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β-Adrenoceptor-mediated effects in rat cultured thymic epithelial cells

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1 Sympathetic nerves were visualized in sections from rat thymus by immunostaining of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis, and by glyoxylic acid-induced fluorescence of catecholamines. Catecholaminergic nerve fibres were detected in close connection to thymic epithelial cells which therefore might be preferred target cells. To evaluate this, rat immunocytochemically defined, cultured thymic epithelial cells were investigated for adrenoceptors and adrenergic effects.

2 In rat cultured thymic epithelial cells mRNA for β_1- and β_2-adrenoceptors was detected by reverse transcription-polymerase chain reaction by use of sequence-specific primers. Specific, saturable binding to the cultivated cells was observed with the β-adrenoceptor agonist CGP 12177.

3 Adrenaline, noradrenaline or the β-adrenoceptor agonist, isoprenaline, increased intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in cultivated thymic epithelial cells dose-dependently about 25 fold. The pharmacological properties revealed that this response was mediated by receptors of the β_1- and the β_2-subtypes. The selective β_3-adrenoceptor agonist BRL 37344 had no effect on cyclic AMP levels. The increase in cyclic AMP was downregulated by preincubation with glucocorticoids like dexamethasone or cortisol which also changed the relative importance of β_1-/β_2-adrenoceptors to the response.

4 Incubation with isoprenaline or the adenylate cyclase activator forskolin decreased basal and serum-stimulated proliferation of thymic epithelial cells. However, adrenergic stimulation of thymic epithelial cells did not induce interleukin 1 production. Since thymic epithelial cells create a microenvironment which influences the maturation and differentiation of thymocytes to T-lymphocytes, their observed capacity to respond to catecholamines provides novel evidence for the suggestion that adrenergic stimulation may interfere with the regulation of immune functions.

Keywords: Adrenergic nerves; β-adrenoceptors; CGP 12177; cyclic AMP; proliferation; neuroimmunology; thymus; thymic epithelial cells; glucocorticoids

Introduction

There is increasing evidence for a communication between the nervous and the immune systems. Potential pathways linking the brain to cells of the immune system include neuroendocrine hormones and direct innervation of lymphoid tissues. The thymus as the major site of T-cell development is exposed to circulating hormones, as well as to neurotransmitters released from innervating aminergic or peptidergic fibres (Felten & Felten, 1989). The complex lympho-stromal structure of the thymus (von Gaudecker, 1991) is contributed to by a variety of cells such as thymocytes, thymic epithelial cells (TEC), mast cells, fibroblasts or cells of the monocyte-macrophage lineage (macrophages, dendritic interdigitating cells). Upon activation by hormones, neurotransmitters or cytokines these cells provide a microenvironment beneficial for the maturation and differentiation of T-cells (Kendall, 1991). TEC may be of major importance for the creation of the thymus microenvironment. However, little is known regarding the activation of TEC by neurotransmitters. Noradrenaline is the main catecholamine in the rat thymus (Kendall *et al*., 1994). In order to identify thymic target cells for noradrenaline and to study its cellular actions, we (i) reinvestigated the morphological connections between adrenergic nerves and thymic cells and found that noradrenergic nerve fibres run in close vicinity to TEC. We then (ii) established highly purified cultures of TEC to evaluate whether or not they express adrenoceptors and respond to catecholamines. The results show that cultured TEC are affected by adrenaline/noradrenaline via β-adrenoceptors, and that the β-adrenoceptor-mediated response is regulated by glucocorticoids.

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Methods

Immunohistochemistry and histochemistry

Immunohistochemistry on tissue sections was performed with thymi of 3-month old female Wistar rats (strain Han:Wist) immersion-fixed in Zamboni's fixative or 4% paraformaldehyde for 24 h and frozen in liquid nitrogen (von Gaudecker *et al*., 1989). Cryostat sections (7 μm) were prepared, and endogenous peroxidase blocked by a solution of Tris-buffered saline, pH 7.6 (TBS), 30% hydrogen peroxide and methanol (ratio 10:1:1). Sections were rinsed with TBS, and aldehydes blocked by $NaBH_4$ (0.8% in water). After endogenous biotin had been inhibited with a blocking kit (Camon, Vector laboratories, code SP-2001) and nonspecific proteins by 10% normal goat serum in TBS, sections were incubated overnight at 4°C with rabbit anti-rat tyrosine hydroxylase (1:800 in TBS supplemented with 5% goat serum; Chemicon International, Temecula, CA, USA; code AB 152). After incubation for 90 min at room temperature with biotinylated goat anti-rabbit IgG (1:400 in TBS supplemented with 5% rat serum; Vector Laboratories INC, Burlingame, CA, USA, code BA-1000) sections were stained with the Vectastain ELITE ABC kit (Serva, Heidelberg, Germany, code PK-6100) and counterstained by Meyer's hemalum (Merck, Darmstadt, Germany).

Catecholamines were stained in frozen cryostat sections (15 μm) of adult rat thymi by sucrose-phosphate-glyoxylic acid-induced fluorescence (de la Torre & Surgeon, 1976). The sections were investigated with a fluorescence microscope (filters Zeiss BP 436/10 FT 460, CP 470 No. 06).

TEC cultivated on poly-D-lysine-coated coverslips were fixed with cold acetone, −20°C, for 10 min and immunostained as described previously (Kurz *et al*., 1996). Anti-rat pan-cytokeratin (DAKO, Glostrup, Denmark; code M821) and several other antibodies served as primary and fluorescein isothiocyanate-conjugated anti-mouse IgG (1 : 50, Vector FI-2000, Serva, Heidelberg) as secondary antibodies. Nuclei were counterstained for 3 min with 0.3% bisbenzimide in phosphate-buffered saline. Green fluorescence of immunostaining and blue fluorescence of nuclei were inspected microscopically with 510–560 nm or 450–490 nm emission filters (excitation filter 390–420 nm).

TEC cultures

TEC were prepared from thymus of 6–8 week old female Wistar rats (strain Han: WIST) by collagenase dissociation. Cultures were purified and freed from fibroblasts by a combination of a pre-plating method, differential trypsin treatment and cultivation in the presence of horse serum. This isolation procedure and the properties of the cells have been described in detail previously (Kurz *et al*., 1996). TEC were cultivated routinely in Dulbecco's modified Eagle medium (DMEM)/ F12 (1 : 1) medium containing 2 mM L-glutamine, 25 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin, 100 u ml^{-1} penicillin and streptomycin and 10% horse serum, and used after 3–20 passages.

