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Induction of Bcl-2 by functional regulation of G-protein coupled receptors protects from oxidative glutamate toxicity by increasing glutathione

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Abstract

Glutamate treatment depletes hippocampal HT22 cells of glutathione, which renders the cells incapable to reduce reactive oxygen species and ultimately cumulates in cell death by oxidative stress. HT22 cells resistant to glutamate displayed increased phosphorylation of cAMP-response-element binding (CREB) and decreased ERK1/2 suggestive of differences in signal transmission. We investigated the amount of candidate G-protein-coupled receptors involved in this resistance and found an increase in mRNA for receptors activated by the vasoactive intestinal peptide VIP ($VPAC_2$, 12.6-fold) and glutamate like the metabotropic glutamate receptor $mGlu_1$ (5.3-fold). Treating cells with VIP and glutamate led to the same changes in protein phosphorylation observed in resistant cells and induced the proto-oncogene Bcl-2. Bcl-2 overexpression protected by increasing the amount of intracellular glutathione and Bcl-2 knockdown by small interfering RNAs (siRNA) increased glutamate susceptibility of resistant cells. Other receptors upregulated in this paradigm might represent useful targets in the treatment of neurological diseases associated with oxidative stress.

Keywords: *HT22 cells, oxidative glutamate toxicity, VIP, mGluR, Bcl-2*

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of cell-surface transmembrane proteins [1]. They are activated by a wide variety of natural ligands and their pharmacological stimulation can protect from neuronal cell death in diverse models of neurological disease like ischemia-induced hippocampal neurodegeneration after global cerebral ischemia [2,3] or 6-hydroxydopamine induced cell death of dopaminergic neurons in mesencephalic primary cultures [4]. We recently demonstrated that the activation of G_s- and G_q-coupled signaling pathways protects primary neurons and the immortalized hippocampal cell line HT22 from oxidative stress [5]. Given the fact that GPCRs constitute the most targeted protein superfamily in pharmaceutical research [6], a screening system able to discriminate protective and detrimental receptors involved in oxidative stress would be helpful in the identification of drug targets especially for neurodegenerative disease like Alzheimer's and Parkinson's disease, where oxidative stress plays a prominent role [7–10].

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HT22 cells are an ideal model system to study oxidative stress. In this paradigm named oxidative glutamate toxicity or oxytosis, increased extracellular glutamate blocks the gradient-driven glutamate/cystine antiporter X^{c-}, which depletes the cells of cystine. Cystine is required for the synthesis of the important antioxidant glutathione (GSH). The sequence of events that leads to glutamate-induced cell death of HT22 cells after depletion of intracellular glutathione [11] involves the activation of 12-lipoxygenase [12], the accumulation of intracellular peroxides [13], and the activation of a cyclic GMP-dependent calcium channel close to the end of the death cascade [14]. The effect of GPCR signaling on cell death by oxidative stress has been studied previously in HT22 cells; stimulation of the dopamine D4 receptor protects from oxidative glutamate toxicity via the regulation of cGMP-operated calcium channels [15] and of metabotropic glutamate receptors by modifying glutathione metabolism [16]. Sagara and Schubert also provided evidence for a protective upregulation of group II metabotropic glutamate receptors in HT22 cells selected for glutamate resistance [17].

In this study, we investigated the mRNA amounts of candidate protective G-protein coupled receptors in glutamate-resistant and sensitive cells by quantitative PCR. The two most prominently upregulated receptors both induced the expression of Bcl-2, which, when overexpressed, protected from glutamate toxicity by increasing cellular glutathione content. We think that this combination of cell selection and expression profiling can help to identify neuroprotective GPCRs, which might represent useful therapeutic targets in the treatment of neurodegenerative diseases or cerebral ischemia.

Material and methods

Reagents

Vasoactive intestinal peptide (VIP) was obtained from Calbiochem–Novabiochem; L-glutamine and penicillin/streptomycin from Invitrogen; fetal calf serum (FCS) from Linaris; Dulbecco's modified essential medium (DMEM) from PAA Laboratories (Austria); dimethyl sulfoxide (DMSO), L-glutamic acid, *N*-(2-hydroxyethyl)-piperazine, monochlorobimane (MCB), *N*-(2-ethanesulfonic acid) (HEPES) and methylthiazoltetrazolium (MTT) from Sigma. (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA) was purchased from Tocris Cookston (USA). Other chemicals were obtained form Merck Eurolab. Small interfering RNA (siRNA) pools were obtained from Dharmacon. Primers and probes were synthesized by MWG Biotech. MTT was reconstituted in DMSO, MCB in ethanol, all other compounds in water. All vendors were from Germany except where otherwise stated.

Glutamate toxicity

HT22 cells were cultured in DMEM supplemented with 5% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (pH 7.2). For selection of glutamate resistant HT22 cells, 6×10^5 cells were seeded in 92 mm culture dishes and 24 h later exposed to 10 mM glutamate for 24 h. Surviving cells were expanded and again exposed to 20 mM glutamate, followed by two additional cycles of 20 mM glutamate with less cells (3×10^5) per dish. Finally, cells remained exposed to 20 mM glutamate for 48 h during the last of the four selections. These cells were further propagated in standard HT22 medium supplemented with 10 mM glutamate and named chronic resistant HT22 cells (HT22R). For glutamate toxicity experiments, 5000 HT22 cells were seeded in 100 μl into 96-well plates and glutamate (250 mM stock solution, pH 7.4) added 24 h later and incubated for 8 h. For experiments with conditioned media, HT22 and HT22R cells were cultured in standard HT22 medium without glutamate for 20 h. This medium was then used to replace the medium of separately grown HT22 cells seeded at the death permitting density at the same time point. Following a brief incubation of 4 h, glutamate was added as usual.

Viability assay

Cell viability was measured by the amount of blue formazan produced by viable cells from the tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) as described previously [5].

