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Stable expression of a functional GluR6 homomeric glutamate receptor channel in mammalian cells

CHARLOTTE K. TYGESEN*, JESPER S. RASMUSSEN†, S. V. PENELOPE JONES‡, ANNETTE HANSEN*, KATE HANSEN†, AND PETER H. ANDERSEN*§

Departments of *Molecular Pharmacology and †Molecular Biology II, Bioscience, Novo Nordisk A/S, Novo Alle, Bldn. 1CS23, DK-2880 Bagsvaerd, Denmark; and ‡Department of Psychiatry, The University of Vermont, College of Medicine, Medical Alumni Building, Burlington, VT 05405-0068

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ABSTRACT **This study demonstrates the stable expression of a functional ionotropic glutamate receptor in a mammalian cell line of non-neuronal origin. The kainate-selective glutamate receptor GluR6 was constitutively expressed under the control of a metallothionein promoter. Clones were isolated expressing ≈3 pmol of receptor per mg of protein. Functionality of the recombinant GluR6 was demonstrated both by electrophysiology and by Ca^{2+} imaging. Application of kainate to the GluR6-transfected cells activated an inward current response at a holding potential of −60 mV. The kainate concentration needed to evoke 50% of the maximal response (EC_{50}) was calculated to be 0.82 ± 0.39 μM. The current–voltage relationship was found to be almost linear, with a reversal potential of −2.5 ± 4.8 mV. Application of kainate also resulted in an increase in the intracellular Ca^{2+} concentration measured by Ca^{2+} imaging. The pharmacological profile of [^{3}H]kainate binding to the recombinant GluR6 resembled the high-affinity [^{3}H]kainate binding sites in rat brain, showing high affinity for domoate (K_i = 5.1 ± 3.0 nM) and kainate (K_d = 12.9 ± 2.4 nM). No decrease in GluR6 expression level was observed over >75 passages of the transfected cells. When domoate, a slowly desensitizing GluR6 agonist, was included in the growth medium for 3 weeks, the number of GluR6 binding sites decreased by 30%, indicating the importance of complete channel closure for stable expression.**

L-Glutamate is the most abundant excitatory neurotransmitter in the central nervous system and is thought to be involved in several neurological disorders. Receptors activated by L-glutamate have been classified as metabotropic and ionotropic receptors. On the basis of pharmacological and electrophysiological studies, the ionotropic glutamate receptors (GluRs) have been grouped into the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors (1, 2). At present, four AMPA receptor subunits (GluR1–4) (3–5) and five KA receptor subunits (GluR5–7, KA-1, and KA-2) (6–10) have been cloned. In addition, a number of splice variants of GluR1–5 have emerged (11, 12). Recombinant homomeric GluR1–4 subunits show high affinity for the agonist AMPA and low affinity for the agonist kainate, and both agonists induce channel openings (4). The kainate receptors all have high affinity for kainate and recombinant GluR5 and GluR6, but not GluR7, KA-1, and KA-2, generate homomeric functional channels activated by kainate (6–10).

The Ca^{2+} permeability of the AMPA receptors is controlled by a single amino acid in the second transmembrane domain (TM2). In GluR2, a glutamine codon is changed to an arginine codon during RNA editing, rendering the receptor Ca^{2+}-impermeable (13–15). In GluR6, the Ca^{2+} permeability is controlled by the same amino acid in TM2. Whether the Ca^{2+} permeability is increased or decreased by the presence of an arginine is controversial (16, 17) and is probably dependent on experimental conditions. In addition, RNA editing of two amino acids in TM1 also influences the Ca^{2+} permeability, leaving eight different GluR6 variants with respect to TM1 and TM2. In contrast to GluR2, which is completely edited, only 65% of all GluR6 subunits found *in vivo* are edited in all three positions (16).

Ligand binding and functional properties of GluR subunits have been studied in transient expression systems such as *Xenopus* oocytes, mammalian cells, and insect cells (3–18). Transient expression in oocytes and mammalian cells is less convenient, and the low expression levels make radioligand binding studies troublesome. The insect cell system has been shown to generate high expression levels with respect to radioligand binding, but the functionality of the receptors still remains to be clarified. Here, we report on the generation of a mammalian cell line stably expressing functional GluR6 at 3 pmol/mg of protein, as measured by binding.

