Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12090–12094, December 1995
Neurobiology

Palmitoylation of the GluR6 kainate receptor

DARRYL S. PICKERING*†, FRANCO A. TAVERNA*†, MICHAEL W. SALTER‡, AND DAVID R. HAMPSON*†§

*Medical Research Council Group on Nerve Cells and Synapses and †Faculty of Pharmacy, University of Toronto, ON Canada M5S 2S2; and ‡Division of Neuroscience, The Hospital for Sick Children, Toronto, ON Canada M5G 1X8

Communicated by William T. Greenough, University of Illinois at Urbana–Champaign, Urbana, IL, September 11, 1995 (received for review May 15, 1995)

ABSTRACT **The G-protein-coupled metabotropic glutamate receptor mGluR1α and the ionotropic glutamate receptor GluR6 were examined for posttranslational palmitoylation. Recombinant receptors were expressed in baculovirus-infected insect cells or in human embryonic kidney cells and were metabolically labeled with [^{3}H]palmitic acid. The metabotropic mGluR1α receptor was not labeled whereas the GluR6 kainate receptor was labeled after incubation with [^{3}H]palmitate. The [^{3}H]palmitate labeling of GluR6 was eliminated by treatment with hydroxylamine, indicating that the labeling was due to palmitoylation at a cysteine residue via a thioester bond. Site-directed mutagenesis was used to demonstrate that palmitoylation of GluR6 occurs at two cysteine residues, C827 and C840, located in the carboxyl-terminal domain of the molecule. A comparison of the electrophysiological properties of the wild-type and unpalmitoylated mutant receptor (C827A, C840A) showed that the kainate-gated currents produced by the unpalmitoylated mutant receptor were indistinguishable from those of the wild-type GluR6. The unpalmitoylated mutant was a better substrate for protein kinase C than the wild-type GluR6 receptor. These data indicate that palmitoylation may not modulate kainate channel function directly but instead affect channel function indirectly by regulating the phosphorylation state of the receptor.**

Receptors for the excitatory neurotransmitter L-glutamate can be grouped into two major families, ionotropic receptors and metabotropic receptors. The metabotropic glutamate receptors are G-protein-linked receptors coupled to effectors such as phospholipase C and adenylyl cyclase, whereas the ionotropic receptors form homomeric and heteromeric ligand-gated ion channels permeable to mono- and divalent cations. A further subdivision of the ionotropic family can be made on the basis of agonist selectivity, giving the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (GluR1–4, also called GluRA–D), kainate (GluR5–7, KA-1, KA-2), and *N*-methyl-D-aspartate (NMDA) classes of receptors (1).

Several studies have examined various aspects of the structure and function of the GluR6 kainate receptor (2–10). The activity of GluR6 channels can be modulated by protein kinase A (6, 7) and protein kinase C (PKC) (8). Site-directed mutagenesis of asparagine-linked glycosylation sites (9–12) and epitope tagging studies (13) have been carried out to derive transmembrane topological models of kainate and AMPA receptors. In these models, a large extracellular amino-terminal domain is followed by either three or five transmembrane domains (TMDs), resulting in either case in a carboxyl terminus that is intracellular.

Palmitoylation is a posttranslational modification whereby a C_{16} fatty acyl chain is covalently bound to a nascent protein in a post-endoplasmic reticulum or pre-Golgi compartment. The fatty acid can be attached to a serine, threonine, or cysteine residue via an ester or thioester bond. In contrast to myristoylation, which occurs cotranslationally and without metabolic turnover (14), a rapid, dynamic turnover of palmitate has been demonstrated in several systems (15–17).

Several G-protein-linked receptors, including rhodopsin (18) and β_2-adrenergic (19), dopamine D_1 (17) and D_{2L} (20), α_{2A}-adrenergic (21, 22), and serotonin $5HT_{1B}$ (23) receptors, have been shown to be palmitoylated. Palmitoylation of ligand-gated ion channels has not been demonstrated, although the nicotinic acetylcholine receptor is fatty acylated (24). In this study, we have examined the palmitoylation state of the G-protein-coupled metabotropic glutamate receptor mGluR1α and the ionotropic GluR6 kainate receptor.

