Volume 308, number 3, 253-257 FEBS 11437 August 1992

Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel

Shigeo Uchino[a], Kenji Sakimura[b], Kenji Nagahari[a] and Masayoshi Mishina[b]

[a]*Biosciences Laboratory, Research Center, Mitsubishi Kasei Corporation, Yokohama 227, Japan* and [b]*Department of Neuropharmacology, Brain Research Institute, Niigata University, Niigata 951, Japan*

Received 18 June 1992; revised version received 6 July 1992

The region preceding putative transmembrane segment M1 of the glutamate receptor (GluR) channel is well conserved among subunits and has been proposed to constitute a part of the agonist binding site. The functional significance of this region was examined by introducing point mutations into charged residues of the α1 subunit of the mouse α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective GluR channel. The dose-response relationships of the mutant receptors were studied after expression in *Xenopus* oocytes by injection of the mutant α1 subunit-specific mRNA together with the wild-type α2-subunit-specific mRNA. Variable changes in the EC_{50} values for different agonists were found for the replacement of glutamic acid 398 by lysine and for the replacement of lysine 445 by glutamic acid. These residues may be involved in selective interaction of the GluR channel with agonists.

Glutamate receptor channel; Agonist binding site; Site-directed mutagenesis

1. INTRODUCTION

GluR channels mediate most of the fast excitatory synaptic transmission in the central nervous system [1] and play a key role in synaptic plasticity, thought to underlie memory and learning as well as development of the nervous system [2,3]. Furthermore, abnormal activation of GluR channels has been suggested to lead neuronal cell death observed in various acute and chronic disorders [4,5].

GluR channels have been classified into three major subtypes, that is, receptors for kainate, AMPA and *N*-methyl-D-asparate (NMDA) based on pharmacological and electrophysiological properties [1,6]. Recent studies on cloning and expression of subunit cDNAs have revealed a great molecular diversity of the GluR channel subunit [7–23]. These GluR channel subunits contain four putative transmembrane segments (M1–M4) characteristic for neurotransmitter-gated ion channels and can be classified into six subfamilies (the α, β, γ, δ, ε and ζ subfamilies) according to the amino acid sequence homology [22,23]. The members of the α subfamily form homomeric and heteromeric channels responsive to L-glutamate, quisqualate, AMPA and kainate [7–11]. The apparent affinities of these channels are higher for quisqualate and AMPA than for kainate, indicating that the α subfamily represents AMPA-selective GluR channels. The β2 (GluR6) subunit forms homomeric channels responsive to L-glutamate and kainate, but not to AMPA [13,17]. The γ2 (KA-2) subunit when expressed together with the β2 or GluR5 subunit yields functional GluR channels selective for kainate [18,19]. Thus the β and γ subfamilies include the subunits of the kainate-selective GluR channel. The members of the ε and ζ subfamilies constitute NMDA receptor channels [15,21–23].

Studies with site-directed mutagenesis have shown that arginine 586 in putative transmembrane segment M2 of the α2 subunit determines the cation permeability of the AMPA-selective GluR channel [24–26]. Functional importance of segment M2 in ion selectivity is in accord with the current transmembrane topology model of the GluR channel [8,10,11] analogous to that of the acetylcholine receptor channel, for which supporting evidence has accumulated indicating that segment M2 forms the transmembrane ion channel [27]. The agonist binding site of the acetylcholine receptor channel has been mapped in the region preceding segment M1 [28]. By analogy, it is reasonable to assume that the glutamate binding pocket of the GluR channel is formed by the region preceding segment M1. In the present investigation, the functional significance of this region of the α1 subunit of the mouse AMPA-selective GluR channel has been examined by site-directed mutagenesis.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction.

Correspondence address: M. Mishina, Department of Neuropharmacology, Brain Research Institute, Niigata University, Asahimachi 1, Niigata 951, Japan. Fax: (81) (25) 225 6458.

