Clathrin-Independent Endocytosis of $GABA_A$ Receptors in HEK 293 Cells†

Hulusi Cinar‡ and Eugene M. Barnes, Jr.*,‡,§

Division of Neuroscience and Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030

Received May 18, 2001; Revised Manuscript Received August 14, 2001

ABSTRACT: The endocytosis of $GABA_A$ receptors was investigated in HEK 293 cells expressing receptor $\alpha1\beta2$- and $\alpha1\beta2\gamma2$-subunit combinations. For assessment of internalized receptors by radioimmunoassay or immunofluorescence, a triple c-myc epitope was introduced into the amino terminus of the $\beta2$ subunit. An assay based on biotin inaccessibility was used for $\alpha1$ subunits. $GABA_A$ $\alpha1\beta2$- and $\alpha1\beta2\gamma2$-subunit receptors were internalized with a $t_{1/2}$ of 5.5 min at 37 °C. With both subunit combinations, phorbol 12-myristate 3-acetate enhanced internalization by nearly 100%. Treatment of the cells with hypertonic sucrose prevented both the basal and phorbol ester-induced endocytosis of $GABA_A$ receptors. GF 109203X, an inhibitor of protein kinase C, blocked the stimulation by phorbol ester but had no detectable effect on basal receptor endocytosis. Coexpression with a dominant-negative mutant of dynamin (K44A) led to a 100% enhancement of $GABA_A$ receptor internalization, while the endocytosis of β_2-adrenergic receptors was completely prevented. The results indicate that the endocytosis of $GABA_A$ $\alpha1\beta2$-subunit receptors in HEK cells is constitutive, positively modulated by activation of protein kinase C, and occurs by a mechanism that requires neither the participation of a $GABA_A$ receptor $\gamma2$ subunit nor a clathrin-mediated pathway.

$GABA_A$[1] receptors are the predominant transducers of fast inhibitory neurotransmission in the brain. By gating an integral Cl^- channel in response to GABA binding, $GABA_A$ receptors provide a shunt for postsynaptic membrane currents. $GABA_A$ receptors are heteropentameric complexes whose major isoforms in the vertebrate brain contain two α subunits, two β subunits, and one γ subunit. These three major families of subunits have several members, six α ($\alpha1-6$), four β ($\beta1-4$), and four γ ($\gamma1-4$) subunits, while other subunit classes have a single representative, ϵ, δ, π, and θ (*1*−*3*). $GABA_A$ receptors also have distinct binding sites for psychoactive modulators such as benzodiazepines, barbiturates, and neurosteroids.

It is well-known that occupancy of a neurotransmitter receptor by agonist induces not only signal transduction but also endocytic and other intracellular transport processes that may be important in the regulation of synaptic strength. In cultured neurons, prolonged occupancy of $GABA_A$ receptors by agonists results in a decrease in the density and function of the surface $GABA_A$ receptors, a process defined as downregulation (*4*). Internalization of $GABA_A$ receptors from the neuronal surface occurs in response to agonist exposure (*5*, *6*), and clathrin-dependent endocytosis has been implicated in this process (*7*, *8*). After heterologous expression in *Xenopus* oocytes or human embryonic kidney (HEK) 293 cells, $GABA_A$ receptors downregulate in response to activation of protein kinase C (PKC). Application of phorbol esters to oocytes expressing $GABA_A$ receptor $\alpha1\beta2$- or $\alpha1\beta2\gamma2$-subunit combinations produces a loss of receptor currents (*9*, *10*). Since this is accompanied by a similar decline in surface fluorescence from $GABA_A$ receptors tagged with green fluorescent protein, it was proposed that receptor internalization in oocytes is induced by PKC activity. Phorbol esters also increased the level of removal of $GABA_A$ receptors from the surface of HEK cells, but the presence of a $GABA_A$ receptor $\gamma2$ subunit was suggested as a requirement for this downregulation (*11*).

Because HEK cells have been extensively used for the study of $GABA_A$ receptor assembly and trafficking (*12*), we have used them for investigation of receptor endocytosis. On the cell exterior, $GABA_A$ receptors were tagged with biotin or with antibodies against epitopes inserted in the $\beta2$ subunit. The internalized fraction of these receptors was detected by biochemical and immunofluorescence techniques. Under these conditions, the endocytosis of $GABA_A$ $\alpha1\beta2$-subunit receptors occurs by a constitutive, clathrin-independent mechanism that is stimulated by PKC activity but does not require the presence of a $GABA_A$ receptor $\gamma2$ subunit. Preliminary reports of some of these findings have been presented previously (*13*, *14*).

EXPERIMENTAL PROCEDURES

Recombinant DNA. Full-length cDNAs of chicken $GABA_A$ receptor subunits were described previously [$\alpha1$ subunit (*15*), $\beta2S$ subunit (*16*), and $\gamma2L$ subunit (*17*)]. These were subcloned into the mammalian expression vector, pcDNA3.

† This work was supported by the National Institutes of Health (Grant NS34253 to E.M.B.).

* To whom correspondence should be addressed: Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4523. Fax: (713) 798-7854. E-mail: ebarnes@bcm.tmc.edu.

‡ Division of Neuroscience.

§ Department of Biochemistry and Molecular Biology.

[1] Abbreviations: β_2AR, β_2-adrenergic receptor; DAG, diacylglycerol; flu, flunitrazepam; GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; PKC, protein kinase C; PMA, phorbol 12-myristate 3-acetate; SE, standard error; TBPS, *tert*-butylbicyclophosphorothionate.

10.1021/bi011025t CCC: $20.00
Published on Web 10/23/2001

Where indicated, the β2S subunit was tagged with three c-myc epitopes ($\beta 2S^{3\times 9E10}$), positioned in tandem between the sixth and seventh amino acids of the mature peptide. These tandem epitopes were lifted by PCR cloning from plasmid pJBN48 provided by J. Bachant. The fidelity of the $\beta 2S^{3\times 9E10}$ construct was verified by DNA sequencing. The expression of functional $GABA_A$ receptors by transfected HEK cells was confirmed by binding of [^{35}S]-*tert*-butylbicyclophosphorothionate ([^{35}S]TBPS) and [^{3}H]flunitrazepam ([^{3}H]flu) to isolated membranes as described by Tehrani et al. (*7*). Plasmids bearing the wild type and the K44A mutant of human dynamin 1aa, tagged with influenza virus hemagglutinin (HA), were a gift from P. De Camilli. B. Knoll provided the β_2-adrenergic receptor (β_2AR) plasmid, β_2AR-pcDNA3.1.

