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Baclofen influences lipopolysaccharide-mediated interleukin-6 release from murine pituicytes

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Abstract

Pituicytes, the glial cells of the neurohypophysis, secrete interleukin-6 upon stimulation with various inflammatory mediators, i.e. lipopolysaccharide. Previous studies have identified several receptors on pituicytes. This study investigates the effect of $GABA_B$ receptor activation on interleukin-6 release from pituicytes. Cultured murine pituicytes were stimulated for 24 h with lipopolysaccharide (0.5 ng/ml) to give a significant interleukin-6 release compared to control. The interleukin-6 release was significantly potentiated by the $GABA_B$ receptor agonist (*R*)-4-amino-3-(4-chlorophenyl) butanoic acid (R-baclofen; 10, 100 or 500 μM). However, R-baclofen itself (10, 100 or 500 μM) did not stimulate the interleukin-6 secretion. Furthermore, the potent $GABA_B$ receptor antagonists 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl]diethoxymethyl) phosphinic acid (CGP52432; 30 or 300 μM) and (*RS*)-3-Amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulphonic acid (2-OH-saclofen; 10 or 100 μM) did not remove the effect of R-baclofen (100 μM). γ-Amino butyric acid (GABA; 30 or 300 μM) did not alter the lipopolysaccharide-mediated interleukin-6 response. After 30 min, intracellular cyclic AMP (cAMP) was higher in cells stimulated with lipopolysaccharide compared to control, and R-baclofen significantly inhibited this increase in cAMP. Nevertheless, neither lipopolysaccharide nor R-baclofen had any effect on intracellular cAMP after 24 h of stimulation. The results suggest that the effect of R-baclofen on lipopolysaccharide-stimulated interleukin-6 secretion is independent of $GABA_B$ receptors.

Keywords: Baclofen; Lipopolysaccharide; Interleukin-6; cAMP; $GABA_B$ receptor antagonist; Pituicyte; Neurohypophysis

1. Introduction

Oxytocin and vasopressin are synthesized in hypothalamic magnocellular neurons in the paraventricular and the supraoptic nuclei, and transported by axons to the neural lobe, where they are secreted into the circulation. The axonal terminals are surrounded and engulfed by modified astrocytes named pituicytes, which stain positive for glial fibrillary acidic protein. Pituicytes are the largest population of cells in the neural lobe (Boersma and van Leeuwen, 1994). Secretion of oxytocin and vasopressin is regulated not only at the level of the hypothalamus, but also at the level of the neural lobe through presynaptic innervation (Pittman et al., 1983; Bicknell, 1988). In the neural lobe, the hormone release may be regulated directly by substances acting on the nerve terminals themselves or indirectly with the pituicytes as an intermediate (Boersma and van Leeuwen, 1994). Therefore, it is of interest to investigate how pituicytes react upon various stimuli.

Interleukin-6 is believed to play an important role during various inflammatory conditions (Gadient and Otten, 1997), and since the neural lobe is situated outside the blood–brain barrier, infectious substances have easy access to this organ compared to other parts of the brain. It has been shown that pituicytes release high amounts of interleukin-6 upon stimulation with interleukin-1β or lipopolysaccharide (Spangelo et al., 1994; Christensen et al., 1999; Hansen et al., 1999). Furthermore, interleukin-1β can increase electric-stimulated secretion of oxytocin and vasopressin from the isolated rat neurohypophysis (Christensen et al., 1990), and interleukin-6 and interleukin-1β stimulate oxytocin and vasopressin secretion in rat hypothalamic explants (Yasin et al., 1994). In addition, Spangelo et al. (1994) found that oxytocin and vasopressin inhibited interleukin-6 release induced by lipopolysaccharide or interleukin-1β in rat, neurointermediate pituitary lobe cells. Hence, the cytokines interleukin-6 and inter-

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leukin-1β have a positive effect on the regulation of oxytocin and vasopressin release, which in turn inhibits interleukin-6 release. Thus, it seems that interleukin-6 is implicated in the modulation of neuroendocrine function in the hypothalamic-pituitary axis, and that interleukin-6 production in the pituicytes may act in a paracrine manner to affect the release of oxytocin and vasopressin.

