Antidepressants Increase β-Arrestin2 Ubiquitinylation and Degradation by the Proteasomal Pathway in C_6 Rat Glioma Cells

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ABSTRACT

β-Arrestins, regulators of G protein-coupled receptor-G protein coupling and receptor desensitization and internalization, function also as scaffolding proteins mediating cellular signaling events. β-Arrestin1 was previously implicated by us in the pathophysiology of depression and in the mechanism of action of antidepressants (ADs). The ubiquitously expressed β-arrestins1 and 2 are structurally highly homologous. There has been extensive investigation of these two proteins to determine whether they serve different roles in receptor signaling. In this study, we show that treatment of C_6 rat glioma cells with ADs of various types for 3 days resulted in decreased β-arrestin2 levels. In contrast, β-arrestin2 mRNA expression was found to be up-regulated by ADs. To unravel the mechanism for these opposite effects several possible β-arrestin2 post-transcriptional events and modifications were examined. C_6 rat glioma cells transfected with β-arrestin1-targeted short hairpin RNA showed similar effects of ADs on β-arrestin2 levels. AD-induced decreases in β-arrestin2 protein levels were not due to cytosolic membrane translocation. Immunoprecipitation experiments showed that ADs were able to increase coimmunoprecipitation of ubiquitin with β-arrestin2. AD-induced increases in β-arrestin2 ubiquitinylation led to its degradation by the proteasomal pathway, as the proteasome inhibitor N-[(phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide (MG-132) prevented antidepressant-induced decreases in β-arrestin2 protein levels.

Theories concerning antidepressant targets of action suggested inhibition of presynaptic monoamine reuptake mechanisms. The delayed therapeutic onset associated with antidepressants led to hypotheses proclaiming regulation of G protein-coupled receptor (GPCRs) functions, including delayed receptor desensitization and down-regulation and induction of adaptive changes in postreceptor signaling cascades and in gene expression, as well as changes in neuroplasticity, cellular resilience, and synaptic plasticity (Racagni and Popoli, 2008).

The basic mechanisms underlying the phenomenon of desensitization and down-regulation of GPCRs involve the activities

of two families of proteins: G protein-coupled receptor kinases and arrestins (Lefkowitz and Shenoy, 2005). β-Arrestins are capable of sterically hindering the G protein coupling of agonist-activated GPCRs, resulting in receptor desensitization and "arrest" of signaling. Recent evidence shows that β-arrestins can also function to activate signaling cascades independently of G protein activation by serving as multiprotein scaffolds for an increasing number of signaling molecules. In addition, new roles for β-arrestins in nuclear functions have been described previously (DeWire et al., 2007).

β-Arrestin1 was suggested to play a role in the pathophysiology of mood disorders as well as in the mechanism of action of antidepressants (Golan et al., 2009). β-Arrestin1 protein and mRNA levels in mononuclear leukocytes of yet untreated patients with major depression were significantly lower than those of healthy subjects and correlated with the severity of depressive symptomatology (Avissar et al., 2004; Matuzany-Ruban et al., 2005). The low β-arrestin1 measures were alleviated by antidepressant treatment within 1 to 3 weeks

ABBREVIATIONS: GPCR, G protein-coupled receptor; HRP, horseradish peroxidase; MG-132, N-[(phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide; G418, antibiotic G418 disulfate salt; RT, reverse transcriptase; PCR, polymerase chain reaction; shRNA, short hairpin RNA; AD, antidepressant; 5-HT, hydroxytryptamine, serotonin; ERK, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein kinase.

(Matuzany-Ruban et al., 2005). In a mouse model of anxiety/depression induced by chronic corticosterone, fluoxetine-reversible decreased expression of β-arrestin1 and 2 was detected in the hypothalamus. Mice deficient in the gene for β-arrestin2 displayed a reduced response to fluoxetine, suggesting that β-arrestin signaling is necessary for the antidepressant effects of fluoxetine (David et al., 2009).

Four arrestin family members have been identified: the retina exclusively expressed arrestin1 and 4 and the ubiquitously expressed β-arrestin1 and 2 (arrestin2 and 3, respectively), sharing 78% amino acid identity. There has been extensive investigation of these two proteins showing differential receptor affinities, differential cellular distribution, differential scaffolding functions, and differential regulation by phosphorylation (Lefkowitz and Shenoy, 2005; DeWire et al., 2007).

