The interaction between dopamine D₂-like and beta-adrenergic receptors in the prefrontal cortex is altered by mood-stabilizing agents

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Abstract
Several studies have suggested the involvement of biogenic monoaminergic neurotransmission in bipolar disorder and in the therapy for this disease. In this study, the effects of the mood-stabilizing drugs lithium, carbamazepine or valproate on the dopaminergic and adrenergic systems, particularly on D₂-like and β-adrenergic receptors, were studied both in cultured rat cortical neurons and in rat prefrontal cortex. In vitro and in vivo data showed that stimulation of β-adrenergic receptors with isoproterenol increased cyclic adenosine monophosphate (cAMP) levels and this effect was significantly inhibited by lithium, carbamazepine or valproate. The activation of dopamine D₂-like receptors with quinpirole decreased the isoproterenol-induced rise in cAMP in control conditions. This inhibition was observed in vivo after chronic treatment of the rats with carbamazepine or valproate, but not after treatment with lithium or in cultured rat cortical neurons after 48 h exposure to the three mood stabilizers. Dopamine D₂ and β₁-adrenergic receptors were found to be co-localized in prefrontal cortical cells, as determined by immunohistochemistry, but western blot experiments revealed that receptor levels were differentially affected by treatment with the three mood stabilizers. These data show that mood stabilizers affect D₂ receptor-mediated regulation of β-adrenergic signalling and that each drug acts by a unique mechanism.

Keywords: adenylate cyclase, β-adrenergic receptors, biogenic monoamines, bipolar disorder, cyclic adenosine monophosphate, dopamine D₂ receptors.


Bipolar disorder is a common psychiatric condition affecting 1% of the world population (Goodwin and Jamison 1990). The most widely prescribed drugs for bipolar disorder are commonly referred to as mood stabilizers, and include lithium, valproate and carbamazepine. Although lithium is the prototypical medication for the treatment of bipolar disorder, there is a substantial group of patients (20–40%) who do not respond to this treatment (Gershon and Soares 1997; Maj 2000). The anti-convulsants carbamazepine and valproate, originally used to treat epileptic seizures, are an alternative or adjunctive to lithium in bipolar disorder treatment (Bowden 1996; Dunn et al. 1998; Post et al. 1998). Despite the widespread clinical use of lithium, valproate and carbamazepine, the molecular mechanism(s) underlying the mood-stabilizing effects are poorly understood (Jope 1999; Manji and Lenox 2000).

Changes of noradrenergic and dopaminergic signalling have been implicated in the pathogenesis and pharmacotherapy of bipolar disorder and related diseases like depression and schizophrenia (Avissar et al. 1996; Sundram et al. 2003;
Wang and Goldman-Rakic 2004). Several lines of evidence indicate that bipolar disorder is associated with a dopaminergic dysregulation. Supporting this hypothesis, drugs that inhibit dopaminergic transmission exert an anti-manic action in bipolar disorder, whereas drugs that stimulate dopamine synthesis, activate dopamine receptors or inhibit dopamine re-uptake, often precipitate mania (Yatham 2002; Schatzberg 2004; Silverstone and Silverstone 2004). Interestingly, whereas conventional neuroleptics and novel anti-psychotics, which are also effective in treating acute mania (McElroy and Keck 2000), act by directly inhibiting dopamine D2-like receptors (Seeman and Lee 1975; Creese et al. 1976), the mood-stabilizing drugs lithium, carbamazepine and valproate do not directly block dopamine D2-like receptors and may therefore act by a different mechanism, such as by dampening the downstream second messenger signalling pathways activated by D2-like receptors (Yatham et al. 2002). On the other hand, good evidence suggests that noradrenaline levels are higher than normal in the body fluids of patients with mania, and drugs such as lithium can blunt noradrenaline signalling through the β-adrenergoreceptors. Moreover, noradrenergic drugs, such as tricyclic antidepressants, are particularly likely to promote manic switches and rapid cycling in bipolar patients (Young 2001). A β-adrenergic hyperfunction has been proposed to underlie manic episodes (Schreiber et al. 1991), whereas bipolar depressed patients showed a hypofunction of β-adrenergic receptors (Avissar et al. 1996).

Dopamine D2-like and β-adrenergic receptors belong to the superfamily of GTP-binding protein (G-protein)-coupled receptors that transduce extracellular stimuli to intracellular signalling events. Agonist activation of the Gαs-coupled β-adrenergic receptor stimulates cyclic adenosine monophosphate (cAMP) production, whereas acute activation of the Gαs/coupled D2-like dopamine receptors inhibits cAMP accumulation to provide antagonistic control on receptor-mediated adenylate cyclase (AC) activity at the cellular level (Watts 2002). Bipolar disorder is associated with an enhanced signalling activity of the cAMP cascade, as shown by higher levels of Gαs protein, stimulation of AC and increased activity of the cAMP-dependent protein kinases (PKA) (Young et al. 1993; Friedman and Wang 1996; Chang et al. 2003). Most of the components of the cAMP signalling pathways have been implicated in the actions of mood-stabilizing agents (Jope 1999; Manji et al. 1995; Mork and Geisler 1995; Montezinho et al. 2004).