RNA extraction and cDNA synthesis

Sensitive and resistant HT22 cells were seeded at a density of 5×10^5 cells in 92 mm cell culture dishes (Nunc) in their respective medium. After 24 h, cells were harvested and total RNA extracted from 10^8 glutamate sensitive and resistant HT22 cells grown at the density permissive to cell death by glutamate toxicity using the TRIzol™ reagent (Life Technologies). Poly-$(A)^+$ RNA was purified over two rounds using DynaBeads (Dynal) affinity capture. All samples were digested with DNase I (Invitrogen) to eliminate residual genomic DNA. Approximately 0.5 μg poly-$(A)^+$ RNA of glutamate sensitive and resistant HT22 cells was reverse transcribed with oligo(dT)-primers using the SUPERSCRIPT™ first strand cDNA synthesis system (Invitrogen).

Quantitative GPCR PCR

To determine the exponential phase of amplification, aliquots of the PCR-reaction were removed after 15,

20, 25, 30, 35 and 40 cycles and visualized on ethidium bromide stained agarose gels. Then, aliquots were removed every other cycle starting five cycles before the first appearance in ethidium bromide stained gels and dot-blotted onto nylon membranes using a 96-well pin tool. Oligonucleotides specific for differentially expressed GPCRs, as judged by ethidium bromide stained gels, were end-labeled with 5 μCi (γ-^{32}P) ATP using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, UK) and hybridized to the membranes overnight at 42°C. Specific activity was $>10^7$ cpm/pmol oligonucleotide. Membranes were washed under high-stringency conditions, exposed to phosphoimaging plates and analyzed with Tina Version 2.10 h (Raytest). The density of each spot on the blot was plotted on a semi logarithmic scale against the cycle number for each reaction to estimate the PCR efficiency. Values were presumed to be valid if compared curves were parallel at a given cycle number, indicating both reactions being in the exponential phase of amplification. The difference in PCR cycles between glutamate sensitive and resistant HT22 cells was multiplied with the PCR efficiency to calculate the magnitude of regulation normalized to β-actin and gapdh expression. All experiments showing no difference between glutamate sensitive and resistant HT22 cells were replicated once; differences were reproduced three times with two different mRNA preparations to calculate the mean of regulation ± SEM. Sequences of primer and probes were: $VPAC_1$ (accession number NM_011703, forward primer (fp) 325–345 bp, reverse primer (rp) 438–457 bp, probe (p) 356–378 bp, Tm 59°C), $VPAC_2$ (NM_009511, fp 897–918, rp 1058–1076, p 985–1007, Tm 59°C), PAC_1 (NM_007407, fp 1004–1022, rp 1124–1144, p 1065–1086, Tm 63°C) AT_2 (NM_007429, fp 645–667, rp 781–803, p 697–717, Tm 59°C), A1 (XM_129465, fp 728–746, rp 865–886 Tm 55°C), A2A (XM_125720, fp 921–940, rp 1247–1267, p 961–980, Tm 63°C), A2B (NM_007413, fp 605–627, rp 743–762 Tm 55°C), A3 (AF069778, fp 5036–5055, rp 5289–5311, Tm 55°C), P2Y1 (NM_008772, fp 481–500, rp 692–712, Tm 55°C), P2Y2 (NM_008773, fp 943–963, rp 1215–1233, p 1002–1019, Tm 55°C), P2Y4 (NM_020621, fp 771–791, rp 910–928, Tm 55°C), P2Y6 (XM_133678, fp 744–764, rp 927–945, p 858–879, Tm 63°C), S1P1 (NM_007901, fp 1117–1139, rp 1216–1233, p 1189–1208, Tm 55°C), LPA1 (BC025425, fp 989–1009, rp 1121–1142, p1007–1023, Tm 55°C), S1P3 (NM_010101, fp 683–702, rp 810–831, p 736–755, Tm 55°C), PAR1 (NM_010169, fp 659–679, rp 1041–1057, p 989–1007, Tm 55°C), PAR2 (NM_007974, fp 463–482, rp 586–605, Tm 55°C), PAR3 (BC037126, fp 1137–1154, rp 1241–1262, p 1181–1203, Tm 55°C), $mGlu_1$ (AF320126, fp 1743–1764, rp 1826–1846, p 1784–1805, Tm 55°C), $mGlu_5$ (U31444, fp 5083–5101, rp 5176–5199, Tm 55°C), $mGlu_3$ (AF170699, fp 395–415, rp 522–541, p 415–437, Tm 59°C), $mGlu_8$ (XM_194317, fp 2910–2931, rp 2973–2993, p 2939–2957, Tm 55°C), CB1 (NM_007726, fp 1073–1091, rp 1276–1296, Tm 55°C), CASR (AF128842, fp 2967–2988, rp 3118–3116, Tm 55°C), GAPDH (XM_122186, fp 1007–1026, rp 1204–1225, p 1110–1130, Tm 55°C), β-ACTIN (NM_007393, fp 817–838, rp 946–967, p 863–884, Tm 59°C)

Quantitative SYBR green I PCR

Bcl-2 and GCLC were quantified on a 7500 Real Time PCR system (Applied Biosystems) using the SYBR green I core kit (Eurogentech) according to the manual. Specific SYBR green I primer pairs for GCLC were obtained from Qiagen (QT00130543) and for Bcl-2 from Applied Biosystems (Mm00477631_m1). Gapdh was quantified using the TaqMan Universal PCR master mix (Roche) with fp 5′-TGCCAGCCTCGTCCCGTAGA-3′ and rp 5′-GCGCCCAATACGGCCAAAT-3′. The Taqman probe was 5′-FAM-AAAATGGTGAAGGTCGGT-GTGAAC-TAMRA-3′

