ISAS - INTERNATIONAL SCHOOL
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Excitatory Amino Acid Evoked Currents and Their Interaction in Rat Cerebellar ranule Cells

Thesis Submitted for the Degree of
Doctor Philosophiae

Candidate:
Xin Zheng

Supervisor:
Dr. Oscar Moran
Co-Supervisor:
Prof. Enrico Cherubini

October 1991

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Fluctuation analysis of the domoic acid evoked currents in cerebellar granule cells.

M. Sciancalepore, Z. Galdzicki, X. Zheng and O. Moran. Fidia Research Foundation Symposium: Excitatory amino acids 1990, Montegrotto Terme, Italy, 1990.

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X. Zheng, M. Sciancalepore and O. Moran. IX Congresso della Societa' Italiana di Biofisica Pura ed Applicata. Isola D'Elba, Italy, 1990

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X. Zheng and O. Moran. 14th Annual Meeting of the European Neuroscience Association, Cambridge, England, 1991

Content

Introduction

Since the pioneering discovery that L-glutamate and a number of other naturally occurring acidic amino acids excited single neurons in the mammalian brain (Curtis et al. 1959), considerable evidence has accumulated that these amino acids and related analogues may function as excitatory neurotransmitters in the mammalian central nervous system (CNS). Consequently, this category of amino acids has been named excitatory amino acids (EAA). The last decade has seen great advances in understanding the physiology and pharmacology of excitatory amino acids. Excitatory amino acid receptors are now generally accepted as the main transmitter receptors mediating synaptic excitation in the vertebrate CNS. In addition to their basic role in synaptic excitation, excitatory amino acid receptors are involved in many physiological phenomena ranging from the processing of sensory information, through coordinated movement patterns, to cognitive processes, learning and memory, synaptic plasticity, etc. (Wroblewski and Danysz 1989). Dysfunction of this system is involved in various neurological disorders including epilepsy, spasticity or neurodegenerative diseases, such as Huntington's and Alzheimer's diseases (Greenamyre et al. 1985b, Young et al. 1988). Furthermore, NMDA receptors are involved in one form of synaptic plasicity, the long-term potentiation (LTP), which is a long-lasting increase in the efficacy of synaptic transmission (Nicoll et al. 1988, Kennedy 1989). LTP is considered as a cellular model for learning and memory. Besides the interest in the research of fundamental

mechanisms of the CNS, the growing evidence that EAA system is causally related to a number of major pathological states has added great impetus to research in EAA receptors. To face the puzzle of the physiology and pathology of EAA receptors, a wide range of techniques, including electrophysiology, pharmacology, biochemistry and molecular biology, are currently used.

The work presented in this thesis is an electrophysiological study of glutamate receptors in the primary cultures of the vertebrate CNS neurons. In Chapter 1 the general features of EAA are briefly reviewed; Chapter 2 introduces the experimental methods; Chapter 3 contains the general electrophysiological properties of cerebellar granule cell in primary cultures, which is the preparation being used in all of our experiments; Chapter 4 deals with the interaction of quisqualate and kainate at the non-NMDA receptor; and Chapter 5 is devoted to a general discussion.

Chapter 1

Excitatory Amino Acids

The excitatory action of L-glutamate on vertebrate CNS neurons has been known for over 30 years. During the last decade, rapid progress has been achieved due to the discovery of sufficiently specific agonists and antagonists and the development of the *in vitro* electrophysiological experimental techniques. The properties of EAA are briefly reviewed in this chapter.

1.1 Agonists

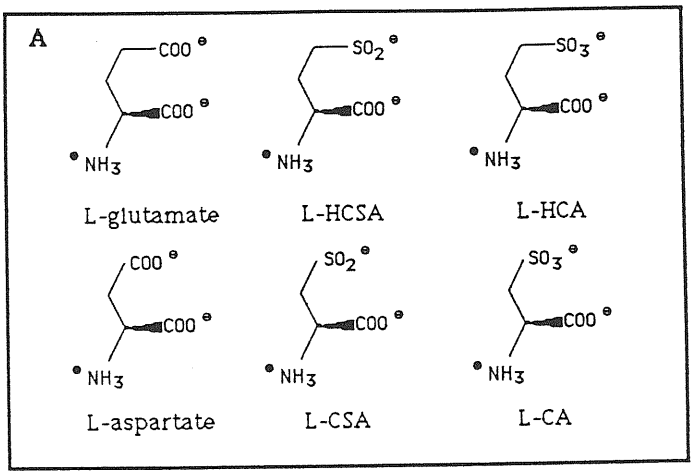
Endogenous Agonists. L-Glutamate, which occurs naturally in brain, was the first EAA recognized in 1950s from its convulsive effects (Hayashi 1954) and its ability to depolarize and excite single central neurons (Curtis et al. 1959). Subsequently, a large number of endogenous compounds have been found to be able to excite CNS neurons through EAA receptors. Some of them have been widely studied and these compounds are L-glutamate (Hayashi 1954, Curtis et al. 1960, 1961), L-aspartate (Hayashi 1954, Curtis et al. 1960, 1961), and their sulfur-containing amino acid analogues: cysteic acid (CA), homocysteic acid (HCA), cysteine sulfinic acid (CSA), homocysteine sulfinic acid (HCSA) and S-sulfocysteine (SCA) (Curtis et al. 1960, 1961, Curtis and Watkins 1963, Mewett et al. 1983). These excitatory amino acids, especially glutamate, are believed to