Reverse transcription-polymerase chain reaction (RT–PCR)

Confluent cultures of TEC, or rat astrocytoma C6 cells (Feindt *et al*., 1995) as positive controls, were rinsed with phosphate-buffered saline (PBS) and lysed in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol and 0.5% sodium lauryl sarconisate. The lysate was homogenized, total RNA isolated by CsCl density centrifugation and RT–PCR was performed as described by Feindt *et al*. (1995) with primers specific for the rat β_1- (Machida *et al*., 1990) and β_2- (Jiang & Kunos, 1995) adrenoceptors. In brief, contaminating genomic DNA was digested by incubation of 1 or 3 μg RNA with 10 units RNase-free DNase I (Boehringer, Mannheim, Germany) in DNase-incubation buffer (Gibco) in a final volume of 10 μl at 25°C for 20 min. DNase was then inactivated by incubation with 1 μl 20 mM EDTA for 15 min at 65°C. Reverse transcription mixture containing 2 μl 10x PCR-buffer IV (Biomol, Hamburg, Germany), 2 μl 10 mM desoxynucleoside triphosphates (Pharmacia, Freiburg, Germany), 2 μl 100 mM dithiothreitol (Gibco, 2 μl 25 mM $MgCl_2$, 1 μl (20 pmol) oligo(dT)$_{15}$-primer (Boehringer), was added to a final volume of 20 μl. After incubation at 65°C for 5 min and at 0°C for 5 min, reverse transcription was performed by adding 100 units of reverse transcriptase (SuperScript, Gibco) and incubation at 37°C for 1.5 h. PCR reaction mixture containing 8 μl 10x PCR-buffer IV, 1 μl (10 pmol) PCR-primers each (for detection of β_1-adrenoceptor mRNA (cDNA) sense: 5′-C-ACG-CTG-CCC-TTT-CGC-TAC-C-3′, corresponding to bases 480 to 499; antisense: 5′-CA-CTT-GGG-GTC-GTT-GTA-GCA-3′, corresponding to bases 644 to 625; for β_2-adrenoceptor sense: 5′-GGA-GCC-ACA-CGG-GAA-TGA-CAG-3′, corresponding to bases 2311 to 2331 of the gene; antisense: 5′-TCC-AGA-ACT-CGC-ACC-AGA-AAT-3′, corresponding to bases 2636-2616 of the gene; oligonucleotides synthesized and purified from Eurogentec, Seraig, Belgium) and 8 μl 25 mM $MgCl_2$ was added to a final volume of 100 μl. After initial denaturation at 95°C for 1 min, addition of 2.5 units Taq-polymerase (Biomol), and 95°C another 5 min, the following temperature cycle was started: 95°C for 1 min, 50°C (β_1) or 54°C (β_2) for 1 min, 72°C for 1.5 min. After 40 cycles, the temperature was maintained at 72°C for 10 min, and finally at 4°C. The products were separated by electrophoresis in 10% polyacrylamide gels or 2% agarose gels. Restriction endonuclease cleavage was performed by incubation with 10 units *Apa 1* (Gibco) or *Fok I* (Appligene-Oncor, Heidelberg, Germany) for 18 h at 37°C. Controls without RNA or reverse transcriptase did not yield products. Inactness of RNA and complete digestion of genomic DNA was analysed by RT–PCR of an intron-spanning sequence of β-actin (Feindt *et al*., 1995).

Binding assays and stimulation experiments for determination of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP)

Confluent layers of TEC in 35 mm Petri dishes were washed twice by incubation for 2 h at 37°C with DMEM/F12-Medium and then for 30 min at 37°C with incubation buffer, consisting of (in mM): NaCl 145, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 20 and HEPES 20 adjusted with NaOH to pH 7.4. The buffer was removed, and 1 ml fresh prewarmed incubation buffer was added. Binding assays with whole cells were performed by addition of the hydrophillic β-adrenoceptor agonist [^{3}H]-CGP 12177 ([5,7-^{3}H]-(−)-4-[3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride) for 20 min at 37°C as described by Lacasa *et al*. (1985); incubations in the presence of 2 μM (−)-propranolol (Sigma) served as controls for non-specific binding. Binding was related to protein content determined from an aliquot of the lysed cells by a modified Coomassie Blue-binding method (Lucius & Mentlein, 1991).

For stimulation experiments, amines (Sigma), agonists and/ or antagonists (Sigma; BRL 37344 ((±)-(R*,R*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid) was purchased from RBI Research Biochemicals, Natick, MA, U.S.A.; ICI 118,551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol hydrochloride) was a gift from ZENECA, Schwetzingen, Germany) were added as 100 fold concentrated stock solutions in water. After 2 min (or longer) at 37°C, incubation medium was withdrawn, and the cells lysed with 700 μl ice-cold 10 mM HEPES buffer, pH 7.4, containing 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma). The cells, placed on ice, were scraped off from the dishes by a rubber policeman, the lysate transferred into Eppendorf caps and mixed thoroughly; 200 μl of the suspension was removed for protein determination, and the remaining 500 μl mixed with 1 ml ice-cold ethanol. This mixture was centrifuged (12,000 *g*, 5 min), the supernatant lyophilized, the residues reconstituted with 100 μl water, and aliquots assayed for cyclic AMP by use of a commercial radioimmunoassay kit (Amersham, Braunschweig Germany, code RPA 509). Protein was assayed as above.

For measuring preincubation effects of steroid hormones or other agonists (human recombinant epidermal growth factor, prolactin from sheep pituitary glands; all from Sigma) on the adrenergic response, TEC in Petri dishes were washed twice by incubation for 2 h each at 37°C with DMEM/F12 medium without horse serum, and then incubated for 24 h in 2 ml fresh, serum-free medium. Agonists were added as 1000 fold concentrated solutions in ethanol (steroids, thyroxine) or water (peptides). After the medium had been replaced by incubation buffer, these cultures were stimulated by 1 μM (−)-isoprenaline without further washings and assayed for cyclic AMP and protein as described above.

Proliferation assays

Cells 10^5 were cultivated for 24 h in 35 mm Petri dishes as described above. After equilibration with DMEM/F12-medium (with or without 10% horse serum as indicated) for 2 h at 37°C, stimulation was performed in 2 ml fresh medium for 48 h with agonists added as 1000 fold concentrated solutions in ethanol (cortisol) or water. After 48 h 1 μCi [^{3}H]-thymidine

(Amersham, Buckinghamshire, U.K., code TRK 565) was added to the medium, and incubation continued for an additional 5 h. The supernatant was then discarded, and the cells successively washed with PBS, two times methanol, water, 10% trichloroacetic acid and water (5 min each time). Cells were then dissolved in 1 ml 0.3 M NaOH (15 min), neutralized with 1 ml 0.3 M HCl, and after addition of 10 ml scintillation liquid (Hydroluma, Baker Chemicals, Gross-Gerau, Germany, code 8584) radioactivity measured in a β-counter.

Cytokine measurements

TEC were cultured in 35 mm culture dishes until confluence and incubated with the cytokines (human recombinant from Saxon Biochemicals, Hannover, Germany) at the concentrations depicted. After 24 h the supernatants were harvested and stored at $-20°C$ in the presence of serum. For preparation of cell lysates, the cells were trypsinized, washed and fresh medium added. The cells were lysed by two freeze thaw cycles. The supernatants or cell lysates were measured in interleukin-1 (IL-1) or IL-6 assays as described below. Briefly, for detection of IL-1 (Loppnow *et al*., 1989a, b) fibroblasts (5,000/well) were cultured in flat bottom microtitre plates (24 h), medium replaced by fresh medium and serial four fold dilutions of samples or IL-1 standard (10 ng ml^{-1} each) were prepared. After 72 h incubation 1 μCi [^{3}H]-thymidine was added for 24 h; cells were trypsinized and harvested onto glass fibre filters and thymidine incorporation measured in an automated liquid scintillation counter. IL-6 was determined with 7TD1-cells according to the method described previously. Briefly, serial four fold dilutions of samples or IL-6 standard were prepared and 4,000 cells added per well. After 72 h 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and the formation of diazolium dye measured in an ELISA reader at 550 nm. The cytokine activity was determined as described previously (Loppnow *et al*., 1994).