Western blotting

Sensitive and resistant HT22 cells were seeded at a density of 5×10^5 cells in 92 mm cell culture dishes (Nunc) in their respective medium and grown for 24 h. For phosphoprotein analysis, cells were quickly washed with PBS and directly lysed in boiling protein lysate buffer. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% skim milk or BSA in 0.1% Tween 20/TBS (TBS-T) and incubated with the respective primary antibodies in blocking solution overnight at 4°C. Primary antibodies used were a monoclonal phospo-MAPK44/42 (1:2000, Cell Signaling Technology), a polyclonal total MAPK44/42 (ERK1/2) (1:1000, Cell Signaling Technology), a monoclonal phospho-cAMP-response-element binding (CREB) (1:2000, Cell Signaling Technology), a monoclonal Bcl-2 clone 100 (1:2000, Upstate) and a monoclonal β-actin (1:5000, Abcam). Subsequently, blots were washed in TBS-T and incubated for 1 h with the secondary antibody goat anti-rabbit (1:700, Promega) or goat anti-mouse HRP-conjugated (1:10.000, Dianova). After a second wash, labeled proteins were detected using the ECL-reagent (Lumi-Phos WB; Pierce).

Transient transfection of plasmid DNA and siRNA pools

Full-length Bcl-2 and $VPAC_2$ cDNA were obtained from RZPD and subcloned in the mammalian

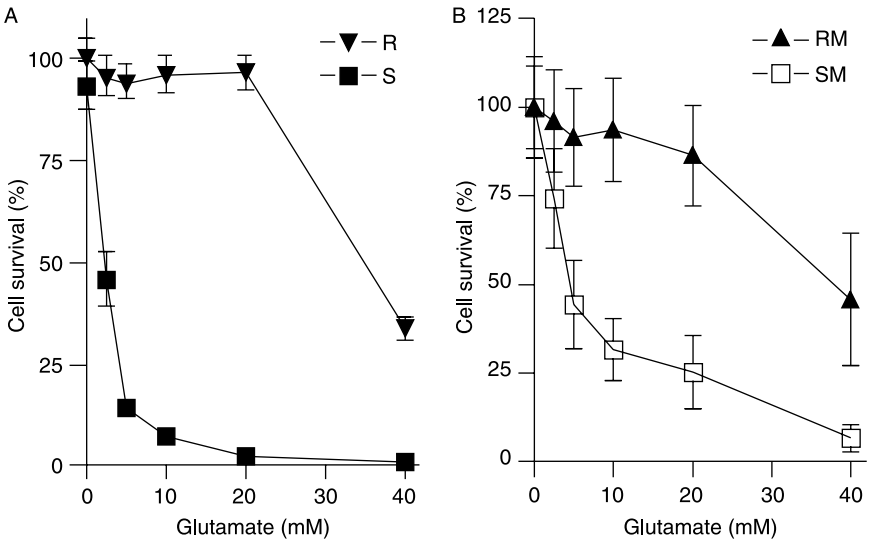

Figure 1. Medium conditioned by glutamate-resistant HT22R cells protects from oxidative glutamate toxicity: (A) 5000 HT22R (R, triangle) or wild type cells (S, square) were seeded into 96-well plates and exposed to the indicated amounts of glutamate for 24 h before assessing viability by MTT assays. (B) HT22 exposed to the indicated amounts of glutamate and medium conditioned by resistant (RM) or sensitive (SM) cells. Each data point shows the mean ± SEM of three pooled independent experiments with $n = 4$.

expression vector pCI-neo (Promega). High purity plasmids were obtained using the Nucleobond AX 500 columns (Machery & Nagel, Düren, Germany) according to the manufacturer. For transfection, confluent HT22 cells were harvested by trypsinization. In the presence of 10 μg plasmid DNA 15×10^6 HT22 cells resuspended in 500 μl of PBS and incubated on ice for 10 min. Empty pCI-neo vector served as control. Electroporation was carried out with a Genepulser (Biorad, Munich, Germany) at 960 F and 250 mA with a time constant of 17–20 s. Immediately after transfection, cells of each electroporation were plated in three 92 mm culture-dishes. Twenty four-hour after plating, cells were trypsinized and 5×10^3 cells were seeded onto 96 well-cell culture plates for toxicity assays or GSH measurement.

For siRNA experiments, HT22 cells were cultured in a 24-well plate and transfected with 50 pmole/well of siRNAs targeting Bcl-2 (M-063933-00) or control (D-001220-01) using Dharmafect 2 siRNA transfection reagent (Dharmacon) according to the manual.

Measuring glutathione content

5000 HT22 and HT22R cells were plated in white non-transparent 96-well-microtiter plates. After 24 h of cultivation, cells were incubated with glutamate or vehicle for 6 h or as indicated. Cells were then carefully washed twice with phenol red-free media and incubated with 20 μM MCB for 5 min at 37° protected from light. MCB fluorescence was then measured by a SpectraMax Gemini (Molecular Devices, Ismaning, Germany) using the SoftmaxPro 3.1.1 software (Softmax, San Diego, USA). Excitation wavelength was 393 nm and emission wavelength was 485 nm. After measurement one volume normal medium and MTT were added immediately and cell viability assessed as described above 2 h later. Results indicate MCB fluorescence per well normalized to the corresponding viability as measured by the MTT-test.

Results

Medium conditioned by glutamate-resistant cells protects from oxidative glutamate toxicity

We selected glutamate-resistant HT22 cells (HT22R) by repeated exposure of the parental cell line HT22 to high concentrations of glutamate and further propagation of the few surviving cells. HT22R cells withstand up to 40 mM glutamate for 24 h with only a ~40% decrease in viability. In contrast, ~90% of wild type HT22 cells die after exposure to only 10 mM glutamate (Figure 1(A)). As protective substances released into the medium could participate or even underlie these differences in viability, we tested the effect of medium conditioned by sensitive- or resistant-cells on glutamate toxicity. A 4-hour exposure to medium from HT22R cells rendered glutamate-sensitive cells less susceptible to large doses of glutamate (Figure 1(B)) and, interestingly, even medium conditioned by other sensitive cells had, in contrast to fresh medium, a minor positive effect on viability (Figure 1(B), open square).