GABA axons terminate synaptically on pituicytes (Buijs et al., 1987), and secretion of GABA from the neural lobe is increased by interleukin-6 under depolarising conditions (De Laurentiis et al., 2000). GABA is found to inhibit secretion of oxytocin and vasopressin from the neural lobe in male rats (Saridaki et al., 1989). Since pituicytes are able to secrete interleukin-6, they may potentiate GABA release in the neural lobe, which in turn could inhibit secretion of oxytocin and vasopressin. Oxytocin and vasopressin terminals in the neural lobe express $GABA_A$ receptors (Zhang and Jackson, 1993), and pituicytes have been proposed to express $GABA_B$ receptors (Boersma and van Leeuwen, 1994). We therefore wanted to investigate whether interleukin-6 secretion from pituicytes could be modulated via $GABA_B$ receptors. The specific $GABA_B$-receptor agonist, (*R*)-4-amino-3-(4-chlorophenyl) butanoic acid (R-baclofen) was used.

Increase in intracellular cyclic AMP (cAMP) is known to be part of the interleukin-1β and lipopolysaccharide-mediated interleukin-6 release (Siegert et al., 1976; Onodera, 1991; Maimone et al., 1993), and activation of $GABA_B$ receptors is known to affect intracellular cAMP levels (Knight and Bowery, 1996; Uzono et al., 1997). For this reason, we also wanted to investigate whether intracellular cAMP was changed during stimulation with lipopolysaccharide and R-baclofen.

2. Materials and methods

2.1. Materials

All solutions used for the cell cultures were passed through filters with 20-kDa cutoff (Ultrasart D20, Sartorius) immediately after dissolution. The lipopolysaccharide content after filtration was below the detection limit in our *Limulus* amoebocyte lysate assay (<1 pg lipopolysaccharide/ml). All laboratory glassware to be used in the handling of cells were rendered pyrogen-free through heating at 180 °C for 6 h. The cells were grown in RPMI 1640 (51800-019, Life Technologies/Gibco) dissolved in pyrogen-free water and supplemented with HEPES (10 mM), 5% fetal bovine serum certified (Life Technologies/Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and mercaptoethanol (50 μM). For stimulation, lipopolysaccharide from *Escherichia coli* 055:B5 (Biotech Line) was used. GABA-receptor agonists and antagonists were obtained as follows: R-baclofen (Sigma-Aldrich), GABA (Sigma), 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl]diethoxymethyl) phosphinic acid (CGP52432; Tocris), and (*RS*)-3-Amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulphonic acid (2-OH-saclofen; Tocris). Stock solutions were made in pyrogen-free water and passed through filters with a 20-kDa cutoff. Subsequently, dilutions were made in supplemented RPMI 1640.

2.2. Primary neurohypophyseal cell cultures

Male albino mice of the NMRI strain, 3–5 weeks old, were used for the experiments. The mice were obtained from M&B breeding facilities, Ry, Denmark. The animals were decapitated and the hypophyses were aseptically removed. The neural lobe was separated from the anterior and intermediate lobe under a stereomicroscope, and 8 to 12 neural lobes were collected in 12 ml supplemented RPMI 1640, which was kept on ice. The tissue was enzymatically digested in two steps. First, the tissue was exposed to trypsin (Fluka 93610, 9068 U/mg) 2.0 mg/ml in unsupplemented RPMI 1640 and incubated for 25 min at 37 °C and 5% CO_2. Trypsin was removed by centrifugation at 155×*g* for 5 min. Second, the pellet was resuspended in pancreatin (Sigma P7545) 2.5 mg/ml in unsupplemented RPMI 1640 and incubated for 5 min (37 °C and 5% CO_2). This suspension was centrifuged for 5 min (155×*g*) and the pellet resuspended in Minimal Essential Medium Eagle (Sigma M4767), containing 10% fetal bovine serum certified, and incubated for 60 min (37 °C and 5% CO_2) to stop the enzymatic reaction. Following centrifugation for 5 min (155×*g*), the tissue was resuspended in 300 μl unsupplemented RPMI 1640 and mechanically disrupted to single cells using a plastic pipette tip. Finally, the volume was adjusted to 3000 μl per neural lobe with supplemented RPMI 1640, and the cells were plated in 24-well culture plates (Nunc, Life Technologies) with 0.5 neural lobe/1500 μl/well. The cells were cultured at 37 °C and 5% CO_2–95% air in a humidified atmosphere. The medium (supplemented RPMI 1640, 1500 μl) was replaced after 7 days and hereafter twice a week.