EXPERIMENTAL PROCEDURES

Receptor Expression. cDNAs containing the open reading frames of GluR6 (2) and GluR6 mutants (see below) were subcloned into the *Xho* I–*Not* I (5′ → 3′) sites, and the mGluR1α cDNA (25) into the *Not* I site, of the multiple cloning region of the expression vector pCis (26). Receptors were expressed transiently in human embryonic kidney 293 (HEK-293) cells by the calcium phosphate method (6). GluR6 and mGluR1α were expressed in *Spodoptera frugiperda* (Sf9) insect cells (3).

Site-Directed Mutagenesis. The cysteine residues C827 and C840 were each replaced with alanine as described (6). The oligonucleotides used were C827A (5′-AAGAGGTCCTTC-GCTAGCGCTATGGT-3′) and C840A (5′-ATGTCCCTGA-AGGCCCAGCGTCGGCT-3′). A double mutant, (C827A, C840A) GluR6, was prepared by subcloning the *Eco*47III–*Not* I fragment from mutant (C840A)GluR6 into the corresponding position of mutant (C827A)GluR6. All mutations were confirmed by dideoxy sequencing.

Palmitoylation in HEK-293 Cells. HEK-293 cells were grown to 75–90% confluency in six-well plates. At 44–48 hr posttransfection, the growth medium was aspirated and 2 ml of minimal essential medium (MEM) without serum or other additives was added per well. This was then aspirated and 1 ml of fresh MEM was added. After incubation for 6 hr (37°C, humidified 5% CO_2 incubator), 10 μl of fetal bovine serum was added with gentle swirling and the cells were incubated for an additional hour. [9,10-^{3}H]Palmitic acid (NEN/DuPont; 37–47 Ci/mmol, 5 mCi/ml in ethanol; 1 Ci = 37 GBq) was concentrated 3- to 5-fold under an argon gas stream, diluted with sterile MEM, and added to each well to give 0.2–0.5 mCi per well, depending upon the dilution factor of the label. Cells were metabolically labeled for 4 hr at 37°C and then the medium containing free label was removed. The cells were rinsed gently with 2 ml of MEM, scraped into ice-cold lysis

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; HEK-293 cells, human embryonic kidney 293 cells; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; TMD, transmembrane domain.

§To whom reprint requests should be addressed at: Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, ON Canada M5S 2S2.

buffer (1 ml per well; 50 mM Tris·HCl/1 mM EGTA/0.1 mM phenylmethanesulfonyl fluoride with trypsin inhibitor at 10 μg/ml, pH 7.4 at 4°C), and microcentrifuged at 4°C for 5 min at 14,000 × *g*.

Palmitoylation in Sf9 Cells. Sf9 cells were grown in six-well plates. At 30 hr postinfection, the medium was aspirated and 1 ml of Grace's medium (without glutamate, antibiotics, or serum) was added per well and the plates were returned to the incubator. At 44–48 hr postinfection, 10 μl of fetal bovine serum was added to each well with gentle swirling and the cells were returned to the incubator. One hour later, [9,10-^{3}H]palmitic acid was added to each well as above with HEK-293 cells, except the label was diluted with Grace's medium (without glutamate, antibiotics, or serum) in place of MEM. After metabolic labeling for 4 h at 26°C, the cells were collected (with the medium) by trituration and transferred into a microcentrifuge tube on ice.

Receptor Solubilization and Immunoprecipitation. Membranes from HEK-293 or Sf9 cells were solubilized in 50 mM Na_2HPO_4/20% (vol/vol) glycerol/10 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride/1 mM DL-benzylsuccinic acid/1 mM L-leucine hydroxamic acid/1 mM iodoacetamide, containing bacitracin (50 units/ml), turkey egg-white trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml), 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer Mannheim), and 0.1% Triton X-100, pH 7.4 at 4°C with NaOH. Membrane pellets were resuspended in 0.5–1 ml of cold (4°C) solubilization buffer per ≈10 cm^2 of cells with a Polytron (setting 5, 2 × 10 s; Brinkmann). The sample was mixed gently for 1 hr on ice then centrifuged for 1 hr at 109,000 × *g* at 4°C. The supernatant was transferred to a 15-ml polystyrene tube and 1 volume of immunoprecipitation buffer (50 mM Tris·HCl/0.2 M NaCl/0.1% Triton X-100, pH 7.4 at 4°C) containing antibody-bound protein A-Sepharose (20–30 μl of resin) was added. After overnight incubation at 4°C with gentle mixing, the resin was pelleted by centrifugation for 10 min at 1000 × *g*. The pellet was washed with 1 ml of cold immunoprecipitation buffer and prepared for SDS/PAGE.