Findings concerning β-arrestin1 involvement in antidepressant mechanism of action and data on the different roles served by β-arrestins1 and 2 in receptor signaling prompted us to carefully explore the roles of β-arrestin2 in the mechanism of action of antidepressants in the C_6 glioma cell line. C_6 rat glioma cells are an established and useful model for studying the phenomena of GPCR desensitization and down-regulation (Fishman and Finberg, 1987) and appear to be a suitable model to study the mechanism of action of antidepressants (Donati and Rasenick, 2005). Unlike neurons in rat brain or primary neuron culture, there are no synaptic structures formed between C_6 cells; thus any alteration observed after drug treatment represents a direct "postsynaptic effect." Moreover, recent studies show that glial plasticity is important for the therapeutic action of antidepressants (Hisaoka et al., 2007) and that antidepressants increase glial cell line-derived neurotrophic factor production in C_6 rat glioma cells (Hisaoka et al., 2007).

Materials and Methods

Materials. Goat polyclonal anti-β-arrestin2 antibody (immunoblot, 1:5000) and rabbit monoclonal anti-Na^+/K^+-ATPase antibody (1:1000) were from Abcam (Cambridge, UK). Mouse monoclonal anti-β-arrestin1 antibody (1:250) was from BD Transduction Laboratories (Franklin Lakes, NJ). Mouse monoclonal anti-ubiquitin (clone FK2; immunoblot, 1:2000; immunoprecipitation, 1:100) antibody was from BIOMOL (Plymouth Meeting, PA). Mouse monoclonal anti-actin antibody (1:50,000) was from MP Biomedicals (Solon, OH). Rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:400) and mouse monoclonal anti-cyclin D1 (1:500) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). HRP-conjugated donkey anti-rabbit antibody (NA934) was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). HRP-conjugated donkey anti-goat antibody was from Santa Cruz Biotechnology, Inc. HRP-conjugated goat anti-mouse antibody was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). MG-132 was from Calbiochem (San Diego, CA). G418 disulfate salt was from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Drug Treatments. C_6 rat glioma cells were maintained in Dulbecco's modified Eagle's medium (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Twenty-four hours after seeding, growth medium was replaced with fresh medium containing the desired antidepressant drug in the presence or absence of 0.1 μM MG-132. Drug-containing medium was replaced every day for 3 days to achieve chronic treatment.

Subcellular Fractionation. C_6 cells were washed three times with ice-cold phosphate-buffered saline, scraped with a rubber policeman into lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, and 20 μl/1 ml protease inhibitor cocktail) and homogenized at 25,000 rpm on a Polytron homogenizer (PT 1200; Kinematica AG, Littau-Lucerne, Switzerland) for 1 min. Cell nuclei and debris were pelleted at 600g for 10 min at 4°C. The supernatant was further centrifuged at 20,000g for 30 min at 4°C, and the cytosolic fraction was collected. To purify the membrane fraction, lysates from two separate 100-mm² plates were combined, homogenized as mentioned above, and centrifuged at 600g for 10 min at 4°C. The supernatant was further centrifuged at 30,000g for 30 min at 4°C. The resulting pellet (membrane fraction) was washed with 500 μl of lysis buffer as above and centrifuged at 30,000g for 30 min at 4°C to produce a clean membranes pellet that was resuspended in 200 μl of lysis buffer and sonicated (Sonicator ultrasonic processor; Misonix, Inc., Farmingdale, NY) for 2 to 3 s.

All fractions were diluted in sample buffer 1:3 (10% v/v glycerol, 20% v/v SDS, 5% v/v β-mercaptoethanol, and 0.05% w/v bromphenol blue, pH 6.8), boiled for 5 min at 95°C, and frozen at −80°C until assayed. Aliquots were taken for protein determination using the Bradford assay. Fraction purity was verified using specific markers: Na^+/K^+-ATPase for membranes and cyclin D1 for cytosol.