2.3. Stimulation of primary neurohypophyseal cell cultures

After 13 days in culture, the cells were confluent and the medium was aspirated and discarded to avoid cross contamination of released interleukin-6 during culturing. On day 13, supplemented RPMI 1640 (1000 μl/well) was added, and after 24 h, the supernatants were aspirated and collected for analysis of unstimulated interleukin-6 release.

2.3.1. Interleukin-6

On day 14, the pituicytes were incubated for 24 h in presence or absence of the test substances (1000 μl/well), and the supernatants were aspirated and collected for analysis of stimulated release. The effect of the compounds was expressed as the ratio of interleukin-6 release day 14/

day 13, and the lipopolysaccharide (0.5 ng/ml) response was standardised as a 100% response.

2.3.2. Cyclic AMP

On day 14, the pituicytes were incubated for 30 min or 24 h in presence or absence of the test substances (1000 μl/well), and the supernatants were discarded. Subsequently, the cells were washed in isotonic sodium chloride, lyzed in 0.1 M hydrochloric acid, frozen (−18 °C), thawed and frozen until used for determination of cAMP. The effect of the compounds was expressed as the ratio of intracellular cAMP at 30 min or 24 h on day 14/unstimulated interleukin-6 release at 24 h on day 13.

2.4. Assay for interleukin-6

The interleukin-6 concentration was determined using a noncompetitive sandwich immunoassay as previously described by Moesby et al. (1997). FluoroNunc™ microtiter plates (Nunc, Denmark) were coated with monoclonal anti-mouse interleukin-6 antibody (2 μg/ml; 100 μl/well, R&D Systems) in phosphate-buffered saline pH 7.4 and incubated overnight at room temperature. After three washes with DELFIA® wash buffer (Wallac, Finland), nonspecific binding was blocked with blocking buffer (300 μl/well; 1% bovine serum albumin, 5% sucrose in phosphate-buffered saline pH 7.4). The plates were shaken in a DELFIA® plate shaker (slow) for a minimum of 1 h at room temperature and washed three times with DELFIA® wash buffer. Standards (recombinant murine interleukin-6, R&D Systems) and test solutions diluted in supplemented RPMI 1640 were added to the wells (100 μl/well) and shaken (slow) for 2 h at room temperature. After three washes, biotinylated polyclonal goat anti-mouse interleukin-6 antibody in Tris-buffered sodium chloride pH 7.4 with 0.1% bovine serum albumin (100 μl/well; 100 ng/ml; R&D Systems) was added to each well and shaken (slow) for 2 h at room temperature. The wells were washed three times and europium-labelled streptavidin (100 μl/well; 100 ng/ml; R&D Systems) diluted in DELFIA® assay buffer was added. The plates were shaken (slow) for 30 min at room temperature and washed six times before the europium was rendered fluorescent with DELFIA® enhancement solution (100 μl/well). The plates were shaken (fast) for 5–10 min and the fluorescence was detected in a 1234 DELFIA® fluorometer. The detection limit of the assay was 12.5 pg interleukin-6/ml. The inter-assay variation was CV%=9.4 (n=9) and the intra-assay variation was CV%=5.1 (n=9) (Christensen et al., 1999).

2.5. Assay for cyclic AMP

The concentration of cAMP was quantified as pmol/ml lysate by using a competitive immunoassay (R&D Systems). The cell lysates were collected and centrifuged at 600×g for 10 min, and the supernatants were assayed immediately. All standards and samples were acetylated (for assay procedure: Cat. No. DE0355, R&D Systems).

2.6. Glial fibrillary acidic protein immunocytochemistry

Cells were grown in 24-well culture plates for 14 days, washed with phosphate-buffered saline pH 7.4 and fixated with methanol. After washing with phosphate-buffered saline pH 7.4, the cells were incubated overnight at 4 °C with rabbit anti-cow GFAP (DAKO) diluted (1:100) in phosphate-buffered saline pH 7.4 containing 0.1% bovine serum albumin. Controls were incubated overnight at 4 °C in phosphate-buffered saline pH 7.4 containing 0.1% bovine serum albumin. Next, the cells were washed with phosphate-buffered saline pH 7.4 and incubated for 90 min at room temperature with fluorescein isothiocyanate-conjungated swine anti-rabbit immunoglobulin (DAKO) diluted (1:40) in phosphate-buffered saline pH 7.4 containing 0.1% bovine serum albumin. Finally, the cells were washed with phosphate-buffered saline pH 7.4 and examined under a Leica fluorescence microscope.

2.7. Statistical analysis

The Mann–Whitney Rank Sum Test was used.