Cell Culture and Transfection. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics, at 37 °C in a CO_2 incubator. Prior to transfection, cells were plated to a density of 2×10^4 cells/cm^2 on fibronectin-coated multiwell plates (Falcon) or 1×10^4 cells/cm^2 on fibronectin-coated glass coverslips. After 20−24 h in culture, the cells were treated with plasmid DNA (per plasmid, 0.1 μg of DNA/cm^2) and FUGENE reagent (0.3 μL/cm^2) according to the manufacturer's instructions (Boehringer Mannheim). Assays were performed 48 h after transfection unless noted otherwise.

^{125}I-Labeled Antibody Binding Internalization Assay. Affinity-purified 9E10 monoclonal antibody against c-myc (Covance, Richmond, CA) was labeled with ^{125}I to a specific activity of 800 Ci/mmol using Bolton-Hunter reagent (NEN Life Science). Saturation binding was studied with cells expressing $GABA_A$ receptor $\alpha 1\beta 2^{3\times 9E10}$ subunits. By Scatchard analysis, the apparent K_m for [^{125}I]9E10 antibody binding was 200 nM. For internalization assays, HEK cells in 12-well plates were incubated for 1 h at 4 °C with 4−10 nM [^{125}I]9E10 in internalization medium [IM (10% FBS, 0.5% BSA, and 25 mM glucose in PBS (pH 7.4) (*18*)]. This solution was aspirated, and the plates were washed once with ice-cold IM. After addition of 0.3 mL of IM containing test compounds at room temperature, the plates were transferred to a block heater at 37 °C for specified times. Control cells were kept at 4 °C until the end of the experiment. To terminate the assay, the medium was aspirated, 1 mL of ice cold IM was added, and plates were placed on a tilt table for 10 min at 4 °C. This washing procedure was repeated once more. Residual [^{125}I]9E10 antibody was stripped from the cell surface by two washes (10 min each, 4 °C) with 0.5% BSA in PBS adjusted to pH 1.5 with HCl. Supernatants from these washes (representing residual surface receptors) were pooled. The stripped cells were lysed in 1% SDS, and aliquots were counted in a γ-counter. A baseline correction using stripped cells held constantly at 4 °C was applied. ^{125}I-labeled antibody internalized at 37 °C, determined in triplicate from stripped cells, was represented as a percentage of the total bound (stripped cells + stripping medium). The level of nonspecific ^{125}I binding was determined with ^{125}I-labeled antibody that was preabsorbed with an excess of the c-myc peptide.

Biotin Inaccessibility Internalization Assay. Transfected cells in six-well plates were washed twice with ice-cold PBS (pH 8.0) and incubated with 1 mL of 1.0 mg/mL sulfo-NHS-SS-biotin (Pierce) in the same buffer for 30 min at 4 °C. After two washes with IM at 4 °C, 1 mL of IM containing test compounds at room temperature was added and the plates were placed on a heating block at 37 °C for 30 min. Cells then washed once with ice-cold IM on a tilt table for 10 min and then stripped of surface biotin by addition of 50 mM 2-mercaptoethanesulfonic acid (HSO_3EtSH, Sigma) in 100 mM NaCl, 2.5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.7 at 4 °C). After a 20 min wash at 4 °C, the stripping medium was removed. This stripping procedure was repeated twice more. After a 10 min incubation with 5 mg/mL iodoacetamide in PBS containing 1% BSA, the cells were washed with PBS and lysed in 2 mL of TBS extraction buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.1% SDS, and "Complete" protease inhibitor cocktail (Boehringer Mannheim, one tablet per 50 mL)]. The amount of protein in each extract was determined by a BCA assay (Pierce). After centrifugation for 10 min at 15000*g*, the clarified extracts were incubated with 50 μL of streptavidin−agarose beads (Sigma) for 45 min at room temperature. The beads were washed three times with TBS extraction buffer and once with 50 mM Tris-HCl (pH 7.4). The beads were extracted with SDS−PAGE sample buffer containing 0.1 M DTT, and the extracts were analyzed by Western blotting using RP4 antibody against the $GABA_A$ receptor α1 subunit as described by Miranda and Barnes (*19*). ECL kits (Amersham) were used for chemiluminescent detection.

Immunofluorescence Microscopy. After being washed with PBS, transfected cells on coverslips were incubated with 40 μg/mL 9E10 monoclonal antibody in IM for 1 h at 4 °C. After the cells had been washed twice with ice-cold IM, test compounds in IM were added at room temperature, and the coverslips were placed on a heating block at 37 °C for 30 min. The cells were washed once with ice-cold IM and once with ice-cold PBS. The subsequent steps were performed at room temperature. The cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed, and then quenched with 0.34% L-lysine and 0.05% sodium periodate in PBS for 20 min. The coverslips were incubated first with PBS containing 0.05% Triton X-100, 10% FBS, and 0.5% BSA for 30 min and then (as indicated) with rabbit anti-HA polyclonal antibody (2 μg/mL; Santa Cruz Biotechnology). After being washed, the cells were incubated with either a goat anti-rabbit IgG−Texas red (5 μg/mL) or horse anti-mouse IgG−FITC conjugate (5 μg/mL). The coverslips were washed three times and mounted on slides with Mowiol. Data from 0.15 μm optical sections was collected with a Zeiss LSM510 laser confocal scanning microscope.