2. MATERIALS AND METHODS

2.1. *Mutagenesis*

Site-directed mutagenesis of the α1 subunit of the mouse GluR channel selective for AMPA was carried out using appropriate synthetic oligonucleotides and DNA fragments derived from the plasmid pSPGR1 [10] by the two-step polymerase chain reaction (PCR) essentially as described [29], except that the α1-K445E mutant was constructed previously by the method of Nakamaye and Eckstein [25,30]. PCR was run for 30 cycles in the first step and for 15 cycles in the second step. PCR buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1 μM each of primers, 200 μM each of four deoxynucleotide triphosphates, 0.5–5 μg/ml template DNA and 25 U/ml *Taq* polymerase. Cycle conditions were heating at 95°C for 1 min, annealing at 55–65°C for 2 min, and synthesis at 72°C for 2 min. PCR products were digested with *Stu*I or *Bam*HI, and resulting *Stu*I (854)–*Stu*I (1,363) or *Bam*HI (1,348)–*Bam*HI (1945) fragments were substituted for the corresponding wild-type fragments in pSPGR1 (Table I). The entire nucleotide sequences of DNA fragments replaced were determined by the dideoxy chain termination method [31] using the GENESIS 2000 DNA Analysis System (Du Pont).

2.2. *Functional analysis*

Messenger RNAs specific for mutant and wild-type GluR channel subunits were synthesized in vitro as described [10]. *Xenopus laevis* oocytes were injected with the wild-type or mutant α1 subunit-specific mRNA (~ 5 pr ~ 10 ng/oocyte) and the wild-type α2 subunit-specific mRNA (~ 1.3 or ~ 2.5 ng/oocyte). The injected oocytes were incubated at 19°C for 2–4 days in modified Barth's medium [32] containing gentamycin (0.1 mg/ml). On the second day, the follicular cell layer was mechanically removed after treatment with 1 mg/ml collagenase (Wako) for 1 h [33]. Whole-cell currents were recorded at −70 mV membrane potential and ~ 20°C with a conventional two-microelectrode voltage clamp; the two pipettes were filled with 3 M KCl. The chamber was continuously perfused with normal frog Ringer's solution composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM Hepes-NaOH (pH 7.2). Agonists were bath-applied for ~ 15 s. Dose–response curves were fitted by the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$, where I is the current response, I_{max} is the maximum response, A is the concentration of agonist and n is the Hill coefficient. When very high concentrations of agonists were applied, small current responses were observed for non-injected control oocytes; 0.4–9 nA for 1–30 mM L-glutamate and 0.6–5 nA for 3–30 mM kainate (averages of measurements on 6–7 oocytes). These values were subtracted from the current responses measured for injected oocytes.

3. RESULTS

The region preceding segment M1 is highly conserved among GluR channel subunits [7–23] and has been proposed to constitute a part of the agonist binding site [10,11]. To test this proposal, we introduced point mutations into charged residues in this region, under the assumption that hydrogen bond interactions through charged residues play a major role in agonist binding to the GluR channel as is the case for the bacterial aspartate receptor [34]. The point mutations introduced into the α1 subunit of the mouse AMPA-selective GluR channel are shown in Fig. 1, together with the alignment of putative agonist binding regions of the mouse and *Drosophila* GluR channel subunits and the chick kainate binding protein [10,17,18,20–22,35,36]. These mutants are named by the wild-type amino acid residue, the