3. Results

3.1. Immunocytochemistry

After 14 days in culture, all cells observed in the phase contrast picture appeared immunoreactive for glial fibrillary acidic protein. There was no immunoreactivity in control cells where the primary antibody had been omitted, which indicates specific staining for glial fibrillary acidic protein.

3.2. Effect of lipopolysaccharide on interleukin-6 release from pituicytes

Lipopolysaccharide is a well-known inflammatory mediator, and in order to get a significant interleukin-6 release, the pituicyte cell cultures were stimulated with various concentrations of lipopolysaccharide. Lipopolysaccharide-stimulated interleukin-6 release in a concentration-dependant manner (Fig. 1). Lipopolysaccharide (0.1, 0.5, 1, and 10 ng/ml) increased the release of interleukin-6 by 1.9-, 6.1-, 9.3- and 17.2-fold, respectively (P=0.002 compared to control). The submaximal dose of lipopolysaccharide (0.5 ng/ml) was chosen as a standard inflammatory mediator in order to see whether other test substances could alter this response. To minimise the variation between experiments,

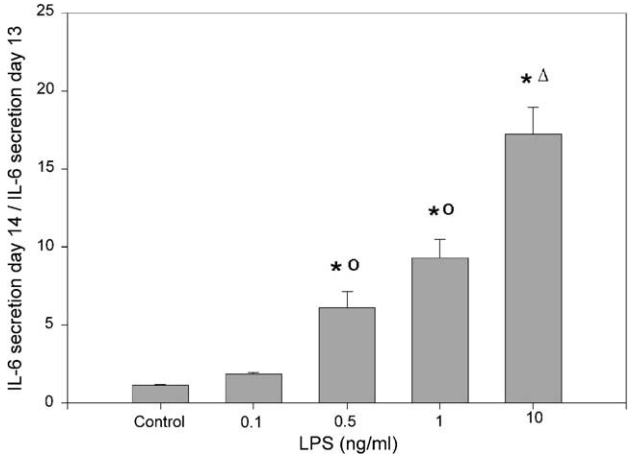

Fig. 1. Effect of lipopolysaccharide on interleukin-6 release from cultured murine neurohypophysial cells. On day 13, the cells were incubated with supplemented RPMI 1640 for 24 h followed by incubation with lipo polysaccharide or supplemented RPMI 1640 (control) for 24 h. The results are expressed as the ratio of interleukin-6 release on day 14 to interleukin-6 release on day 13. Each value is the mean±S.E.M. of six observations. *P=0.002 compared to control, OP=0.002 compared to lipopolysaccharide (0.1 ng/ml), ΔP=0.009 compared to lipopolysaccharide (1 ng/ml) (Mann–Whitney Rank Sum Test). Control=RPMI 1640 supplemented; LPS=lipopolysaccharide; IL-6=interleukin-6.

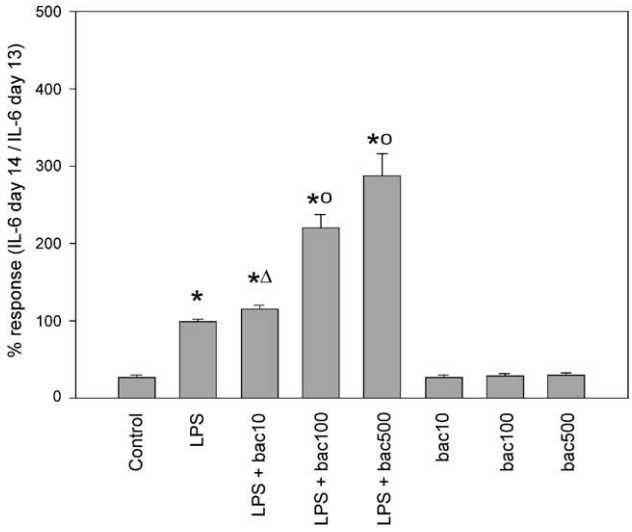

Fig. 2. Effect of lipopolysaccharide and R-baclofen on interleukin-6 release from cultured murine neurohypophysial cells. On day 13, the cells were incubated with RPMI 1640 supplemented for 24 h followed by incubation with test substances for 24 h. The results are expressed as the ratio of interleukin-6 release on day 14 to interleukin-6 release on day 13. The lipopolysaccharide response (0.5 ng/ml) is standardised as a 100% response. Each value is the mean±S.E.M. of 9 to 11 observations. *P≤0.001 compared to control or R-baclofen, ΔP=0.027 compared to lipopolysaccharide (0.5 ng/ml), OP=0.009 compared to lipopolysaccharide (0.5 ng/ml) or lipopolysaccharide (0.5 ng/ml)+R-baclofen (10 μM) (Mann–Whitney Rank Sum Test). Control=RPMI 1640 supplemented; LPS=lipopolysaccharide (0.5 ng/ml); LPS+bac10–500=lipopolysaccharide (0.5 ng/ml)+R-baclofen (10–500 μM); bac10–500=R-baclofen (10–500 μM); IL-6=interleukin-6.