Immunoblotting. Cytosolic and membrane fractions were thawed on the day of assay. Protein aliquots (30 μg/lane) were taken for protein separation by SDS-polyacrylamide gel electrophoresis as described previously (Avissar et al., 2004). Semiquantitative analysis was performed using a computerized image analysis system (EZQuant-Gel 2.11; EZQuant Biology Software Solutions Ltd., Tel Aviv, Israel). To ensure that equal amounts of total protein were loaded to each lane, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase or actin was measured using a proper antibody.

Isolation of RNA and RT-PCR. Isolation and purification of total RNA from C_6 cells was performed using an EZ-RNA Kit (Biological Industries Ltd.). One-step RT-PCR was performed with oligonucleotide primers selected from the highly conserved nucleotide sequences of rat β-actin (forward primer 5′-AGCCATG-TACGTAGCCATCC-3′ and reverse primer 5′-CTCTCAGCTGTGGTG-GTGAA-3′; amplified product 228 base pairs) and rat β-arrestin2 (forward primer 5′-GTTCATCGCCACCTACCAG-3′ and reverse primer 5′-AGGAAGTGGCGTGTGGTTT-3′; amplified product 345 base pairs). Primers were synthesized by Sigma Genosys (Revohot, Israel). One microgram of total RNA was used for RT-PCR in a 25-μl reaction volume. After a denaturation step for 2 min at 95°C, thermal cycling was performed at 95°C for 20 s, 60°C for 30 s, and 72°C for 1 min, with a total number of 30 cycles for β-actin and β-arrestin2 gene products. After staining with ethidium bromide, amplified DNA fragments were separated by gel electrophoresis in 1% agarose. The relative density of the bands imprinted on the autoradiographic films was measured using a computerized image analysis system (EZQuant-Gel 2.11) and was normalized to β-actin.

Short Hairpin RNA Transfection. C_6 cells were stably transfected with β-arrestin1-targeted short hairpin RNA (shRNA) or scrambled shRNA using a SureSilencing shRNA kit (Superarray Bioscience Corporation, Frederick, MD) according to the manufacturer's instructions. The pGeneClip-Neo expression vector in which the desired shRNA was cloned conveys neomycin resistance. Therefore, to establish stable expression in the C_6 glioma cell line, 48 h after transfection, transfected cells undergo selection with full growth medium containing 800 μg/ml G418 disulfate salt solution (Sigma-Aldrich) for 14 days. After selection, individual cells were removed and plated in 24-well

plates, maintaining antibiotic selection pressure. Individual colonies were screened for shRNA expression using Western blot analysis. Stably transfected cells were maintained by growing them in the presence of G418 at 400 μg/ml.

Immunoprecipitation. C_6 cells were lysed and homogenized as described above in lysis buffer supplemented with 10 mM *N*-ethylmaleimide, a deubiquitination inhibitor). Cytosolic proteins (500 μg) were incubated with a specific monoclonal anti-ubiquitin antibody that does not cross-react with free ubiquitin molecules (FK2, 0.5 μl) overnight at 4°C on a rotator. The next day 20 μl of protein G plus agarose (Santa Cruz Biotechnology, Inc.) was added and further incubated for 1 h at 4°C on a rotator. Immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4°C. Pellets were washed four times with 1 ml of PBS followed by a centrifugation step. After a final wash pellets were resuspended with 40 μl of sample buffer, boiled for 5 min at 95°C, and centrifuged to discard beads. Immune complexes were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were probed as described above.

Data Analysis. One-way analysis of variance followed by Bonferroni corrections (for multiple comparisons among all groups) was used for the statistical analyses of antidepressant drug effects.

Results

Antidepressant concentrations used in the present study were selected from dose-response experiments and represent the minimal concentrations inducing a maximal response. A representative dose-response relationship for the effect of 3 days of citalopram treatment on cytosolic β-arrestin2 levels in C_6 rat glioma cells is presented in Fig. 1A. Treatment of C_6 rat glioma cells with antidepressants (ADs) of various types (serotonin selective, norepinephrine selective, and nonselective reuptake inhibitors, as well as a monoamine oxidase inhibitor) for 3 days, resulted in decreased cytosolic β-arrestin2 protein levels (Fig. 1B). In contrast, mRNA expression of β-arrestin2 was found to be unregulated by ADs as shown by RT-PCR analysis (Fig. 1C).

