosine is enhanced in hypothyroidism

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1. Synaptosomal membranes were isolated from rats made hypothyroid by treatment with propylthiouracil and a low iodine diet. 2. When assayed in the presence of 100 mM-Na⁺, inhibition of forskolin-stimulated adenylate cyclase by GTP was enhanced in membranes from hypothyroid animals. 3. Hypothyroidism also enhanced inhibition of adenylate cyclase by phenylisopropyladenosine (with 100 mM-Na⁺ and 10 μ M-GTP present). 4. Hypothyroidism did not increase binding of the A₁ adenosine receptor agonist phenylisopropyladenosine to synaptosomal membranes; rather, the maximum binding was slightly decreased without any change in the K_D. 5. The effect of GTP in modifying the displacement of the antagonist [³H]diethylphenylxanthine from synaptosomal membranes by unlabelled phenylisopropyladenosine was more pronounced in the hypothyroid state. 6. These findings are consistent with hypothyroidism causing modification of the brain adenylate cyclase system at the level of the coupling protein G_i.

INTRODUCTION

Adenylate cyclase is regulated by GTP in a biphasic fashion. Low concentrations of the guanine nucleotide are stimulatory, whereas higher concentrations inhibit the enzyme (Londos *et al.*, 1979, 1981; Cooper, 1983). It is now recognized that these effects of GTP are mediated by the guanine-nucleotide-binding proteins (G-proteins) G_s and G_i (Casey & Gilman, 1988). In addition, in the presence of GTP, G_i is able to mediate the inhibition of adenylate cyclase that occurs when various cell surface receptors are occupied by agonists. A number of studies have shown that these inhibitory events can be modified in different physiological states. In adipose tissue, hypothyroidism enhances inhibition of adenylate cyclase both by GTP and by adenosine acting through A₁-type adenosine receptors (Malbon *et al.*, 1985). This change is not brought about by an increase in adenosine receptor numbers (Chohan *et al.*, 1984; Malbon *et al.*, 1985) but is associated with an increased abundance of the α -subunit of G_i in fat-cell membranes (Milligan *et al.*, 1987; Ros *et al.*, 1988). By contrast, decreased G_i-mediated inhibition of rat liver adenylate cyclase by guanylyl 5-imidodiphosphate is found in streptozotocin diabetes (Gawler *et al.*, 1987) and in the (*fa/fa*) type of inherited obesity (Houslay *et al.*, 1989).

In the central nervous system, many presynaptic receptors that mediate inhibition of neurotransmitter release, including A₁ adenosine, α_2 -adrenergic, opiate, GABA_B, muscarinic cholinergic and dopamine D₂ receptors, are also able to mediate inhibition of adenylate cyclase and lowering of cyclic AMP levels (Fredholm & Dunwiddie, 1988). In the case of the A₁ adenosine receptor, inhibition of adenylate cyclase has been noted in several regions of the brain, with the effect being seen in both neuronal and glial cell types (Cooper *et al.*, 1980; Ebersolt *et al.*, 1983).

The extent to which lowering of cyclic AMP contributes to inhibition of neurotransmitter release by adenosine is unresolved (see reviews by Silinsky, 1986; Dunwiddie & Proctor, 1987; Fredholm & Dunwiddie, 1988) and other mechanisms such as inhibition of voltage-sensitive Ca²⁺ channels or increasing K⁺ conductance are also implicated (Proctor & Dunwiddie, 1983; Trussell & Jackson, 1985, 1987; Dolphin *et al.*, 1986; Scott & Dolphin, 1987).

Following findings from this and other laboratories that hypothyroidism enhances the inhibitory effects of G_i-coupled agonists in fat cells (Malbon *et al.*, 1985; Saggerson, 1986; Milligan *et al.*, 1987; Ros *et al.*, 1988), and in view of known behavioural changes in hypothyroidism (Gold *et al.*, 1981; Reus, 1986), we have investigated whether similar changes in the regulatory properties of the adenylate cyclase system are also seen in the central nervous system in this state. Using purified rat synaptosomal membranes, it is shown that inhibition of adenylate cyclase by GTP and by the A₁ receptor agonist phenylisopropyladenosine (PIA) is enhanced.