SDS/PAGE, Immunoblotting, and Autoradiography. HEK-293 and Sf9 membrane samples and immunoprecipitated receptors were analyzed by SDS/8% PAGE, and the separated proteins were transferred to nitrocellulose or Immobilon-P (Millipore) for immunoblot analysis (9). GluR6 was detected with an antibody raised against the carboxyl terminus of this subunit (4); mGluR1α was detected with an anti-peptide antibody (37). Receptor bands were visualized with an alkaline phosphatase-conjugated anti-rabbit secondary antibody (Promega) or a horseradish peroxidase-conjugated anti-rabbit secondary antibody (ECL detection; Amersham Canada). Autoradiograms were obtained by exposing the blot to Hyperfilm-^{3}H (Amersham) for 1–5 weeks at −75°C prior to incubation with antibodies.

Electrophysiological Recordings. Forty-eight hours after transfection of HEK-293 cells with GluR6 and or (C827A,C840A)GluR6 cDNA, the cells were washed with extracellular recording solution (140 mM NaCl/5.4 mM KCl/25 mM Hepes/1.3 mM $CaCl_2$/1 mM $MgCl_2$/33 mM glucose, pH 7.35). The extracellular solution was supplemented with wheat-germ agglutinin (10 μg/ml) to inhibit the rapid desensitization of kainate receptors (2, 6, 27). Recording pipettes were filled with a standard intracellular solution (140 mM CsCl/10 mM Hepes/10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA)/2 mM $MgCl_2$/4 mM dipotassium ATP, pH 7.25. Recordings were made in the standard whole-cell patch clamp configuration (28). The holding potential was −60 mV unless otherwise indicated. Kainic acid (500 μM) was applied by pressure from a pipette positioned 20–30 μm from the cell being studied.

PKC-Dependent Phosphorylation. At 48 hr posttransfection, HEK-293 cells were collected for immunoaffinity purification of receptors as described above. The purified receptors (bound to the resin) were resuspended in 50 μl of 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/0.1 mM $CaCl_2$. The following cofactors were added to achieve maximal enzymatic activity (29): L-α-phosphatidylserine (100 μg/ml; bovine spinal cord; Calbiochem) and 1,2-diolein (20 μg/ml; Sigma). The cofactors were prepared by drying down stock solutions (under nitrogen) in a preweighed tube and reconstituting them in an appropriate volume of 4% (vol/vol) Triton X-100 to obtain 40× stocks. [γ-^{32}P]ATP was added at 25 μM (12 Ci/mmol, from 3000 Ci/mmol stock; New England Nuclear). PKC (2.8 units in 2 μl; rat brain; Calbiochem) was added to start the reaction. After 30 min at 30°C, the reaction was stopped by addition of SDS sample buffer.

RESULTS

Labeling of GluR6 and mGluR1α. Baculovirus-infected Sf9 cells expressing recombinant mGluR1α or GluR6, or HEK-293 cells transiently transfected with GluR6 cDNA, were metabolically labeled with [9,10-^{3}H]palmitic acid. Despite the high level of expression, no labeling of mGluR1α was observed in samples immunoprecipitated from Sf9 cells infected with the recombinant mGluR1α baculovirus (data not shown). In contrast, robust labeling of GluR6 was observed in crude membrane preparations of Sf9 cells infected with the recombinant GluR6 baculovirus and in samples of Sf9 cells or HEK-293 cells subjected to immunoprecipitation (Fig. 1). No immunoprecipitated labeled (or unlabeled) bands were seen from uninfected Sf9 cells or from untransfected or vector-only-transfected HEK-293 cells with the anti-GluR6 antibody (data not shown).

Chemical Nature of Labeled Sites. Palmitoylation may occur at serine and threonine residues via an ester linkage or at cysteine residues via a thioester linkage. In addition, [^{3}H]palmitic acid can