Internalization Assay for β2-Adrenergic Receptors. Assays for endocytosis of β_2ARs were carried out as described by Moore et al. (*20*). HEK cells transfected with β_2ARs were incubated with 10 μM isoproterenol (omitted in controls) in DMEM-H [DMEM containing 20 mM HEPES (pH 7.4)] at 37 °C for 30 min, washed four times with ice-cold buffer, and then incubated with 6 nM [^{3}H]CGP12177 in DMEM-H at 4 °C for 90 min to measure the levels of surface receptors. The cells were washed twice with DMEM-H, lysed in 0.1% Triton X-100, and counted by liquid scintillation. The level of nonspecific binding was defined with 3 μM propranolol. The isoproterenol-dependent decline in the level of ligand binding, representing β_2AR internalization, was expressed

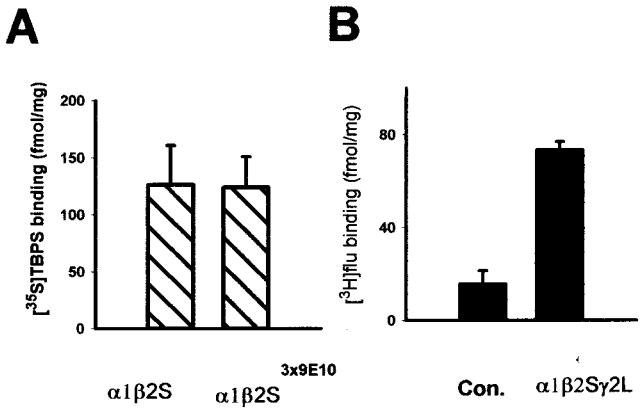

FIGURE 1: Ligand binding to recombinant $GABA_A$ receptors. Membranes from HEK cells expressing the indicated receptor subunits were assayed for ligand binding as described in Experimental Procedures. (A) Specific binding of [^{35}S]TBPS (10 nM). Results are means ± SE from three preparations. (B) Specific binding of [^{3}H]flu (2.5 nM). Results are means ± SE from three experiments.

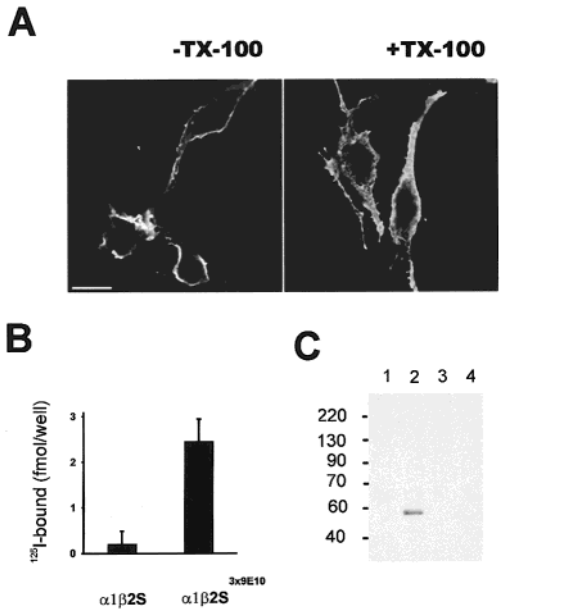

FIGURE 2: Surface expression of recombinant $GABA_A$ receptors. (A) Immunofluorescence assay. Cells expressing $\alpha1\beta2S^{3\times9E10}$ subunits were incubated at 4 °C with 9E10 primary antibody, washed and fixed, and then labeled with a horse anti-mouse IgG−FITC conjugate and processed for confocal immunofluorescence microscopy as described in Experimental Procedures. Secondary antibody was added to unpermeabilized (left panel) or Triton X-100-permeabilized (right panel) cells. Untransfected cells did not exhibit significant fluorescence (not shown). The scale bar is 10 μm long. The results are from an experiment that was repeated twice. (B) Radioimmunoassay. Cells, transfected as indicated, were incubated with ^{125}I-labeled 9E10 antibody for 1 h at 4 °C and then washed, harvested, and assayed for ^{125}I as described in Experimental Procedures. The results are means ± SE of three experiments. (C) Biotinylation assay. Cells expressing the indicated $GABA_A$ receptor subunits were incubated with sulfo-NHS-SS-biotin at 4 °C for 30 min. Cell extracts were purified on streptavidin−agarose beads and analyzed by Western blotting using RP4 antibody against the $GABA_A$ receptor α1 subunit as described in Experimental Procedures: lane 1, $\alpha1\beta2S$ subunits, unbiotinylated control; lane 2, $\alpha1\beta2S$ subunits, biotinylated; lane 3, $\beta2S$ subunit only, biotinylated; and lane 4, untransfected, biotinylated. Molecular weight markers are shown at the left. The results are from an experiment that was repeated twice.

as a percentage of the level of specific binding to untreated cells.

RESULTS

Characterization of Recombinant $GABA_A$ Receptors. Chicken $GABA_A$ receptor α1, $\beta2S$, and $\gamma2L$ subunit cDNAs were transiently transfected into HEK 293 cells. To facilitate immunochemical analyses, the $GABA_A$ receptor $\beta2S$ subunit was tagged using three tandem epitopes of the anti-c-myc monoclonal antibody 9E10. These epitopes were inserted by PCR cloning into the $\beta2S$ subunit between amino acids 6 and 7 of the mature subunit to create a $\beta2S^{3\times9E10}$ subunit. The functional effects of this modification were tested by binding of [^{35}S]TBPS to membranes from transfected HEK cells. $GABA_A$ receptors containing $\alpha1\beta2S^{3\times9E10}$-subunit combinations had levels of [^{35}S]TBPS binding that were comparable to those of $\alpha1\beta2S$-subunit receptors (Figure 1A). This indicates that the epitope insertion in the $\beta2S^{3\times9E10}$ subunit did not impair the assembly of the $GABA_A$ receptor Cl^- channel. Since the presence of a γ subunit is a prerequisite for formation of the $GABA_A$ receptor benzodiazepine-binding site (*1*), incorporation of the $\gamma2$ subunit into the recombinant receptors could be verified by [^{3}H]flu binding to membranes from cells expressing an $\alpha1\beta2S\gamma2L$-subunit combination (Figure 1B). The level of [^{3}H]flu binding to $\alpha1\beta2S^{3\times9E10}\gamma2L$-subunit receptors (78 ± 15 fmol/mg of membrane protein) was nearly identical (not shown).