Statistical analysis

Statistical evaluation was performed with the BMDP PC90 programme 7d (Dixon *et al*., 1990). First, golbal null hypotheses (equality of means) were tested either by classical ANOVA (assuming equality of variances) or by non-classical Welch test (not assuming equality of variances). The adequate procedure was selected after testing equality of variances by Levene test. Null hypotheses were rejected at 95% significance level ($P<0.05$). Subsequently, the group means of all treatments were compared to the corresponding control group mean by application of the Dunnett's control group comparison test. All data are expressed as mean values $\pm$ s.e.mean for n tested samples.

Results

Adrenergic innervation of TEC

In sections of rat thymus adrenergic nerves were visualized by immunostaining for tyrosine hydroxylase, the rate-limiting enzyme for synthesis of catecholamines, and by applying sucrose-phosphate-glyoxylic acid-induced fluorescence for direct visualization of catecholamines (Figure 1). The nerve fibres run in septa which divide the thymic lobes in which the thymocytes develop to mature T-cells. Frequently, the nerves were found in direct neighbourhood to subcapsular TEC (Figure 1a). Very rarely nerves enter the thymus parenchyme where they may have contact with thymocytes. This location indicates that TEC might be the preferred targets for sympathetic nerves in the thymus. Therefore, we evaluated whether cultured TEC might express adrenoceptors and respond to catecholamines. Since TEC create a microenvironment which is important for the maturation of T-cells, catecholamine-controlled TEC products upon stimulation might in turn influence thymocytes.

Adrenoceptors on cultured TEC

In order to provide further evidence for this hypothesis, we used a cell culture approach. We established highly purified cultures of rat TEC which were investigated for the presence of adrenoceptors and were challenged by adrenoceptor agonists. TEC grew in culture as a confluent monolayer adherent to plastic or poly-D-lysine-coated coverslips. The squamous cells were tightly attached to each other, and yielded a polygonal pattern in phase contrast imaging (Figure 2a). Cultures were more than 95% immunopositive for the epithelial cell marker cytokeratin (Figure 2b) and completely negative for the CD5-

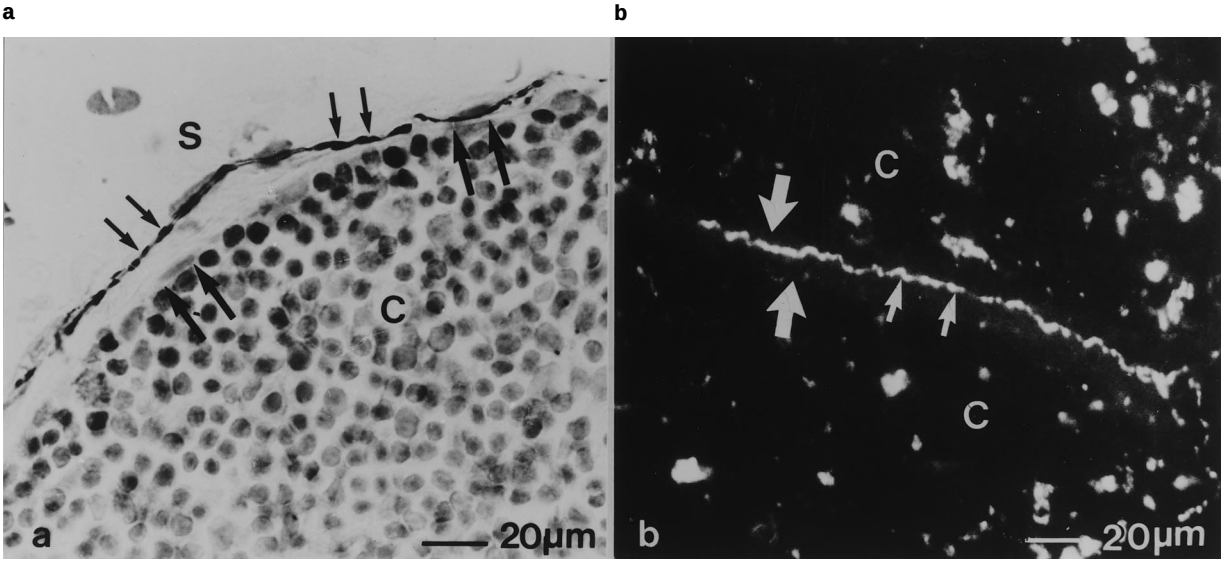

Figure 1 Adrenergic innervation of the rat thymus. (a) A cryostat section of a thymus from a 3-month old female rat stained with anti-tyrosine hydroxylase showing an immunopositive nerve fibre (small arrows) in a septum (S) in direct neighbourhood to TEC (big arrows), which separate the septum from thymic cortex (C). (b) Catecholamines in a nerve fibre (small arrows) detected by sucrose/phosphate/glyoxylic acid-induced fluorescence. The catecholamine positive nerve fibre passes a small septum that is separated from thymic cortex (C) by TEC (big arrows). Fluorescent spots in the cortex may result from autofluorescence (e.g. macrophages) or impurities. Original magnifications: (a) 350x, (b) 300x.

antigen as marker for thymocytes/T-cells. The analysis of the cultures for subtypes of TEC found *in situ* with a panel of specific antibodies has been described previously (Kurz *et al*., 1996). Markers for subcapsular/perivascular and medullary TEC were preferentially expressed, but a subset of the cultivated TEC stained positive for cortical markers. No changes in the adrenoceptor-mediated effects described below were detected in different cultures between the third to twentieth passage.

The presence of adrenoceptors on these cultivated TEC was investigated by RT–PCR and by binding assays. Cultured TEC, as well as rat C6 astrocytoma cells which served as a positive control (Krisch & Mentlein, 1994; Hosoda *et al*., 1994), expressed mRNA for β_1- and β_2-adrenoceptors as measured by RT–PCR with sequence specific primers (Figure 3). The PCR products showed the expected size of 165 bp (β_1) or 326 bp (β_2), and yielded expected restriction fragments of 113 and 52 bp (β_1, Figure 3b) or 231 and 95 bp (β_2, not shown) after cleavage with the endonucleases *Apa 1* or *Fok I*.

Expression of β-adrenoceptors on the cell surface of cultured TEC was detected by binding assays with the radiolabelled β-adrenoceptor agonist CGP 12177 which is non-selective for β-subtypes (Figure 4). TEC in culture displayed a specific, saturable binding with a dissociation constant of 5 nM and a maximal binding of 51 fmol mg^{-1} total cellular protein. Thus, mRNA coding for β_1- and β_2-adrenoceptors as well as receptor proteins are present in cultured TEC providing some of the essential prerequisites for activation by catecholamines.

