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Stable expression of homomeric AMPA-selective glutamate receptors in BHK cells

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Abstract

cDNAs encoding glutamate receptor glu_1, glu_2 (Q and R) or glu_4 under control of a constitutively active metallothionine promoter, were transfected into baby hamster kidney cells. Following the addition of selection agent, transfectants expressing high levels of glutamate receptor as measured by [^{3}H]α-amino-3-hydroxy-5-methyl-isoxalazole-4-propionate (AMPA) binding, were selected for further studies. Using glutamate receptor antibodies, the receptor proteins were visualized in Western blotting as having a molecular weight of approximately 100 kDa. [^{3}H]AMPA binding to the glutamate receptor expressing cell lines revealed that glu_1, glu_2 (Q), and glu_4 receptors displayed a single site in Scatchard analysis with K_d values of 12, 15.7 and 21 nM, respectively. However, the Ca^{2+} impermeable variant of the glu_2 receptor, glu_2 (R) displayed a curvilinear Scatchard plot. Computer resolution suggested the presence of a high and low affinity state (K_H = 2.9 nM; K_L = 40.7 nM). The pharmacological profile of the [^{3}H]AMPA binding to these recombinant receptors resembled the high affinity [^{3}H]AMPA binding site in rat brain showing high affinity for glutamate, quisqualate, and medium affinity for 6-cyano-7-nitro-quinoxaline-2,3-dione, CNQX; 6,7-dinitro-quinoxaline-2,3-dione, DNQX; and 6-nitro-7-sulphanyl-benzo(f)quinoxaline-2,3,dione, NBQX. Kainate displayed low affinity and *N*-methyl-D-aspartate (NMDA), was inactive in inhibiting specific [^{3}H]AMPA binding. These cell lines will prove to be important tools in the study of glutamate receptors.

Keywords: Glutamate receptor; AMPA (α-amino-3-hydroxy-5-methyl-isoxalazole-4-propionate); Subunit; BHK cell; cDNA

1. Introduction

Glutamate is the most widespread excitatory neurotransmitter in the mammalian central nervous system. Glutamatergic transmission is believed to play an important role in memory and learning, epilepsy, stroke and Alzheimers dementia (Bliss and Collingridge, 1993; Olney, 1990).

Glutamate receptors have been classified into the NMDA, AMPA/kainate and the metabotropic class of receptors (Monaghan et al., 1989). Molecular cloning studies in rat identified four AMPA preferring glutamate receptor subunits, each of which can be alternatively spliced to the flip or flop versions. (Boulter et al., 1990; Hollmann et al., 1989; Keinänen et al., 1990; Sommer et al., 1990). The topography of the glutamate receptor family may resemble that of other ligand-gated ion channels, e.g., the nicotinic, glycine and GABA receptors. However, recent data suggest that a five or three transmembrane model better explains certain experimental observations (Hollmann et al., 1994; Jørgensen et al., 1995; Wo and Oswald, 1994).

Investigations into the functional and ligand binding properties of the glutamate receptors have been accomplished so far by transient expression in *Xenopus* oocytes, insect cells or in mammalian cell lines (Hollmann et al., 1989; Keinänen et al., 1990, 1994; Lomeli et al., 1992; Bettler et al., 1992). However, transient expression is less convenient and reproducible than permanent expression, like stable cell lines. We have previously reported on the generation of stable glu_6 receptor cell lines and on the use

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of this system in characterizing glutamate receptor chimers (Tygesen et al., 1994, 1995). Here we report on generation of cell lines stably expressing high levels of glu_1, glu_2 (R and Q) and glu_4 receptors. These data have previously been presented in a preliminary form (Houamed et al., 1992; Rasmussen et al., 1992).

2. Materials and methods

2.1. Cloning and expression constructs

1st strand cDNA was prepared from rat cerebellar or hippocampal RNA isolated by standard guanidinium/CsCl gradient centrifugation. Using this 1st strand cDNA as a template and oligonucleotides based on previously published sequences (Boulter et al., 1990; Hollmann et al., 1989; Keinänen et al., 1990; Sommer et al., 1990), glu_{1-4} flip and flop receptors were cloned by the polymerase chain reaction (PCR). The oligotides used were synthesized on an Applied Biosystem 394 DNA synthesizer and ethanol precipitated before use.