The aim of this study was to identify the mechanisms by which the mood stabilizers lithium, valproate and carbamazepine affect the interaction of dopamine D2-like and β-adrenergic signalling, both in vitro and in vivo. The effects of the mood stabilizers on dopamine D2 and β1-adrenergic protein levels were studied by immunoblotting of both cultured cortical neurones and rat prefrontal cortex. The cAMP levels produced by the isoproterenol-sensitive AC, mimicking β-adrenergic activity, as well as the ability of D2-receptor stimulation to block the increase of isoproterenol-stimulated cAMP levels, were measured in vitro in cultured cortical neurones and in vivo by microdialysis in the rat prefrontal cortex after treatment with mood-stabilizing drugs.

Materials and methods

Materials

The cAMP radioimmunoassay kits, [8-3H] and [125I], the polyvinylidene difluoride (PVDF) membrane, the alkaline phosphatase-linked anti-mouse and anti-rabbit secondary antibodies, the enhanced chemiluminescence (ECL) reagent and the low-fat milk were obtained from Amersham Biosciences (Little Chalfont, UK). Other reagents used in immunoblotting experiments were purchased from Bio-Rad (Hercules, CA, USA). Neurobasal medium, B27 supplement and trypsin (USP grade) were purchased from Gibco Invitrogen (Glasgow, UK). The 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, 3-isobutyl-1-methylxanthine and the bicinchoninic acid reagent were obtained from Biomol (Plymouth Meeting, PA, USA), Aldrich-Chemie (Steinheim, Germany) and Pierce (Rockford, IL, USA.), respectively. Primary mouse monoclonal and rabbit polyclonal antibodies against the dopamine D2 receptors (sc-5303) and the β1-adrenergoreceptor (sc-568), respectively, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cy3-conjugated anti-rabbit and cy2-conjugated anti-mouse antibodies were from Jackson Immuno Laboratories (West Grove, PA, USA). Guide cannulas and microdialysis probes were from CMA/Microdialysis AB (Stockholm, Sweden). All other reagents were from Sigma Chemical Company (Madrid, Spain) or from Merck (Darmstadt, Germany).

Preparation of rat prefrontal cortical neuronal cultures

Primary rat cortical neurones were prepared as previously described (Montezinho et al. 2004). Cortical neurones were cultured in Neurobasal medium supplemented with 2 mM l-glutamine, 2% B27 supplement, penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were plated on poly d-lysine (0.1 mg/mL)-coated multwell plates, at a density of 0.15 × 10⁶ cells/cm², and the cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Experimental treatments on cultured cortical neurones

After 5 days in culture, cortical neurones were pre-exposed to therapeutic relevant doses of LiCl (1 mM), valproate (0.05 mM) or carbamazepine (0.5 mM), for 2 days. Non-exposed cultures served as a control. After this time period, neurones were pre-incubated with the cAMP phosphodiesterase inhibitor, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-201724) 25 µM, 15 min (Reeves et al. 1987) and then treated or not (basal cAMP levels) with isoproterenol (10 µM; 15 min), or isoproterenol (10 µM; 15 min) after pre-treatment with quinpirole (10 µM, 5 min). The medium was then removed and the cells were scraped off the plates in 1 mL 50 mM Tris/4 mM EDTA buffer, pH 7.35, after washing with an ice-cold phosphate-buffered saline (PBS) solution. The extracts were boiled at 90°C for 4 min, then centrifuged at 14 000 g for 5 min at 4°C. Supernatant fluids were used to quantify cAMP levels. Levels of cAMP were normalized to the protein concentration using the
bicinchoninic acid assay and expressed as a percentage relative to the control cAMP levels. For western blot analysis of receptor expression, cortical neurones exposed to different treatments for 48 h were washed twice with PBS and then lysed at 4°C with lysis buffer [in mM: 50 KCl, 50 Piperazone-1,4-bis(2-ethanesulfonic acid; PIPES), 10 EGTA, 2 MgCl₂, pH 7.4] supplemented with 0.2% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 1 mM diithreitol, 1 mg/mL chymostatin, 1 mg/mL leupeptin, 1 mg/mL antipain and 5 mg/mL pepstatin. The lysates were collected in microcentrifuge tubes, sonicated for 10 s, and centrifuged at 14,000 g for 10 min. Pellets were resuspended in 5% sodium dodecyl sulfate (SDS) and stored at –80°C for further use. Protein content was determined by the bicinchoninic acid assay, using bovine serum albumin (BSA) as standard.

Animals and drug treatments
Male Wistar rats, initially weighing 200–250 g, housed two per cage under a 12 h light/dark cycle were used (lights on at 06:00 hours). Food and water were available ad libitum. The lithium-treated group received 50 mM LiCl/kg diet for 3 weeks with free access to NaCl during the treatment period. The rats fed with the lithium-supplemented diet achieved a plasma lithium level (determined by flame photometry) of 0.84 ± 0.11 mM. The carbamazepine-treated rats were fed with 5 g carbamazepine/kg diet, whereas the intraperitoneal dosage of valproate was 250 mg/kg once daily for 3 weeks. The doses of carbamazepine and valproate used were previously shown to produce the therapeutic drug levels found in the plasma (Mørk and Jensen 2000). Control rats received standard diet, and vehicle-treated rats received intraperitoneal injections with sterile-filtered saline solution (NaCl 0.9%) once daily for 3 weeks. Ethical permissions for the studies were granted by the animal welfare committee appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with the EC directive 86/609/EEC and with the Danish law regulating experiments on animals.