MATERIALS AND METHODS

Chemicals

These were obtained from the Boehringer Corporation (London) Ltd. (Lewes, East Sussex, U.K.) or from the Sigma Chemical Company Ltd. (Poole, Dorset, U.K.) with the exception of radiochemicals, which were from Amersham International plc (Little Chalfont, Bucks., U.K.) or from NEN Research Products (Stevenage, Herts., 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

Abbreviations used: B_{max}, maximum binding capacity at a given site; DPX, 1,3-diethyl-8-phenylxanthine; PIA, N⁶-L-2-phenylisopropyladenosine; G-protein, guanine-nucleotide-binding protein; GABA, γ -aminobutyric acid.

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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 and 19:00 h. Rats to be made hypothyroid 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.

Isolation of synaptosomal membranes

Rats were stunned and then killed by decapitation. Forebrains were rapidly removed and collected in ice-cold 0.32 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4) and 1 mM-EDTA. They were then weighed, cut into small pieces and washed with the same medium to remove blood. Homogenates were prepared in a Potter–Elvehjem glass homogenizer using a Teflon pestle (six up-and-down strokes at 500 rev./min with a radial clearance of 0.2 mm). The homogenate was diluted with the ice-cold sucrose medium to give a final concentration of 11–15% (w/v) and then fractionated to obtain synaptosomes essentially as described by Booth & Clark (1978) with some minor modifications. After an initial centrifugation at 1500 g_{av} for 3 min, the resulting supernatant was centrifuged at 18000 g_{av} for 10 min. The pellet was 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)/1 mM-EDTA]. Portions (5 ml) of these suspensions were pipetted into 14 ml centrifuge tubes followed by a 2.5 ml layer of 7% Ficoll medium [7% (w/w) Ficoll/0.32 M-sucrose/10 mM-Tris/HCl buffer (pH 7.4)/1 mM-EDTA] and then a 2.5 ml layer of 0.32 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4) and 1 mM-EDTA. After centrifugation at 110000 g_{av} , the synaptosomal fraction, which banded at the lower interface, was removed and resuspended in 5 mM-Tris/HCl buffer (pH 8.0) containing 1 mM-EDTA. After sonication for 30 s, the suspension was left on ice for 30–45 min to achieve lysis of the synaptosomes, followed by centrifugation at 105000 g_{av} for 45 min. The resulting synaptic membrane fraction was resuspended in 2–3 ml of 50 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-EDTA, and stored at -80°C . Fractions were standardized by measurement of protein using the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Assay of adenylate cyclase (EC 4.6.1.1)

This was a minor modification of the method of Cooper & Londos (1979), which in turn was derived from the original method of Salomon *et al.* (1974). Assays were performed at 24°C in a final volume of 0.1 ml containing 30 mM-Tris/HCl buffer (pH 7.5), 4 mM- MgCl_2 , albumin (0.4 mg/ml), 10 μM -forskolin, 0.1 mM- $[\alpha\text{-}^{32}\text{P}]$ deoxyATP (0.1 $\mu\text{Ci/nmol}$), 0.1 mM-cyclic deoxy-AMP, 10 μM -papaverine, 0.25 units of adenosine deaminase, 2 mM-phosphocreatine and 2.5 units of creatine kinase. Other additions are indicated in the legends to individual Figures. In all cases, reactions were initiated