To unravel the mechanism underlying the contrasting effects of ADs on β-arrestin2 protein and mRNA levels, several possible β-arrestin2 post-transcriptional events and modifications were examined as follows:

1. To reject the possibility of β-arrestin1 involvement in AD-induced alterations in β-arrestin2 levels, C_6 rat glioma cells were transfected with β-arrestin1-targeted shRNA or with scrambled shRNA, and the impact of

β-arrestin1 knockdown on β-arrestin2 protein levels was measured by Western blot analysis. Figure 2A clearly shows that β-arrestin1 knockdown is due to the knockdown itself and not an experimental method because the scrambled shRNA did not affect β-arrestin1 levels either in the presence or absence of various ADs. β-Arrestin1 knockdown did not affect the ability of ADs to lower β-arrestin2 protein levels (Fig. 2B), indicating that this protein is not involved in AD effects on β-arrestin2.

2. The AD-induced decrease in cytosolic β-arrestin2 protein levels was not due to cytosolic-membrane translocation, as the antidepressant citalopram did not cause any change in membrane β-arrestin2 protein levels (Fig. 3).

3. ADs were able to increase coimmunoprecipitation of ubiquitin with β-arrestin2 (Fig. 4), indicating that ADs increased ubiquitinylation of β-arrestin2.
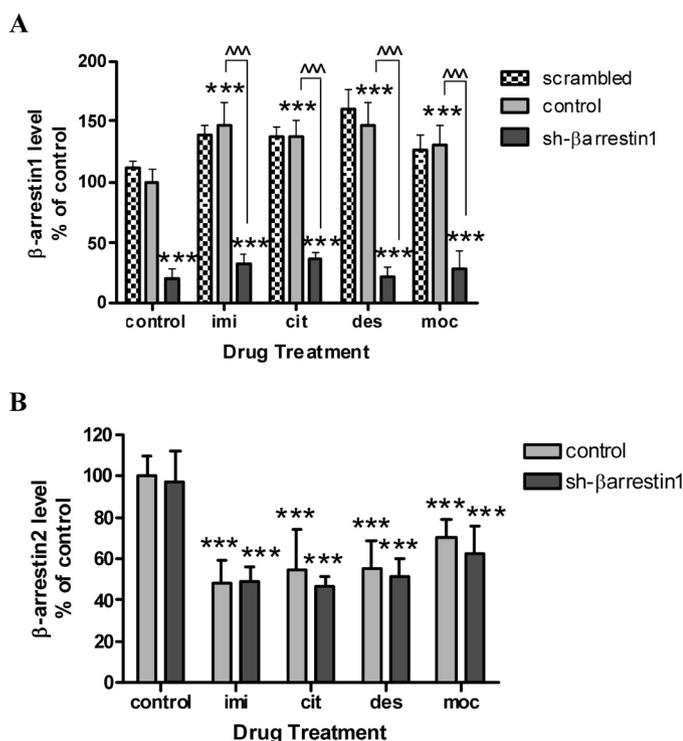
The AD-induced ubiquitin-mediated degradation of β-arrestin2 suggests a mechanism for the post-transcriptional decrease in cytosolic β-arrestin2 protein levels induced by ADs. To verify this line of evidence, inhibition of protein proteasomal degradation by the proteasome inhibitor MG-132 was undertaken. Indeed, MG-132 caused inhibition of protein proteasomal degradation, as indicated by the accumulation of ubiquitinylated proteins in MG-132-treated cells (Fig. 5A). Furthermore, MG-132, as expected, abolished the ability of ADs to induce a decrease in cytosolic β-arrestin2 protein levels (Fig. 5B). These findings point to the involvement of the proteasomal pathway in the AD-induced post-transcriptional decrease in cytosolic β-arrestin2 protein levels through ubiquitinylation in C_6 rat glioma cells.