Surgery for microdialysis
Two days before completing the treatment, rats were anaesthetized with 2 mL/kg Hypnorm/Dormicium (fentanyl citrate, 0.079 mg/mL; fluanisone, 2.5 mg/mL; midazolam, 1.25 mg/mL) and intracerebral guide cannulas (CMA/12) were stereotaxically implanted into the brain, positioning the dialysis probe tip in the prefrontal cortex (coordinates: 3.2 mm anterior to bregma; lateral, –0.8 mm; 3.3 mm ventral to dura mater) (Paxinos and Watson 1986). Anchor screws and acrylic cement were used for fixation of the guide cannulas. The body temperature of the animals was monitored by a rectal probe and maintained at 37°C. The rats were allowed to recover from surgery for 2 days, housed individually in cages.

Microdialysis procedure
On the day of the experiment, a microdialysis probe (CMA/12, 0.5 mm diameter, 2 mm length) was inserted through the guide cannula. The probes were connected via dual channel swivel to a microinjection pump. Perfusion of the microdialysis probe with filtered Ringer’s solution [in mM: 145 NaCl, 3 KCl, 1 MgCl₂, 6H₂O, 1.2 CaCl₂, 2H₂O, 0.25 3-isobutyl-1-methylxanthine (IBMX), pH 7.4] was initiated shortly before insertion of the probe into the brain and continued for the duration of the experiment, at a constant flow of 1.36 μL/min. After 180 min of stabilization, the experiments were initiated. A 30 min sampling regime was used throughout the experimental period. Time points were corrected for lag time of the perfusate from the microdialysis site to the probe outlet. Basal cAMP levels were taken as the average cAMP concentration in four consecutive samples. Thereafter, the agent of interest (100 μM quinpirole, or 2.5 mM isoproterenol, or 100 μM quinpirole simultaneously with 2.5 mM isoproterenol) dissolved in Ringer’s solution was infused through the probe for 30 min and seven further samples were collected. The dialysates were stored at –80°C until cAMP determination.

Tissue preparation for immunoblotting
The rats were killed by decapitation and the brains rapidly removed. The cerebral prefrontal cortices were dissected at 4°C and homogenized in 5 volumes of 10 mM Tris (pH 7.2), 1:100 protease inhibitor cocktail, 0.1 mM phenylmethylsulfonyl fluoride and 1% orthovanadate. Nonidet P-40 (1%) was added for 30 min and then homogenates were centrifuged at 18,000 g, 4°C, for 20 min. Supernatant fluids (cytoplasmic fraction) were collected and stored at –80°C until further use. SDS (1%) was added to the pellet for 30 min and this was further centrifuged at 18,000 g, 4°C, for 20 min. Supernatant fluids (membrane fraction) were collected and stored at –80°C for further use. Protein content was determined by the bicinchoninic acid assay with BSA as standard.

Quantification of cAMP levels
The content of cAMP in cortical lysates and microdialysis dialysates was determined in duplicate using the [8-3H] and [125I] radio-immunoassay kits, respectively, following the manufacturer’s instructions. In the microdialysis dialysates, the high-sensitivity method following acetylation of the samples was used. The lower limit of detection for cAMP was 2 fmol per tube and basal levels were at least four times the detection limit.

Immunohistochemistry
Male Wistar rats were anaesthetized with 680 mg/kg avertin intraperitoneally and perfused transcardially with PBS followed by phosphate-buffered 4% paraformaldehyde, for 10 min. The brains were removed, post-fixed overnight in the same fixative and then embedded in paraffin. Prefrontal cortex sections of 4 μm were picked up on gelatin-coated glass slides. Following xylene deparaffinization and alcohol rehydration, the slides were heated in a microwave oven for antigen retrieval (Cattoretti et al. 1993; Shi et al. 1995) at 700 W for 10 min (2 x 5 min) in 0.01 citrate buffer, pH 6.0. Sections were blocked for 20 min with 5% normal swine serum in PBS supplemented with 1% BSA and 0.3% Triton X-100, and then incubated overnight at 4°C with the specific primary antibodies mouse anti-dopamine D₂ receptor and rabbit anti-β₁-adrenoreceptor diluted 1:100 and 1:500, respectively. These antibodies were previously used in immunohistochemistry (anti-dopamine D₂; Zou et al. 2005) and immunoblotting (anti-β₁; Dincer et al. 2001). Sections were thoroughly washed in PBS with 0.25% BSA and 0.1% Triton X-100. Then, secondary Cy3-conjugated anti-rabbit and Cy2-conjugated antimouse antibodies were applied to sections after dilution of 1:250 and 1:100 in PBS, respectively, and incubated for
60 min at 22–25°C. Slides were washed with PBS and then coveredslipped with Dako (Carpinteria, CA, USA) fluorescent mounting medium. As negative controls, adjacent sections were incubated without primary antibody. Brain sections were analysed using the LSM 510 Meta confocal system (Carl Zeiss AG, Göttingen, Germany).