MATERIALS AND METHODS

Cloning and Expression Constructs. First-strand cDNA was synthesized from rat hippocampal total RNA with SuperScript RNase H$^-$ reverse transcriptase (BRL) and oligo(dT) primer in a 20-μl reaction mixture (19). PCR mixtures included first-strand cDNA as template and oligonucleotides based on the published sequence (GenBank/EMBL accession no. Z11548) as primers. The primers were synthesized on an Applied Biosystems 394 DNA synthesizer and are referred to according to the corresponding base pairs in the gene: 1–15, 5′-CACGGAGGTACCATGAAGATTATTTCC-3′; 1223–1194, 5′-GCTGGATCCCAAGTTCCAATCTTCTCTAGA-3′; 1194–1223, 5′-TCTAGAGAAGATTGGAACTTGGGATCCAGC-3′ (contains a silent point mutation at bp 1197 that generates an *Xba* I site); 2727–2713, 5′-TGGCCTTCTAGATTCATGCCATGGTTTC-3′.

PCRs were carried out with AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) for 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The receptor cDNA fragments were subcloned into pBluescript II SK(+) (Stratagene) and the DNA sequence was verified to be the fully edited version of GluR6 (16). The GluR6 cDNA was assembled from two fragments into a mammalian expression vector that utilizes the constitutively active mouse metallothionein promoter for expression and the dihydrofolate reductase gene for selection of stable transformants (20). For transfection, DNA was purified by CsCl centrifugation.

Generation of Cell Line. The expression construct was transfected into BHK570 baby hamster kidney cells by

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline.

§To whom reprint requests should be addressed.

Lipofectin (BRL) (21). After application of 1 μM methotrexate, surviving colonies were isolated by conventional cloning cylinders. To assure that monoclonal cell lines were generated, the selected clones were subjected to several rounds of further subcloning. After 10–14 days the selection pressure was raised to 5 μM methotrexate and the selected clones were then screened for [^{3}H]kainate binding. Wild-type BHK cells showed no specific [^{3}H]kainate binding, whereas the GluR6-transfected cells showed high levels of specific [^{3}H]kainate binding. Colonies showing the highest level of radioligand binding were selected for further characterization.

The GluR6 cell line was grown in standard high-glucose Dulbecco's modified Eagle's medium with Glutamax (L-glutamate free medium, GIBCO/BRL catalogue no. 31966-021) and supplemented with 10% fetal bovine serum and 5 μM methotrexate.

[^{3}H]Kainate Binding. Confluent cells on 14.5-cm plates were washed twice with phosphate-buffered-saline at 25°C, and 10 ml of 10 mM EDTA was added. After 30 min of incubation, the cells were scraped off and centrifuged at 30,000 × *g* for 10 min at 4°C. The pellet was homogenized [Ultra-Turrax (Janke and Kunkel), 20 sec] in 50 mM Tris/citrate buffer (pH 7.0) and centrifuged as above. This step was repeated twice. The resulting pellet was rehomogenized in buffer to a final protein concentration of 0.2 mg/ml.

Binding was carried out in a final volume of 0.55 ml containing 500 μl of membranes, 25 μl of [^{3}H]kainate (NEN, DuPont), and 25 μl of water (total binding), L-glutamate (680 μM in assay mixture) (nonspecific binding), or test compound. The incubation time was 40 min at 0°C. The reaction was terminated by addition of 6 ml of ice-cold buffer followed by rapid filtration through Whatman GF/C glass-fiber filters. The filters were washed with 6 ml of buffer and then transferred to scintillation vials for measurement of radioactivity.

[^{3}H]AMPA Binding. Confluent cells were scraped off and centrifuged 10 min at 30,000 × *g*. The pellet was homogenized (Ultra-Turrax, 20 sec) in 10 ml of ice-cold 30 mM Tris·HCl, pH 7.1/2.5 mM $CaCl_2$ and centrifuged as before. This homogenization/centrifugation procedure was repeated. All steps were carried out at 0–4°C. The pellet was homogenized in buffer, incubated at 37°C for 30 min, and centrifuged. The pellet was rehomogenized once in buffer and frozen on dry ice for 30 min. After thawing, the homogenate was centrifuged and the final pellet was homogenized in buffer with 100 mM KSCN. The final protein concentration was 0.15 mg/ml.