Surface Expression of $GABA_A$ Receptors. The subcellular distribution of the $GABA_A$ receptors expressed in HEK cells was determined by indirect immunofluorescence confocal microscopy (Figure 2A). After application of the 9E10 antibody to unpermeabilized cells coexpressing α1 and $\beta2S^{3\times9E10}$ subunits, only plasma membranes were stained (left panel), while prominent intracellular staining was observed after permeabilization (right panel). This is consistent with previous findings (*21*). Surface expression of $GABA_A$ receptors was also examined by radioimmunoassay. The [^{125}I]-9E10 antibody was bound to cells transfected with $\alpha1\beta2S^{3\times9E10}$ subunits, but not to those expressing $\alpha1\beta2S$ subunits (Figure 2B). In a third approach, HEK cells containing $\alpha1\beta2$-subunit receptors were incubated at 4 °C with sulfo-NHS-SS-biotin, an impermeant, SH-cleavable reagent. Cell extracts were absorbed with streptavidin beads, and eluates from the beads were analyzed on Western blots probed with the RP4 antibody directed against the $GABA_A$ receptor α1 subunit. As shown in Figure 2C, α1 subunits (54 kDa polypeptide) were biotinylated extracellularly in cells expressing $\alpha1\beta2S$ subunits. However, these subunits were not recovered from unbiotinylated control cells or from biotinylated cells that were untransfected or mock-transfected. The recovery of biotinylated α1 subunits from cells expressing $\alpha1\beta2S$ and $\alpha1\beta2S^{3\times9E10}$ subunits was similar (data not shown), suggesting that the epitope tag does not introduce abnormalities in the surface targeting of $GABA_A$ receptors. Due to a much lower sensitivity, it was not possible to reliably detect $\beta2S^{3\times9E10}$ subunits on immunoblots using the 9E10 antibody.

Constitutive Endocytosis of $GABA_A$ Receptors. To measure the apparent rates of $GABA_A$ receptor internalization, HEK cells transfected with $\alpha1\beta2S^{3\times9E10}$ and $\alpha1\beta2S^{3\times9E10}\gamma2L$ subunits were incubated with the [^{125}I]9E10 antibody at 4 °C and then placed at 37 °C for the time shown in Figure 3A. Then the cells were stripped of ^{125}I-labeled antibody that

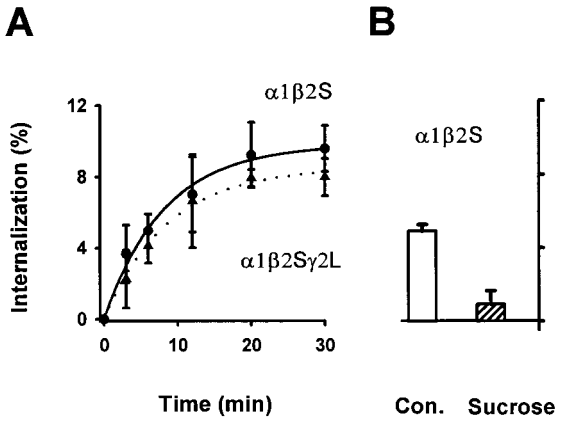

FIGURE 3: Quantitation of GABA$_A$ receptor internalization with ^{125}I-labeled 9E10 antibody. (A) Cells transfected with α1β2S$^{3\times9E10}$ or α1β2S$^{3\times9E10}\gamma$2L subunits were labeled with ^{125}I-labeled 9E10 antibody as described in the legend of Figure 2B, and then placed in fresh medium at 37 °C for the times shown. After the cells had been stripped at pH 1.5, cell extracts and stripping medium were counted as described in Experimental Procedures. The ^{125}I associated with stripped cells is expressed as a percentage of the total activity per well, corrected for binding to cells held continuously at 4 °C. The results are means ± SE from three experiments. The lines through the data are single exponentials ($t_{1/2}$ = 5.5 min). (B) Cells were surface labeled and incubated at 37 °C with 0.45 M sucrose (as indicated) for 6 min as in panel A. The results are means ± SE from three experiments.

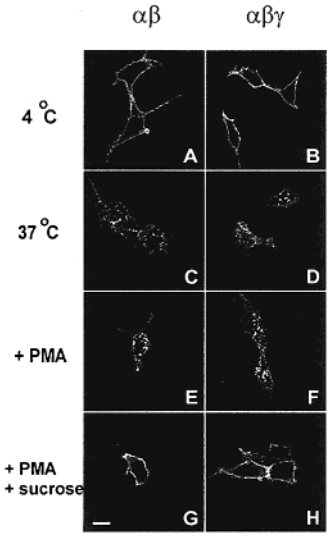

FIGURE 4: Immunofluorescence assay of GABA$_A$ receptor internalization. Cells transfected with α1β2S$^{3\times9E10}$ or α1β2S$^{3\times9E10}\gamma$2L subunits were incubated with 9E10 antibody for 1 h at 4 °C and then with fresh medium containing 150 nM PMA or 0.45 M sucrose (as indicated) at 37 °C for 30 min. Fixed, Triton-treated cells were processed for confocal immunofluorescence microscopy as in Figure 2A: left, α1β2S$^{3\times9E10}$ subunits; and right panels, α1β2S$^{3\times9E10}\gamma$2L subunits. The scale bar is 10 μm long.

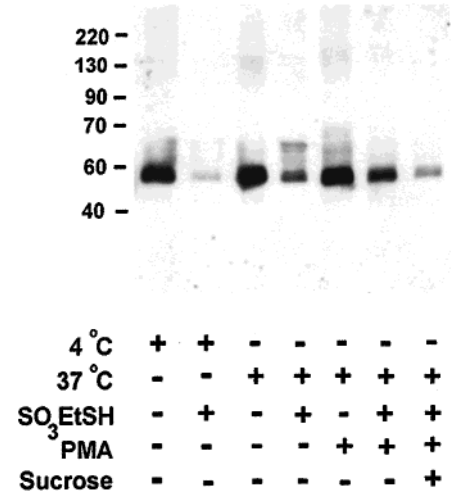

FIGURE 5: Assay of GABA$_A$ receptor internalization by biotin inaccessibility. Cells expressing α1β2S subunits were biotinylated as in Figure 2C and then incubated for 30 min at 37 °C in medium containing 0.45 M sucrose or 150 nM PMA (as indicated). Surface biotin was removed by treatment with HSO_3EtSH as described in Experimental Procedures. Cell extracts were analyzed as described in the legend of Figure 2C. Molecular weight markers are shown at the left.