Western blot
For western blot analysis, 60 μg of each protein sample were used, after addition of a sixfold concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) and boiling for 5 min at 95°C. Proteins were separated by electrophoresis on 7.5% acrylamide/bisacrylamide gels and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes which were subsequently blocked for 1 h at 22–25°C in Tris-buffered saline (in mM: 137 NaCl and 20 Tris-HCl, pH 7.5) containing 0.1% Tween 20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies (mouse antidopamine D2 receptor, diluted 1 : 100, or rabbit anti-β1-adrenergic receptor, diluted 1 : 1000, in TBS-T with 1% low-fat milk) was performed overnight at 4°C. After extensive washing in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature (22–25°C) with alkaline phosphatase- or horseradish peroxidase (HRP)-linked secondary antibodies (anti-mouse IgG diluted 1 : 5000 and anti-rabbit IgG diluted 1 : 10 000 in TBS-T with 1% low-fat milk). Protein immunoreactivity was visualized by chemiluminescence on a Fuji Imager 4400 chemiluminescence detection unit (Fujifilm Danmark A/S, Vedbaek, Denmark) or by chemiluminescence on a Storm 860 Gel and Blot Imaging System (Amersham Biosciences).

Statistical analysis
The levels of cAMP in the dialysates were calculated as fmol/20 μL dialysate. The average of the four basal values prior to isoproterenol and quinpirole plus isoproterenol infusions was taken as baseline and set to 100%. The changes in cAMP levels occurred mostly during the first two samples collected after the beginning of isoproterenol and quinpirole plus isoproterenol infusions. Therefore, the maximal release of cAMP, measured after 30 min post-infusion with different treatments, was determined and differences were analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. However, in some cases the extracellular accumulation of cAMP was slower, the total amount of cAMP produced during 150 min post-infusion (area under the curves) was also calculated, and the results were expressed as a percentage of the total amount of cAMP produced during 150 min post-infusion with isoproterenol in control or vehicle-treated rats. Differences were analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. The density of the bands in the western blots was quantified using QUANTITY ONE 1D-ANALYSIS Software, then data were expressed as percentage of control or vehicle, and analysed using one-way ANOVA, followed by the Bonferroni test for multiple comparisons, as indicated in the figure captions. The data obtained were expressed as means ± SEM values and p < 0.05 was considered significant.

Results

Determination of intracellular cAMP levels in cerebrocortical neurones
Figure 1 shows the cAMP accumulation in cultured cortical cells pre-exposed or not (control) to lithium (1 mM), carbamazepine (0.05 mM) or valproate (0.5 mM), during

![Graph showing cAMP levels in different conditions](image)

Fig. 1 Effects of lithium, valproate and carbamazepine on the intracellular cAMP levels in cortical neurones under basal conditions and after stimulation with isoproterenol or quinpirole plus isoproterenol. Neurones were pre-exposed or not (control) to 1 mM lithium, 0.05 mM carbamazepine or 0.5 mM valproate, for 48 h. After this period, neurones were incubated with Ro-201724 (25 μM) and were then treated or not (basal) with isoproterenol (10 μM; 15 min), or with isoproterenol together with quinpirole (10 μM; 15 min). In the latter experimental conditions, the cells were pre-treated with 10 μM quinpirole for 5 min. The cAMP levels were measured as described in the Methods section. The average value of basal cAMP level for all cells tested (101.4 ± 21.0 nmol/μg protein) was set to 100%. Data are means ± SEM, for the indicated number of independent experiments, performed in duplicate. Data were analysed by one-way ANOVA, followed by posthoc Bonferroni’s test for multiple comparisons. ##p < 0.001, #p < 0.05, compared with control isoproterenol-treated cells; *p < 0.05, **p < 0.01, compared with control quinpirole plus isoproterenol-stimulated cells; ***p < 0.001, compared with control of untreated cells; *p < 0.05, **p < 0.01, compared with neurones pre-exposed to LiCl and valproate, respectively.
48 h. After pre-treatment with the mood-stabilizing agents, cells were exposed to isoproterenol (10 μM), for 15 min, in the absence or presence of quinpirole (10 μM), and then intracellular cAMP levels were determined. The average value of basal cAMP level for all cells tested (101.4 ± 21.0 nmol/μg protein) was set to 100%. Isoproterenol-stimulated cAMP production was twofold above basal levels (264.6 ± 14.3%, p < 0.001). Lithium, carbamazepine or valproate had no effect on the basal cAMP production, but partially inhibited isoproterenol-induced cAMP accumulation (lithium: 160.7 ± 25.6%, p < 0.001; carbamazepine: 143.9 ± 13.1%, p < 0.001; valproate: 195.4 ± 21.8%, p < 0.05). The agonist of dopamine D2-like receptors, quinpirole, had no effect on the basal cAMP accumulation (data not shown) but inhibited isoproterenol-enhanced cAMP levels in untreated neurones (158.4 ± 16.8%, p < 0.001). In neurones pre-exposed to lithium, carbamazepine or valproate, the ability of quinpirole to reduce the isoproterenol-evoked rise in cAMP was decreased (lithium: 249.1 ± 28.4%, p < 0.05; valproate: 259.0 ± 18.4%, p < 0.01; carbamazepine: 243.8 ± 21.5%, p < 0.05).