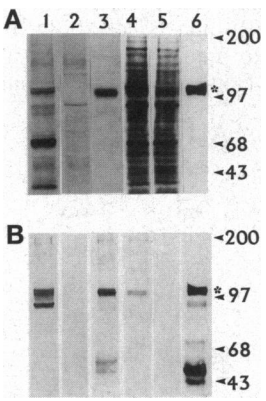

FIG. 1. [^{3}H]Palmitic acid labeling of GluR6 in Sf9 and HEK-293 cells. Sf9 cells infected with recombinant GluR6 baculovirus and HEK-293 cells transiently transfected with GluR6 cDNA were metabolically labeled with [9,10-^{3}H]palmitic acid. Whole membranes either were prepared directly for SDS/PAGE or were solubilized and GluR6 was immunoprecipitated prior to SDS/PAGE, electrophoretic transfer and autoradiography for 2–5 weeks at −75°C. Films were then processed (*A*) and the immunoblots were developed with an anti-GluR6 antibody (*B*). Lane 1, membranes from GluR6-infected Sf9 cells; lane 2, membranes from uninfected Sf9 control cells; lane 3, GluR6 immunoprecipitated from Sf9 membranes; lane 4, membranes from GluR6-transfected HEK-293 cells; lane 5, membranes from untransfected HEK-293 cells; lane 6, GluR6 immunoprecipitated from HEK-293 membranes. All the lanes of crude membranes contained 20–25 μg of protein. Arrowheads indicate molecular size markers: myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). Asterisk indicates the position of GluR6.

be metabolically converted into [^{3}H]myristic acid by cells so that resultant receptor labeling could be due to myristoylation instead of palmitoylation. These possibilities were distinguished by chemical tests. Myristoylation occurs via amide linkages and is stable to base and strong nucleophiles such as hydroxylamine. Esters are hydrolyzed by base but not by hydroxylamine at neutral pH. Thioesters are the most labile of the three types of bonds and are hydrolyzed by treatment with base or hydroxylamine at neutral pH (30). Labeling of GluR6 was completely eliminated by hydroxylamine treatment (Fig. 2*A*, lane 3) and almost completely eliminated by strong base treatment (lane 2) but was resistant to reducing conditions (lane 4).

Palmitoylation in the Presence of Agonist. Some GluR6 palmitoylation experiments in Sf9 cells were also conducted in the presence of 0.1 mM kainate during the last 5 or 30 min of the 4-hr metabolic labeling. GluR6 was solubilized and immunoprecipitated and the resulting autoradiograms and immunoblot films (ECL chemiluminescent development) were scanned with a microcomputer video imaging device (Imaging Research, St. Catherine's, ON, Canada) to measure the band intensities in units of area × optical density. The normalized, background-subtracted band intensities were calculated as follows (n = 5): (autoradiogram band − background) ÷ (ECL band − background). No statistically significant difference from the control (no kainate) was observed for either period of agonist activation (data not shown).

Palmitoylation of the GluR6 Cysteine Mutants. Since palmitoylation of GluR6 was sensitive to hydroxylamine at neutral pH, cysteine was implicated as the residue to which the palmitate group was covalently linked. In the G-protein-coupled receptors, palmitoylation occurs carboxyl-terminal to the last presumed TMD (19). In GluR6, both C827 and C840 are carboxyl-terminal to the last putative TMD; consequently these residues were examined as potential palmitoylation sites. Three GluR6 mutants—C827A, C840A, and C827A,C840A were transiently expressed in HEK-293 cells, labeled with [^{3}H]palmitic acid, immunoprecipitated, and subjected to SDS/PAGE and electrophoretic transfer. The single cysteine mutants, (C827A)GluR6 and (C840A)GluR6, were labeled by [^{3}H]palmitic acid to approximately the same extent as the wild-type receptor. However, complete elimination of receptor

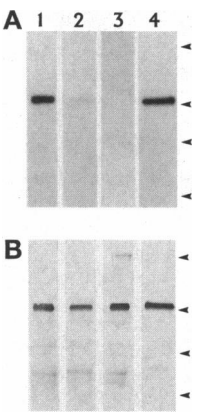

FIG. 2. Determination of the chemical nature of the labeled site in GluR6. Recombinant GluR6 baculovirus-infected Sf9 insect cells were metabolically labeled with [9,10-^{3}H]palmitic acid and the membranes were solubilized. The solubilized samples were treated with 1 M hydroxylamine (at pH 7–7.4 with NaOH) or 1 M NaOH on ice for 3 hr. After neutralization and immunoprecipitation, the samples were subjected by SDS/PAGE followed by electrophoretic transfer and autoradiography (*A*) for 17 days at −75°C before immunoblot detection (*B*). Three such experiments were conducted with similar results. Lane 1, untreated control; lane 2, NaOH-treated; lane 3, hydroxylamine-treated; lane 4, control treated with 1 M 2-mercaptoethanol for 1 hr at room temperature just prior to SDS/PAGE. Arrowheads indicate size markers as in Fig. 1.