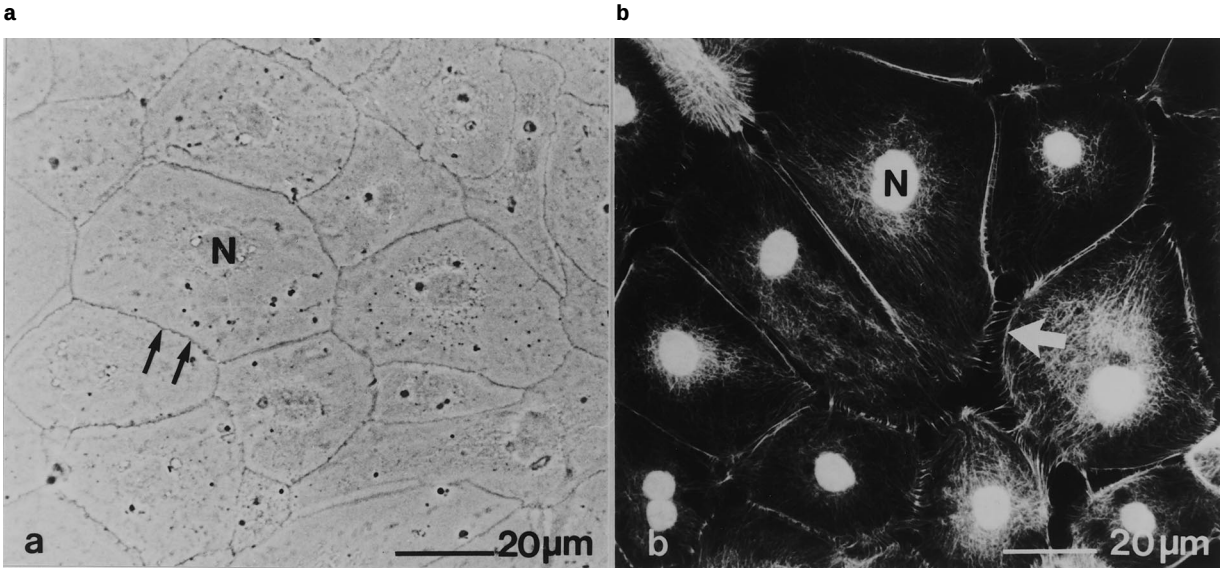

Figure 2 Characterization of TEC after four months of subcultivation (12 passages). (a) Phase-contrast micrograph showing a confluent flat layer of polygonal cells. Their surface membranes are tightly connected to each other (arrows) and organelles and some vacuoles accumulate around the nuclei (N). (b) Immunostaining for the epithelial cell marker cytokeratin. Tonofilaments stained by anti-pan-cytokeratin form a network in the cytoplasm and converge to desmosomes which connect adjacent cells to each other (arrow). Immunofluorescence is shown in combination with bisbenzimide fluorescence of nuclei (N). Original magnifications: (a) 560x, (b) 600x.

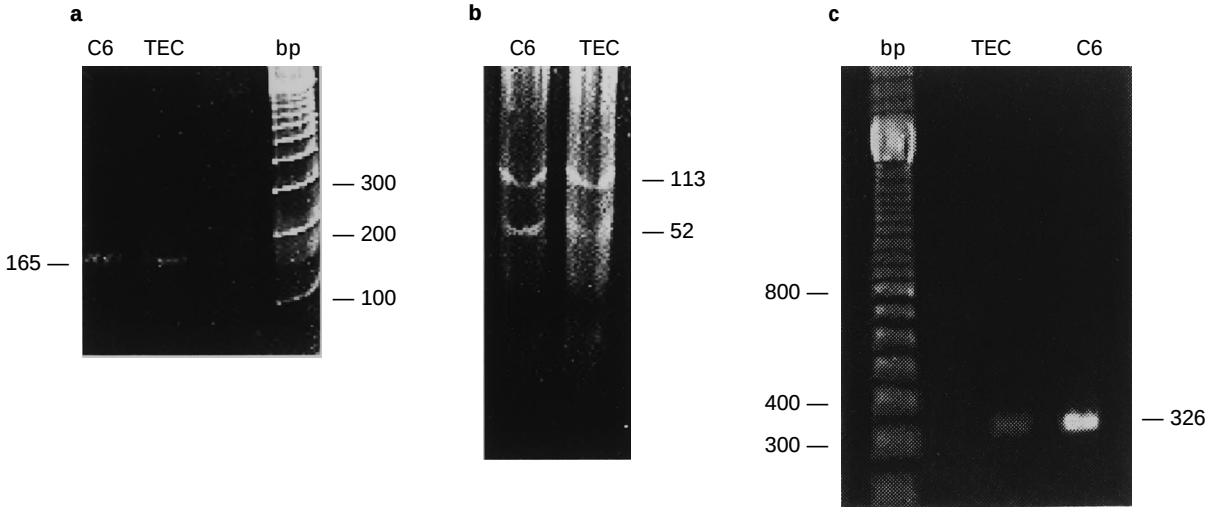

Figure 3 Rat TEC express mRNA for the β_1- (a) and β_2- (c) adrenoceptors as detected by RT–PCR. (a). Reversed transcribed RNA of cultured rat C6 astrocytoma cells (C6, positive control) and TEC was amplified with primers specific for β_1-adrenoceptors and products were separated in 10% polyacrylamide gels. They show the expected size of 165 base pairs (bp). (b) Restriction analysis of both amplified products with restriction endonuclease *Apa 1*. Comparison with markers show the expected sizes of 113 bp and 52 bp for the fragments. (c) RT–PCR with primers specific for β_2-adrenoceptors. The products separated in 2% agarose gels show the expected size of 326 bp. Two independent cultures (subcultures 10 and 20) yielded identical results.

Effect of adrenergic stimulation on cyclic AMP levels

In the next step we determined whether these β-adrenoceptors on cultured TEC can also transduce signals into the cells. As far as is known, activation of β-adrenoceptors results in the elevation of intracellular cyclic AMP levels (Weiner & Molinoff, 1994). Consistent with this, adrenaline, noradrenaline and β-adrenoceptor agonists, such as (−)-isoprenaline, potently stimulated cyclic AMP levels in a dose-dependent manner (Table 1, Figure 5). This proves the presence of functionally active, signal-transducing β-adrenoceptors on cultured TEC. The β_3-selective agonist BRL 37344 did not elicit cyclic AMP levels indicating the absence of functionally active β_3-adrenoceptors on cultured TEC (Table 1). The dose-response curve for noradrenaline was shifted to about 10 fold higher concentrations compared to isoprenaline and adrenaline (Figure 5). This can be explained by the presence of the β_2-receptor subtype in addition to the β_1-subtype, since β_2-receptors respond better to adrenaline and isoprenaline than to noradrenaline at the concentrations used, whereas β_1-receptors are stimulated equally by all three agonists (Weiner & Molinoff, 1994).