Regulation of cAMP levels in prefrontal cortex
In order to confirm the data obtained with cultured cortical neurones, parallel in vivo experiments were performed on prefrontal cortex of freely-moving rats using microdialysis. Extracellular concentration of cAMP can be monitored in the prefrontal cortex of freely-moving animals by microdialysis (Masana et al., 1991, 1992). It has been demonstrated that a fraction of intracellular cAMP generated by activation of AC is extruded into the extracellular fluid in proportion to its accumulation in cells. Therefore, the efflux of cAMP can be used to study the cAMP second messenger system in intact brains, using in vivo microdialysis (Mørk and Geisler 1994).

Preliminary experiments showed that extracellular levels of cAMP declined to stable values within 180 min (data not shown). Therefore, the experiments were started 180 min after the insertion of the probes and the baseline values were stable throughout the experiment (Fig. 2). The average basal cAMP levels for all animals tested was 9.68 ± 2.10 fmol/20 μL. Average basal levels were not different in valproate-treated rats but were significantly increased in lithium-treated rats, to 13.17 ± 1.21 fmol/20 μL (p < 0.05), and decreased in carbamazepine-treated rats, to 7.82 ± 0.34 fmol/20 μL (p < 0.01) (Fig. 2).

The effect was then determined of local infusion of isoproterenol, or quinpirole together with isoproterenol, on the extracellular cAMP levels in control or vehicle-treated rats, and in lithium-, carbamazepine- or valproate-treated rats. In a preliminary series of experiments, it was confirmed that infusion of isoproterenol in the rat prefrontal cortex (500 μM, 1 mM, 2.5 mM, 5 mM and 10 mM) increased cAMP production in a concentration-dependent manner, and this effect was inhibited by quinpirole (50 μM, 100 μM, 500 μM, 1 mM and 2.5 mM) (data not shown). Accordingly, the concentrations of isoproterenol and quinpirole used in all subsequent experiments were 2.5 mM and 100 μM, respectively, which were found to be the minimal concentrations that produced an observable effect under our experimental conditions (data not shown). These concentrations of isoproterenol and quinpirole cannot be directly compared with those used in in vitro experiments and with extracellular levels obtained in the brain (5-15% of the initial concentrations), since the extracellular concentration achieved in brain tissue under the present conditions depends on diffusion across the dialysis membrane (Mørk and Geisler 1994).

The infusion of isoproterenol in the prefrontal cortex of control and vehicle-treated rats for 30 min increased cAMP levels to approximately 234.6 ± 12.2% (Fig. 3a)
Effects of mood stabilizers on $D_2$ and $\beta$ receptors

and 272.1 ± 12.0% (Fig. 3d) of the basal values, and the extracellular amount of cAMP returned to basal values by 150 min post-infusion. In lithium-, carbamazepine- or valproate-treated rats there was a significant decrease in the isoproterenol-stimulated cAMP levels determined after 30 min of infusion (lithium: 131.9 ± 5.8%, $p < 0.01$; carbamazepine: 159.0 ± 8.5%, $p < 0.01$; valproate: 137.9 ± 8.7%, $p < 0.01$) (Figs 3b, c and e), as well as a decrease in the total evoked increase in extracellular cAMP (lithium: 85.1 ± 3.7%, $p < 0.01$; carbamazepine: 87.5 ± 1.6%, $p < 0.05$; valproate: 69.5 ± 2.4%, $p < 0.001$), when compared with the effect observed in control or vehicle-treated rats (Fig. 3f). Local infusion of the $D_2$-like receptor agonist, quinpirole, significantly inhibited the effect of isoproterenol on the extracellular cAMP, measured 30 min after the infusion of the agonist (control rats: 132.5 ± 5.8%, $p < 0.01$; vehicle-treated rats: 141.0 ± 9.1, $p < 0.01$) (Figs 3a and d). The total increase in extracellular cAMP evoked by isoproterenol within 150 min was also significantly decreased in the presence of quinpirole (control rats: 77.0 ± 1.6%, $p < 0.001$; vehicle-treated rats: 74.9 ± 2.7, $p < 0.001$) (Fig. 3f). As observed in cultured cortical neurones, in lithium-treated rats the activation of $D_2$-like receptors did not inhibit the isoproterenol-induced increase in extracellular cAMP. Instead, there was an increase in extracellular cAMP measured 30 min after the infusion (188.2 ± 19.1%, $p < 0.01$) (Fig. 3b) when compared with the amount of the cyclic nucleotide determined in control rats under the same conditions (132.5 ± 5.8%) (Fig. 3a) or in animals treated with lithium and infused only with isoproterenol (131.9 ± 5.8%) (Fig. 3b). Treatment with lithium also increased the total extracellular accumulation of cAMP induced by quinpirole and isoproterenol, and produced within 150 min (77.0 ± 1.6 vs. 94.7 ± 4.4%, $p < 0.01$) (Fig. 3f). In contrast to what was observed with cultured cortical neurones and lithium-treated rats, in carbamazepine- or valproate-treated rats, quinpirole infusion did not significantly change the isoproterenol-induced increase in extracellular cAMP produced 30 min post-infusion (Figs 3c and e) (176.9 ± 8.3% and 158.0 ± 11.7%, respectively), when compared with the effect observed in control and vehicle-treated rats under the same conditions (132.5 ± 5.8% and 137.9 ± 8.7%, respectively) (Figs 3a and d), or in animals treated with carbamazepine or valproate and infused only with isoproterenol (159.0 ± 8.5% and 137.9 ± 8.7%, respectively) (Figs 3c and e). Treatment with carbamazepine or valproate did not significantly change the total evoked extracellular accumulation of cAMP measured during 150 min following infusion with quinpirole and isoproterenol (84.7 ± 3.5% and 85.9 ± 2.2%, respectively), when compared with the values obtained in control and vehicle-treated rats, 77.0 ± 1.6% and 74.9 ± 2.7%, respectively (Fig. 3f). In carbamazepine-treated rats, the activation of $D_2$-like receptors did not significantly interfere with the total isoproterenol-induced increases in extracellular cAMP, although the effect occurred at a slower rate.