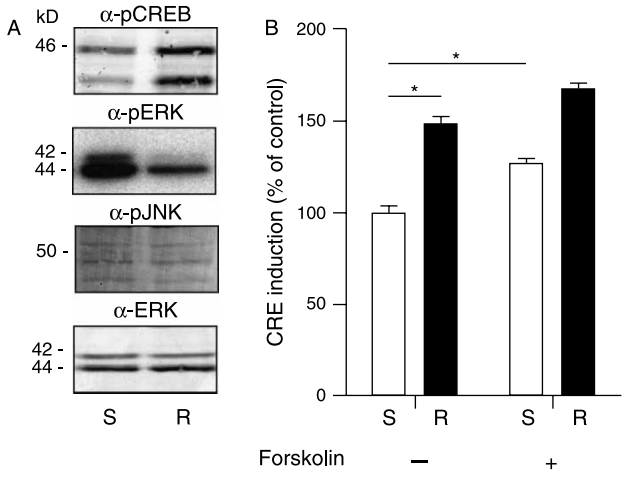

Figure 2. Increased CREB signaling in glutamate-resistant HT22R cells: (A) Western blot analysis using phosphorylation-specific antibodies against pCREB, pERK1/2 and pJNK and total ERK in wt (S) and HT22R (R) cells. (B) HT22R and wt cells were transfected with CRE–EGFP or CMV–EGFP expressing constructs and fluorescence measured by FACS analysis at 488 nM. Data represent the mean ± SEM CRE–EGFP expression normalized to CMV–EGFP from three independent experiments. Statistical significance was calculated using a two-paired Mann–Whitney test with $\star = p < 0.05$.

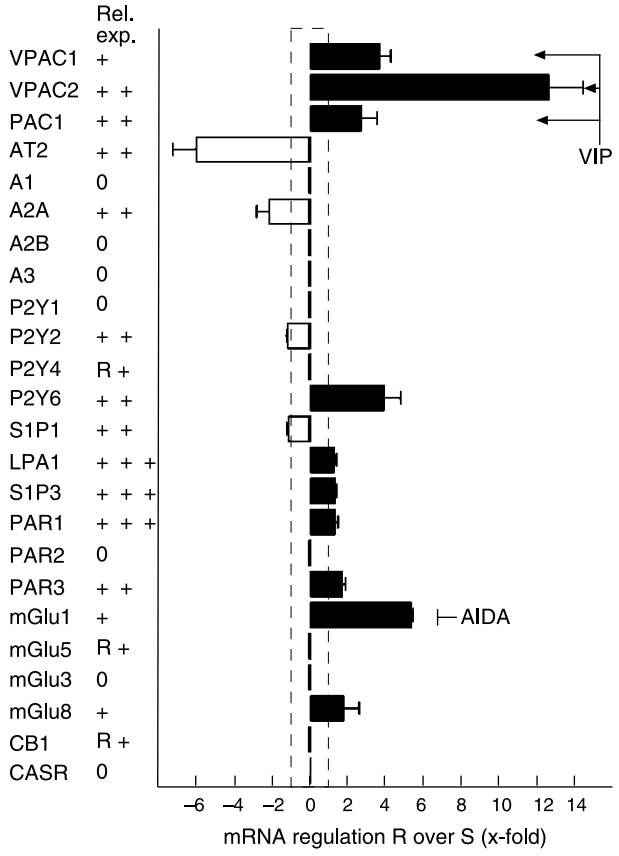

Figure 3. Regulation of G-protein coupled receptor mRNA in glutamate-resistant HT22R cells: Bar graphs correspond to the mean ± SEM of three independent quantitative PCR experiments with two different mRNA preparations. Regulation was calculated by ΔCT R and S normalized to gapdh and β-actin expression. The dashed line indicates equal expression. Relative expression level is depicted to the left with 0, no expression; R, expression in resistant cells only; +, first detection at 40 cycles PCR; ++, at 35 cycles; +++, at 30 cycles.

Glutamate-resistant cells demonstrate an increase in G_s-coupled signaling

We therefore hypothesized that HT22R cells release protective substances into the medium and previous studies by others [11,18] and us [5] suggested that these substances might protect by activating G-protein coupled membrane receptors. For that reason, we compared the activation of signal transduction pathways involved in GPCR signaling in glutamate-sensitive and resistant cells with phosphorylation-specific antibodies and found constitutively more phosphorylated CREB protein) and decreased phosphorylation of extracellular-signal regulated protein kinases 1 and 2 (ERK1/2) in glutamate-resistant cells. The amount of phosphorylated c-Jun N-terminal kinases/stress-activated protein kinases, total protein and total ERK, in contrast, remained unchanged (Figure 2(A)). CREB is mainly phosphorylated in response to the activation of G_s-coupled receptors; ERK1/2 phosphorylation can occur downstream of receptor tyrosine kinase activation, through G_i-coupled mechanisms or via activation of protein kinase C (PKC) by G_q coupled receptors (for review see [19]).

We next quantified the assumed increase in CRE activation in HT22R cells by transient transfection of a reporter vector containing a CRE element controlled EGFP [20] into HT22 and HT22R cells. HT22R cells exhibited a 50% increase in CRE activation as judged by mean CRE–EGFP dependent fluorescence normalized to control experiments with a constitutively active CMV-driven EGFP (Figure 2(B)). Forskolin treatment served as positive control for CRE activation and induced CRE–EGFP fluorescence to a similar extent in both cell lines. In summary, HT22R cells demonstrate an activation of G_s-coupled signal transduction pathways, which might contribute to their resistance against oxidative stress.