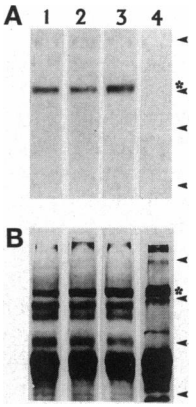

FIG. 3. Analysis of GluR6 cysteine-to-alanine mutants. HEK-293 cells transiently transfected with wild-type GluR6 (lane 1), (C827A)GluR6 (lane 2), (C840A)GluR6 (lane 3), or (C827A,C840A)GluR6 (lane 4) were labeled with [^{3}H]palmitic acid. Membranes were solubilized before immunoprecipitation with an anti-GluR6 antibody. Following separation of the receptor–antibody complex by SDS/PAGE, and electrophoretic transfer onto nitrocellulose, the transfer membranes were apposed to Hyperfilm-^{3}H for 2–4 weeks at −75°C (*A*). Immunoblots were developed with the same anti-GluR6 antibody (*B*). Identical results were obtained in three separate experiments. Arrowheads indicate size markers as in Fig. 1. Asterisk indicates position of wild-type and mutant GluR6.

[^{3}H]palmitoylation was observed for the double cysteine mutant, (C827A,C840A)GluR6 (Fig. 3).

Electrophysiological Properties of GluR6 and (C827A, C840A)GluR6. Application of 500 μM kainate elicited a rapid-onset inward current in cells transfected with GluR6 (Fig. 4). The kainate-evoked currents were reproducible in all cells tested (n = 5). Similar currents in response to kainate were observed in cells transfected with the double cysteine mutant (n = 6). The current–voltage relationship for the responses was examined by applying kainate when the membrane potential was varied from −80 to +60 mV (Fig. 4*B*). The reversal potential of the currents was near 0 mV with the cells expressing either mutant or wild-type receptors, indicating that the driving force for the currents was similar with both types of receptors. Also, the current–voltage relationship showed outward rectification for kainate-evoked responses with the mutant as well as the wild-type receptor.

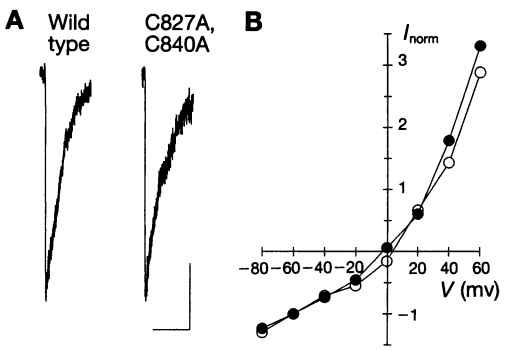

FIG. 4. Kainate-evoked responses in cells expressing double palmitoylation mutant or wild-type GluR6 receptors. (*A*) Individual traces of membrane current for two single cells transfected with wild-type GluR6 (*Left*) or (C827A,C840A)GluR6 (*Right*). The inward currents were induced by applying kainate (500 μM, 100 ms). Scale bars: horizontal, 20 s; vertical, 40 pA (*Left*) or 20 pA (*Right*). (*B*) Normalized peak inward current (I_{norm}) is plotted versus membrane holding potential (V). Data are from individual cells expressing wild-type (●) or mutant (○) receptors. For each point the peak amplitude of the current was normalized by dividing by the magnitude of the current elicited at a membrane potential of −60 mV.

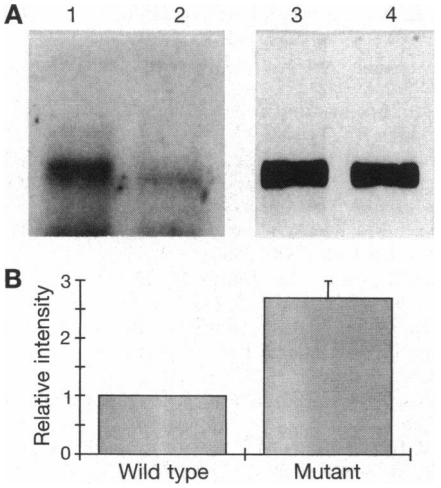

FIG. 5. Phosphorylation of GluR6 and (C827A,C840A)GluR6 by PKC. (*A*) Receptors immunoprecipitated from HEK-293 cells expressing GluR6 (lanes 2 and 4) or (C872A,C840A)GluR6 (lanes 1 and 3) were incubated with PKC in the presence of [γ-^{32}P]ATP and cofactors. Autoradiogram (lanes 1 and 2) and corresponding immunoblot (lanes 3 and 4) are shown. (*B*) Quantitation of the relative level of PKC phosphorylation of the wild-type and C827A,C840A mutant receptors.