The activation of $D_2$-like receptors had no effect on basal cAMP levels under control conditions (control rats: 100.4 ± 18.4%; vehicle-treated rats: 104.0 ± 11.3%). However, in lithium-treated rats, the activation of dopamine $D_2$-like receptors, with quinpirole, significantly increased cAMP levels to 155.1 ± 9.3%, $p < 0.05$. In contrast, in carbamazepine- and valproate-treated rats, the infusion of quinpirole did not significantly change basal cAMP levels (114.4 ± 8.9% and 98.7 ± 13.6%, respectively, vs. 100.4 ± 18.4% and 104.0 ± 11.3%) (Fig. 4).

Immunohistochemistry

The dopamine $D_2$ receptor is the predominant member of the family of $D_2$-like dopamine receptors in the brain (Emilien et al. 1999) and the $\beta_1$-adrenergic receptors are the most abundant members of $\beta$-adrenergic receptors in the cerebral cortex (Rainbow et al. 1984). To confirm that dopamine $D_2$ and $\beta_1$-adrenergic receptors are co-localized in cells of the prefrontal cortex, allowing the interactions at the second messenger level, immunohistochemistry studies were performed. Double-labelling experiments confirmed the co-existence of dopamine $D_2$ and $\beta_1$-adrenergic receptors in the majority of the cells (Fig. 5). The prefrontal cortex of sections prepared after treating rats for 3 weeks with lithium, carbamazepine or valproate suggested a loss of dopamine $D_2$ receptors. In contrast, in valproate-, carbamazepine- and lithium-treated rats the $\beta_1$-adrenergic receptors seemed to be down-regulated, up-regulated or not changed, respectively (Fig. 5). Immunohistochemical controls were prepared by omitting addition of the primary antibody in some slices, and it was observed that no significant amount of secondary binding occurred.

Immunoblotting

In order to confirm the immunostaining data, the dopamine $D_2$ and $\beta_1$-adrenergic receptor protein levels were determined in membranes prepared from cultured rat cortical neurones and from rat prefrontal cortex, pre-treated or not with the mood-stabilizing agents, by western blot analysis, using the same anti-$D_2$ and anti-$\beta_1$ specific antibodies used for immunohistochemistry. A strong immunoreactivity was observed at approximately 50 kDa and 64 kDa, which are the predicted molecular weights for the dopamine $D_2$ and $\beta_1$-adrenergic receptor proteins, respectively (Fig. 6a). The two bands obtained with the dopamine $D_2$ antibody could be due to the two isoforms of the receptor, $D_2$ long ($D_{2L}$) and short ($D_{2S}$), which are encoded by splice variants of a single gene and detected by this antibody. Densitometry analysis of the bands at approximately 50 kDa showed that lithium, valproate and carbamazepine treatments decreased the immunoreactivity of the dopamine $D_2$ receptor protein band,
by 87.6 ± 3.0% (p < 0.01), 90.5 ± 2.7% (p < 0.01) and 86.5 ± 3.1% (p < 0.01), respectively, in membranes from cultured rat cortical neurones, when compared with the control (103.9 ± 1.7%) (Fig. 6b). Similar results were obtained in the prefrontal cortex tissue, where lithium, valproate and carbamazepine reduced D₂ protein levels by 69.6 ± 3.9% (p < 0.01), 97.9 ± 6.2% (p < 0.01) and 76.5 ± 5.0% (p < 0.05), respectively, when compared with control (97.9 ± 4.7%) and vehicle-treated rats (101.9 ± 4.3%) (Fig. 6c). In cultured cerebrocortical neurones, only
Effect of the local infusion of isoproterenol (2.5 mM) or quinpirole had an effect on extracellular cAMP levels in the prefrontal cortex of control, vehicle-, lithium-, carbamazepine- or valproate-treated rats. Baseline levels of cAMP were taken as the average cAMP content in the four consecutive samples collected from 3 h after the insertion of the probe. Thereafter, 100 μM of quinpirole, dissolved in Ringer’s solution, were infused through the probe for 30 min, and six samples were collected post-infusion. Extracellular cAMP levels in the dialysates were measured by radioimmunoassay analysis and expressed as percentages of the baseline. Results are means ± SEM, from four independent experiments. The rats were treated or not with doses yielding therapeutic plasma levels of lithium, carbamazepine or valproate, for 3 weeks, as indicated in the legend of Fig. 2. Data were analysed by one-way ANOVA, followed by posthoc Bonferroni’s test for multiple comparisons. *p < 0.05 compared with the cAMP produced 30 min after the infusion of quinpirole in control rats. Valproate had an effect on β1-adrenergic receptors by decreasing the amount of the receptor present (101.9 ± 4.3%, p < 0.05), compared with control (105.6 ± 6.7%) (Fig. 6b, graph). In contrast, in vivo treatment of rats with carbamazepine, valproate or lithium up-regulated (129.6 ± 7.0%, p < 0.01), down-regulated (70.8 ± 4.4%, p < 0.01) or had no effect (100.4 ± 3.6%) on β1-adrenergic receptor levels, respectively, when compared with control (101.3 ± 1.6%) and vehicle-treated (100.9 ± 4.6%) rats (Fig. 6c).