Binding was carried out in 0.55 ml containing 500 μl of membranes, 25 μl of [^{3}H]AMPA (NEN/DuPont), and 25 μl of water (total binding) or L-glutamate (680 μM in assay mixture) (nonspecific binding). After incubation for 30 min at 0°C, the reaction was terminated by addition of 10 ml of ice-cold 0.9% NaCl and rapid filtered through Whatman GF/C glass-fiber filters. The filters were washed with 10 ml of NaCl and then transferred to scintillation vials for radioactivity measurement (22).

All binding data were analyzed with EBDA-SCARFIT (Elsevier Biosoft, Cambridge, U.K.). Data are expressed as mean ± SD.

Western Blot Analysis. Samples were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (4–20% gradient gel) and transferred to nitrocellulose by electroblotting (200 mA, 2 hr) as described (23). The blot was blocked (30 min) with 3% nonfat dry milk in Tris-buffered saline (TBS) at room temperature and then incubated for 2 hr at room temperature with affinity-purified GluR6 anti-peptide antibody (aa 862–877, kindly supplied by Craig Blackstone) (24) diluted 1:200 in 1.5% nonfat dry milk in TBS. After washing for 20 min (five intermediate changes) in TBS/0.1% Tween 20, the blot was incubated for 1 hr at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Dako P217) diluted 1:2000 in 1.5% nonfat dry milk in TBS. After several washes in TBS/0.1% Tween 20, immunoreactive protein was visualized with ECL (Amersham) (25).

Protein Determination. Protein concentrations were determined by the Bradford (Bio-Rad) assay using gamma globulin as standard.

Electrophysiology. Cells were plated on 3.5-cm culture dishes, washed twice in extracellular recording solution (150 mM NaCl/5 mM KCl/0.1 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes/25 mM glucose, adjusted to pH 7.4 with NaOH; 325 mOsm), and then incubated for 3 min at 22°C in the same solution containing concanavalin A (Con A; Sigma type IV) at 1 mg/ml to inhibit receptor desensitization (7). After incubation, the cells were washed thoroughly with recording solution and then placed on an inverted phase-contrast microscope for electrical recordings at 22°C. Patch pipettes (4–7 MΩ) were filled with 5 mM cesium, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'* tetraacetate (BAPTA)/5 mM potassium BAPTA/130 mM KCl/10 mM Hepes/0.1 mM $CaCl_2$/1 mM $MgCl_2$/0.1 Li-GTP/1 mM Mg-ATP (pH 7.2 with KOH; 315 mOsm). Membrane currents were recorded with the whole-cell patch-clamp technique (List EPC-7 amplifier, Darmstadt, Germany) (26), filtered at 1 KHz, displayed on a thermal array recorder (200 Hz; Graphtec), and also digitized (NBMIO-16, National Instruments, Austin, TX) at a sampling rate of 2 KHz by a Macintosh Quadra 800 using AXODATA (Axon Instruments, Foster City, CA). Series resistance and capacitance compensation were applied prior to breakthrough into the whole-cell mode. Cells were voltage clamped at a holding potential of −60 mV, and control currents and the currents activated by kainate were recorded. Various concentrations of kainate were applied by a flow pipe (100 μm) placed adjacent to the cell. Current–voltage (*I–V*) relationships were constructed from the currents measured at the end of 120-msec steps to a variety of potentials ranging from −160 mV to +30 mV, in the presence or absence of agonist. Data are expressed as mean ± SEM.