PKC-Dependent Phosphorylation of GluR6 and (C827A, C840A)GluR6. Membranes from HEK-293 cells transfected with GluR6 or the double cysteine mutant were collected and solubilized 48 hr after transfection. After immunoprecipitation with the GluR6 antibody, the wild-type and mutant receptors were subjected to phosphorylation by PKC. The double cysteine mutant receptor was phosphorylated to a greater degree than the wild-type receptor (Fig. 5*A*). The densitometric scans of the autoradiograms were normalized for the amount of receptor by scanning the corresponding immunoblots. In four such experiments, the PKC-mediated phosphorylation of the unpalmitoylated mutant receptor was increased 2.7 ± 0.33-fold (mean ± SEM) compared with the wild-type receptor.

DISCUSSION

Many proteins involved in signal transduction undergo post-translational palmitoylation. Although it would be of interest to investigate the palmitoylation of glutamate receptors in brain tissue or neurons in culture, we chose to study recombinant receptors expressed in Sf9 cells and HEK-293 cells because the analysis of receptor palmitoylation requires relatively high levels of protein expression (31) and because recombinant systems are amenable to the study of mutant receptors.

Since several G-protein-linked receptors are known to be palmitoylated, we wished to examine the metabotropic glutamate receptor mGluR1α for palmitoylation. mGluR1α was not palmitoylated when expressed in Sf9 cells; the absence of palmitoylation was also observed when mGluR1α was expressed in baby hamster kidney cells (32). In contrast to mGluR1α, the ionotropic glutamate receptor GluR6 was labeled after incubation with [^{3}H]palmitate when expressed in Sf9 cells and in HEK-293 cells. The chemical sensitivity of the palmitate label to hydroxylamine treatment in the GluR6 protein implied covalent linkage to cysteine via a thioester bond.

GluR6 contains two cysteine residues (C827 and C840) in the putative intracellular carboxyl-terminal domain of the molecule that are flanked by several positively charged amino acid residues. Although no consensus sequence for palmitoylation has been definitively established, this flanking motif containing positively charged amino acids is present at the palmitoylated cysteine of several G-protein-coupled receptors. Thus two GluR6 mutants, C827A and C840A, were constructed and tested for palmitoylation. Both of these single mutants were palmitoylated to approximately the same extent as the wild-type receptor. However, the double cysteine mutant (C827,C840)GluR6 was not palmitoylated. We do not know whether both cysteine residues are simultaneously palmitoylated in the native wild-type receptor. Since both cysteines exist in the native receptor, both could be palmitoylated, although it is possible that one of the cysteines may be a better substrate than the other for the palmitoyltransferase; additional quantitative kinetic studies will be required to resolve this issue. Precedents for doubly palmitoylated proteins do exist; examples include the G-protein subunit α_q (33), rhodopsin (18), and the transferrin receptor (34). In α_q, palmitoylation occurs on two adjacent cysteine residues near the amino terminus and is required for membrane association and stimulation of phospholipase C (33). Activation-induced depalmitoylation correlated with translocation of α_q from the membrane to the cytosol (35).

Sequence alignment of GluR6 with other members of the ionotropic glutamate receptor family revealed that C827 of the GluR6 protein is conserved in all of the kainate receptors (i.e, GluR6 and -7, KA-1, KA-2, and frog and chicken kainate-binding proteins) except GluR5. C840 is conserved only in the mammalian kainate receptors (GluR6, GluR7, KA-1, and KA-2) with the exception of GluR5. Neither cysteine is conserved among the AMPA, NMDA-R1, or NMDA-R2 families of receptors. This observation suggests that palmitoylation of the ionotropic glutamate receptors may be unique to the kainate subclass of glutamate receptor subunits.

In the dopamine D_1, β_2-adrenergic, and transferrin receptors (17, 19, 34) and the G-protein subunits α_s and α_q (33, 35), dynamic, stimulation-induced turnover of palmitate takes place. We did not observe any such agonist-induced changes in the overall palmitoylation state of GluR6. Although we did not detect a significant change in the palmitoylation of GluR6 after kainate treatment, the absence of an effect under the conditions we employed does not preclude the possibility that agonist-induced changes may occur under other experimental conditions.