Discussion

In the present study, we show that β-arrestin2 protein levels in C_6 rat glioma cells were decreased, whereas its mRNA levels were increased by ADs. To unravel the mechanism for these opposite effects several possible β-arrestin2 post-transcriptional events or modifications were examined. Previous studies have demonstrated that ADs induced increases in β-arrestin1 protein and mRNA levels in rat brain (Avissar et al., 2004) and human mononuclear leukocytes (Avissar et al., 2004; Matuzany-Ruban et al., 2005). To reject the possibility of β-arrestin1 influences on AD-induced alterations in β-arrestin2 levels, C_6 rat glioma cells were transfected with β-arrestin1-
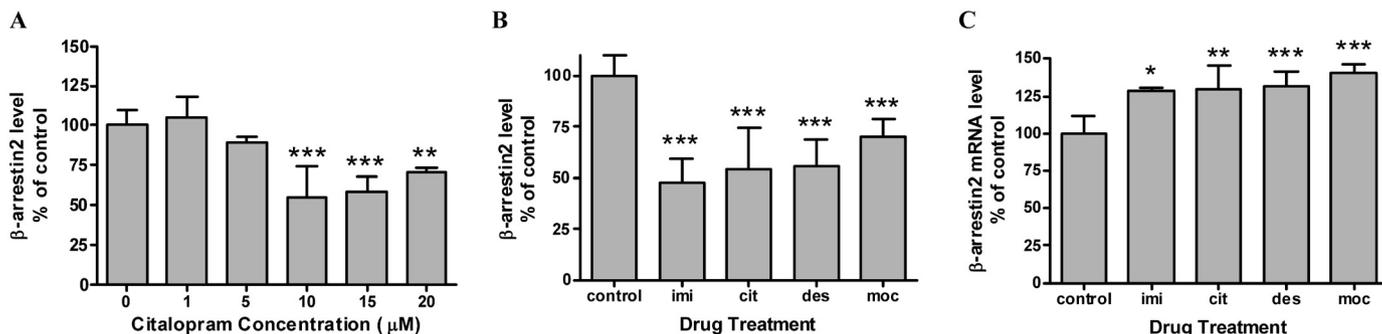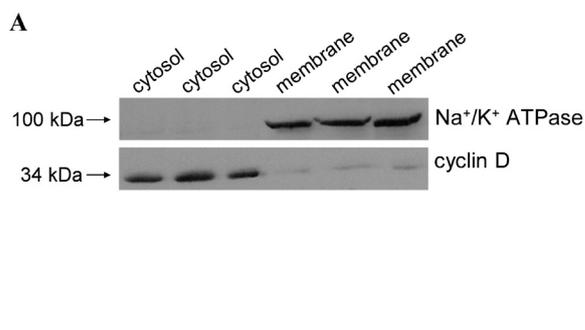
Fig. 1. Inverse AD effects on β-arrestin2 protein and mRNA levels. Twenty-four hours after C_6 cell seeding, growth medium was replaced with fresh medium containing the following drugs: 10 μM citalopram (cit), 10 μM imipramine (imi), 10 μM desipramine (des), or 20 μM moclobemide (moc). Drug-containing medium was replaced every day for 3 days to achieve chronic treatment. A, a representative dose-response experiment for the effect of citalopram treatment on cytosolic β-arrestin2 levels. B, antidepressant treatments decrease cytosolic β-arrestin2 protein levels. C, antidepressant treatments increase β-arrestin2 mRNA levels. Protein and mRNA levels are given as a percentage of control levels; Data are means ± S.D. of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control cells.

Fig. 4. Chronic AD treatment effect on β-arrestin2 ubiquitinylation. Twenty-four hours after C_6 cell seeding, growth medium was replaced with fresh medium containing the desired drug: 10 μM citalopram (cit), 10 μM imipramine (imi), 10 μM desipramine (des), or 20 μM moclobemide (moc). Drug-containing medium was replaced every day for 3 days to achieve chronic treatment. Cells were lysed and immunoprecipitated with an ubiquitin (Ub)-specific antibody (FK2). Western blots were performed with antibody to detect immunoprecipitated β-arrestin2. Six percent of the total lysate used for immunoprecipitation was loaded as input signal. Protein levels are given as a percentage of control levels. Data are means ± S.D. of at least three independent experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$, compared with control cells.

To our knowledge, this is the first report on antidepressant-induced β-arrestin2 ubiquitinylation.