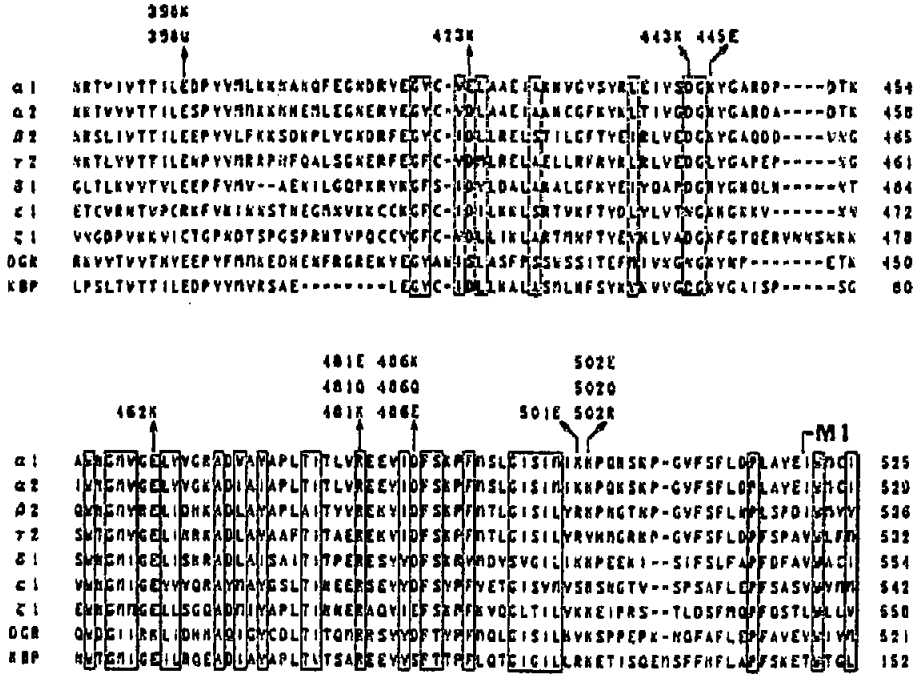

Fig. 1. Point mutations introduced into the α1 subunit of the mouse GluR channel and alignment of putative agonist-binding regions of the mouse and *Drosophila* GluR channel subunits and the chick kainate binding protein. The amino acid substitutions and putative transmembrane segment M1 are indicated. Sets of identical or conservative residues are enclosed. Conservative amino acid groups are defined as follows [38]: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W. Numbers of the amino acid residues are given at the end of the individual lines. Amino acid sequences are taken from [10] (α1, α2), [17] (β2), [18] (γ2), [20] (δ1), [22] (ε1), [21] (ζ1), [35] (*Drosophila* DGluR-II, DGR), and [36] (chick kainate binding protein, KBP).

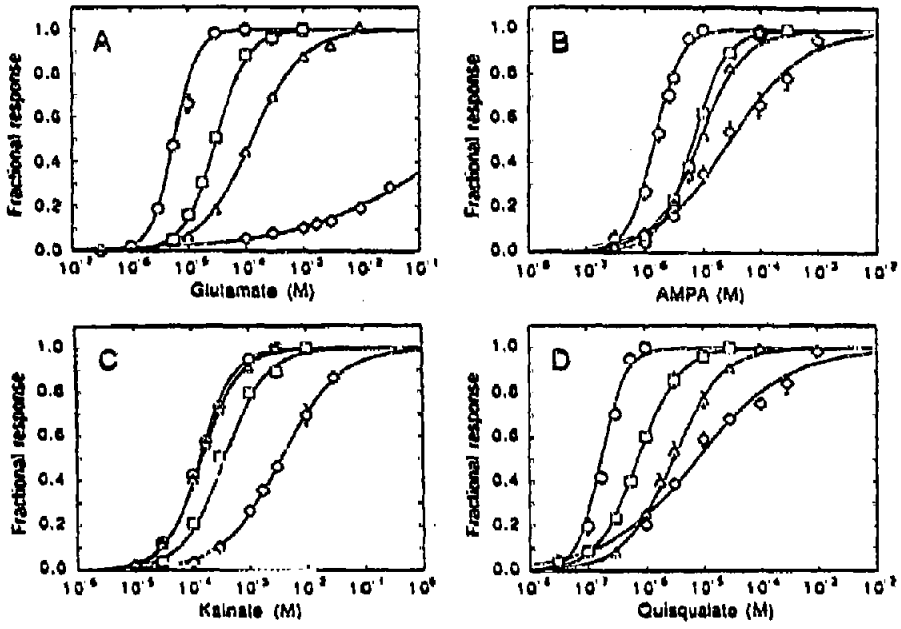

Fig. 2. Effects of point mutations on dose-response curves for L-glutamate (A), AMPA (B), kainate (C) and quisqualate (D). Each point represents the mean fractional responses obtained from measurements on 3-5 oocytes at −70 mV membrane potential; the maximum current responses for the mutation α1-E398K were estimated by fitting to the theoretical curve. S.E.M. are indicated by bars when larger than the symbols. ○, wild type; ◇, α1-E398K; □, α1-D443K; △, α1-K445E. The largest current responses measured were as follows. (A) 23–138 nA (○), 14–57 nA (◇), 21–63 nA (□) and 47–172 nA (△). (B) 16–42 nA (○), 17–44 nA (◇), 25–45 nA (□) and 19–103 nA (△). (C) 123–350 nA (○), 42–115 nA (◇), 184–298 nA (□) and 120–875 nA (△). (D) 22–36 nA (○), 13–37 nA (◇), 16–23 nA (□) and 16–36 nA (△).

residue number and the substituted residue. For example, α1-E398K represents the α1 subunit mutant in which glutamic acid 398 is replaced by lysine. Because the heteromeric α1/α2 GluR channel exhibits much larger current responses than the homomeric α1 channel [10], the effects of the point mutations on dose–response relationships for L-glutamate, AMPA, kainate and quisqualate were examined after co-expression of the mutant α1 subunit and the wild-type α2 subunit in *Xenopus* oocytes by injection of the respective subunit-specific mRNAs. The wild-type heteromeric α1/α2 channel shows higher apparent affinities for AMPA and quisqualate than for kainate, but exhibits larger current responses to kainate than to L-glutamate, AMPA and quisqualate ([10]; Fig. 2, circles).