the interleukin-6 response to lipopolysaccharide (0.5 ng/ml) was standardised as a 100% response.

3.3. Effect of R-baclofen on interleukin-6 release from pituicytes

R-baclofen (10–500 μM) alone did not have any effect on interleukin-6 release from pituicytes (Fig. 2). However, when added in combination with lipopolysaccharide (0.5 ng/ml), R-baclofen showed a significant potentiation of the lipopolysaccharide-mediated interleukin-6 release. R-baclofen (10, 100, and 500 μM) potentiated the lipopolysaccharide-mediated (0.5 ng/ml) interleukin-6 release from 100% to 116%, 221%, and 289%, respectively (P=0.027, P<0.001, P<0.001 compared to lipopolysaccharide).

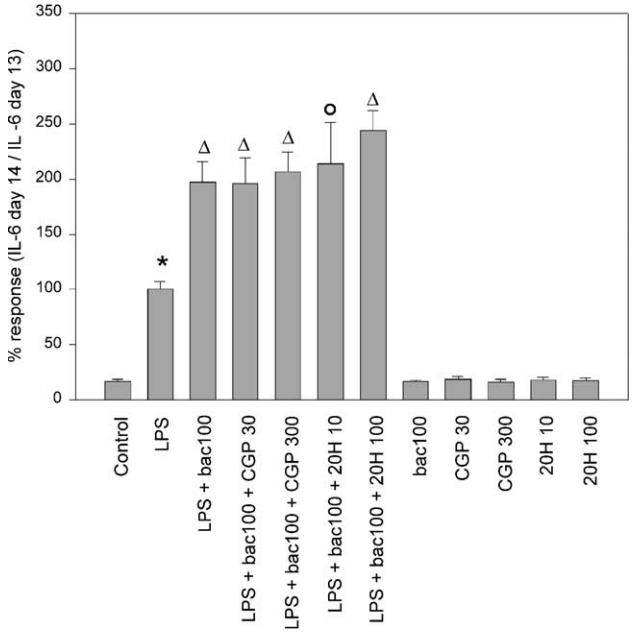

Fig. 3. Effect of the $GABA_B$ receptor antagonists CGP52432 (30 or 300 μM) and 2-OH-saclofen (10 or 100 μM) on the R-baclofen (100 μM) potentiation of the lipopolysaccharide-mediated (0.5 ng/ml) interleukin-6 release from cultured murine neurohypophysial cells. On day 13, the cells were incubated with RPMI 1640 supplemented for 24 h followed by incubation with test substances for 24 h. The results are expressed as the ratio of interleukin-6 release on day 14 to interleukin-6 release on day 13, and the lipopolysaccharide response (0.5 ng/ml) is standardised as a 100% response. Each value is the mean±S.E.M. of six to seven observations. *P≤0.002 compared to control, baclofen, CGP52432 and 2-OH-saclofen. ΔP≤0.002 compared to lipopolysaccharide, OP=0.041 compared to lipopolysaccharide (Mann–Whitney Rank Sum Test). Control=RPMI 1640 supplemented; LPS=lipopolysaccharide (0.5 ng/ml); LPS+bac100=lipopolysaccharide (0.5 ng/ml)+R-baclofen (100 μM); LPS+bac100+CGP 30 or 300=lipopolysaccharide (0.5 ng/ml)+R-baclofen (100 μM)+CGP52432 (30 or 300 μM); LPS+bac100+2OH 10 or 100=lipopolysaccharide (0.5 ng/ml)+R-baclofen (100 μM)+2-OH-saclofen (10 or 100 μM); bac100=R-baclofen (100 μM); CGP (30 or 300 μM)=CGP52432, (30 or 300 μM); 2OH (10 or 100 μM)=2-OH-saclofen (10 or 100 μM); IL-6=interleukin-6.