remained at the surface by washing at pH 1.5. The internalized fraction of ^{125}I-labeled antibody increased over a period of 15−20 min. A fit of the data to single-exponential functions (Figure 3A) yielded $t_{1/2}$ values of 5.5 min for internalization of both α1β2S$^{3\times9E10}$- and α1β2S$^{3\times9E10}\gamma$2L-subunit receptors. Likewise, the extent of receptor internalization (8−9% of the total) after 30 min was similar for both subunit combinations. To evaluate the possible contribution of recycling of receptor−antibody complexes to the steady-state level of internalization, 30 min incubations were carried out in the presence of 300 μM chloroquine or 0.25 μM bafilomycin A. However, neither of these recycling inhibitors produced a detectable effect on GABA$_A$ receptor internalization (not shown).

Consistent with a process of endocytosis, more than 85% of GABA$_A$ receptor internalization was blocked by treatment of the cells with hypertonic sucrose (Figure 3B). But the internalization of GABA$_A$ receptors was not significantly affected by addition of GABA to the medium (not shown). Exposure to filipin (5 μg/mL) or to 12 mM methyl-β-cyclodextrin also had no detectable effect (not shown).

To assess internalization morphologically, cells expressing α1β2S$^{3\times9E10}$ and α1β2S$^{3\times9E10}\gamma$2L subunits were labeled as in Figure 2A and incubated in fresh medium at 37 °C for 30 min. Then the cells were fixed, permeabilized, and stained as before. As shown in panels A and B of Figure 4, fluorescence was confined to the surface of cells held continuously at 4 °C, but at 37 °C punctate intracellular staining was apparent with both α1β2S$^{3\times9E10}$- and α1β2S$^{3\times9E10}$-γ2L-subunit receptors (Figure 4C,D). The extent of vesicular immunofluorescence was similar with both combinations.

To avoid possible pitfalls due to antibody-induced receptor clustering and the resulting endocytosis (*22*), cells expressing α1β2S subunits were tagged with sulfo-NHS-SS-biotin as in Figure 2C and then the receptors were internalized by incubation at 37 °C for 30 min. After the residual surface biotin had been stripped with an impermeant reducing agent, HSO_3EtSH, cell extracts were affinity-purified and analyzed for GABA$_A$ receptor α1 subunits as before. In cells held continuously at 4 °C, nearly all of the biotin-labeled α1 subunits were SH-stripped, while a readily detectable fraction of this label became inaccessible after 30 min at 37 °C (Figure 5). Although an accurate quantitation of chemiluminescence from these immunoblots was not possible, to a rough approximation the amounts of GABA$_A$ receptor internalization by biotin assay were comparable to those obtained by radioimmunoassay (Figure 3A).

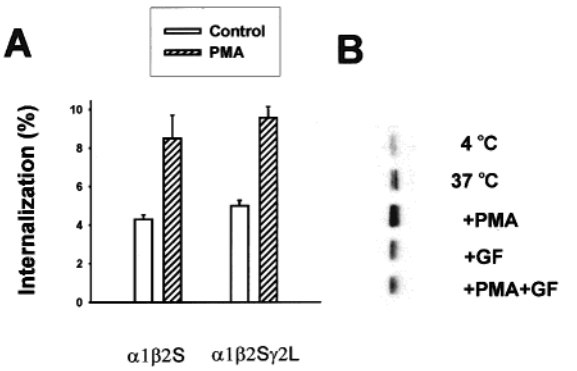

FIGURE 6: PKC activation increases the level of $GABA_A$ receptor internalization. (A) The amount of [^{125}I]9E10 antibody internalized by cells expressing $\alpha1\beta2S^{3\times9E10}$ or $\alpha1\beta2S^{3\times9E10}\gamma2L$ subunits was determined as described in the legend of Figure 3B. PMA (150 nM) was added as indicated. The results are means ± SE from three experiments. (B) Cells expressing $\alpha1\beta2S$ subunits were biotinylated, incubated with 1 μM GF109203X or 150 nM PMA as indicated, and then stripped and assayed as described in the legend of Figure 5. The results are from a typical experiment that was repeated five times.

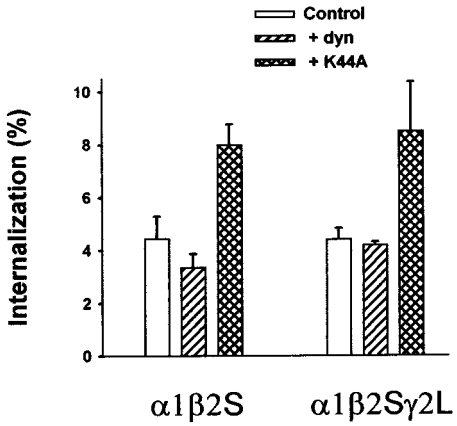

FIGURE 7: K44A dynamin stimulates $GABA_A$ receptor internalization. Cells expressing $\alpha1\beta2S^{3\times9E10}$ or $\alpha1\beta2S^{3\times9E10}\gamma2L$ subunits were cotransfected as indicated with wild-type or K44A dynamin. The receptors were tagged with ^{125}I-labeled antibody, incubated, and processed as described in the legend of Figure 3B. The results are means ± SE from three experiments.

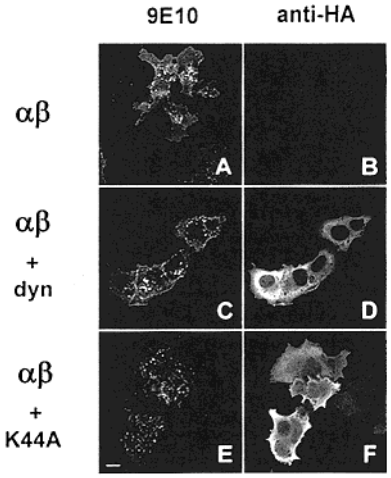

FIGURE 8: Dynamin constructs and $GABA_A$ receptors are expressed in the same HEK cells. Cultures cotransfected with $\alpha1\beta2S^{3\times9E10}$ subunits and HA-tagged wild-type (dyn) or K44A dynamin were tagged with 9E10 primary antibody and then incubated at 37 °C for 30 min with 150 nM PMA as indicated. The cells were fixed, incubated either with an anti-mouse IgG−FITC conjugate (left column) or with a rabbit anti-HA primary antibody followed by an anti-rabbit IgG−Texas red conjugate (right column) and processed for fluorescence microscopy as described in the legend of Figure 4.