Table I

Construction of point mutations in the GluR α1 subunit

Wild type		Mutant		cDNA fragment replaced
Amino acid	Codon	Amino acid	Codon	
Glutamic acid-398	GAA	Lysine	AAA	*Stu*I
Glutamic acid-398	GAA	Glutamine	CAA	*Stu*I
Glutamic acid-423	GAA	Lysine	AAA	*Stu*I
Aspartic acid 443	GAC	Lysine	AAA	*Stu*I
Lysine 445	AAA	Glutamic acid	GAA	*Stu*I
Glutamic acid-462	GAG	Lysine	AAG	*Bam*HI
Arginine 481	CGG	Glutamic acid	GAG	*Bam*HI
Arginine 481	CGG	Glutamine	CAG	*Bam*HI
Arginine 481	CGG	Lysine	AAG	*Bam*HI
Aspartic acid 486	GAC	Lysine	AAG	*Bam*HI
Aspartic acid 486	GAC	Glutamine	CAG	*Bam*HI
Aspartic acid 486	GAC	Glutamic acid	GAG	*Bam*HI
Lysine 501	AAG	Glutamic acid	GAG	*Bam*HI
Lysine 502	AAG	Glutamic acid	GAG	*Bam*HI
Lysine 502	AAG	Glutamine	CAG	*Bam*HI
Lysine 502	AAG	Arginine	AGG	*Bam*HI

*Stu*I and *Bam*HI represent the *Stu*I (854)-*Stu*I (1,363) and *Bam*HI (1348)-*Bam*HI (1,945) fragments from pSPGR1, respectively.

Replacement of lysine 501 by glutamic acid (the mutation α1-K501E) did not appreciably affect dose–response relationships for agonists (Table II) and current amplitudes. Similarly, the mutations α1- E423K, α1-K445E and α1-E462K exerted little effect on dose–response relations for kainate (Table II). The α1- K445E mutation, however, resulted in reduction of the apparent affinities for L-glutamate (22-fold), AMPA (6- fold) and quisqualate (17-fold) as shown in Fig. 2 (triangles). The mutation thus differentially affects the EC_{50} values for respective agonists. The effects of the mutations α1-E423K and α1-E462K on the affinity for L- glutamate were not examined because of low channel activities. The efficient assembly of the two subunits, expression on the cell surface or gating of the channel may be hindered by the mutations α1-423K and α1- E462K.

Replacement of negatively charged glutamic acid 398 or aspartic acid 443 by positively charged lysine (the mutations α1-E398K and α1-D443K) decreased appar-

ent affinities for all the agonists examined (Fig. 2, diamonds and squares). The effects of the mutation α1-E398K were so strong that the current responses did not reach a plateau even at the highest concentrations of the agonists tested. The EC_{50} values for agonists were estimated by extrapolating the fitting of the dose–response curves. The decrease in the apparent affinity for L-glutamate could be ~ 100,000-fold, whereas the extents of reduction were ~ 20 to ~ 40 fold for AMPA, kainate and quisqualate (Table II). The Hill coefficient values were less than unity. On the other hand, the effects of the mutation α1-D443K were rather small (3–5-fold reduction) and no significant differences in the extents of decrease were found among agonists (Table II). Neutralization of glutamic acid 398 (the mutation α1-E398Q) exerted no appreciable effect on the apparent affinity for kainate (Table II), but the current amplitudes observed were smaller.

Substitution of lysine for aspartic acid 486 (the mutation α1-D486K) resulted in vanishment of any detectable responses. When aspartic acid 486 was replaced by glutamine or glutamic acid (the mutations α1-D486Q and α1-D486E), a small response to kainate was detected without any appreciable alteration of the apparent affinity (Table II). The effects of these substitutions on the affinities for L-glutamate, AMPA and quisqualate were not examined because of low channel activities. Replacement of lysine 502 by glutamic acid or glutamine (the mutations α1-K502E and α1-K502Q) also resulted in failure of functional receptor formation. Only arginine could replace lysine 502 without significant change in the apparent affinity for kainate (the mutation α1-K502R, Table II), but the current amplitudes were small. Arginine 481 could not be substituted for by any of glutamic acid, glutamine and lysine without loss of measurable responses (the mutations α1-R481E, α1-R481Q and α1-R481K), suggesting the importance of this arginine residue.