Glutamate-resistant and sensitive HT22 cells dramatically differ in their GPCR expression pattern

This activation could theoretically occur via para- or auto-crine activation of protective GPCRs by their cognate ligands or by an increase in the amount of protective GPCRs as described by Sagara and Schubert in 1998 for group I metabotropic glutamate receptors [17]. Because of its feasibility, we quantified the relative mRNA expression of candidate receptors in wild type and HT22R cells by quantitative PCR. Twenty-four receptors were chosen due to their previously reported involvement in cell differentiation,

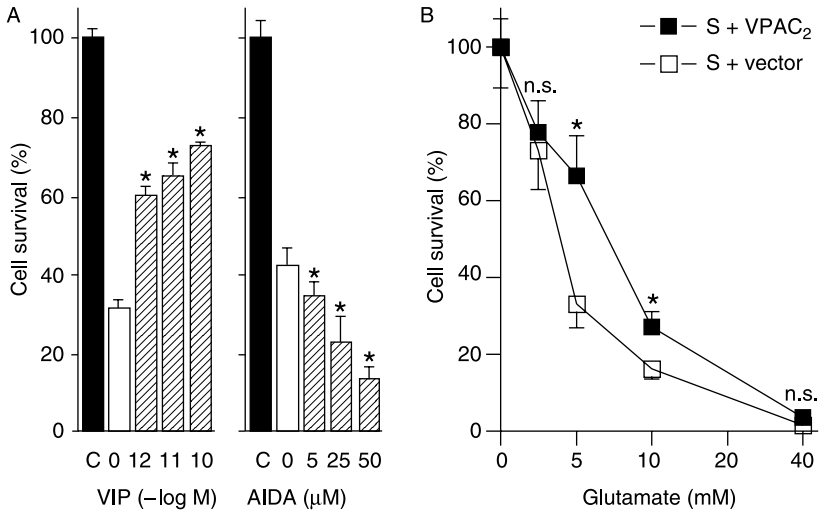

Figure 4. Mimicking the regulation of $VPAC_2$ receptor pharmacologically and by overexpression protects from glutamate toxicity: (A) HT22 exposed to vehicle (C) or 2.5 mM glutamate for 8 h in the presence of VIP or the $mGlu_1$ antagonist AIDA at the indicated concentrations. Viability was assessed by MTT assays after 24 h. Shown is mean ± SEM of two pooled independent experiments with $n = 4$. (B) HT22 cells were transfected with $VPAC_2$ or empty vector and 24 h later exposed to the indicated amounts of glutamate for 8 h. Viability was assessed by MTT assays 24 h after glutamate treatment. Each data point shows the mean ± SEM of three pooled independent experiments with $n = 4$. Statistical significance was calculated using a two-paired Mann–Whitney test with $\star = p < 0.05$.

survival, and apoptosis or like the metabotropic glutamate receptors because their ligand obviously plays a role in glutamate-mediated cell death. We observed that three out of 24 receptors were reproducibly regulated more than 4-fold. The receptors for the VIP $VPAC_2$ (12.6-fold, SEM 1.8) and metabotropic glutamate receptor $mGlu_1$ (5.3-fold, SEM 0.11) were found more prominently expressed in resistant cells, and the angiotensin receptor AT_2 (6-fold, SEM 1.23) in sensitive cells. Four receptors were upregulated less than 5-, but more than 2-fold. Two of these, $VPAC_1$ (3.6-fold, SEM 0.59) and PAC_1 receptor (2.7-fold, SEM 0.85) are also activated by VIP. The nucleotide receptor P2Y6 (3.9-fold, SEM 0.86) was found upregulated and the adenosine receptor A2A (2.2-fold, SEM 0.66) downregulated in resistant cells. The nucleotide receptor P2Y4, the metabotropic glutamate receptor $mGlu_5$ and the cannabinoid receptor CB1 were found newly, although very scarcely, expressed in resistant cells. No receptor was expressed in sensitive cells only. The relative amount of receptor expression is depicted in Figure 3 to the left. The following receptors were either not, or less than 2-fold regulated: nucleotide receptor P2Y2, lipid receptors S1P1, LPA1 and S1P3, protease-activated receptor PAR1, and metabotropic glutamate receptor $mGlu_8$. Adenosine receptors A1, A2B and A3, nucleotide receptor P2Y1, protease-activated receptor PAR2, metabotropic glutamate receptor $mGlu_3$, and calcium-sensing receptor; CASR could not be detected at all. In summary, resistant cells clearly expressed a dramatically changed pattern of GPCR mRNAs.

Pharmacological stimulation of GPCRs reflects their regulation

Next, we examined whether the changes in mRNA expression of regulated GPCRs might have functional consequences on cell survival. We pharmacologically simulated the regulation of all receptors upregulated more than 4-fold and investigated the effect on glutamate toxicity and CREB/ERK phosphorylation. In the case of $VPAC_2$, the natural ligand VIP was used and where this was not possible, as in the case of metabotropic glutamate receptors, the specific antagonist AIDA. Conditions with less glutamate were chosen that allowed us to see an increase in survival, or an exacerbation of glutamate-induced cell death in parallel experiments. As shown in Figure 4(A), activating the receptors for VIP with its natural ligand resulted in increased cell survival, whereas antagonizing the signaling of the upregulated $mGlu_1$ receptor with AIDA was associated with increased cell death as shown previously [16]. Thus, the pharmacological data support our hypothesis that protective receptors are upregulated in HT22R cells. However, VIP did not protect in conditions that lead to complete cell death, which in this model depends largely on the amount of serum. We suspected an increased degradation of VIP through proteases contained in the serum and therefore used the opposite approach of overexpressing the most-regulated receptor $VPAC_2$ in sensitive cells and exposed these cells to increasing amounts of glutamate in the presence of sufficing amounts of serum. Here, $VPAC_2$ protected against larger doses of glutamate but was again not able to