PKC Stimulates $GABA_A$ Receptor Internalization. Because previous studies suggested the involvement of PKC in the intracellular trafficking of $GABA_A$ receptors, we examined the effects of phorbol 12-myristate 3-acetate (PMA), a potent activator of this protein kinase. When HEK cells expressing $\alpha1\beta2S^{3\times9E10}$ or $\alpha1\beta2S^{3\times9E10}\gamma2L$ subunits were incubated with PMA for 6 min at 37 °C, the amount of ^{125}I-labeled antibody internalization nearly doubled in comparison to controls (Figure 6A). A similar 92−99% enhancement in ^{125}I uptake was observed with a 30 min internalization period in the presence of PMA compared to controls (not shown).

Using immunofluorescence imaging, we observed larger and more intensely stained vesicles after PMA treatment (Figure 4E,F). Judged by biotin inaccessibility, PMA also increased the level of endocytosis of $GABA_A$ receptor $\alpha1$ subunits (Figure 5). To examine the mechanism of PMA action, we used GF109203X, a potent, specific inhibitor of PKC that competes with ATP at the catalytic site. As indicated in Figure 6B, GF109203X prevented the enhancement of $GABA_A$ receptor endocytosis by PMA, but alone the PKC inhibitor had little effect. Thus, as expected, the action of PMA on $GABA_A$ receptor endocytosis seems to be through activation of PKC. The basal levels of PKC-dependent protein phosphorylation make little contribution to receptor internalization.

$GABA_A$ Receptor Endocytosis Is Clathrin-Independent. To investigate the mechanism of $GABA_A$ receptor internalization, $\alpha1\beta2S^{3\times9E10}$- or $\alpha1\beta2S^{3\times9E10}\gamma2L$-subunit combinations were cotransfected with either wild-type dynamin or a dominant-negative mutant (K44A). It was previously shown that K44A dynamin blocks the endocytosis of many receptors and leads to the accumulation of clathrin-coated pits in the plasma membrane (*23*). Surprisingly, coexpression of K44A dynamin did not prevent internalization of $GABA_A$ receptors, but rather enhanced by 90−100% the endocytosis of both $\alpha1\beta2S^{3\times9E10}$- and $\alpha1\beta2S^{3\times9E10}\gamma2L$-subunit combinations (Figure 7). On the other hand, no significant effects were produced by receptor coexpression with wild-type dynamin. In Figure 7, a 6 min period of internalization was used to provide an estimate of the endocytic rate. Using a 30 min period to reflect the extent of $\alpha1\beta2S^{3\times9E10}$ subunit endocytosis at steady state (not shown), 13.8 ± 1.5% of surface receptors were internalized in cells coexpressing wild-type dynamin, while with K44A dynamin, 21.9 ± 1.2% of the receptors underwent endocytosis. Similar results were obtained with $GABA_A$ receptors formed from the $\alpha1\beta2S^{3\times9E10}$-$\gamma2L$-subunit combination.

In parallel immunofluorescence assays (Figure 8), the pattern of vesicular staining of internalized $GABA_A$ receptors was similar in controls and cotransfections with wild-type and K44A dynamin. The coexpression of dynamin in these cells was verified by the use of a polyclonal antibody against an HA epitope that the dynamin constructs carry in their N-termini (Figure 8). To show that K44A dynamin retained dominant-negative properties under these conditions, we cotransfected HEK cells with β_2-adrenergic receptors (β_2-ARs). As expected, K44A dynamin completely prevented the agonist-evoked endocytosis of β_2ARs (Figure 9). Col-

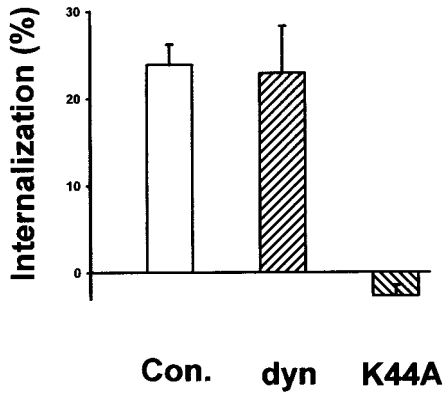

FIGURE 9: K44A dynamin prevents β_2-adrenergic receptor internalization. Cells cotransfected with β_2AR and either wild-type (dyn) or K44A dynamin were exposed to 10 μM isoproterenol for 30 min at 37 °C. The level of surface receptors was determined by binding of [^{3}H]CGP12177 at 4 °C as described in Experimental Procedures. The internalized fraction of ligand binding sites is expressed as a percentage of the total ligand bound. The results are means ± SE from three experiments.

lectively, these findings suggest that GABA$_A$ receptor endocytosis in HEK cells occurs by a clathrin-independent mechanism.

DISCUSSION

By transfection of both wild-type and epitope-tagged GABA$_A$ receptor subunits, we were able to directly assess receptor endocytosis by HEK 293 cells using a combination of biochemical and immunological techniques. We found that the endocytosis of a GABA$_A$ receptor $\alpha1\beta2$-subunit combination in these cells is constitutive, positively modulated by activation of PKC, and occurs by a mechanism that requires neither the participation of a GABA$_A$ receptor $\gamma2$ subunit nor a clathrin-mediated pathway. The constitutive internalization of GABA$_A$ receptors was suggested by previous studies of HEK cells (*11*), but this was inferred from measurements of the stability of surface receptors.