Imaging Analysis. Cells were plated on a glass coverslip and incubated for 15 min at room temperature in 2 μM fura-2 acetoxymethyl ester in a modified Ca^{2+} Hepes buffer: 140 mM *N*-methyl-D-glucamine/5.5 mM glucose, 10 mM Hepes/5 mM KCl/1 mM $MgSO_4$/10 mM $CaCl_2$ (pH 7.4 with HCl). The cells were rinsed twice in buffer, incubated for 15 min at 22°C in buffer with Con A (1 mg/ml), and rinsed as before. The coverslip was mounted in a perfusion chamber and placed on the stage of an inverted multimode fluorescence microscope (BDS). The cells were viewed with a ×40 oil-immersion objective (n.a. 1.3; Zeiss Achrostigmat) and were continuously perfused with either buffer or buffer containing test compound. The cells were sequentially excited with 340-nm and 380-nm UV light, and the emitted fura-2 fluorescence (505 nm) was collected with an intensified video rate camera. The video signals were digitized by a Macintosh Quadra computer and data analysis was performed with EXCEL (Microsoft).

RESULTS

Western Blot. Membrane preparations of the GluR6-expressing cells, the wild-type BHK cells, and rat brain were subjected to SDS/PAGE and immunoblot analysis. When incubated with an anti-peptide antibody which recognizes the GluR6 subunit, a protein band of ≈100 kDa was detected in the GluR6-expressing cells and in rat brain, whereas no band was detected in the wild-type cell line (Fig. 1). The apparent size of this band is in accordance with a previous report on GluR6 expression in a transient expression system (24).

Radioligand Binding Studies. We investigated the binding of [^{3}H]kainate to membranes of the GluR6-transfected BHK cells. The binding was saturable, with a K_d of 12.9 ± 2.4 nM (n = 4) (Fig. 2; Table 1). Displacement profiles for the

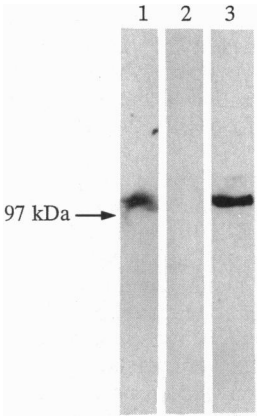

FIG. 1. Western blot showing GluR6 expression in transfected BHK cells. Lane 1, 20 μg of membrane protein from rat brain; lane 2, 20 μg of membrane protein from wild-type BHK570 cells; lane 3, 10 μg of membrane protein from GluR6-transfected BHK cells. Membrane preparations were subjected to SDS/PAGE and immunoblotted with a GluR6 anti-peptide antibody.

non-NMDA antagonists CNQX, DNQX, and NBQX (27) and for the agonists domoate, L-glutamate, quisqualate, and kainate were determined (Table 1). Domoate was the most potent inhibitor of [^{3}H]kainate binding, followed by quisqualate, DNQX, L-glutamate, CNQX, and NBQX. Nonradioactive AMPA was not able to inhibit [^{3}H]kainate binding (K_i >> 15 μM). In all displacement experiments, the Hill slope was close to unity, indicating no cooperativity in binding. Scatchard analysis with various concentrations of CNQX (data not shown) revealed that the blocking effect of CNQX could be surmounted by high concentrations of [^{3}H]kainate, indicating a competitive mode of action.

No specific [^{3}H]AMPA binding (50 nM) could be detected in membrane preparations from the GluR6-expressing BHK cells.

Electrophysiology. Following Con A treatment, application of kainate induced a sustained, dose-dependent increase in whole-cell inward current when the cells were held at a potential of −60 mV. The EC_{50} of kainate for GluR6 was calculated to be 0.82 ± 0.39 μM (n = 4) (Fig. 3). Application of CNQX shifted the dose–response curve to the right (data not shown), in accordance with a competitive mode of action. The *I–V* curve was almost linear, with whole-cell conductances of 0.4 ± 0.1 nS in control solution which were increased to 32.3 ± 4.8 nS (n = 4) in the presence of 3 μM kainate (Fig. 3). The reversal potential was measured to be −2.5 ± 4.8 mV (n = 4). These findings are consistent with previous studies on the fully edited version of GluR6 (7, 16).