Previous studies have demonstrated that ubiquitinylation of other proteins is selectively increased by antidepressants. Expression of the *kf-1* gene, which mediates ubiquitin-conjugating enzyme-dependent ubiquitinylation, is elevated in rat brain after chronic antidepressant treatments (Yamada et al., 2000). Mice lacking the *kf-1* gene were suggested to provide an animal model for elucidating molecular mechanisms of anxiety/depression and for screening novel anxiolytic/antidepressant compounds (Tsujimura et al., 2008). Hypericin was found to enhance ubiquitinylation of heat shock protein 90, constituting a mechanism for generating mitotic cell death in cancer cells (Blank et al., 2003).

Glial cells, especially astrocytes, the most abundant cells in human brain, are dynamic partners with neurons in synaptogenesis and with adult neural progenitor cells in neurogenesis (Christopherson et al., 2005; Nishida and Okabe, 2007). A growing body of evidence from human postmortem studies have shown modifications in corticolimbic glial cell density (Ongür et al., 1998; Rajkowska
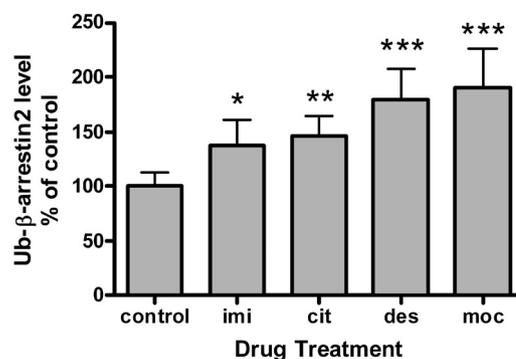

Fig. 2. AD-induced decreases in β-arrestin2 protein levels in β-arrestin1 knockdown C_6 cells. C_6 cells were transfected with either control shRNA (scrambled) or β-arrestin1-specific shRNA. Stably transfected cells were treated with the desired drug: 10 μM citalopram (cit), 10 μM imipramine (imi), 10 μM desipramine (des), or 20 μM moclobemide (moc). Drug-containing medium was replaced every day for 3 days to achieve chronic treatment. Protein levels were measured using Western blot analysis. A, β-arrestin1. B, β-arrestin2. Protein levels are given as a percentage of relevant control levels. Data are means ± S.D. of at least four independent experiments. ∗∗∗, $P < 0.001$ compared with control cells; ∧∧∧, $P < 0.001$, compared with indicated column.

targeted shRNA or with scrambled shRNA, and the impact of β-arrestin1 knockdown on β-arrestin2 protein levels was measured. β-Arrestin1 is not the primary target for ADs because its knockdown did not affect ability of ADs to lower β-arrestin2 protein levels. On the other hand, ADs were able to increase coimmunoprecipitation of ubiquitin with β-arrestin2. AD-induced β-arrestin2 ubiquitinylation led to its degradation by the proteasomal pathway, as MG-132 abolished the ability of ADs to induce a decrease in cytosolic β-arrestin2 protein levels.

Fig. 3. Citalopram does not induce β-arrestin2 membrane translocation. Twenty-four hours after C_6 cell seeding, growth medium was replaced with fresh medium containing 10 μM citalopram (cit). Drug-containing medium was replaced every day for 3 days to achieve chronic treatment. A, purity of the different fractions was verified using the proper markers as described under *Materials and Methods*. B, β-arrestin2 protein levels in the cytosol and membrane fractions of C_6 cells. Protein levels are given as a percentage of control cytosolic levels. Data are means ± S.D. of at least three independent experiments.
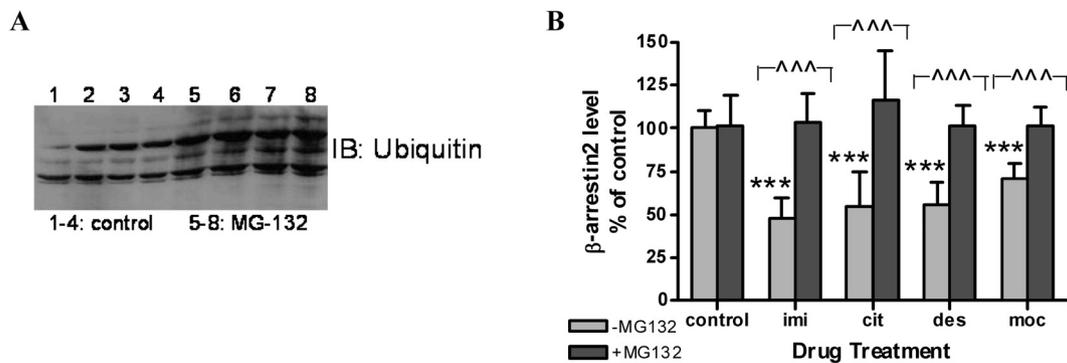
A