3.4. Effect of $GABA_B$ receptor antagonists on lipopolysaccharide and R-baclofen-mediated interleukin-6 release from pituicytes

The $GABA_B$ receptor antagonists CGP52432 (30 or 300 μM) and 2-OH-saclofen (10 or 100 μM) were tested for their ability to reduce the R-baclofen (100 μM) potentiation of the lipopolysaccharide-mediated (0.5 ng/ml) interleukin-6 (197%) response (Fig. 3). Neither of the antagonists had any effect alone nor in removing the effect of R-baclofen on the lipopolysaccharide-mediated response.

3.5. Effect of GABA on interleukin-6 release from pituicytes

The $GABA_A$ and $GABA_B$ receptor agonist GABA (30 or 300 μM) did not alter the lipopolysaccharide-mediated interleukin-6 release nor did GABA have any effect alone (Fig. 4).

3.6. Effect of lipopolysaccharide and R-baclofen on intracellular cyclic AMP concentration in pituicytes

In order to examine the effect of lipopolysaccharide and R-baclofen on intracellular cAMP, the concentration of intracellular cAMP in the pituicytes was determined after 30 min or 24 h on day 14. After 30 min of stimulation, the intracellular cAMP concentration was significantly higher in pituicytes stimulated with lipopolysaccharide (0.5 ng/ml) compared to control cells, P=0.017 (Fig. 5). Pituicytes stimulated with lipopolysaccharide (0.5 ng/ml) and R-baclofen (100 μM) had a significantly lower concentration of intracellular cAMP than cells stimulated with lipopolysaccharide alone, P=0.002 (Fig. 5). After 24 h of stimulation, neither lipopolysaccharide (0.5 ng/ml) and R-baclofen (100 μM) nor lipopolysaccharide (0.5 ng/ml) alone changed the intracellular concentration of cAMP in pituicytes as compared to control (results not shown).

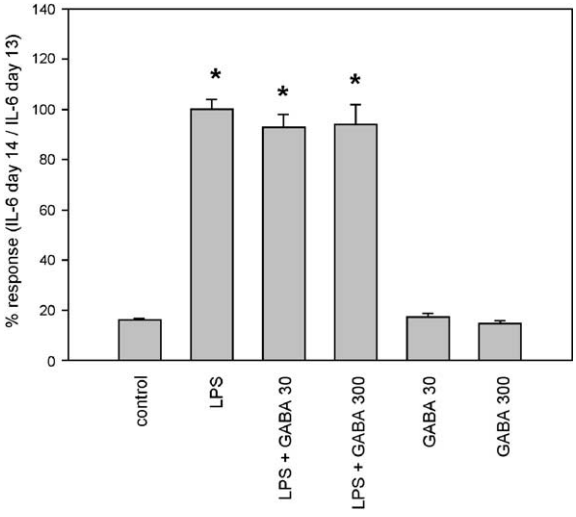

Fig. 4. Effect of GABA (30 or 300 μM) on the lipopolysaccharide-mediated (0.5 ng/ml) interleukin-6 release from cultured murine neurohypophysial cells. On day 13, the cells were incubated with RPMI 1640 supplemented for 24 h followed by incubation with test substances for 24 h. The results are expressed as the ratio of interleukin-6 release on day 14 to interleukin-6 release on day 13, and the lipopolysaccharide response (0.5 ng/ml) is standardised as a 100% response. Each value is the mean±S.E.M. of 7 to 13 observations. *$P \leq 0.001$ compared to control, and GABA (30 or 300 μM) (Mann–Whitney Rank Sum Test). Control=RPMI 1640 supplemented; LPS=lipopolysaccharide (0.5 ng/ml); LPS+GABA (30 or 300 μM)=lipopolysaccharide (0.5 ng/ml)+GABA (30 or 300 μM); GABA (30 or 300 μM)=GABA (30 or 300 μM); IL-6=interleukin-6.