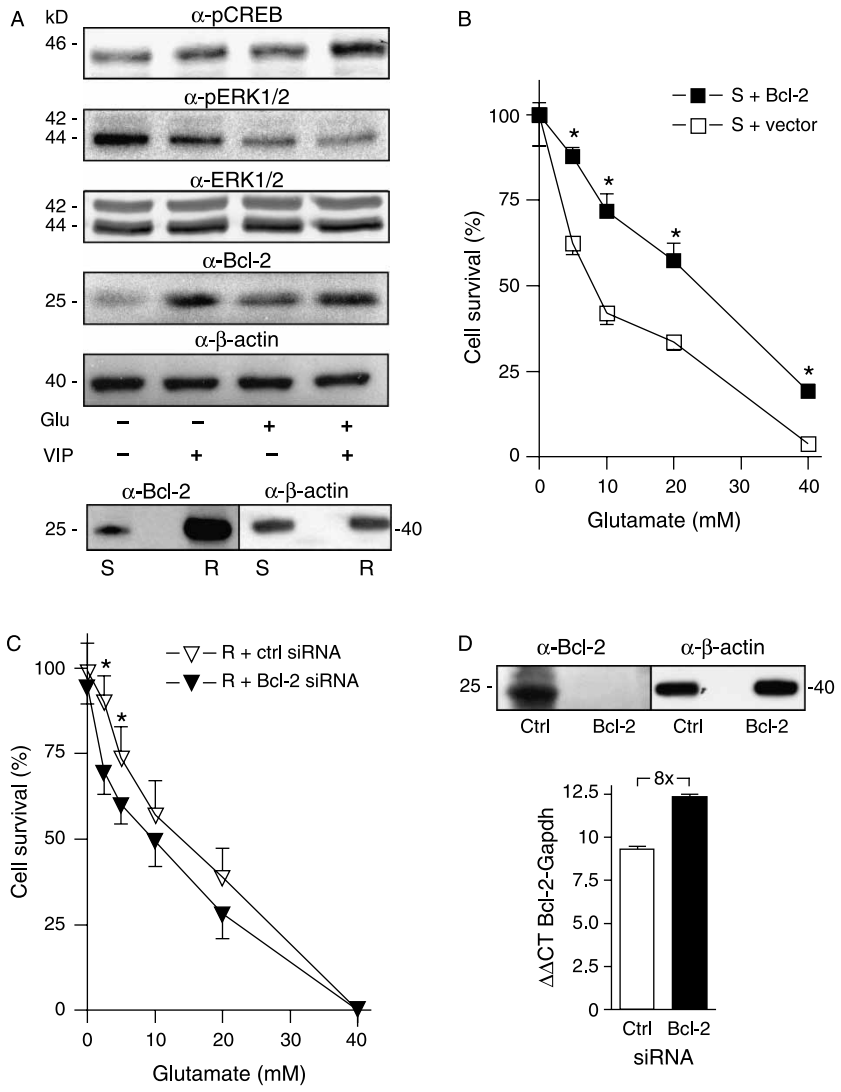

Figure 5. Bcl-2 is induced by the ligands of upregulated receptors and protects from glutamate toxicity: (A) Western blot analysis of HT22 cells treated with VIP (10^{-8} M), glutamate (2.5 mM) or both for 20 min using phosphorylation-specific antibodies against pCREB, pERK1/2 and Bcl-2. Lower panel shows constitutive Bcl-2 expression in wt (S) and HT22R (R) cells. Total ERK and β-actin serve as loading controls. (B) and (C) HT22 cells were transfected with Bcl-2 or empty vector, HT22R cells were transfected with Bcl-2 and control siRNA pools, before exposure to the indicated amounts of glutamate for 8–24 h later. Viability was assessed by MTT assays 24 h after glutamate treatment. Each data point shows the mean ± SEM of three pooled independent experiments with $n = 4$. Statistical significance was calculated using ANOVA and Bonferroni post-test with $\star = p < 0.05$. (D) Expression analysis of Bcl-2 in HT22R cells treated with Bcl-2 and control siRNA pools for 48 h using Western blotting and quantitative real-time PCR. Regulation was calculated by ΔCT normalized to gapdh expression.

protect in conditions that kill all cells like 40 mM glutamate (mock 1.67%, SEM 0.88, $n = 12$ vs. $VPAC_2$ 3.70%, SEM 0.99, $n = 12$, (Figure 4(B))).

Activation of VIP receptors and metabotropic glutamate receptors leads to a phosphorylation pattern constitutively observed in HT22R cells

We next examined the effect of VIP and glutamate on CREB and ERK phosphorylation to assess their share of the constitutive activation or inhibition of signaling pathways observed in glutamate-resistant cells. The effect of VIP on sensitive HT22 cells was studied under serum-free conditions to exclude the influence of VIP contained in the serum. Western blot analyses of HT22 cells treated with VIP showed increased CREB and decreased ERK1/2 phosphorylation. Treating the cells with glutamate for 20 min, thus without eliciting cell damage had the same effect, probably mediated by metabotropic glutamate receptors as these cells do not express ionotropic glutamate receptors. VIP and glutamate together acted synergistically. Therefore, stimulating these receptors with their ligands resulted in the same phosphorylation pattern constitutively observed in glutamate resistant HT22R cells (Figure 5(A), compare with Figure 2(A)).