by addition of 10 μg of synaptosomal membrane protein. After 20 min the reaction was terminated by addition of 0.1 ml of a 'stopping mixture' consisting of 10% (w/v) SDS solution containing 10 mM-EDTA and cyclic $[8\text{-}^3\text{H}]$ deoxyAMP (approx. 70000 d.p.m.) to monitor recovery of the cyclic $[^{32}\text{P}]$ deoxyAMP. This reaction product was separated by sequential chromatography on Dowex and alumina columns essentially as described by Cooper & Londos (1979). ^{32}P and ^3H were then measured by dual-label liquid scintillation counting in a cocktail consisting of 4 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)-thiophen/litre in toluene/Triton X-100 (2:1, v/v). Preliminary experiments established that the assay procedure was satisfactory in our hands with a 63% average recovery of cyclic deoxyAMP that had negligible contamination by deoxyATP or deoxyAMP. The ^{32}P -labelled reaction product co-chromatographed with authentic cyclic deoxyAMP on t.l.c. using a solvent mixture of propan-2-ol: ethyl acetate: 8 M-ammonia (9:4:3, by vol.) (Arch & Newsholme, 1976). Assays were linear for at least 20 min in the presence and absence of forskolin, GTP or Na^+ in both the euthyroid and the hypothyroid states.

Assay of radioligand binding to membranes

The methods were basically those described by Lohse *et al.* (1984). Prior to assays, synaptosomal membranes were diluted to a concentration of 1 mg/ml using 50 mM-Tris/HCl buffer (pH 7.4) and preincubated for 30 min at 37°C with adenosine deaminase (3 units/ml) to remove endogenous adenosine. To measure saturation binding of PIA, membranes (100 μg of protein) were then incubated with the required concentration of $(-)\text{N}^6\text{-R-}[G\text{-}^3\text{H}]$ phenylisopropyladenosine (0.25–50 nM) in a total volume of 1.0 ml of 50 mM-Tris/HCl buffer (pH 7.4) for 45 min at 37°C . The membranes were separated from unbound radioactivity by vacuum filtration through Whatman GF/B glass fibre filters. The filters were immediately washed twice with 5 ml of ice-cold 50 mM-Tris/HCl buffer (pH 7.4) and then counted for radioactivity in a cocktail consisting of 4 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)-thiophen/litre plus 80 g of naphthalene/litre dissolved in toluene/2-methoxyethanol (3:2, v/v). Non-specific binding of $[^3\text{H}]$ PIA was determined in the presence of an additional 50 μM of unlabelled PIA and generally amounted to 30–50% of total binding. Study of the displacement of 1,3-diethyl-8- $[4\text{-}^3\text{H}]$ phenylxanthine ($[^3\text{H}]$ DPX) by PIA was carried out in essentially the same way except that the final incubation volume was reduced to 0.25 ml, and labelled ligand ($[^3\text{H}]$ DPX) was present throughout at 10 nM and unlabelled PIA was varied over the concentration range 0.1 nM–0.1 mM. Two washes of only 3 ml of ice-cold 50 mM-Tris/HCl buffer (pH 7.4) were used to wash the filters, and incubation with ligands was performed for 15 min at 37°C . Non-specific binding was not determined experimentally, but was calculated from computer analysis of displacement curves (see below).

Statistical methods

Statistical significance was evaluated by Student's *t* test for paired or unpaired samples as appropriate. Radioligand binding data were analysed by a non-linear weighted least-squares curve-fitting procedure using a search-type minimization method (Colquhoun, 1971). On input of data as means $\pm$ S.E.M., the program

estimated K_D and B_{max} , and, in the case of competition curves, non-specific binding. Statistical analysis to assess the appropriateness of one- or two-site models of binding was performed using a partial F test (Munson & Rodbard, 1980).

RESULTS AND DISCUSSION

Membrane preparations

Preliminary experiments (Mazurkiewicz & Saggerson, 1989) established that the synaptosomal preparations were relatively free from contamination by marker enzymes for myelin and mitochondria (2',3'-cyclic nucleotide phosphodiesterase and succinate dehydrogenase respectively). Furthermore, hypothyroidism did not alter these relative distributions.