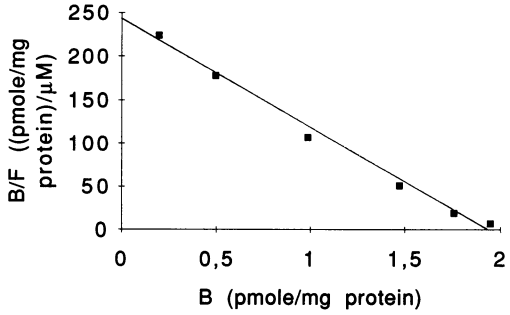

FIG. 2. Scatchard analysis of [^{3}H]kainate binding to GluR6-transfected BHK cells. The plot is from a single experiment performed in duplicate. [^{3}H]Kainate concentrations were from 1 to 300 nM. Protein concentration was 0.2 mg/ml. Nonspecific binding was defined in the presence of 680 μM L-glutamate. B, bound; F, free.

Table 1. Ligand binding properties of GluR6 expressed in BHK cells

Drug	K_d, nM	K_i, nM
Kainate	12.9 ± 2.4	10.5 ± 3.8
Domoate		5.1 ± 3.0
Quisqualate		203 ± 114
L-Glutamate		355 ± 74
CNQX		528 ± 65
DNQX		351 ± 130
NBQX		866 ± 118

Values represent mean ± SD. The K_d value is derived from four saturation experiments performed in duplicate. K_i values are derived from three displacement experiments (10 nM [^{3}H]kainate) performed in duplicate. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline.

Imaging Analysis. Following Con A treatment, stimulation of GluR6-expressing BHK cells with 5 μM kainate in modified Ca^{2+} Hepes buffer resulted in a marked increase in the ratio 340 nm/380 nm, which after a short time reached a plateau. This increase in the ratio reflects an increase in the intracellular Ca^{2+} concentration (Fig. 4). The maximum Ca^{2+} concentration varied from cell to cell, indicating individual differences in the number of receptors presented on the cell surface. The variation in Ca^{2+} response could not be explained by insufficient subcloning of the cell line, as further subcloning resulted in a cell line responding in a similar manner. After washout of kainate, the Ca^{2+} concentration declined to the basal level (≈100 nM) and the cells could be restimulated. More than 75% of the cells responded to kainate (n = 58), whereas no response was seen in wild-type cells (n = 25). Addition of 10 μM kainate to cells not pretreated with Con A also failed to elicit a response (n = 23). This is in accordance with reports on the inhibiting effect of Con A on receptor desensitization (7, 28) and the low time resolution of the Ca^{2+} imaging system. When cells were depolarized by 100 mM KCl, no response was seen (n = 22), indicating that voltage-gated Ca^{2+} channels did not contribute to the observed increase in intracellular Ca^{2+}.

Stability of the Cell Line. The GluR6 expression level was followed by assaying [^{3}H]kainate binding at cell passages 12, 24, 32, 38, 51, 59, and 76 (≈150 days). The maximal number of binding sites (B_{max}) was found to be stable for this period of time and was estimated to be ≈3 pmol/mg of protein. The cells could be frozen and thawed with no change of expression level. To test whether the stability was dependent on continuous agonist exposure, cells were grown for 3 weeks in

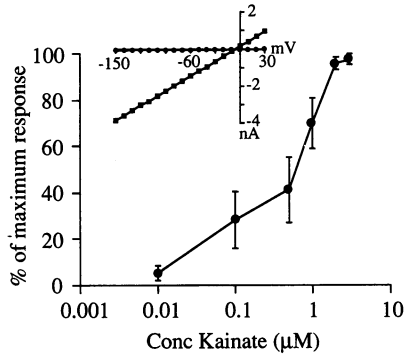

FIG. 3. Dose–response relation for kainate in BHK cells expressing GluR6, obtained by using the patch-clamp technique. Points shown are mean ± SEM for four cells. All responses are correlated to the maximal current (at 3 μM kainate) in the cell from which they were measured. *Inset* shows a representative *I–V* relationship in control solution (●) and in the presence of 3 μM kainate (■).