Neither dopamine nor 5-hydroxytryptamine affected the cyclic AMP levels in TEC (Table 1). Somatostatin and neuropeptide Y (via Y_2 receptors) that are known to reduce elevated cyclic AMP levels in their target cells (Krisch & Mentlein, 1994) had no effect on their own or in combination with isoprenaline (Table 1).

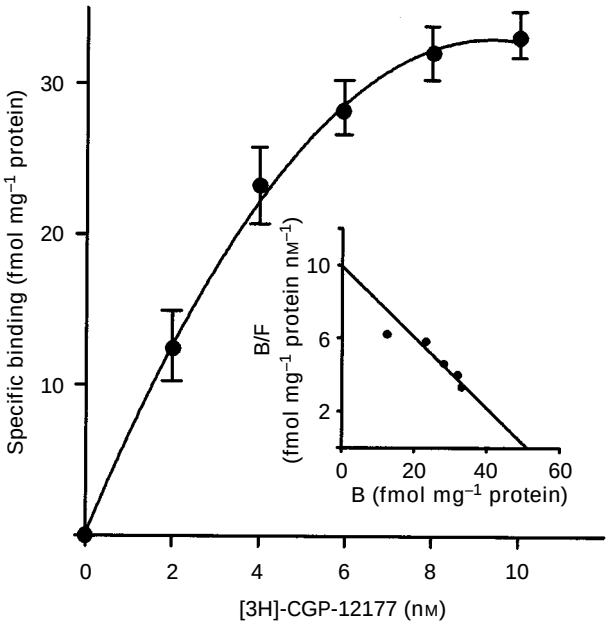

Figure 4 Specific binding of the hydrophillic β-adrenoceptor agonist [^{3}H]-CGP 12177 to cultivated rat TEC. Inset: Scatchard-plot of the binding data. TEC were incubated for 20 min at 37°C with the indicated concentrations of [^{3}H]-CGP 12177, bound radioactivity and protein were measured as described in Methods section. Non-specific binding was determined in the presence of 1 μM (−)-propranolol and subtracted from total binding. Each point reflects the mean of triplicate measurements (subculture 10). The maximal binding and the dissociation constant were calculated by linear regression analysis from the Scatchard-plot to be $B_{max} = 51$ fmol mg^{-1} protein and $K_D = 5$ nM.

Table 1 Induction of cyclic AMP levels in cultivated TEC by adrenoceptor agonists

Agonist (A)	*[A]* (μM)	*Cyclic AMP* (fmol μg^{-1} protein)	
None (control)	–	64±17	(23)
(−)-Adrenaline	1	1520±80	(3)
(−)-Noradrenaline	1	575±50	(3)
(−)-Isoprenaline	1	1479±183	(4)
BRL 37344	1	63±11	(3)
	10	97±17	(3)
Dopamine	1	74±35	(2)
5-Hydroxytryptamine	1	53±6	(2)
Somatostatin	0.1	64±10	(2)
(−)-Isoprenaline + somatostatin	1±0.1	1482±150	(4)
Neuropeptide Y	0.1	104±10	(2)
(−)-Isoprenaline + neuropeptide Y	1±0.1	1579±50	(2)

TEC (subcultures 5 to 15) were incubated with various agonists for 2 min at 37°C and cyclic AMP measured after lysis. Data shown are means±s.e.mean of n results (number in parentheses).

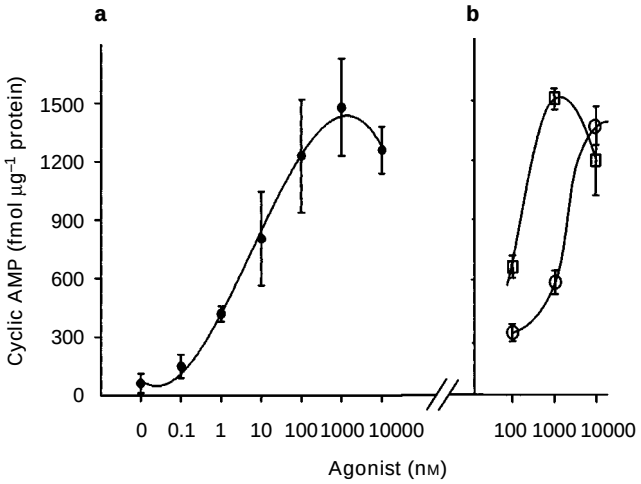

Figure 5 The β-adrenoceptor agonist (−)-isoprenaline (●, a), adrenaline (□, b) and noradrenaline (○, b) elicit cyclic AMP levels in rat cultivated TEC dose-dependently. TEC (subculture 10) were incubated with the agonists for 2 min at 37°C and cyclic AMP measured after lysis ($n = 4$); vertical lines show s.e.mean.

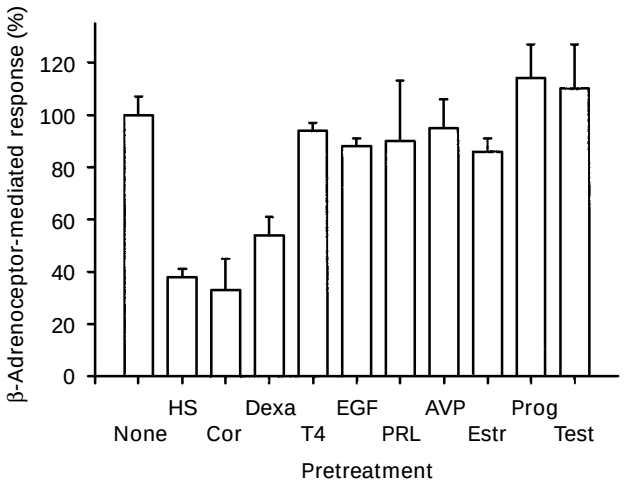

Figure 6 Glucocorticoids downregulate the β-adrenoceptor-mediated responsiveness of TEC to isoprenaline. TEC were pretreated with various hormones for 24 h in serum-free medium (DMEM/F12 supplemented with glutamine and transferrin) before stimulation with isoprenaline. Cells were then challenged for 2 min at 37°C without (= 0%, basal = 64 fmol cyclic AMP/μg protein) or with the β-adrenergic agonist (none = 100%, 1 μM (−)-isoprenaline = 1498 fmol cyclic AMP μg^{-1} protein) assayed for cyclic AMP and protein. Pretreatment: HS, horse serum (10%); Cor, cortisol (1 μM); Dexa, dexamethasone (1 μM); T4, thyroxine (1 μM); EGF, epidermal growth factor (10 ng ml^{-1}); PRL, prolactin (1 μM); AVP, arginine vasopressin (1 μM); Estr, oestrogen (1 μM); Prog, progesterone (1 μM); Test, testosterone (1 μM). TEC subcultures 10 to 20; $n = 4$. Statistical significance versus control: $P < 0.01$ for horse serum, cortisol and dexamethasone, $P < 0.05$ for EGF, other agonists response not significantly different.