Table II

Effects of point mutations in the GluR α1 subunit on EC_{50} values

α1 subunit	EC_{50} values (μM)			
	L-Glutamate	AMPA	Kainate	Quisqualate
Wild type	5.8 (2.2)	1.4 (2.0)	130 (1.4)	0.17 (2.0)
E398K	600,000 (0.3)	30 (0.7)	3,500 (0.8)	7.6 (0.5)
E398Q	ND	ND	170 (1.2)	ND
E423K	ND	NT	180 (1.0)	NT
D443K	31 (1.5)	6.6 (1.5)	370 (1.2)	0.73 (1.2)
K445E	130 (1.0)	8.4 (1.1)	150 (1.3)	2.9 (1.0)
E462K	ND	NT	120 (1.2)	NT
D486Q	ND	ND	100 (1.0)	ND
D486E	ND	ND	140 (1.2)	ND
K501E	6.5 (1.7)	1.8 (1.7)	94 (1.3)	0.14 (1.8)
K502R	ND	ND	140 (1.1)	ND

EC_{50} values for respective agonists were calculated from dose–response curves obtained from measurements on 2–8 oocytes; the dose–response curves did not reach a plateau for the mutation of α1-E398K and the EC_{50} values were estimated by fitting to a theoretical curve. Hill coefficient values are indicated in parentheses. ND, not determined because of low channel activies. NT, not tested.

4. DISCUSSION

Functional significance of a putative agonist-binding region preceding segment M1 of the α1 subunit was examined by site-directed mutagenesis and analysis of dose–response relationships of mutant GluR channels expressed from cDNAs. Several charged residues in this region have been identified to be important for responses to agonists. Among mutants examined, the strongest effect on the apparent affinities for agonists as well as the Hill coefficient values was observed for the mutation α1-E398K. It is possible that heteromeric channel formation or subunit cooperativity may be hindered by the mutation. However, the EC_{50} values were much larger than those for the homomeric α1 channel [10]. Decrease in the apparent affinity caused by the mutation was remarkably larger for L-glutamate than for AMPA, kainate and quisqualate. Differences in the extents of reduction among agonists were also found for the mutation α1-K445E. This mutation exerted no appreciable effect on the apparent affinity for kainate, but strongly decreased the apparent affinities for L-glutamate, AMPA and quisqualate. Glutamic acid 398 is highly conserved among non-NMDA receptor channels [7–14, 16–19] and kainate binding proteins [36,37], but is not found in NMDA receptor channels [15,21–23]. It is interesting that lysine 445 is also well conserved among GluR channels [7–13,15–17,20–23] and kainate binding proteins [36,37] except for the γ subfamily of the kainate-selective GluR channel [14,18,19]. These residues may be involved in selective interaction of the GluR channel with different agonists.

Arginine 481, aspartic acid 486 and lysine 502 are located in a highly conserved domain in the putative agonist binding region preceding segment M1 (Fig. 1). Conversion of these residues to oppositely charged residues abolished the formation of responsive GluR channels. Remarkably, a subtle change from arginine to lysine at the position 481 also resulted in the failure of functional receptor production. Among charged residues found in a putative agonist-binding region preceding segment M1, arginine 481 is the only one that is completely conserved among GluR channels [7–23,35] and kainate binding proteins [36,37]. It is possible that these residues are essential for proper folding and assembly of the subunit polypeptide or gating of the channel. An alternative possibility is that the charged residues are key elements of the agonist binding site of the GluR channel. In this respect, it is to be noted that the highly charged ligand binding pocket of the *Salmonella typhimurium* aspartate receptor is formed by three arginine residues, which interact with aspartate by hydrogen bonds [34].

Acknowledgements: We thank Dr. Mikio Nakazawa for advice. This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Institute of Physical and Chemical Research, the Ministry of Health and Welfare of Japan, and the Yujin Memorial Grant.