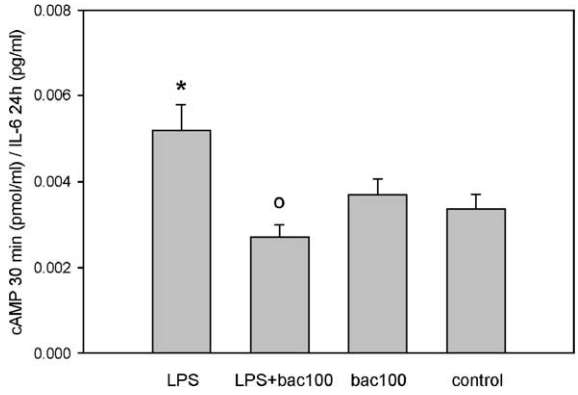

Fig. 5. Effect of lipopolysaccharide and R-baclofen (100 μM) on intracellular cAMP concentration in pituicytes after 30 min of stimulation. On day 13, the cells were incubated with RPMI 1640 supplemented for 24 h followed by incubation with test substances for 30 min on day 14. The results are expressed as the ratio of intracellular cAMP on day 14 to interleukin-6 release on day 13. Each value is the mean±S.E.M. of 8 to 12 observations. *P=0.017 compared to control and ○P=0.002 compared to lipopolysaccharide (Mann–Whitney Rank Sum Test). Control=RPMI 1640 supplemented; LPS=lipopolysaccharide (0.5 ng/ml); LPS+bac100=lipopolysaccharide (0.5 ng/ml)+R-baclofen (100 μM); bac100=R-baclofen (100 μM); IL-6=interleukin-6.

4. Discussion

Pituicytes are defined as modified astrocytic glial cells that stain positive for glial fibrillary acidic protein. Other nucleated cells in the neural lobe are microglia and endothelium cells, but these cells does not stain positive for glial fibrillary acidic protein. Since all cells in this study were immunoreactive for glial fibrillary acidic protein, we consider them to be pituicytes.

$GABA_A$ receptors are present on nerve endings secreting oxytocin and vasopressin (Zhang and Jackson, 1993), and $GABA_B$ receptors are suggested to be present on pituicytes (Boersma and van Leeuwen, 1994). Since interleukin-6 can directly stimulate (Yasin et al., 1994), and indirectly (through GABA) inhibit (Saridaki et al., 1989) secretion of oxytocin and vasopressin, we were interested in showing whether activation of $GABA_B$ receptors on pituicytes could modulate interleukin-6 secretion from pituicytes.

Our data support earlier findings that lipopolysaccharide induces the secretion of interleukin-6 from posterior pitui-

tary lobe cell cultures (Hansen et al., 1999) and neurointermediate pituitary lobe cell cultures (Spangelo et al., 1994). We believe this is the first study to show that the $GABA_B$ receptor specific agonist R-baclofen potentiates lipopolysaccharide-induced interleukin-6 secretion from pituicytes and that R-baclofen does not modulate interleukin-6 secretion by itself (Fig. 2). However, if the effect of R-baclofen is mediated through $GABA_B$ receptors, this effect should be blocked or inhibited by $GABA_B$ receptor antagonists. This is not the case in the present study. Neither the potent antagonist CGP52432 nor the well-known antagonist 2-OH-saclofen is able to inhibit the potentiation by R-baclofen on the lipopolysaccharide-mediated interlukin-6 release (Fig. 3). Furthermore, it is expected that the $GABA_A$ and $GABA_B$ receptor ligand GABA has an effect similar to R-baclofen. GABA and R-baclofen are usually equipotent at the $GABA_B$ receptor (Bowery, 1993; Barthel et al., 1996), but in contrast to R-baclofen GABA does not have an effect on the lipopolysaccharide-induced interleukin-6 release from pituicytes in the present study (Fig. 4). Consequently, it appears that the effect of R-baclofen on the lipopolysaccharide-induced interleukin-6 response is not mediated via $GABA_B$ receptors. However, this does not exclude that $GABA_B$ receptors may be present on pituicytes.

We have further investigated these unexpected results replacing the pituicytes with the macrophage cell line Mono Mac 6 (Ziegler-Heitbrock et al., 1988). In one series of experiments, we stimulated Mono Mac 6 cells with lipopolysaccharide, known to mediate its cytokine-inducing effect via the CD14-Toll-like receptor 4 (CD14-TLR4) receptor complex (Chow et al., 1999; Shimazu et al., 1999). In another series of experiments, Mono Mac 6 cells were stimulated with interleukin-1β known to induce interleukin-6 secretion via the interleukin-1 receptor. In Mono Mac 6 cells (unpublished results), R-baclofen alone had no effect on the interleukin-6 secretion. R-baclofen interfered with both lipopolysaccharide and interleukin-1β-induced interleukin-6 secretion, however this action was inhibitory. Neither CGP52432 nor 2-OH-saclofen could block the effect of R-baclofen.