Glu_1 receptor:

1–21: TTAGGATCCACCATGCCGTACATCTTTGCCTTT;

849–820: CCAGTCCACTCGAGTATGGTCTCGGGAGTC;

829–858: GACCATACTCGAGTGGACTGGAAGAGGCCA;

2001–1972: GCCTGCCTCGAGTGTCCCATAAGCAATTTC;

1981–2010: TATGGGACACTCGAGGCAGGCTCCACTAAG;

2721–2701: AATTCTAGATTACAATCCTGTGGCTCCCAAGGG;

Glu_2 receptor:

1–23: AATAATGTCGACCATGCAAAAGATTATGCATATTTC;

942–916: AAGGTTACGGAAAGCTTCAGTCATCAC;

916–942: GTGATGACTGAAGCTTTCCGTAACCTT;

1526–1491: ACCTCTTCTCTCACTAGTGTGATAGTTAATGGAGA;

1491–1526: TGCTCCATTAACTATCACACTAGTGAGAGAAGAGGT;

2649–2627: TTATTAGGTACCTCAAATTTTAACACTCTCGGCCAT;

Glu_3 receptor:

1–21: TTAGGATCCACCATGGGGCAAAGCGTGCTCCGG;

766–739: CATGACTCTCTCGAGTAAAATGTCAGT;

739–766: ACTGACATTTTACTCGAGAGAGTCATG;

1775–1747: TCAGGAGGGCTTTGTGGGTCTCGAGGTTC;

1747–1775: GAACCTCGAGACCCACAAAGCCCTCCTGA;

2664–2644: AATTCTAGATTAGATCTTAACACTTTCTGTTCC;

Glu_4 receptor:

1–21: TTAGGATCCACCATGAGGATTATTTGCAGGCAG;

1074–1045: AGTCAGCCCTTGAATTCGAACCTGCTTCAG;

1045–1074: CTGAAGCAGGTTCGAATTCAAGGGCTGACT;

1761–1732: GTCACTGGGCCCTTCCTTCCCATCCTCAGG;

1732–1761: CCTCAGGATGGGAAGGAAGGGCCCAGTGAC;

2706–2686: AATTCTAGATTATGGTAGGTCCGATGCAATGAC)

The PCRs were carried out using Taq polymerase as described by the manufacturer (Perkin Elmer/Cetus) for 25–40 cycles of 1 min at 94°C, 1 min at 50°C and 1–3 min at 72°C. Each receptor subunit cDNA was assembled from three PCR fragments ranging from 0.8 to 1.5 kb. The DNA sequence was verified to be correct and then subcloned into a mammalian expression vector under the control of a constitutively active metallothionine promoter. For selection of stable transformants, the vector contained the dihydrofolate reductase gene (Petersen et al., 1990). For transfection, DNA was purified by CsCl gradient centrifugation. The expression constructs were transfected into baby hamster kidney (BHK) cells using lipofectin (BRL).

2.2. Membrane preparation and ligand binding

Confluent cells were scraped off and centrifuged at $30\,000 \times g$ for 10 min. The cell pellet was homogenized (Ultra-Turrax for 20 s) in 10 ml ice-cold 30 mM Tris · HCl (pH 7.1) containing 2.5 mM $CaCl_2$ and centrifuged at $30\,000 \times g$ for 10 min at 4°C. This homogenization/centrifugation step was repeated. The pellet was homogenized in buffer, incubated at 37°C for 30 min and centrifuged for 10 min at $30\,000 \times g$ in 4°C. The pellet was rehomogenized in buffer and frozen on dry ice for 30 min. After thawing, the homogenate was centrifuged as above, and the final pellet was homogenized in buffer with 100 mM KSCN (potassium thiocyanid) to yield a final protein concentration of approximately 100–150 μg/ml.

Binding assay consisted of 0.5 ml of membranes, 25 μl [^{3}H]AMPA (final concentration 5 nM or as indicated) and 25 μl H_2O/10 μM glutamate/test compound (total binding/nonspecific binding/compound testing) and were incubated for 30 min at 37°C. The reaction was terminated by rapid filtration through Whatman GF/C glass fiber filters which were subsequently washed with 2×10 ml 0.9% NaCl. The filters were transferred to scintillation vials, scintillation cocktail was added, and the filters were counted in a conventional scintillation counter. All values, K_d, K_i and B_{max} are means from 3–9 independent experiments and were calculated using Radlig (Elsevier Biosoft).