Through analysis of GABA$_A$ receptor currents and surface fluorescence of tagged subunits, PMA-induced receptor internalization was observed in *Xenopus* oocytes (*9*, *10*). By site-directed mutagenesis, it was shown that the PKC phosphorylation sites required for PMA activation of receptor internalization in oocytes do not reside on GABA$_A$ receptor subunits. Chapell et al. (*9*) also found a similar degree of PMA-dependent internalization in oocytes expressing GABA$_A$ receptor $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subunits. In concordance, we observed that both constitutive and PMA-induced endocytosis in HEK cells occurred to the same extent with $\alpha1\beta2$- and $\alpha1\beta2\gamma2$-subunit receptors. On the other hand, it has been reported that PMA-induced downregulation of GABA$_A$ receptors in HEK cells was obtained only by coexpression of an $\alpha1\beta2\gamma2$-subunit combination (*11*). Differences in methodology between the latter study and our own may account for this disagreement.

These effects of PMA cannot be attributed to a generalized process of membrane retrieval (cf. pinocytosis). The surface stability of nicotinic acetylcholine receptors on oocytes (*10*) or β_2ARs on HEK cells (*24*) is not perturbed by PMA.

In our study of HEK cells, PMA produced a similar enhancement of both the apparent rate and the extent of GABA$_A$ receptor internalization. If PMA were acting as an inhibitor of receptor recycling, a more pronounced effect level would be expected in the longer incubations. Furthermore, general inhibitors of recycling (chloroquine and bafilomycin) failed to enhance the internalization of GABA$_A$ receptor−antibody complexes in HEK cells. Accordingly, we favor the interpretation that PMA enhances GABA$_A$ receptor endocytosis.

PMA mimics the positive modulatory action of diacylglycerol (DAG) by binding to the same regulatory site on PKC (*25*). However, it was recently shown that phorbol ester responses previously attributed to PKC isozymes may proceed through PKC-independent pathways (*26*). Because of this new complexity, results obtained with agents that act through the DAG-binding site, including phorbol esters and PKC inhibitors such as calphostin C (*9*), should be interpreted cautiously. In contrast, GF109203X inhibits PKC by competing with ATP at the catalytic site (*27*), presumably distinguishing the PKC-specific effects of PMA from those in other signaling pathways. Since we found that GF 109203X blocked the activation of GABA$_A$ receptor endocytosis by PMA, PKC is implicated more clearly as a regulator of this process.

Since GABA$_A$ receptor endocytosis in HEK cells was not prevented by coexpression of the K44A dominant-negative mutant of dynamin, we conclude that this process follows a clathrin-independent pathway. In mammalian cells, expression of K44A dynamin blocks clathrin-dependent endocytosis and leads to the accumulation of clathrin-coated pits in the plasma membrane (*23*). Thus, K44A dynamin inhibits the endocytosis of most G-protein-coupled receptors (GPCRs), including β_2-adrenergic and M_1-muscarinic acetylcholine receptors in HEK cells (*28*, *29*). We used a similar inhibition of β_2AR endocytosis as a control in our study. In contrast, the endocytosis of several other GPCRs, including M_2-muscarinic (*30*, *31*), D2 dopamine (*32*), and α_{2B}-adrenergic receptors (*33*), proceeds unabated in cells coexpressing K44A dynamin. This dynamin mutant has thus become a diagnostic reagent for classification of clathrin-independent pathways of receptor endocytosis. Such pathways are not limited to GPCRs, as it was recently demonstrated that local recycling of synaptic vesicle components at nerve terminals involves a clathrin-independent endocytic process that is necessary for efficient synaptic transmission (*34*).

We actually observed a 100% increase in the constitutive endocytosis of GABA$_A$ receptors as a result of K44A dynamin coexpression. A comparable enhancement of D2 receptor endocytosis was also observed in HEK cells (*32*). It has been suggested that blockade of clathrin-dependent endocytosis with K44A dynamin may cause a compensatory enhancement in clathrin-independent pathways (*35*). The mechanism of this compensation is unknown.

We also found that exposure of HEK cells to hypertonic sucrose prevented both constitutive and PMA-induced GABA$_A$ receptor endocytosis. While it was originally thought that this inhibition is a unique characteristic of the clathrin-mediated endocytic pathway (*36*), later work suggests that this is not the case. The clathrin-independent endocytosis of secretin receptors (*37*) and M_2 receptors (*38*) is also blocked by hypertonic sucrose treatment. The lack of a significant effect of methyl-β-cyclodextrin or filipin on GABA$_A$ receptor endocytosis is inconsistent with a role of caveolae (*39*, *40*).

Several studies have implicated a clathrin-dependent mechanism for $GABA_A$ receptor endocytosis in neural tissues. A small fraction of $GABA_A$ receptors is copurified with clathrin-coated vesicles during isolation from mammalian brain (*7*, *41*, *42*). In addition, an association of the clathrin-adapter protein, AP2, with $GABA_A$ receptor subunits and a role for dynamin−amphiphysin interactions in the stability of surface receptors in hippocampal neurons have been proposed (*8*). However, the participation of these components in clathrin-independent processes has not been tested. Given our findings in HEK cells, the possibility that clathrin-independent endocytosis of $GABA_A$ receptors may occur in the brain should be examined.

Another characteristic of $GABA_A$ receptor endocytosis in neurons is a requirement for ligand occupancy. The internalization of $GABA_A$ receptor α1 subunits in cultured cortical neurons required the presence of $GABA_A$ agonists. Little or no constitutive endocytosis was detected (*18*). Similarly, Meyer et al. (*6*) reported that application of GABA agonist to hippocampal neurons from newborn rats evoked the internalization of $GABA_A$ receptor α2 subunits. Thus, the GABA insensitivity of $GABA_A$ receptor endocytosis in HEK cells is puzzling, particularly since normal GABA-gated currents are observed in these cells after expression of either wild-type or epitope-tagged $GABA_A$ receptor subunits (*11*). It may be that the constitutive endocytosis in HEK cells resembles that reported by Kittler et al. (*8*) for $GABA_A$ receptors in hippocampal neurons from rat embryos. This controversy illustrates the need for further examination of the mechanism of $GABA_A$ receptor endocytosis, in both neurons and heterologous cells.