Regulation of the β-adrenoceptor-mediated response by glucocorticoids

The β-adrenoceptor-mediated response elicited by isoprenaline was constant over a number of different subcultures of TEC. However, the presence of glucocorticoids in the culture medium for 1–2 days before stimulation with 1 μM (−)-isoprenaline significantly decreased cyclic AMP levels to 40% (cortisol) or 50% (dexamethasone; Figure 6). Similar downregulation was observed by pretreatment with horse serum. The sexual steroid hormones oestrogen, progesterone, testosterone as well as prolactin, thyroxine, epidermal growth factor and vasopressin did not (or slightly) regulate the β-adrenoceptor-mediated response (Figure 6).

The possible contributions of β_1- and β_2-adrenoceptors to the cyclic AMP response in TEC was measured by coincubation with an optimal dose of the general β-adrenoceptor agonist (−)-isoprenaline (1 μM) together with increasing concentrations of either a β_1- or β_2-selective antagonist (atenolol or ICI 118,551). In serum-free, washed cells, both antagonists were similarly potent in suppressing the elevation of cyclic AMP (Figure 7) indicating that β_1- and β_2-receptors must be active in TEC. In cells pretreated with glucocorticoids for 24 h, the adrenergic response – apart from being overall lower (Figure 6) – was reduced nearly totally by the β_2-antagonist, but not by the β_1-antagonist which had only a minor effect. This shows that the β_1-adrenoceptor-mediated response (and probably this receptor subtype) is drastically downregulated by glucocorticoids, whereas the effects of glucocorticoids on the β_2-adrenoceptor-mediated response are comparably less pronounced.

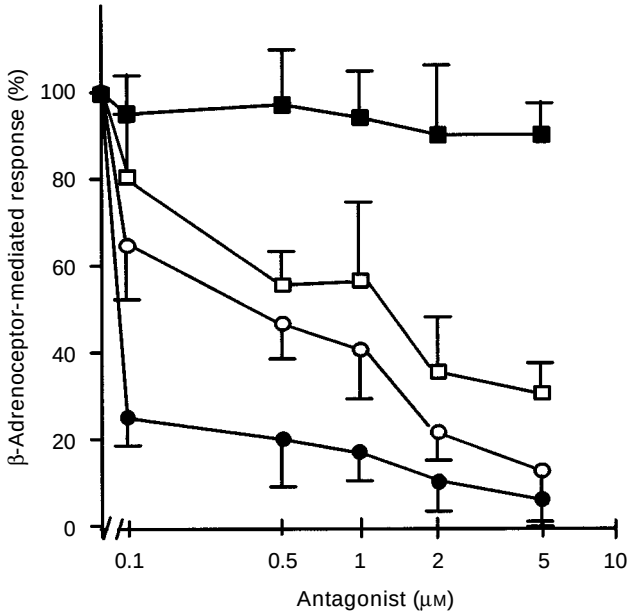

Figure 7 The glucocorticoid cortisol regulates the reduction of the responsiveness of TEC to isoprenaline by downregulation of the β_1-response. Cultured TEC were pretreated for 24 h without (open symbols) or with cortisol (+Cor, 1 μM, solid symbols) in serum-free medium. Cells were then challenged for 2 min with an optimal dose of the non-selective β-adrenoceptor agonist (−)-isoprenaline (1 μM) in the presence of increasing concentrations of the β-selective antagonist atenolol (□, ■) or the β_2-selective antagonist ICI 118,551 (○, ●) and assayed for intracellular cyclic AMP and protein. TEC subcultures 10 to 15; $n=4$.

β-Adrenoceptor-mediated regulation of TEC proliferation

As examples of the effects of catecholamines on TEC beyond the second messenger level we investigated the effects of the β-adrenoceptor agonist (−)-isoprenaline on the proliferation of TEC and the production of some cytokines. Proliferation of TEC was measured by incorporation of [^{3}H]-thymidine. Isoprenaline and the adenylate cyclase activator, forskolin inhibited the basal proliferation of TEC cultivated in serum-free or horse serum supplemented medium by 40% or 90%, respectively (Figure 8). As in many other cell types, the glucocorticoid, cortisol also reduced the incorporation of thymidine in TEC to about 60% (data not shown).

Effect of catecholamines on cytokine production

Since TEC are known to produce cytokines that have the potential to regulate the proliferation of thymocytes, we also investigated the effect of catecholamine-induced stimulation on IL-1 and IL-6 production in cultured TEC. Isoprenaline-

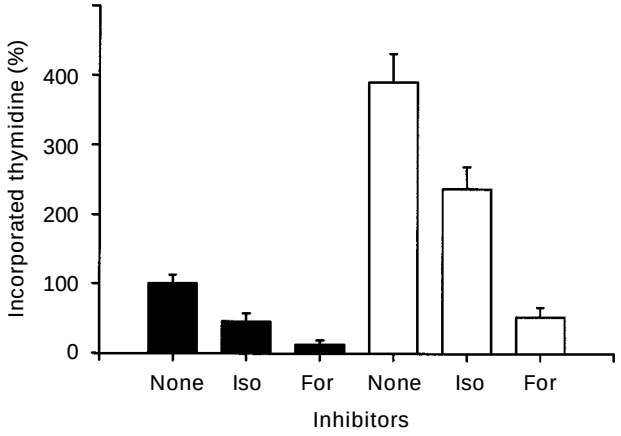

Figure 8 Isoprenaline reduces the proliferation of TEC. Cells were cultured in basal medium (DMEM/F12 supplemented with glutamine and transferrin) in the absence (solid columns) or presence (open columns) or 10% horse serum. Cells were incubated without (None) or with different agents (Iso, isoprenaline, 1 μM; For, forskolin, 50 μM) for 48 h, then [^{3}H]-thymidine was added and incorporation measured after an additional 5 h. Subculture 15; $n=4$; $P<0.01$ for isoprenaline and forskolin versus control.

Table 2 Isoprenaline-stimulated TEC do not produce interleukin-1 (IL-1)

Stimuli[1]	*Concentrations*	*Supernatant*[2] (pg ml^{-1})	*Cell-associated*[3] (pg)
Medium	–	0	0
(−)-Isoprenaline	1 μM	0	0
Forskolin	3 μM	0	0248±95
TNF-α	10 ng ml^{-1}	336±90	0
LPS	1 μg ml^{-1}	299±24	13,300±1,800
LS+Isoprenaline	1 μg ml^{-1}+1 μM	614±186	15,900±4,700

[1]TNF-α, tumour necrosis factor-α: LPS, lipopolysaccharide from *Salmonella friedenau*. [2]TEC (subculture 12) were incubated in 2 ml basal medium and stimulated for 24 h with the stimuli. The supernatants were harvested and tested in fibroblast assays. [3]The remaining monolayers were trypsinized, fresh medium added and cells lysed by two freeze thaw cycles before measurement.

stimulated TEC did not release significant levels of IL-6 (data not shown) as measured in three independent experiments. Furthermore, cultured TEC did not produce significant amounts of IL-1 upon stimulation with either isoprenaline, forskolin or tumour necrosis factor. However, the endotoxin of *Salmonella friedenau* (1 μg ml^{-1}) induced IL-1 synthesis and, to less extent, release (Table 2). Isoprenaline had a small, but not statistically significant effect on IL-1 production and release induced by the lipopolysaccharide (Table 2).