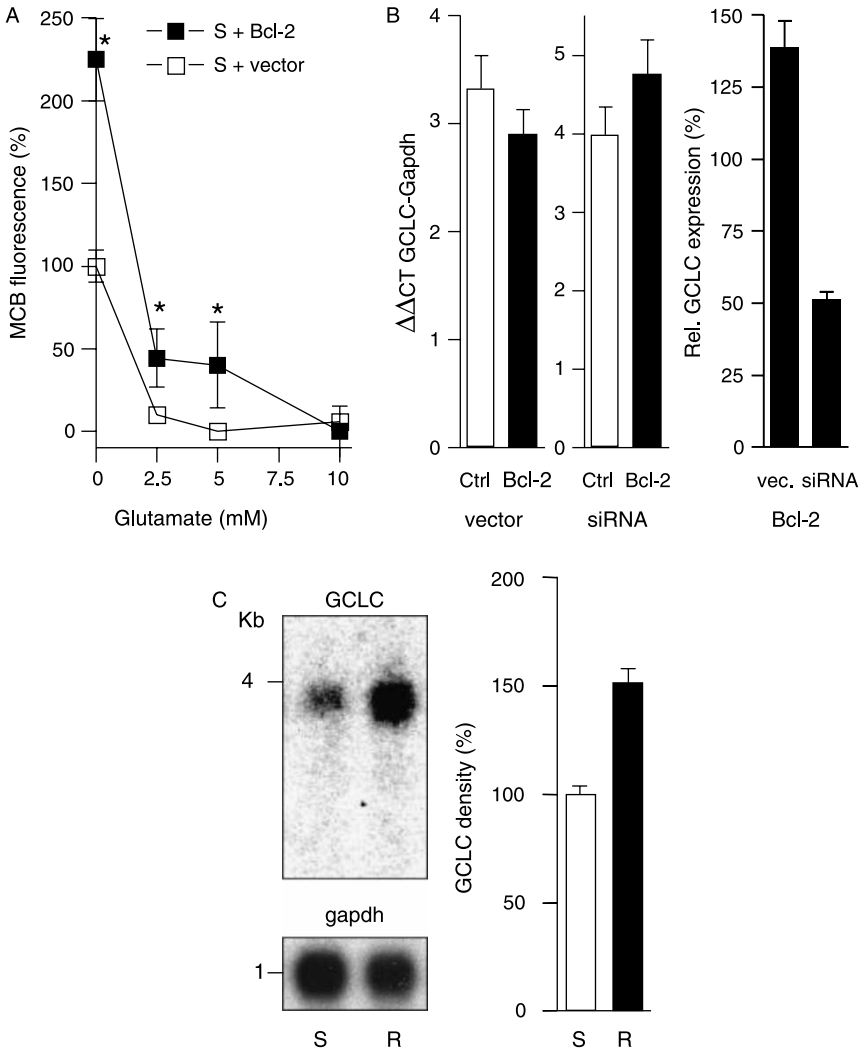

Figure 6. Bcl-2 protects from glutamate toxicity by increasing the intracellular glutathione content: (A) Glutathione content of HT22 cells transiently transfected with Bcl-2 or empty vector was measured by MCB fluorescence 6 h after glutamate treatment and normalized to cell viability as measured by MTT. Each data point shows the mean ± SEM of three pooled independent experiments with $n = 4$. Statistical significance was calculated using a ANOVA and Bonferroni post-test with $\star = p < 0.05$. (B) GCLC expression in wt cells transfected with Bcl-2 or empty vector, respectively HT22R cells transfected with Bcl-2 and control siRNA pools, was measured by quantitative real-time PCR and normalized to controls (right panel). (C) Northern blot loaded with 5 μg total RNA from HT22R (R) and HT22 (S) cells probed with GCLC and quantification of expression in mean fold regulation ± SEM R over S from two blots normalized to the housekeeping gene *gapdh*.

VIP and glutamate synergistically induce Bcl-2, which protects from glutamate toxicity

In HT22 cells the antiapoptotic protein Bcl-2 is upregulated in a cAMP-dependent manner [5] and is capable of suppressing formation of ROS [21,22]. It could thus represent a suitable downstream mediator of GPCR signaling. We investigated by Western blotting whether VIP, glutamate or both induce Bcl-2 at the protein level; VIP increased the amount of Bcl-2 and so did glutamate, although to a lesser extent. There was, however, no synergistic effect when the two agents were applied together. Bcl-2 was also clearly increased in resistant cells, whereas the amount of the housekeeping protein β-actin remained unchanged (Figure 5(A) lower panel). We therefore assume that VIP and glutamate signaling converge at least in part on Bcl-2. To test if this induction confers resistance against glutamate, we transiently overexpressed Bcl-2 in glutamate-sensitive HT22 cells. We observed an explicit increase in viability up to 5-fold in cells treated with 40 mM glutamate (mock 3.79% survival (SEM 0.89, $n = 12$) vs. Bcl-2 19.15% (SEM 1.99, $n = 12$)), which was clearly superior to $VPAC_2$ overexpression (Figure 5(B)). The reverse experiment, meaning knockdown of Bcl-2 by siRNA in resistant cells, increased their susceptibility to glutamate (Figure 5(C)). This effect was however not very prominent and using ANOVA and Bonferroni post-test only statistically significant at lower

concentrations of glutamate, although Bcl-2 was efficiently knocked down approximately 8-fold at the mRNA level and undetectable at the protein level (Figure 5(D)).

Bcl-2 increases cellular glutathione content and induces γ-glutamylcysteine synthetase mRNA

Bcl-2 overexpression and protection was accompanied and probably at least in part caused by a significant increase of 224% (SEM 24, $n = 22$) in intracellular glutathione content as measured by MCB fluorescence. MCB itself is non-fluorescent and reacts with free GSH to form a highly fluorescent derivative. GSH content decreased after a 6-hour treatment with increasing doses of glutamate but remained significantly higher in Bcl-2-expressing cells (Figure 6(A)). Jang et al. showed recently that ectopic expression of Bcl-2 increased the cellular glutathione level and the expression of the rate-limiting enzyme in glutathione synthesis γ-glutamylcysteine ligase (GCLC) [23]. In our hands, GCLC mRNA was also upregulated by 139% (SEM 9.1, $n = 6$) after transient expression of Bcl-2. This in itself not very convincing result is substantiated by a downregulation of 51% (SEM3, $n = 6$) after siRNA-mediated knockdown (Figure 6(B)), and also by the similar increase in GCLC expression observed by Northern blotting in HT22R cells (152% (SEM 7.7, $n = 2$), Figure 6(C)).