Our experiments measuring whole-cell currents in GluR6- and (C827A,C840A)GluR6-transfected HEK-293 cells did not reveal any differences in the current properties between the wild-type and the double mutant homomeric channels. Preliminary experiments conducted in *Xenopus* oocytes that were coinjected with cDNAs for GluR6 plus KA-2 or (C827A, C840A)GluR6 plus KA-2 have not revealed any differences in the currents mediated by these heteromeric receptor complexes (K.-Y. Yue, J. F. MacDonald, and D.R.H., unpublished observations). It is possible that more subtle differences in channel function, such as changes in the desensitization kinetics or single-channel parameters were present and were masked by the presence of wheat-germ agglutinin; further experiments are required to explore these possibilities.

Recent studies have documented interactions between the palmitoylation state and the phosphorylation state of G-protein-coupled neurotransmitter receptors (16, 36). Moffett *et al.* (36) reported that a mutant β_2-adrenoceptor in which the palmitoylation site was eliminated had a basal level of phosphorylation about 4 times greater than that of the wild-type receptor. This suggests that in the absence of palmitoylation, access of the kinase(s) to phosphorylation site(s) in the β_2-adrenergic receptor is increased. Since phosphorylation of the β_2-adrenergic receptor increases the functional uncoupling of the receptor and facilitates rapid desensitization, the presence or absence of the palmitate modification may influence receptor desensitization (36).

The GluR6 sequence contains two carboxyl-terminal PKC consensus sites, S815 and S837, near the palmitoylation sites at

C827 and C840. Pertinent to this observation is the finding that the functional activity of GluR6 expressed in *Xenopus* oocytes is regulated by PKC-dependent phosphorylation (8). In contrast to the potentiation of GluR6-mediated currents after application of protein kinase A (6, 7), peak kainate-activated responses were inhibited by the application of a phorbol ester, and this inhibition could be blocked by a selective PKC inhibitor (8). Thus, an attractive prospect based on the studies noted above with the G-protein-coupled receptors is that the palmitoylation status of GluR6 could determine the accessibility of PKC to one or both of these sites.

We tested this hypothesis by comparing the ability of the wild-type GluR6 and the unpalmitoylated mutant receptor to act as substrates for PKC-dependent phosphorylation. Consistent with previous results on the β_2-adrenergic receptor (36), we observed greater PKC-mediated phosphorylation with the unpalmitoylated receptor than with the wild-type receptor. In our experiments, PKC-mediated phosphorylation was tested on partially purified (immunoprecipitated) receptors. Our observation that this effect is apparent on detergent-solubilized receptors in solution lends further support to the notion that access of the kinase to the phosphorylation site may be blocked by the presence of the palmitate moiety. Together, these findings suggest that an important role for palmitoylation in both ionotropic and G-protein-coupled receptors may be to influence the functional properties of these molecules indirectly by controlling the ability of kinases, and possibly other proteins, to directly modulate receptor function.

We thank Dr. Brian F. O'Dowd for helpful comments on the manuscript and Xi-ping Huang for conducting the transfections. This work was supported by a Group Grant from the Medical Research Council of Canada (to D.R.H.). D.S.P. was supported by a Natural Sciences and Engineering Research Council Postdoctoral Fellowship. F.A.T. was supported by an Ontario Graduate Scholarship.