2.3. Western blot analysis

Samples (20 μg protein) were subjected to sodium-dodeyl sulphate polyacrylamid gel electrophoresis (SDS/PAGE) (4–20%) and transferred to nitrocellulose by electroblotting (250 mA for 2 h) as described previously (Towbin et al., 1979). Blots were blocked (30 min) with 3% nonfat dry milk in Tris-borate saline buffer (TBS) at room temperature and then incubated with a glutamate receptor subunit antibody (kindly supplied by Dr Blackstone). After washing for 20 min (five changes of buffer) in TBS with 0.1% Tween-20, blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1 : 2000, Dako P217) for 1 h at room temperature. Following several washes with TBS containing 0.1% Tween-20, immunoreactive proteins were visualized with ECL (Amersham).

2.4. Protein determination

Protein concentrations were determined by the Bio-Rad protein assay using γ-globulin as the standard.

3. Results

3.1. Generation of cell lines

Baby hamster kidney (BHK) cells were transfected with the glu_{1-4} flip or flop receptors subunit cDNA constructs. Following the addition of methotrexate, surviving colonies were subcloned using conventional cloning cylinders and screened for [^{3}H]AMPA binding. Wild type BHK cells had no [^{3}H]AMPA binding while methotrexate resistant colonies, transfected with either the glu_1, glu_2 or glu_4 receptor cDNAs all showed high levels of [^{3}H]AMPA binding. Colonies from cells transfected with glu_3 receptor were no different from the wild type cell with respect to [^{3}H]AMPA binding although Southern blots indicated incorporation of the glu_3 DNA. Colonies showing the highest level of [^{3}H]AMPA binding were selected and subjected to increasing methotrexate levels (0.1–20 μM) and to a number of subclonings in order to obtain cell lines originally representing only a single cell.

These cell lines have been cultivated in standard high glucose Glutamax-DMEM supplemented with 1 μM methotrexate. Their growth rate is slightly higher than the wild type BHK cell, showing a doubling time of 14–16 h compared to 20–24 h for the wild type. The selected cell lines have proven stable for more than 30 passages. However, freezing/thawing of the cells resulted in a massive loss of binding sites (see Table 1). This loss of binding sites was not affected by the inclusion of 6-nitro-7-sulfanyl-benzo(*f*)quinoxaline-2,3,dione (NBQX) (1 μM) in the media (not shown).

3.2. Western blot

Membranes from transfected and untransfected BHK cells and from rat brain were subjected to SDS/PAGE followed by immunoblot analysis. Glutamate receptor antibodies recognized a band of approximately 100 kDa in transfected cells and rat brain whereas no band was detected in the nontransfected cells or in glu_3 receptor transfected cells (see Fig. 1). In some of the cell lines, faint bands of slightly higher molecular weights were detected. In glu_4 receptor cell lines an additional band on approximately 200 kDa was detected.

3.3. Ligand binding properties of the cell lines

Scatchard analysis of [^{3}H]AMPA binding to cells expressing glu_1 and glu_4 receptors revealed only a single high affinity binding site with K_d values of 12 and 21 nM,

Table 1
Recombinant glutamate receptors stably expressed in BHK cells, [^{3}H]AMPA affinity, expression level and effect of freezing and thawing

	Receptor						
	glu_1 flip	glu_1 flop	glu_2 (R) flip	glu_2 (R) flop	glu_2 (Q) flip [a]	glu_4 flip	glu_4 flop
K_d [b] (nM)	12 ± 3.2	13 ± 1.7	K_H 2.9 ± 1.3 K_L 40.7 ± 6.9	K_H 2.5 ± 1.1 K_L 43.9 ± 8.2	15 ± 4.5	22 ± 3.4	20 ± 3.2
B_{max} [b] (pmol/mg protein)	0.6 ± 0.2	2.5 ± 0.3	R_H 1.1 ± 0.4 R_L 6.2 ± 1.4	R_H 1.1 ± 0.4 R_L 6.2 ± 1.4	0.3–2.5	5.0 ± 1.1	0.6 ± 0.2
K_d [c] (nM)	10–18	12–20	16.25	12–25	NT	20 [d]	31 [d]
B_{max} [c] (pmol/mg protein)	0.05–0.06	0.1–0.2	0.2–0.5	0.1–0.3	NT	0.7 [d]	0.03 [d]