Activation of adenylate cyclase by forskolin

Rather than study the effects of GTP and PIA on basal adenylate cyclase, we chose to use forskolin-activated enzyme as the test system. It was therefore necessary at the outset to investigate whether hypothyroidism altered the effect of forskolin on the enzyme. This was not found to be so. Dose-response curves for activation by forskolin are shown in Fig. 1. Hill coefficients of less than 1 were observed in all cases, consistent with high- and low-affinity effects of the diterpene reported previously (Green & Clark, 1982; Seamon *et al.*, 1984; Seamon & Daly, 1985). Although Na^+ was found to decrease adenylate cyclase activity, this effect being more pronounced in the euthyroid case, neither hypothyroidism nor addition of 100 mM-NaCl appreciably altered the Hill coefficients or the calculated EC_{50} values. In addition, hypothyroidism did not significantly alter adenylate cyclase activity at any forskolin concentration in the

presence or absence of Na^+ . All further studies of the cyclase in either state were performed using 10 μM -forskolin.

Effects of GTP upon adenylate cyclase activity

In the absence of Na^+ , GTP at concentrations in excess of 1 μM significantly decreased forskolin-stimulated cyclase activity (Fig. 2a). By contrast with the fat-cell system (Malbon *et al.*, 1985), hypothyroidism did not enhance the inhibitory effect of GTP in the absence of Na^+ . Although quantitative comparisons cannot easily be made between different systems, there is general agreement that Na^+ attenuates the inhibitory effect of GTP (Cooper *et al.*, 1980, 1982; Londos *et al.*, 1981; Koski *et al.*, 1982; Jacobs *et al.*, 1984). By contrast, Na^+ amplifies the degree of inhibition seen in the presence of receptor agonists and GTP (Blume *et al.*, 1979, 1980; Lichtshtein *et al.*, 1979; Jacobs *et al.*, 1981; Londos *et al.*, 1981). Fig. 2(b) indeed shows that addition of 100 mM-NaCl abolished the inhibitory effect of GTP in membranes from euthyroid rats. However, in membranes from hypothyroid animals, GTP was still able to inhibit adenylate cyclase, i.e. GTP was able to overcome the attenuating effect of Na^+ .

Effects of PIA upon adenylate cyclase activity

In order to facilitate the detection of an inhibitory action of PIA, the assays of the cyclase were performed throughout using deoxyATP as the substrate (Cooper & Londos, 1979) and in the presence of a high concentration of adenosine deaminase. These precautions minimize effects due to endogenous adenosine. Fig. 3 shows that PIA significantly decreased forskolin-stimulated cyclase activity when GTP and Na^+ were present. As found previously with brain membranes (Cooper *et al.*, 1980;

Fig. 1. Effect of forskolin and Na^+ upon adenylate cyclase activity in synaptosomal membranes from euthyroid and hypothyroid rats

The values are means $\pm$ S.E.M. of between three and five separate experiments and are expressed relative to those with 100 μM -forskolin in the absence of Na^+ (100% activity). These were 124 ± 20 and 99 ± 9 pmol/min per mg of protein in the euthyroid and hypothyroid cases respectively. Basal cyclase activities in the absence of Na^+ were 39 ± 8 and 36 ± 2 pmol/min per mg of protein (euthyroid and hypothyroid states respectively). (a) Euthyroid: ●, without Na^+ (Hill coefficient = 0.73, $\text{EC}_{50} = 0.63 \mu\text{M}$); ○, with 100 mM- Na^+ (Hill coefficient = 0.62, $\text{EC}_{50} = 0.83 \mu\text{M}$). (b) Hypothyroid: ●, without Na^+ (Hill coefficient = 0.82, $\text{EC}_{50} = 1.54 \mu\text{M}$); ○, with 100 mM- Na^+ (Hill coefficient = 0.68, $\text{EC}_{50} = 0.89 \mu\text{M}$). Where S.E.M. bars are not shown, these lie within the symbol.