ACKNOWLEDGMENT

We thank Dr. Mark Darlison for providing $GABA_A$ receptor subunit cDNAs and Dr. Brian Knoll for helpful discussions.

REFERENCES

1. Macdonald, R. L., and Olsen, R. W. (1994) *Annu. Rev. Neurosci. 17*, 569−602.
2. Rabow, L. E., Russek, S. J., and Farb, D. H. (1995) *Synapse 21*, 189−274.
3. Whiting, P. J., Bonnert, T. P., McKernan, R. M., Farrar, S., le Bourdelles, B., Heavens, R. P., Smith, D. W., Hewson, L., Rigby, M. R., Sirinathsinghji, D. J., Thompson, S. A., and Wafford, K. A. (1999) *Ann. N.Y. Acad. Sci. 868*, 645−653.
4. Tehrani, M. H., and Barnes, E. M., Jr. (1991) *J. Neurochem. 57*, 1307−1312.
5. Barnes, E. M., Jr. (2000) *Life Sci. 66*, 1063−1070.
6. Meyer, D. K., Olenik, C., Hofmann, F., Barth, H., Leemhuis, J., Brunig, I., Aktories, K., and Norenberg, W. (2000) *J. Neurosci. 20*, 6743−6751.
7. Tehrani, M. H., Baumgartner, B. J., and Barnes, E. M., Jr. (1997) *Brain Res. 776*, 195−203.
8. Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G., and Moss, S. J. (2000) *J. Neurosci. 20*, 7972−7977.
9. Chapell, R., Bueno, O. F., Alvarez-Hernandez, X., Robinson, L. C., and Leidenheimer, N. J. (1998) *J. Biol. Chem. 273*, 32595−32601.
10. Filippova, N., Sedelnikova, A., Zong, Y., Fortinberry, H., and Weiss, D. S. (2000) *Mol. Pharmacol. 57*, 847−856.
11. Connolly, C. N., Kittler, J. T., Thomas, P., Uren, J. M., Brandon, N. J., Smart, T. G., and Moss, S. J. (1999) *J. Biol. Chem. 274*, 36565−36572.
12. Barnes, E. M., Jr. (2001) *Int. Rev. Neurobiol. 48*, 1−29.
13. Cinar, H., and Barnes, E. M., Jr. (1999) *Soc. Neurosci. Abstr. 25*, 1711.
14. Cinar, H., and Barnes, E. M., Jr. (2000) *Soc. Neurosci. Abstr. 26*, 1657.
15. Bateson, A. N., Harvey, R. J., Wisden, W., Glencorse, T. A., Hicks, A. A., Hunt, S. P., Barnard, E. A., and Darlison, M. G. (1991) *Brain Res. Mol. Brain Res. 9*, 333−339.
16. Liu, S. C., Parent, L., Harvey, R. J., Darlison, M. G., and Barnes, E. M., Jr. (1998) *Eur. J. Pharmacol. 354*, 253−259.
17. Glencorse, T. A., Bateson, A. N., and Darlison, M. G. (1990) *Nucleic Acids Res. 18*, 7157.
18. Calkin, P. A., and Barnes, E. M., Jr. (1994) *J. Biol. Chem. 269*, 1548−1553.
19. Miranda, J. D., and Barnes, E. M., Jr. (1997) *J. Biol. Chem. 272*, 16288−16294.
20. Moore, R. H., Tuffaha, A., Millman, E. E., Dai, W., Hall, H. S., Dickey, B. F., and Knoll, B. J. (1999) *J. Cell Sci. 112* (Part 3), 329−338.
21. Connolly, C. N., Krishek, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1996) *J. Biol. Chem. 271*, 89−96.
22. Pelchen-Matthews, A., Parsons, I. J., and Marsh, M. (1993) *J. Exp. Med. 178*, 1209−1222.
23. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol. 127*, 915−934.
24. Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J. A., Carsrud, N. D., Dickey, B. F., and Knoll, B. J. (1995) *J. Cell Sci. 108* (Part 9), 2983−2991.
25. Nishizuka, Y. (1988) *Nature 334*, 661−665.
26. Kazanietz, M. G. (2000) *Mol. Carcinog. 28*, 5−11.
27. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., et al. (1991) *J. Biol. Chem. 266*, 15771−15781.
28. Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature 383*, 447−450.
29. Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K. H., and van Koppen, C. J. (1999) *J. Biol. Chem. 274*, 12333−12338.
30. Pals-Rylaarsdam, R., Gurevich, V. V., Lee, K. B., Ptasienski, J. A., Benovic, J. L., and Hosey, M. M. (1997) *J. Biol. Chem. 272*, 23682−23689.
31. Vogler, O., Bogatkewitsch, G. S., Wriske, C., Krummenerl, P., Jakobs, K. H., and van Koppen, C. J. (1998) *J. Biol. Chem. 273*, 12155−12160.
32. Vickery, R. G., and von Zastrow, M. (1999) *J. Cell Biol. 144*, 31−43.
33. Schramm, N. L., and Limbird, L. E. (1999) *J. Biol. Chem. 274*, 24935−24940.
34. Daly, C., Sugimori, M., Moreira, J. E., Ziff, E. B., and Llinas, R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6120−6125.
35. Damke, H., Baba, T., van der Bliek, A. M., and Schmid, S. L. (1995) *J. Cell Biol. 131*, 69−80.
36. Daukas, G., and Zigmond, S. H. (1985) *J. Cell Biol. 101*, 1673−1679.
37. Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G., and Shetzline, M. A. (1999) *J. Biol. Chem. 274*, 31515−31523.
38. Werbonat, Y., Kleutges, N., Jakobs, K. H., and van Koppen, C. J. (2000) *J. Biol. Chem. 275*, 21969−21974.
39. Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B., and Sandvig, K. (1999) *Mol. Biol. Cell 10*, 961−974.
40. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994) *J. Cell Biol. 127*, 1217−1232.
41. Tehrani, M. H., and Barnes, E. M., Jr. (1993) *J. Neurochem. 60*, 1755−1761.
42. Tehrani, M. H., and Barnes, E. M., Jr. (1997) *J. Pharmacol. Exp. Ther. 283*, 384−390.

BI011025T