B

Fig. 5. MG-132 abolished AD effects on β-arrestin2. Twenty-four hours after C_6 cell seeding, growth medium was replaced with fresh medium containing the desired drug: 10 μM citalopram (cit), 10 μM imipramine (imi), 10 μM desipramine (des), or 20 μM moclobemide (moc) in the presence or absence of 0.1 μM MG-132. Drug-containing medium was replaced every day for 3 days to achieve chronic treatment. A, MG-132 inhibits degradation of ubiquitinylated proteins. B, the cytosol of C_6 cells was obtained by fractionation and analyzed by Western blot for effects of proteasome inhibition on β-arrestin2. Protein levels are given as a percentage of control levels. Data are means ± S.D. of at least three independent experiments. ∗∗∗, $P < 0.001$ compared with control cells; ∧∧∧, $P < 0.001$, compared with indicated column. IB, immunoblot.

and Miguel-Hidalgo, 2007) and morphology and expression of glial fibrillary acidic protein (Rajkowska and Miguel-Hidalgo, 2007), associated with major depressive disorder. Importantly, studies on laboratory animal models of stress and mood disorders have also supported the concept of an astrocytic deficit in depression (Leventopoulos et al., 2007).

Fluoxetine (Czéh et al., 2006) and clomipramine (Liu et al., 2009) treatments reversed glial pathology in a chronic unpredictable stress-induced animal model of depression. ADs were found to normalize elevated S100B protein, suggested as a serum marker of glial pathology in major depression (Schroeter et al., 2008). Moreover, ADs were found to increase glial cell line-derived neurotrophic factor production in C_6 rat glioma cells (Hisaoka et al., 2007).

Relatively few studies have addressed β-arrestin function in glia. The existence of β-arrestin-dependent signaling to ERK has been demonstrated for many GPCRs in different cell types and was recently demonstrated in immortalized and primary astrocyte cultures (McLennan et al., 2008; Miyatake et al., 2009). The present study is the first report on AD effects on β-arrestins in a glioma cell line.

A role for monoaminergic mechanisms in the pathogenesis of mood disorders has been formulated in the catecholamine, indoleamine, and other biogenic amine hypotheses (for review see Golan et al., 2009). These hypotheses did not provide an adequate explanation for the lag period of 10 to 20 days in the therapeutic actions of ADs. Thus, in the mid-1970s, theories on the mode of action of ADs shifted the emphasis from acute presynaptic events to delayed postsynaptic adaptations at the level of receptors. ADs are well known for their potential to modulate the density of functional neurotransmitter receptors such as β-adrenergic receptors in the brain (Banerjee et al., 1977; Peroutka and Snyder, 1980), as well as in cultured cells (Honegger et al., 1986; Fishman and Finberg, 1987). The mechanisms for this reduction in receptor numbers are not completely understood. More importantly, the onset of both down-regulation and clinical effectiveness requires 10 to 20 days of AD treatment. It has been proposed that the reduction in the number of functional β-adrenergic and other receptors could be a regulatory response to the enhanced presence of the neurotransmitter in the synaptic

cleft. The elevated concentration of the neurotransmitter is induced by acute inhibition of its reuptake or of monoamine oxidase activity by ADs (Banerjee et al., 1977; Wolfe et al., 1978). However, some clinically effective ADs neither influence norepinephrine or serotonin reuptake nor inhibit MAO activity but still cause receptor down-regulation, e.g., iprindole and rolipram (Hindmarch, 2001). Decreased monoamine receptors densities after treatment with ADs can also be seen in cell culture systems lacking a presynaptic input (Fishman and Finberg, 1987). Thus, monoamine receptor down-regulation may result directly from postsynaptic actions of ADs.