The action of interleukin-1β is known to be independent of the CD14-Toll-like receptor 4 receptor complex. However, the cytoplasmic part of Toll-like receptor 4 share striking homology to the type 1 interleukin-1 receptor (IL-1R) and both activate the MyD88/IRAK signalling cascade leading to activation of NF-κB (Means et al., 2000). Therefore, it is very unlikely that the action of R-baclofen is at the CD14-Toll-like receptor 4 receptor complex. It is more likely that the action of R-baclofen is on the signalling cascade leading to NF-κB activation since lipopolysaccharide and interleukin-1β share this pathway. Furthermore, the $GABA_B$ receptor antagonists CGP52432 or 2-OH-saclofen had no effect on the action of R-baclofen in Mono Mac 6 cells, just like in pituicytes. Therefore, the effect of R-baclofen observed in this study is not mediated via $GABA_B$ receptors.

An explanation for the effect of R-baclofen may be the ability of this compound to alter the concentration of intracellular cAMP (Uezono et al., 1997; Knight and Bowery, 1996). Lipopolysaccharide increases cAMP (Siegert et al., 1976; Onodera, 1991) possibly via CD14-Toll-like receptor 4 receptor complex (Wright et al., 1990; Beutler and Poltorak, 2001). An increase in intracellular cAMP leads to interleukin-6 synthesis and later interleukin-6 release in astrocytes (Van Wagoner and Benveniste, 1999; Mattson and Camandola, 2001). Should R-baclofen somehow potentiate lipopolysaccharide stimulated increase in intracellular cAMP, this would explain an increase in interleukin-6 synthesis and finally, interleukin-6 secretion. Stimulation of β_2-adrenoreceptors has also been shown to induce secretion of interleukin-6 from murine pituicytes (Christensen et al., 1999). R-baclofen potentiates cAMP induced by β_2-adrenoreceptors in *Xenopus* oocytes and this potentiation occurs via $GABA_B$ receptors (Uezono et al., 1997). Upon stimulation of β_2-adrenoreceptors, activation of G_s G-protein occurs. This leads to activation of $G_{\alpha s}$ and hereby activation of adenylate cyclase type II (ACII), which increases the intracellular concentration of cAMP. $GABA_B$ receptors couple to G_i or G_0 G-proteins, and stimulation of the receptor leads to activation of $G_{\beta\gamma}$, which potentiates $G_{\alpha s}$ and thus activation of adenylate cyclase type II leading to further increased intracellular cAMP (Uezono et al., 1997; Knight and Bowery, 1996). Activation of $G_{\beta\gamma}$ alone does not affect adenylate cyclase type II and thus cAMP, since $G_{\alpha s}$ has to be activated simultaneously. Other compounds like noradrenaline and adrenaline increase cAMP and are known to have a synergistic action with interleukin-1β on interleukin-6 secretion in astrocytes and pituicytes, respectively (Norris and Benveniste, 1993; Christensen et al., 1999).

However, our experiments in pituicytes show that R-baclofen in combination with lipopolysaccharide decreases intracellular cAMP after 30 min (Fig. 5) and has no effect on intracellular cAMP after 24 h. This observation was unexpected and the role of cAMP for the observed effect of R-baclofen in the present study is not clear. A decrease in intracellular cAMP by R-baclofen is not totally unexpected since R-baclofen inhibits cAMP induced by forskolin (Knight and Bowery, 1996). Consequently, R-baclofen has the potential to either potentiate or inhibit cAMP depending on the primary inducer. Normally, a change in concentration of intracellular cAMP would be apparent after 30 min of stimulation, and the additional time period of 24 h in the above experiments was chosen because this is the normal timespan of the experiments. Nevertheless, 24 h is a long period of time and cAMP could be downregulated at the beginning (30 min), then overcompensated as the $GABA_B$ receptor desensitises, and at the end (24 h) return to normal levels.

In summary, our results demonstrate that R-baclofen potentiates lipopolysaccharide-induced interleukin-6 release from isolated murine pituicytes. This effect does not seem to be mediated via $GABA_B$ receptors as $GABA_B$ receptor

antagonists have no effect on the response and as GABA cannot mimic the effect of R-baclofen. Moreover, our results show that after 30 min of stimulation, R-baclofen does inhibit intracellular increases in cAMP induced by lipopolysaccharide.

The above strongly suggest that the effect of R-baclofen on the lipopolysaccharide-mediated interleukin-6 release is not mediated via $GABA_B$ receptors. Thus, the mechanism of action for R-baclofen in our system has to be further investigated.

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