[a] Data previously presented in Tygesen et al. (1995). [b] Data are means ± S.D. from 3–9 separate experiments using 5–10 different concentrations of [^{3}H]AMPA. [c] Analysis of [^{3}H]AMPA binding sites following one cycle of freezing/thawing. Data shown are a range from two independent experiments or from [d] single experiments. K_d and B_{max} estimated from inhibition of [^{3}H]AMPA (10 nM) with AMPA. NT not tested. K_H and K_L denotes K_d values for the high or low affinity sites/states of the receptor. R_H and R_L denotes B_{max} values for the high or low affinity sites/states of the receptor.

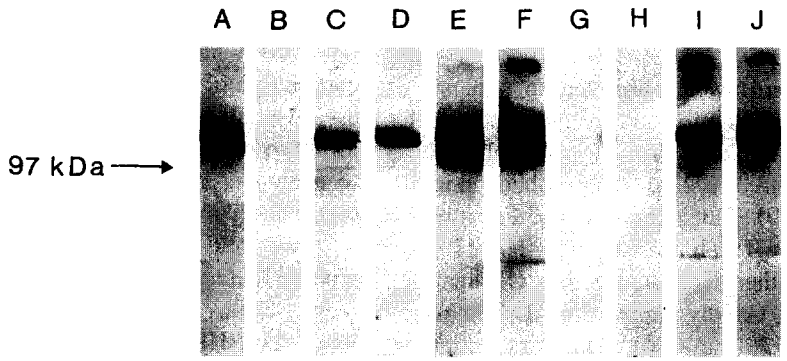

Fig. 1. Western blot of glutamate receptor expressing BHK cell lines. 20 μg protein were loaded per lane: (A) rat brain; (B) wild type BHK cells and BHK cells stably expressing: (C) glu_1 flip receptor; (D) glu_1 flop receptor; (E) glu_2 flip receptor; (F) glu_2 flop receptor; (I) glu_4 flip receptor; (J) glu_4 flop receptor. In lanes G and H 20, μg protein were loaded from cells transfected with cDNA encoding glu_3 flip or flop receptor, respectively. See Materials and methods for experimental details.

respectively. No significant difference was observed between K_d values for the flip and flop variants. The corresponding B_{max} values were 0.6 and 2.5 pmol receptor/mg protein for the glu_1 flip or flop receptor and 5 and 0.6 pmol receptor/mg protein for the glu_4 flip or flop receptor. Scatchard analysis of cells expressing the calcium permeable variant of the glu_2 receptor, glu_2 (Q) also displayed a linear Scatchard plot with $K_d = 15.7$ nM, but with high variability of the expression level ($B_{max} = 0.3$–2.5 pmol/mg protein, $n = 3$) (see Table 1). Surprisingly, a similar analysis of the Ca^{2+} impermeable variant, glu_2 (R) receptor indicated the presence of more binding sites. Computer-assisted analysis of the saturation experiments indicated two sites with K_d values of 2.9 and 40.7 nM. The corresponding B_{max} values were 1.4 and 7.1 and 0.8 and 4.2 pmol receptor/mg protein, respectively for the high and low affinity site in glu_2 (R) flip or flop receptor (see Table 1).

To ensure the pharmacological identity of the stably expressed recombinant receptors, the effect of relevant reference compounds were tested on specific [^{3}H]AMPA binding. Quisqualate, glutamate, 6-cyano-7-nitro-quinoxaline-2,3-dione, CNQX, 6,7-dinitro-quinoxaline-2,3-dione, DNQX and 6-nitro-7-sulfanyl-benzo(*f*)quinoxaline-2,3,dione, NBQX were all capable of inhibiting specific [^{3}H]AMPA binding with fairly high affinity. Kainate also inhibited specific [^{3}H]AMPA binding but with low potency. NMDA had no effect on the [^{3}H]AMPA binding. The results from these experiments are presented in Table 2.