C_6 glioma cells endogenously express $β_1$- and $β_2$-adrenergic receptors as well as 5-HT$_{2}$A receptors, which are, respectively, desensitized by catecholamines (norepinephrine and epinephrine) (Guerrero et al., 1995) and by indoleamines (5-HT) (Hanley and Hensler, 2002). 5-HT-induced desensitization of the 5-HT$_{2}$A receptor in C_6 glioma cells was found to involve receptor internalization through a clathrin- and dynamin-dependent process because it was prevented by concanavalin A and monodansylcadaverine and by expression of the dominant-negative mutants of β-arrestin and dynamin (Hanley and Hensler, 2002). Antidepressants are known to cause β-adrenergic (Fishman and Finberg, 1987; Prenner et al., 2007) and 5-HT$_{2}$A receptor (Kagaya et al., 1996) desensitization in C_6 glioma cells. Unlike neurons in rat brain or primary neuron culture, there are no synaptic structures formed between C_6 cells; thus, any alteration observed after drug treatment represents a direct postsynaptic effect. Therefore, receptor desensitization effects of antidepressants in these cells are postsynaptic and are not due to elevated concentrations of monoamines. Accordingly, the effect of ADs shown in the present study of reduced β-arrestin2 protein levels through AD-induced increases in β-arrestin2 ubiquitinylation, leading to its degradation by the proteasomal pathway, should also be considered a pure postsynaptic effect of ADs. ADs are supposedly acting at an intracellular site not likely to be mediated by actions at noradrenaline/serotonin transporters. As basic lipophilic compounds (Daniel, 2003) ADs can cross the plasma membrane and therefore could have direct intracellular actions such as the effects presented in this study on β-arrestin2.

It is known that β-arrestin2 forms complexes with individual members of a particular MAP kinase cassette, retaining the activated MAP kinase in the cytoplasm, thereby directing phosphorylation of specific cytoplasmic substrates (DeFea et al., 2000; Luttrell et al., 2001). Coordinately, it inhibits phosphorylation of nuclear transcription factors, thus inhibiting ERK-dependent transcription (Tohgo et al., 2003). This ability of β-arrestin2 to retain activated scaffolded MAP kinases in the cytoplasm is apparently related to the presence of a leucine-rich nuclear export signal in the C terminus of β-arrestin2 but not of β-arrestin1 (Scott et al., 2002; Wang et al., 2003). Accordingly, a model for the antidepressant mechanism of action is suggested: before antidepressant treatment β-arrestin2 binds activated scaffolded MAP kinases and retains them in the cytoplasm, thereby inhibiting the activation of nuclear transcription factors. Antidepressant treatments, as described in the present study, reduce the levels of β-arrestin2 in the cytosol, thus enabling translocation of these regulatory proteins to the nucleus where they are able to affect transcription processes.

The findings on the involvement of β-arrestin1 in the pathophysiology and treatment of depression (Avissar et al., 2004; Matuzany-Ruban et al., 2005; Golan et al., 2009), together with the present findings on AD effects on β-arrestin2, may have extended implications concerning a possible switch in postreceptor signaling related to tyrosine kinases, Src, and MAP kinase and a variety of other signaling molecules that may characterize major depressive disorder or be induced by antidepressant treatment. Recent findings on reduced activation and expression of ERK1/2 in postmortem brain of depressed suicidal subjects (Dwivedi et al., 2001) and on MAP kinase activation by fluoxetine in cultured rat astrocytes (Mercier et al., 2004) support this suggestion. It is presently not known whether the effects of ADs on β-arrestins described in the present study are directly related to these proteins as molecular targets for ADs or secondary to AD effects elsewhere in the intricate signaling pathway. Similarly it is still not known whether the plethora of AD effects on signal transduction elements in which β-arrestins serve as signaling scaffold proteins and on transcription factors and cofactors in which β-arrestins serve in mediated regulation of transcription are directly related to AD effects on β-arrestins as target sites. In any case, the emergence of G protein-independent signaling pathways, through β-arrestins, changes the way in which GPCR signaling is evaluated, from a cell biological to a pharmacological and pharmaceutical perspective and raises the possibility of the development of pathway-specific therapeutic and monitoring tools, e.g., antidepressant medications targeting β-arrestin regulatory and signaling proteins (Golan et al., 2009).

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