Discussion

Postganglionic sympathetic nerves enter the thymus along with blood vessels of the capsule and septa (Bellinger *et al*., 1988; Felten & Felten, 1989). Although the medullary region receives relatively sparse innervation, the blood vessels of the corticomedullary junction are richly innervated. The outer zones of the cortex where immature thymocytes are developing also contain a substantial catecholaminergic innervation, and Felten & Felten (1989) and recently Vizi *et al*. (1995) demonstrated some nerve fibres distributed among thymocytes. We visualized by two independent methods – immunochemical detection of the transmitter-synthesizing enzyme and catecholamine-induced fluorescence – that catecholaminergic nerves also run in close contact to TEC. Since TEC form the blood-thymus barrier in the outer thymic cortex (Raviola & Karnowsky, 1972; Nieuwenhuis, 1990), these cells may be targets for circulating catecholamines from the adrenals or those from perivascular nerves. In the thymus the adrenaline concentration has been demonstrated to be negligible as compared to that of noradrenaline (Kendall *et al*., 1994). Therefore, noradrenaline can be supposed as the main transmitter of sympathetic nerves in the thymus. Changes of noradrenergic innervation of the thymus during pregnancy and with age (Kendall *et al*., 1994) suggest a role for noradrenaline in thymic functions.

As a target for this sympathetic transmitter, we identified β-adrenoceptors in cultured TEC at the mRNA and protein level. Upon stimulation with adrenaline, noradrenaline or β-adrenoceptor agonists TEC respond with increased intracellular cyclic AMP levels. This signal transduction is generally linked with β-receptors (subtype 1, 2 and 3), whereas stimulation of α_2-receptors inhibits adenylate cyclase and stimulation of α_1-adrenoceptors leads to hydrolysis of phosphoinositides and/or Ca^{2+}-channel opening (Weiner & Molinoff, 1994). β_1-Receptors are stimulated equally by adrenaline and noradrenaline, whereas β_2-receptors display a higher affinity for adrenaline than for noradrenaline. Both are present on cultured TEC.

We found the β-adrenoceptor-mediated response of cultured TEC to be suppressed by glucocorticoids. It is known from other studies that glucocorticoid receptors are present in cultured TEC (Dardenne *et al*., 1989). Glucocorticoids are known to have differential effects on the expression of β-adrenoceptor subtypes through glucocorticoid-responsive elements in the 5′-flanking region of their genes (Collins *et al*., 1993). Glucocorticoids increase the rate of β_2-receptor gene transcription in many cells (Hadcock & Craig, 1991), but they reduce the density of β_1-receptors in other cells, such as C6 rat astroglioma cells (Zhong & Minnemann, 1993). Although glucocorticoids do not alter the total responsiveness to catecholamines in C6 cells due to an inverse regulation of the β_1-/β_2-subtypes (Zhong & Minnemann, 1993), they reduced the total responsiveness in cultured TEC as investigated by us. In the case of cultured TEC, β_2-adrenoceptors are upregulated and β_1-receptors appear to be downregulated by glucocorticoids. However, this does not compensate for the reduction of the β_1-mediated response.

Among thymic cells a response to adrenergic stimulation has been detected previously with suspensions of whole thymocytes (Singh & Owen, 1976; Singh *et al*., 1979; Vizi *et al*., 1995). Noradrenaline has been shown to influence the expression of Thy-1 and TL antigens on 14-day foetal thymic stem cells of the mouse (Singh & Owen, 1976). A subsequent study correlated this effect to the elevation of cyclic AMP in thymocytes, although only cell suspensions of total thymi without cell purification or characterization were stimulated by isoprenaline (Singh *et al*., 1979). We showed clearly that immunocytochemically defined cultured TEC also respond to this agonist. Thus, TEC have to be added to the list of immune cells, which possess β-adrenoceptors; this includes mature lymphocytes, monocytes/macrophages, neutrophils and mast cells (Felten & Felten, 1989).

Considering the sparse innervation of the thymus parenchyme in comparison to the rich innervation in the septa, at least in the outer thymic cortex neuronally released noradrenaline should preferably reach TEC, but not thymocytes. Also circulating catecholamines transported in the blood vessels which run in the septa should only affect TEC, not thymocytes in the outer cortex, since TEC form the blood-thymus barrier here (Raviola & Karnowsky, 1972; Nieuwenhuis, 1990). Upon stimulation with (nor)adrenaline, TEC may release factors which in turn influence the differentiation of thymocytes. TEC are known to produce cytokines (interleukin-1, interleukin-3, interleukin-6, interleukin-7, granulocyte-macrophage colony-stimulating factor and others), thymic peptide hormones (thymulin, thymosin α1, thymopoietin) and neuropeptides/peptide hormones (oxytocin, vasopressin, calcitonin gene-related peptide) which can act on thymocytes (Hadden *et al*., 1991; Kendall, 1992; Screpanti *et al*., 1993; Kendall & Strebbings, 1994; Kurz *et al*., 1995). However, the conditions for the release of these bioactive factors from TEC *in vivo* are mostly unknown. Therefore, we measured the effect of β-adrenoceptor stimulation on the production and release of two cytokines *in vitro*. However, both were not affected. Further studies are needed to analyse the effect of adrenergic stimulation on the production of other TEC-derived mediators.

It is known that the thymus morphology changes with age. Its size decreases after puberty or during lactation (von Gaudecker, 1991; Kendall, 1991). This so called physiological involution in children can be induced by accidents, heat shock or other stress situations. From our results, the inhibitory effect of (nor)adrenaline on TEC proliferation may be considered as one of the stress factors affecting thymus reduction. It may also be speculated whether β-blockers may affect the immune system via TEC.

In conclusion, glucocorticoids and (nor)adrenaline are effectors of stress reactions at dual sites: the hypothalamic-hypophysial-adrenal and the neuronal axis. Their mutual influence on TEC is an example of the complex regulation of immune responses by stress molecules.

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References

BELLINGER, D.L., FELTEN, S.Y. & FELTEN, D.L. (1988). Maintenance of noradrenergic sympathetic innervation in the involuted thymus of the aged Fischer 344 rat. *Brain Behav. Immunity*, **2,** 133–150.

COLLINS, S., OSTROWSKI, J. & LEFKOWITZ, R.J. (1993). Cloning and sequence analysis of the human β_1-adrenergic receptor 5′-flanking promoter region. *Biochim. Biophys. Acta*, **1172,** 171–174.

DARDENNE, M., SAVINO, W., GAGNERAULT, M., ITOH, T. & BACH, J. (1989). Neuroendocrine control of thymic hormonal production. I. Prolactin stimulates *in vivo* and *in vitro* the production of thymulin by human and murine epithelial cells. *Endocrinology*, **125,** 3–12.

DIXON, W.J., BROWN, M.B., ENGELMANN, L. & JENNRICH, R.I. (1990). *BMDP Statistical Software Manual*. Berkley, CA, U.S.A.: University of California Press.