REFERENCES

[1] Mayer, M.L. and Westbrook, G.L. (1987) Prog. Neurobiol. 28, 197–276.
[2] Collingridge, G.L. and Bliss, T.V.P. (1987) Trends Neurosci. 10, 288–293.
[3] McDonald, J.W. and Johnston, M.V. (1990) Brain Res. Rev. 15, 41–70.
[4] Choi, D.W. (1988) Neuron 1, 623–634.
[5] Olney, J.W. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 47–71.
[6] Monaghan, D.T., Bridges, R.J. and Cotman, C.W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 365–402.
[7] Holmann, M., O'Shea-Greenfield, A., Rogers, S.W. and Heinemann, S. (1989) Nature 342, 643–648.
[8] Keinänen, Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. and Seeburg, P.H. (1990) Science 249, 556–560.
[9] Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C. and Heinemann, S. (1990) Science 249, 1033–1037.
[10] Sakimura, K., Bujo, H., Kushiya, E., Araki, K., Yamazaki, M., Yamazaki, M., Meguro, H., Warashina, A., Numa, S. and Mishina, M. (1990) FEBS Lett. 272, 73–80.
[11] Nakanishi, N., Shneider, N.A. and Axel, R. (1990) Neuron 5, 569–581.
[12] Bettler, R., Boulter, J., Hermans-Borgmeyer, L., O'Shea-Greenfield, A., Deneris, E.S., Moll, C., Borgmeyer, U., Hollmann, M. and Heinemann, S. (1990) Neuron 5, 583–595.
[13] Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I. and Heinemann, S. (1991) Nature 351, 745–748.
[14] Werner, P., Voigt, M., Keinänen, K., Wisden, W. and Seeburg, P.H. (1991) Nature 351, 742–744.
[15] Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1991) Nature 354, 31–37.
[16] Bettler, B., Egebjerg, J., Sharma, G., Pecht, G., Hermans-Borgmeyer, I., Moll, C., Stevens, C.F. and Heinemann, S. (1992) Neuron 8, 257–265.
[17] Morita, T., Sakimura, K., Kushiya, E., Yamazaki, M., Meguro, H., Araki, K., Abe, T., Mori, K.J. and Mishina, M. (1992) Mol. Brain Res. 14, 143–146.
[18] Sakimura, K., Morita, T., Kushiya, E. and Mishina, M. (1992) Neuron 8, 267–274.
[19] Herb, A., Burnashev, N., Werner, P., Sakmann, B., Wisden, W. and Seeburg, P.H. (1992) Neuron 8, 775–785.
[20] Yamazaki, M., Araki, K., Shibata, A. and Mishina, M. (1992) Biochem. Biophys. Res. Commun. 183, 886–892.
[21] Yamazaki, M., Mori, H., Araki, K., Mori, K.J. and Mishina, M. (1992) FEBS Lett. 300, 39–45.
[22] Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K. and Mishina, M. (1992) Nature 357, 70–74.
[23] Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M. and Mishina, M. (1992) Nature 358, 36–41.
[24] Hume, R.I., Dingledine, R. and Heinemann, S.F. (1991) Science 253, 1028–1031.
[25] Mishina, M., Sakimura, K., Mori, H., Kushiya, E., Harabayashi, M., Uchino, S. and Nagahari, K. (1991) Biochem. Biophys. Res. Commun. 180, 813–821.
[26] Burnashev, N., Monyer, H., Seeburg, P.H. and Sakmann, B. (1992) Neuron 8, 189–198.
[27] Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K. and Numa, S. (1988) Nature 335, 645–648.
[28] Galzi, J.-L., Revah, F., Bessis, A. and Changeux, J.-P. (1991) Annu. Rev. Pharmacol. 31, 37–72.
[29] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[30] Nakamaye, K.L. and Eckstein, F. (1986) Nucleic Acid Res. 14, 9679–9698.
[31] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[32] Gurdon, J.B. (1974) The Control of Gene Expression in Animal Development, Clarendon, Oxford.
[33] Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S. and Sakmann, B. (1986) Pflügers Arch. 407, 577–588.
[34] Milburn, M.V., Prive, G.G., Milligan, D.L., Scott, W.G., Yeh, J., Jancarik, J., Koshland, D.E. Jr and Kim, S.-H. (1991) Science 254, 1342–1347.
[35] Schuster, C.M., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B. and Betz, H. (1991) Science 254, 112–114.
[36] Gregor, P., Mano, I., Maoz, I., Mekeown, M. and Teichberg, V.I. (1989) Nature 342, 689–692.
[37] Wada, K., Dechesne, C.J., Shimasaki, S., King, R.G., Kusano, K., Buonanno, A., Hampson, D.R., Banner, C., Wenthold, R.J. and Nakatani, Y. (1989) Nature 342, 684–689.
[38] Dayhoff, M.O., Schwartz, R.M. and Orcutt, B.C. (1978) in: Atlas of Protein Sequence and Structure (Dayhoff, M.O., Ed.) vol. 5, pp. 345–352, National Biomedical Research Foundation, Silver Spring, Maryland.