Discussion

The effects of mood-stabilizing drugs on the dopaminergic and adrenergic systems were studied via analysis of the intracellular cAMP accumulation induced by activation of β-adrenergic and/or dopamine D2-like receptors. The change in the levels of dopamine D2 and β1-adrenergic receptors under the same experimental conditions was also investigated. In the present study, we showed that there is a crosstalk between dopamine D2-like and β-adrenergic receptor activities in the rat brain cortical region, which is differentially affected by therapeutic concentrations of the mood-stabilizing drugs lithium, carbamazepine and valproate. Indeed, in vivo and in vitro data showed that activation of dopamine D2-like receptors inhibits β-adrenergic receptor-stimulated cAMP production. In vitro this inhibition was attenuated by lithium, carbamazepine and valproate, whereas in vivo only lithium had such an effect. Consistent with this regulatory role on AC activity, dopamine D2 and β1-adrenergic receptors are co-localized in the rat prefrontal cortex and their protein levels are changed by mood stabilizers, as determined by immunohistochemistry and immunoblotting, respectively.

In vivo chronic treatment with lithium increased the intracellular cAMP content in the rat prefrontal cortex by 40%. This type of effect of lithium may be attributed to the inhibition of Gi, which is the G-protein preferentially activated under basal conditions (Masana et al. 1991, 1992; Jope 1999; Montezinho et al. 2004). However, our results showing a functional cross-talk between β-adrenergic and
control

Lithium

Carbamazepine

Valproate

$\beta_1$AR  DAD$_2$R  merge
dopamine D$_2$-like receptors, and a selective down-regulation of the dopamine D$_2$ receptors by chronic treatment with lithium, indicate that the loss of these receptors, which are coupled to the inhibition of AC, may also account for the increase in the basal cAMP levels. The decrease in dopamine D$_2$ receptor levels following lithium treatment might also have contributed to the lack of effect of D$_2$ receptor stimulation on isoproterenol-evoked intracellular cAMP accumulation in the in vitro and in vivo studies. Recent evidence has implicated cortical D$_2$ receptors as the important sites of action of anti-psychotics. Psychotic and manic symptoms may result from the supersensitivity of a subset of cortical pyramidal neurones to stimulation of D$_2$ receptors (Yatham et al. 2002; Schatzberg 2004; Silverstone and Silverstone 2004). Therefore, part of the anti-manic action of chronic lithium treatment could be mediated by a decrease in D$_2$ receptor levels shown in this study. In contrast to lithium, in vivo chronic treatment with carbamazepine inhibited basal cAMP levels by 20%. This is in agreement with previous findings showing that carbamazepine decreases the basal concentrations of cAMP in mouse cerebral cortex and cerebellum (Palmer et al. 1979), as well as in rabbit CSF (Myllyla 1976). In contrast with the results obtained with lithium, the in vivo effects of carbamazepine on the intracellular cAMP content under resting conditions do not correlate with the down-regulation of dopamine D$_2$ receptors and the up-regulation of $\beta$-adrenergic receptors under the same conditions. These changes in AC-coupled receptor would be expected to increase the intracellular cAMP content. This discrepancy may be explained by the fact that the action of carbamazepine, at therapeutically relevant concentrations, is independent of the phosphodiester-
erase activity and is mediated by a G_{i}-independent mechanism (Chen et al. 1996a). Thus, the inhibitory effect of carbamazepine on cAMP levels may be exerted primarily at the level of cAMP production, acting directly on AC and/or through factor(s) that are tightly associated with this enzyme (Chen et al. 1996a). A direct inhibitory action of carbamazepine on AC could also explain our finding that in vivo chronic carbamazepine treatment attenuated the isoproterenol-evoked cAMP production, despite an increase in the β₁-receptor levels and the lack of a further effect of D₂ receptor activation. The changes in receptor levels could represent a compensatory mechanism in the in vivo conditions, although this was not observed in cultured cortical neurones. Recently, it was proposed that the β-adrenoreceptors mediating cAMP efflux after norepinephrine stimulation are localized predominantly on glial cells in the rat brain cortex (Stone et al. 1990; Stone and John 1991). This is not the case in the neuronal cultures used in the present work, since they contain a very small percentage of astrocytes (Brewer et al. 1993). Identification of the cells that contribute to the cAMP responses observed both in vivo and in vitro, and the regulating mechanisms involved, remain to be clarified. In addition, Khan et al. found that approximately one-third of the total D₂ receptor binding sites in the cortex are associated with astroglia (Khan et al. 2001). In vivo chronic administration of valproate significantly reduced both the β₁-adrenoreceptor and dopamine D₂ receptor immunoreactivity in the rat prefrontal cortex. The down-regulation of both receptor types did not affect the basal levels of cAMP, but the decrease in the density of β-adrenoreceptors is likely to be reflected in the decrease in isoproterenol-evoked, receptor-mediated cAMP production. Furthermore, dopamine D₂ receptor stimulation did not further attenuate cAMP production, which could be related to the lower levels of receptor protein after chronic treatment with valproate. Supporting our findings, previous studies showed that valproate selectively down-regulates β₁-adrenergic receptors in vitro, in rat C6 glioma cells (Chen et al. 1996b).