4. Discussion

As compared to transient expression systems, stable cell lines offer a far more convenient and reproducible system. However, stable expression of glutamate receptors has been very troublesome as these receptors seem to be toxic to cells. We have identified BHK cells as being capable of expressing glutamate receptors stably and have also previously reported a glu_6 receptor expressing cell line (Tygesen et al., 1994). Here we extend the previous study and report on the stable expression of the AMPA receptor subfamily glu_1, glu_2 and glu_4 flip and flop receptors.

In these BHK cells, expression of glu_1, glu_2 and glu_4 flip and flop receptors can be maintained for more than 30

Table 2
Recombinant glutamate receptors stably expressed in BHK cells; potency (K_i, nM) of selected compounds

Compounds	Receptor						
	glu_1 flip	glu_1 flop	glu_2 (R) flip	glu_2 (R) flop	glu_2 (Q) flip	glu_4 flip	glu_4 flop
Glutamate	111	157	170	185	477	180	110
Quiqualate	1.8	4.1	3.5	3.6	10.3	9.7	13.7
Kainate	310	220	2200	1650	3054	1350	1330
NBQX	90	115	34	34	394	77	90
CNQX	120	115	150	235	184	460	290
DNQX	250	220	115	90	454	200	190
NMDA	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000

Potency, K_i, determined by inhibition of specific [^{3}H]AMPA (5 nM) binding to recombinant glutamate receptors stably expressed in BHK cells. Data are means (S.D.s were 12–45% of mean) from 3–6 separate experiments. NBQX, 6-nitro-7-sulfanyl-benzo(*f*)quinoxaline-2,3,dione; DNQX, 6,7-dinitro-quinoxaline-2,3-dione; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-isoxalazole-4-propionate. > indicates less than 10% inhibition of specific binding at the concentration indicated.

passages. Contrary to glu_6 receptor, however, expression of the AMPA receptor family is not fully stable as freezing and thawing results in a massive loss of binding sites (Table 1). As discussed previously (Tygesen et al., 1994), the reason could be the presence of a nondesensitizing current in the AMPA receptors. This nondesensitizing current is not present in glu_6 receptor homomeric channels (Hollmann et al., 1991; Köhler et al., 1993; Verdoorn et al., 1991).

The pharmacological profile of the expressed receptors closely resembles the profile of the AMPA high affinity binding site in rat brain (Honoré and Drejer, 1988). Thus, agonists – quisqualate and AMPA – had high (1–20 nM), glutamate medium (100–200 nM) and kainate low (200–2000 nM) affinity. NMDA, a selective NMDA receptor agonist had no effect on the expressed AMPA receptors as labeled with [^{3}H]AMPA. The antagonists CNQX, DNQX and NBQX all displayed medium affinity (30–500 nM). No subtype selectivity was observed for any of the compounds tested. In general, the affinity observed is in close agreement with previous reports from transient expression in either mammalian or insect cells (Keinänen et al., 1990, 1994; Lomeli et al., 1992).

Surprisingly, the glu_2 (R) flip and flop receptors as the only subunits, appeared to exist in two states; a high and low affinity form. A single amino acid change (R to Q) in the putative transmembrane region 2 of the glu_2 receptor changed the two state form of the glu_2 receptor into a single affinity form. In addition, the affinity for both agonists and antagonists dropped 2–10-fold. At present, the molecular mechanism underlying this observation is not understood. It is, however, interesting that this particular amino acid affects the ligand binding properties of the glutamate receptor, as it has previously been demonstrated to convey the ion selectivity of the glutamate receptor ion channel (Hume et al., 1991; Verdoorn et al., 1991).

Even though it was attempted to express the glu_3 receptor, we were unsuccessful, since neither specific [^{3}H]AMPA binding, glu_3 receptor protein nor glu_3 receptor mRNA (not shown) could be demonstrated in BHK cells transfected with glu_3 receptor constructs. The reason for this is unknown at present.

In summary, we have presented data demonstrating that homomeric AMPA receptor channels can be expressed in BHK cells. The functionality of these receptors has previously been demonstrated (Houamed et al., 1992). These cell lines will prove to be valuable tools in novel high throughput screens and in characterizing the molecular regulation of these receptors.

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