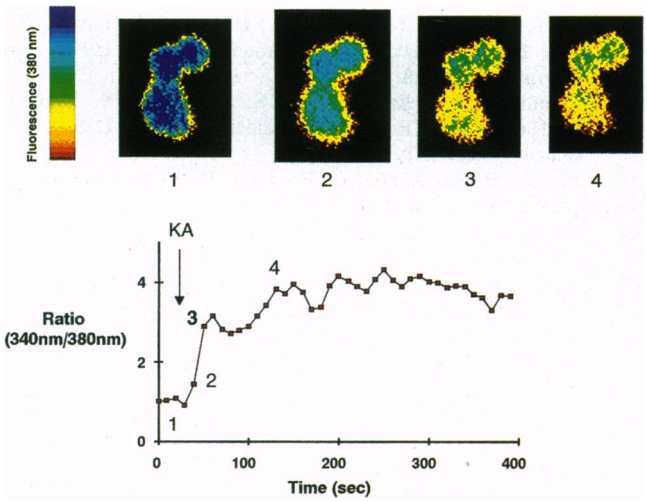

FIG. 4. Kainate-induced increase in the ratio of fluorescence (505 nm) with excitation at 340 nm to fluorescence with excitation at 380 nm in a single representative GluR6-expressing BHK cell loaded with fura-2. The increase in ratio represents an increase in intracellular Ca^{2+} from 100 nM to 2 μM. Arrow indicates application of kainate (KA, 5 μM). The cell is continuously perfused with agonist. After washout of kainate the Ca^{2+} concentration declines to the basal level.

the standard medium with addition of 3 μM domoate. This treatment resulted in a significant decrease in the total number of binding sites (Student's paired t test; $P < 0.05$) from 3.6 ± 0.5 pmol/mg of protein to 2.5 ± 0.4 pmol/mg of protein. In contrast, addition of 100 μM L-glutamate did not cause any decrease in the number of binding sites.

DISCUSSION

The present study shows that it is possible to generate a stable mammalian cell line expressing homomeric functional channels of the ionotropic glutamate receptor GluR6. Previous attempts to generate cell lines stably expressing functional GluR1, -2, and -4 (29, 30) have been only partly successful, with freezing and thawing often resulting in massive losses of binding sites. As previous experiments clearly have demonstrated functionality of these receptors (30), this loss could be the result of an activation of the GluRs and subsequent cell death caused by L-glutamate present in the medium or generated from glutamine. The presence of glutamine in the medium has been shown to cause cell damage in primary neuronal cell cultures through activation of NMDA receptors by generated L-glutamate (31). Recombinant GluR1, -3, and -4 desensitize rapidly when continuously exposed to L-glutamate, leaving, however, a nondesensitizing steady-state inward current (at negative holding potentials) (14, 32). In contrast, GluR6 (fully edited version) desensitizes completely with L-glutamate, whereas application of domoate results in a slowly desensitizing current, leaving a nondesensitizing steady-state component (16). The finding that addition of domoate to the medium of the GluR6-expressing cells decreased the number of binding sites suggests that complete desensitization is important for the survival of cells expressing high numbers of receptors and thus is important for stable expression of ionotropic GluRs.

The pharmacological profile of the GluR6 expressed in the BHK cells resembles the one reported from other cell systems and from rat brain membranes, showing high affinity for domoate (K_i = 5.1 nM) and kainate (K_d = 12.9 nM) and with the rank order of potency being domoate > kainate > quisqualate > L-glutamate > CNQX > AMPA (8, 33, 34). However, the affinities are higher than reported previously. In human embryo kidney 293 cells and in HeLa cells the K_d for kainate has been reported to 36 nM and 95 nM, respectively (8, 33). This indicates that agonist affinities may vary with the cell system or assay conditions.

The observed Ca^{2+} permeability of the fully edited GluR6 is consistent with previous studies (16, 17), as is the EC_{50} for kainate obtained by electrophysiology. In *Xenopus* oocytes, Egebjerg *et al.* (7) reported an EC_{50} value of 1.0 μM for kainate. Also, the *I–V* curve being linear and the reversal potential being close to zero are consistent with reports on the fully edited GluR6 version in low extracellular Ca^{2+} concentrations (7, 16, 17).

In summary, we have presented data indicating that fully functional GluR6 channels can be stably expressed in BHK570 cells. This cell line offers an easy and convenient way of studying GluR6 in a reproducible biological system.

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