Each of the mood-stabilizing drugs modulated dopamine D₂ and β-adrenergic receptor-regulated cAMP levels by a distinct mechanism in vivo. The effects of carbamazepine are most likely due to direct inhibition of AC; lithium may act by affecting dopamine D₂ receptor-mediated signalling and valproate, by down-regulating β-adrenergic transmission. Interestingly, the results obtained in vitro with cultured cerebrocortical neurones did not fully correlate with the in vivo findings. In cultured cortical neurones, lithium, valproate and carbamazepine did not significantly affect basal cAMP levels. The increase in β-adrenergic receptor-mediated production of cAMP was attenuated by all three drugs both in vitro and in vivo. This attenuation was reversed by dopamine D₂-like receptor stimulation in controls and after chronic treatment of the rats with carbamazepine or valproate, but not after treatment with lithium or in cultured cortical neurones after pre-exposure to the three mood-stabilizing drugs. Therefore, we propose that in in vitro conditions, the three mood-stabilizing drugs used interfere with the ability of quinpirole to blunt isoproterenol effects, which may result from the action of these drugs downstream of second messenger signalling pathways activated by D₂-like receptors (Yatham et al. 2002). The overall results obtained in the present work suggest that additional mechanisms are operative in vivo compared with in vitro. It can be speculated that dopaminergic and noradrenergic neurotransmitter levels released by afferent projections participate in the regulation of these monoaminergic systems. In agreement with this hypothesis, a decrease in the dopamine concentration in prefrontal cortex (Baptista et al. 1993) and an increase in striatum (Dziedzicka-Wasylewska et al. 1996) were reported after the in vivo intragastrical administration of lithium. In contrast, it has been reported that therapeutic concentrations of carbamazepine or valproate enhanced basal releases of dopamine in the prefrontal cortex (Ichikawa and Meltzer 1999). In addition, carbamazepine and valproate are metabolized in vivo, which might contribute to the differences observed in the effects of these drugs in vitro and in vivo. Moreover, the presence of glia cells expressing β-adrenergic (Stone et al. 1990; Stone and John 1991) and D₂ receptors (Khan et al. 2001) in rat brain cortex increases the complexity of the regulation of the cAMP levels in vivo when compared with the in vitro data. Lastly, the other D₃-like receptor subtypes, namely the D₁ and D₃ receptors, and the β₁β₂- and β₁β₃-adrenergic receptors, may also play a role in the regulation of cAMP levels, and the expression pattern of these receptors in vitro may be different from that observed in vivo. Although the in vivo system is rather complex, providing results that may be affected by multiple cell–cell interactions, it reflects the effects of the drugs in the brain, which is also a complex system. Comparison with data from cell culture experiments is useful, to some extent, for distinguishing the effect of drugs within the cell from those resulting from multiple cell–cell interactions.

In this study we provide evidence that the three mood stabilizers act by different mechanisms on the dopamine D₂-like and β-adrenergic receptor-mediated regulation of cAMP levels both in vitro and in vivo. Furthermore, we show that the effects of the mood stabilizers are different in vivo and in vitro, highlighting the importance of the in vivo studies. The different effects of the three mood-stabilizing drugs in the in vivo studies suggest that they have distinct mechanisms of action. This can be related to differences in the efficacy of the treatment of manic episodes in clinical cases, where lithium, but not carbamazepine or valproate, has been shown to be effective as an acute treatment (Silverstone and Silverstone 2004). In conclusion, we have shown that lithium, carbamazepine and valproate affect differentially the basal and evoked cAMP levels produced by the balance between β-adrenergic and dopamine D₂ receptors, both
in vitro and in vivo. However, the results did not lead to a unifying hypothesis for the mechanism of action of these drugs in bipolar disorder. A common effect on the interaction between β-adrenergic and dopamine D2 receptors does not seem to explain the therapeutic efficacy of these drugs, suggesting that additional signalling systems are involved as well.

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