Discussion

Our results demonstrate that cell selection and mRNA quantification can be used to identify neuroprotective G-protein coupled receptors by their expression pattern. We assume that the ligands of the most regulated neuroprotective receptors in this study, VIP and glutamate, are present in the serum or produced by the cells themselves in an auto- or para-crine manner. Constitutive activation of the VIP receptors has only been reported after intentional mutagenesis or ectopic expression. In the case of metabotropic glutamate receptors, constitutive activity is controlled by Homer proteins, which bind directly to the receptors' carboxy-terminal intracellular domains [24]. It is however unlikely that this plays an eminent role in the paradigm used herein as the ligand glutamate is present in excess. Signaling through these receptors is most probably involved in the phenotype of HT22R cells as treating wild type cells with VIP and glutamate increased CREB and decreased ERK phosphorylation similar to changes observed constitutively in glutamate-resistant HT22R cells. Both effects were even synergistically enhanced if both agents were applied simultaneously.

The contribution of the observed decrease in ERK phosphorylation to the glutamate-resistant phenotype is difficult to assess, as previous work on the role of ERK activation in glutamate toxicity is inconclusive. In one study, a 6-hour glutamate exposition activated ERKs and inhibition of the ERK-activating MEK-1 and -2 kinases with U0126 protected HT22 cells from glutamate toxicity [25]. In another study, an 8-hour treatment with glutamate reduced the amount of phosphorylated ERK1/2. There the phorbol ester tetradecanoylphorbol acetate TPA protected HT22 cells via activation of ERKs and inhibition of MEKs with PD98059 drastically reduced this protection [18]. A third study reproduced the beneficial effects of U0126 and the deleterious effects of PD98059 [26]. Therefore, the differences might rather be due to lack of specificity of these substances. We only stimulated HT22 cells for 20 min to exclude an effect associated with oxidative stress; therefore the observed reduction by glutamate cannot be compared with the previous studies. However, the constitutive reduction in ERK phosphorylation observed in HT22R cells rather hints to a deleterious effect of ERKs in glutamate toxicity.

On the other hand, the protection conferred by the observed increase in CREB phosphorylation at Ser133 is rather self-evident, as this has been shown to be protective in diverse paradigms of neuronal cell death through the induction of survival promoting proteins (for review see [27]). One of these genes is the antiapoptotic protein Bcl-2, which is upregulated in HT22 cells in a cAMP-dependent manner [5]. Indeed, VIP increased the amount of Bcl-2 protein and so did glutamate, and again the same pattern was observed in glutamate-resistant HT22R cells. Transient overexpression of Bcl-2 in glutamate-sensitive HT22 cells increased viability and was accompanied by a significant increase in intracellular glutathione content. This corresponded to an increase in mRNA of the rate-limiting enzyme in glutathione synthesis GCLC, which was not very prominent but roughly equivalent to the increase observed semiquantitatively by comparing the density of PCR bands described by Jang et al. [23]. In this publication, Bcl-2 induction of GCLC was attributed to an increased activation of the transcription factor NF-κB, but it remained unclear how exactly Bcl-2 increased the observed retention of p65 (the functionally active subunit of NF-κB) in the nucleus. Bcl-2 overexpression also attenuates calcium transients induced by the activation of G_q-coupled receptors by reducing the calcium content of the endoplasmic reticulum [28,29] and one could speculate that changes in signal transduction downstream of GPCRs or other InsP3-coupled receptors underlie the increased activation of NF-κB.

We doubt that the rather minor effect on GCLC expression fully explains the prominent protection conferred by transient Bcl-2 overexpression. Bcl-2 is also supposed to suppress ROS formation by protecting mitochondrial respiratory function thus preventing superoxide production and to scavenge superoxides directly as an antioxidant (for review see

[30]). The above-mentioned reduction of ER calcium also lowers the mitochondrial calcium accumulation that, in the case of cell-death stimuli, leads to irreversible opening of the permeability transition pore, ATP loss and generation of reactive oxygen species [29]. This multitude of protective actions probably accounts for the prominent protection observed. In addition, we suspect that Bcl-2 is not the only protective gene increased in HT22R cells, as its knockdown by siRNA did not reconstitute full susceptibility to glutamate.

It remains also unclear, why other receptors capable of increasing cAMP through a G_s-coupled stimulation of adenylyl cyclase, e.g. the slightly downregulated adenosine receptor A2A [31] are not similarly upregulated. In this case, G_s-coupling has been shown in kidney HEK293 cells and it is possible that A2A does not increase cAMP in HT22 cells. In the case of the most regulated receptor $VPAC_2$ it can be argued that dual coupling to two protective pathways explains the most prominent regulation observed in HT22R cells. $VPAC_2$ activates adenylate cyclase and in addition phospholipase C [32–34], which leads to PKC activation. PKC activation by phorbol esters was shown previously to protect in this paradigm by an activation of mitogen-activated kinase and down-regulation of certain PKC isoforms [18].

In summary, we conclude that the signaling cascade of VIP and glutamate receptors in HT22 cells probably involves CREB phosphorylation, CRE-dependent induction of Bcl-2 transcription and protection from glutamate toxicity by increasing the amount of the intracellular antioxidant glutathione. We think that this method of cell selection and expression profiling can be used to identify the neuroprotective qualities of other, yet uncharacterized G-protein coupled receptors and thus help to identify potentially interesting drug targets.

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