Fig. 2. Effects of GTP upon forskolin-activated adenylate cyclase in the presence and absence of Na^+

All assays contained $10 \mu\text{M}$ -forskolin with or without 100 mM - NaCl . The values are means $\pm$ S.E.M. and are expressed relative to those in the absence of GTP (100%). Significant effects of GTP are indicated by: * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$ (paired differences). (a) Without Na^+ : ●, euthyroid ($n = 8$); ○, hypothyroid ($n = 5$). (b) With Na^+ : ●, euthyroid ($n = 8$); ○, hypothyroid ($n = 4$).

Fig. 3. Effect of PIA on adenylate cyclase activity in synaptosomal membranes from euthyroid and hypothyroid rats

All assays contained $10 \mu\text{M}$ -forskolin, $10 \mu\text{M}$ -GTP and 100 mM - NaCl . The values are means $\pm$ S.E.M. of four to eight experiments and are expressed relative to those in the absence of PIA (100%). Significant effects of PIA are indicated by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (paired differences). In addition, the effect of $10 \mu\text{M}$ -PIA was significantly greater in hypothyroidism than in the euthyroid state ($P < 0.05$ by an unpaired test). ●, Euthyroid; ○, hypothyroid.

Ebersolt *et al.*, 1983), inhibitory effects of PIA were relatively slight compared with the effect seen in the fat-cell system (Malbon *et al.*, 1985). Nevertheless, hypothyroidism significantly enhanced the inhibitory effect of PIA upon the brain adenylate cyclase activity (Fig. 3).

Saturation binding of [^3H]PIA to membranes

Fig. 4 shows that [^3H]PIA bound to synaptosomal membranes from normal animals with a B_{max} value of 600 fmol/mg. Values of 810 fmol/mg and 740 fmol/mg have previously been reported by Schwabe & Trost (1980) and Lohse *et al.* (1984) respectively. The binding was fitted to a single class of binding site by computer analysis. This conclusion is supported by the linearity of the Scatchard plot ($r = -0.96$, $P < 0.001$). The K_D of 3.3 nM was not dissimilar to the values of 5.1 nM and 1.4 nM previously reported by Schwabe & Trost (1980) and Lohse *et al.* (1984) respectively. Hypothyroidism did not appreciably change the K_D for [^3H]PIA binding (3.8 nM) and insignificantly decreased the B_{max} by 14% to 518 fmol/mg. Again, a one-site model of binding was found to be the most appropriate ($r = -0.94$, $P < 0.001$ for the Scatchard plot). These findings imply that the increased responsiveness of the cyclase to inhibition by PIA cannot be attributed to an increase in A_1 adenosine receptor number; rather, the implication is that hypothyroidism has brought about a post-receptor change.

In this respect it is noteworthy that [^3H]PIA binding to isolated fat-cell plasma membranes is also decreased in this animal model of hypothyroidism (Chohan *et al.*, 1984). Re-examination of the data in Fig. 4 of Chohan *et al.* (1984) using the present computer model shows that hypothyroidism decreased the total amount of specific binding of [^3H]PIA to fat cell membranes from 1230 to 1040 fmol/mg (a 15% decrease). It is noteworthy that Malbon *et al.* (1985) also observed a small (15%) decrease in [^3H]cyclohexyladenosine binding to fat-cell membranes in hypothyroidism.

Fig. 4. Specific binding of [3 H]PIA to synaptosomal membranes from euthyroid and hypothyroid rats

The values are means $\pm$ S.E.M. The curves are computer estimates of best fit to a single-site binding model (see the Materials and methods section). Estimates of binding parameters were (means $\pm$ S.D.): euthyroid, $K_D = 3.3 \pm 0.4$ nM, $B_{max} = 600 \pm 33$ fmol/mg of protein; hypothyroid, $K_D = 3.8 \pm 0.5$ nM, $B_{max} = 518 \pm 28$ fmol/mg of protein. ●, Euthyroid ($n = 3-5$); ○, hypothyroid ($n = 6-12$).