1. Hollmann, M. & Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17,** 31–108.
2. Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I. & Heinemann, S. (1991) *Nature (London)* **351,** 745–748.
3. Ross, S. M., Taverna, F., Pickering, D. S., Wang, L.-Y., MacDonald, J. F., Pennefather, P. S. & Hampson, D. R. (1994) *Neurosci. Lett.* **173,** 139–142.
4. Wenthold, R. J., Trumpy, V. A., Zhu, W.-S. & Petralia, R. S. (1994) *J. Biol. Chem.* **269,** 1332–1339.
5. Herb, A., Burnashev, N., Werner, P., Sakmann, B., Wisden, W. & Seeburg, P. H. (1992) *Neuron* **8,** 775–785.
6. Wang, L.-Y., Taverna, F. A., Huang, X.-P., MacDonald, J. F. & Hampson, D. R. (1993) *Science* **259,** 1173–1175.
7. Raymond, L. A., Blackstone, C. D. & Huganir, R. L. (1993) *Nature (London)* **361,** 637–641.
8. Dildy-Mayfield, J. E. & Harris, R. A. (1994) *J. Neurochem.* **62,** 1639–1642.
9. Taverna, F. A., Wang, L.-Y., MacDonald, J. F. & Hampson, D. R. (1994) *J. Biol. Chem.* **269,** 14159–14164.
10. Roche, K. W., Raymond, L. A., Blackstone, C. & Huganir, R. L. (1994) *J. Biol. Chem.* **269,** 11679–11682.
11. Wo, Z. G. & Oswald, R. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7154–7158.
12. Hollmann, M., Maron, C. & Heinemann, S. (1994) *Neuron* **13,** 1331–1343.
13. Bennett, J. A. & Dingledine, R. (1995) *Neuron* **14,** 373–384.
14. Johnson, D. R., Bhatnagar, R. S., Knoll, L. S. & Gordon, J. I. (1994) *Annu. Rev. Biochem.* **63,** 869–914.
15. Mumby, S. M., Kleuss, C. & Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2800–2804.
16. Mouillac, B., Caron, M., Bonin, H., Dennis, M. & Bouvier, M. (1992) *J. Biol. Chem.* **267,** 21733–21737.
17. Ng, G. K. Y., Mouillac, B., George, S. R., Caron, M., Dennis, M., Bouvier, M. & O'Dowd, B. F. (1994) *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **267,** 7–19.
18. Ovchinnikov, Y. A., Abdulaev, N. G. & Bogachuk, A. S. (1988) *FEBS Lett.* **230,** 1–5.
19. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. (1989) *J. Biol. Chem.* **264,** 7564–7569.
20. Ng, G. Y. K., O'Dowd, B. F., Caron, M., Dennis, M., Brann, M. & George, S. R. (1994) *J. Neurochem.* **63,** 1589–1595.
21. Kennedy, M. E. & Limbird, L. E. (1993) *J. Biol. Chem.* **268,** 8003–8011.
22. Eason, M. G., Jacinto, M. T., Theiss, C. T. & Liggett, S. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11178–11182.
23. Ng, G. Y. K., George, S. R., Zastawny, R. L., Caron, M., Bouvier, M., Dennis, M. & O'Dowd, B. F. (1993) *Biochemistry* **32,** 11727–11733.
24. Olson, E. N., Glaser, L. & Merlie, J. P. (1984) *J. Biol. Chem.* **259,** 5364–5367.
25. Houamed, K. M., Kuijper, J. L., Gilbert, T. L., Haldeman, B. A., O'Hara, P. J., Mulvihill, E. R., Almers, W. & Hagan, F. S. (1991) *Science* **252,** 1318–1321.
26. Gorman, C. M., Gies, D. R. & McCray, G. (1990) *DNA Protein Eng. Technol.* **2,** 3–10.
27. Wang, L.-Y., Salter, M. W. & MacDonald, J. F. (1991) *Science* **253,** 1133–1135.
28. Salter, M. W. & Hicks, J. L. (1994) *J. Neurosci.* **14,** 1563–1575.
29. Bell, R. M., Hannum, Y. & Loomis, C. (1986) *Methods Enzymol.* **124,** 353–359.
30. Fujimoto, T., Stroud, E., Whatley, R. E., Prescott, S. M., Muszbek, L., Laposata, M. & McEver, R. P. (1993) *J. Biol. Chem.* **268,** 11394–11400.
31. Bouvier, M., Chidiac, P., Herbert, T. E., Loisel, T. P., Moffett, S. & Mouillac, B. (1995) *Methods Enzymol.* **250,** 300–314.
32. Alaluf, S., Mulvihill, E. R. & McIlhinney, R. A. J. (1995) *J. Neurochem.* **64,** 1548–1555.
33. Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J. & Bourne, H. R. (1993) *J. Biol. Chem.* **268,** 25001–25008.
34. Alvarez, E., Gironès, N. & Davis, R. J. (1990) *J. Biol. Chem.* **265,** 16644–16655.
35. Wedegaertner, P. B. & Bourne, H. R. (1994) *Cell* **77,** 1063–1070.
36. Moffett, S., Mouillac, B., Bonin, H. & Bouvier, M. (1993) *EMBO J.* **12,** 349–356.
37. Pickering, D. S., Thomsen, C., Suzdak, P. D., Fletcher, E. J., Robitaille, R., Salter, M. W., MacDonald, J. F., Huang, X.-P. & Hampson, D. R. (1993) *J. Neurochem.* **61,** 85–92.