FEINDT, J., BECKER, I., BLÖMER, U., HUGO, H.-H., MEHDORN, H.M., KRISCH, B. & MENTLEIN, R. (1995). Expression of somatostatin receptor subtypes in cultured astrocytes and gliomas. *J. Neurochem.*, **65,** 1997–2005.

FELTEN, D.L. & FELTEN, S.Y. (1989). Innervation of the thymus. *Thymus Update*, **2,** 3–88.

VON GAUDECKER, B. (1991). Functional histology of the human thymus. *Anat. Embryol.*, **183,** 1–15.

VON GAUDECKER, B., LARCHE, M., SCHUURMAN, H.-J. & RITTER, M.A. (1989). Analysis of the fine distribution of thymic epithelial microenvironmental molecules by immuno-electron microscopy. *Thymus*, **13,** 187–194.

HADCOCK, J.R. & CRAIG, C.C. (1991). Regulation of receptor expression by agonists: transcriptional and post-transcriptional events. *Trends Neurosci.*, **14,** 242–247.

HADDEN, J.W., HADDEN, E.M. & COFFEY, R.G. (1991). First and second messengers in the development and function of thymus-dependent lymphocytes. In *Psychoneuroimmunology*, 2nd Edn. ed. Ader, R., Felten, D.L. & Cohen, N. pp. 529–560. San Diego: Academic Press.

HOSODA, K., FEUSSNER, G.K., RYDELED-FITZGERALD, L., FISHMAN, P.H. & DUMAN, R.S. (1994). Agonist and cyclic AMP-mediated regulation of β_1-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells. *J. Neurochem.*, **63,** 1635–1645.

JIANG, L. & KUNOS, G. (1995). Sequence of the 5′ regulatory domain of the gene encoding the rat beta 2-adrenergic receptor. *Gene*, **163,** 331–332.

KENDALL, M.D. (1991). Functional anatomy of the thymus microenvironment. *J. Anat.*, **177,** 1–29.

KENDALL, M.D. (1992). The thymus: new views of an old gland. *Endeavour*, **16,** 158–163.

KENDALL, M.D., ATKINSON, B.A., MUNOZ, F.J., DE LA RIVA, C., CLARKE, A. & VON GAUDECKER, B. (1994). The noradrenergic innervation of the rat thymus during pregnancy and in the post partum period. *J. Anat.*, **185,** 617–625.

KENDALL, M.D. & STREBBINGS, R.J. (1994). The endocrine thymus. *Endocrine J.*, **2,** 333–339.

KRISCH, B. & MENTLEIN, R. (1994). Neuropeptide receptors and astrocytes. *Int. Rev. Cytol.*, **148,** 119–169.

KURZ, B., VON GAUDECKER, B., KRANZ, A., KRISCH, B. & MENTLEIN, R. (1995). Calcitonin gene-related peptide and its receptor in the thymus. *Peptides*, **16,** 1497–1503.

KURZ, B., VON GAUDECKER, B., KRISCH, B. & MENTLEIN, R. (1996). Rat thymic epithelial cells *in vitro* and *in situ*: characterisation by immunocytochemistry and morphology. *Cell Tissue Res.*, **283,** 221–229.

LACASA, D., AGLI, B. & GIUDICELLI, Y. (1985). Direct assessment of β-adrenergic receptors in intact rat adipocytes by binding of [^{3}H]CGP 12177. Evidence for agonist high-affinity binding complex and for β_1 and β_2 receptor subtypes. *Eur. J. Biochem.*, **146,** 339–346.

LUCIUS, R. & MENTLEIN, R. (1991). Degradation of the neuropeptide somatostatin by cultivated neuronal and glial cells. *J. Biol. Chem.*, **266,** 18907–18913.

LOPPNOW, H., BRADE, H., DÜRRBAUM, I., DINARELLO, A., KUSUMOTO, S., RIETSCHEL, E.T. & FLAD, H.-D. (1989a). IL 1 induction capacity of defined lipopolysaccharide partial structures. *J. Immunol.*, **142,** 3229–3238.

LOPPNOW, H., BRADE, H., RIETSCHEL, E.T. & FLAD, H.-D. (1994). Induction of cytokines in mononuclear and vascular cells by endotoxin and other bacterial products. *Methods Enzymol.*, **236,** 3–10.

LOPPNOW, H., FLAD, H.-D., DÜRRBAUM, I., MUSEHOLD, J., FETTING, R., ULMER, A.J., HERZBECK, H. & BRANDT, E. (1989b). Detection of interleukin 1 with human dermal fibroblasts. *Immunobiology*, **179,** 283–291.

MACHIDA, C.A., BUNZOW, J.R., SEARLES, R.P., VAN TOL, H., TESTER, B., NEVE, K.A., TEAL, P., NIPPER, V. & CIVELLI, O. (1990). Molecular cloning and expression of the rat β_1-adrenergic receptor gene. *J. Biol. Chem.*, **265,** 12960–12965.

NIEUWENHUIS, P. (1990). Self-tolerance induction and the blood-thymus barrier. *Thymus Update*, **3,** 31–51.

RAVIOLA, E. & KARNOVSKY, M.J. (1972). Evidence for a blood-thymus barrier using electron-opaque tracers. *J. Exp. Med.*, **136,** 466–497.

SCREPANTI, I., MODESTI, A. & GULINO, A. (1993). Heterogeneity of thymic stromal cells and thymocyte differentiation: a cell culture approach. *J. Cell Sci.*, **105,** 601–606.

SINGH, U. & OWEN, J.J.T. (1976). Studies on the maturation of thymus stem cells. The effect of catecholamines, histamine and peptide hormones on the expression of T cell alloantigens. *Eur. J. Immunol.*, **6,** 59–62.

SINGH, U., MILLSON, D.S., SMITH, P.A. & OWEN, J.J.T. (1979). Identification of β-adrenoceptors during thymocyte ontogeny in mice. *Eur. J. Immunol.*, **9,** 31–35.

DE LA TORRE, J.C. & SURGEON, J.W. (1976). A methodological approach to rapid and sensitive monoamine histofluorescence using a modified glyoxylic acid technique: the SPG-method. *Histochemistry*, **49,** 81–93.

VIZI, E.S., ORSÓ, E., OSIPENKO, O.N., HASKÓ, G. & ELENKOV, I.J. (1995). Neurochemical, electrophysiological and immunocytochemical evidence for a noradrenergic link between the sympathetic nervous system and thymocytes. *Neuroscience*, **68,** 1263–1276.

WEINER, N. & MOLINOFF, P.B. (1994). Catecholamines. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 5th Edn. ed. Siegel, G.J., Agranoff, B.W., Albers, R.W. & Molinoff, P.B. pp. 261–281. New York: Raven Press.

ZHONG, H. & MINNEMANN, K.P. (1993). Close reciprocal regulation of β(1)-adrenergic and β(2)-adrenergic receptors by dexamethasone in C6 glioma cells–effects on catecholamine responsiveness. *Mol. Pharmacol.*, **44,** 1085–1093.

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