Fig. 5. Competition for [3 H]DPX binding to rat synaptosomal membranes by PIA in the presence and absence of GTP

Membranes were incubated with 10 nM-[3 H]DPX in the absence or presence of 100 μ M-GTP and with 24-26 different concentrations of unlabelled PIA over the range 0.1 nM-100 μ M. The values are means of three to seven separate experiments and are presented in the form of Hill plots. Displacement at a given concentration of PIA is represented by d and the maximum displacement by D . (a) Hypothyroid: ●, without GTP (Hill coefficient = 0.32, $r = 0.95$); ○, with GTP (Hill coefficient = 0.98, $r = 0.99$). (b) Euthyroid: ●, without GTP (Hill coefficient = 0.48, $r = 0.98$); ○, with GTP (Hill coefficient = 0.75, $r = 0.95$).

Displacement of [3 H]DPX from synaptosomal membranes by PIA

To further explore the possibility that hypothyroidism was bringing about post-receptor changes, perhaps at the

level of receptor/cyclase coupling, we investigated the effect of GTP in modifying the profile of displacement of the antagonist [3 H]DPX by unlabelled PIA. In the absence of GTP, Lohse *et al.* (1984) found that these displacement curves fit to a two-site model. The same was true in the

present study ($P < 0.005$ and < 0.01 in the euthyroid and hypothyroid cases respectively for comparison of one- and two-site fitting using the F test). A displacement model more complex than one involving a single site is also indicated by the low values of the Hill coefficients (Fig. 5). On addition of $100 \mu\text{M}$ -GTP, the displacement curve in the hypothyroid case became that expected for a single binding site after a change in Hill coefficient from 0.32 to 0.98. By contrast, in the euthyroid state, this concentration of GTP only changed the Hill coefficient from 0.48 to 0.75. These qualitative findings imply that this chosen concentration of GTP is more effective in changing the affinity of agonist binding in membranes from hypothyroid animals.

General discussion and conclusions

This study shows that hypothyroidism increases the effectiveness of an inhibitory agonist at brain adenylate cyclase. This is achieved without any increase in the abundance of the agonist receptor and without any change in the basal or the forskolin-stimulated adenylate cyclase activity. The changes that are observed, i.e. enhanced inhibition of the cyclase by GTP (in the presence of Na^+) and increased effectiveness of GTP in modifying agonist displacement curves, suggest that altered function or abundance of the coupling G-protein is involved. In the case of the fat-cell system, an increased abundance of the α -subunit of G_i is already established in hypothyroidism. Further studies should be directed towards investigating this possibility in the synaptosomal membrane system. If the coupling G-protein is the major locus of change in hypothyroidism, then it is to be expected that inhibition of brain adenylate cyclase through receptors other than the A_1 adenosine receptor [e.g. opiate (Blume *et al.*, 1979, 1980; Cooper *et al.*, 1982; Koski *et al.*, 1982) or muscarinic cholinergic (Lichtstein *et al.*, 1979)] would also be enhanced in this state. This may have general consequences with regard to pre-synaptic inhibition of neurotransmitter release and could contribute to the coexistence of depression with thyroid dysfunction (Gold *et al.*, 1981; Reus, 1986). With specific regard to the effects of adenosine, the extracellular production of this agonist is also likely to be enhanced in hypothyroidism, at least in some brain regions, since the activity of the 5'-nucleotidase ectoenzyme in synaptosomal membranes is considerably increased (Mazurkiewicz & Saggerson, 1989). Increased availability of the agonist together with more effective coupling of its receptor to the adenylate cyclase would be expected to enhance the neuromodulatory effects of adenosine in hypothyroidism.

D.M. was supported by a studentship from the Medical Research Council.

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Received 28 April 1989/13 June 1989; accepted 20 June 1989