play the roles of mediating the excitatory neurotransmission in the synapses (see **1.2**).

The structure-activity studies of excitatory amino acids established that the presence of one basic and two acidic groups in the molecule is optimally required for such activity (Curtis and Watkins 1960). The structure of the above mentioned endogenous compounds are shown in Fig. 1.1A.

Exogenous Agonists. Besides the endogenous EAA agonists, some synthetic or/and naturally occurring substances, which do not exist in brain, can also excite CNS neurons by acting on EAA receptors. Some examples are: N-methyl-D-aspartate (NMDA, Curtis and Watkins 1963), kainate (KA, Shinozaki and Konishi 1970), quisqualate (QA, Biscoe et al. 1975), α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate (AMPA, Krogsgaard-Larsen et al. 1980), domoate (DA, Biscoe et al. 1975) and ibotenate (Johnston et al. 1968). Fig 1.1B shows the structure of these compounds.

Among these exogenous agonists, NMDA, KA and QA are prototypic agonists. NMDA is a synthetic analogue of aspartate (Watkins 1962). It was shown to be a potent neuronal excitant in the spinal cord in 1963 (Curtis and Watkins 1963). KA, a glutamate analogue, was found to be a powerful excitant of rat cortical neurons in 1970 (Shinozaki and Konishi 1970). QA was first described as a potent excitant at the crayfish neuromuscular junction (Shinozaki and Shibuya 1974) and then was shown to be a excitant in frog and rat spinal cord (Biscoe et al. 1975). All three agonists depolarize the membrane of vertebrate CNS neurons. However, the patterns of the activations of NMDA, KA and QA are different. As reported by Herrling in cat caudate neurons, NMDA causes repetitive long-duration depolarizations and burst activity while quisqualate causes only repetitive firing (Herrling et al. 1983). Based on these observations, the EAA receptors were initially divided into two categories named NMDA and non-NMDA respectively.



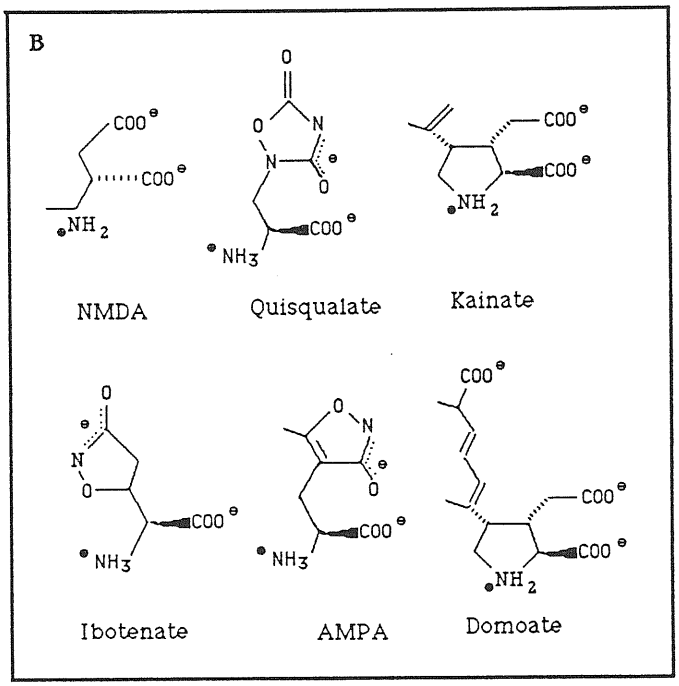


Fig.1.1 A Endogenous excitatory amino acid agonists. Abbreviations: L-HCSA, L-homocysteine sulphinic acid; L-HCA, L-homocysteic acid; L-CSA, L-cysteine sulphinic acid; L-CA, L-cysteic acid. B Exogenous excitatory amino acid agonists.

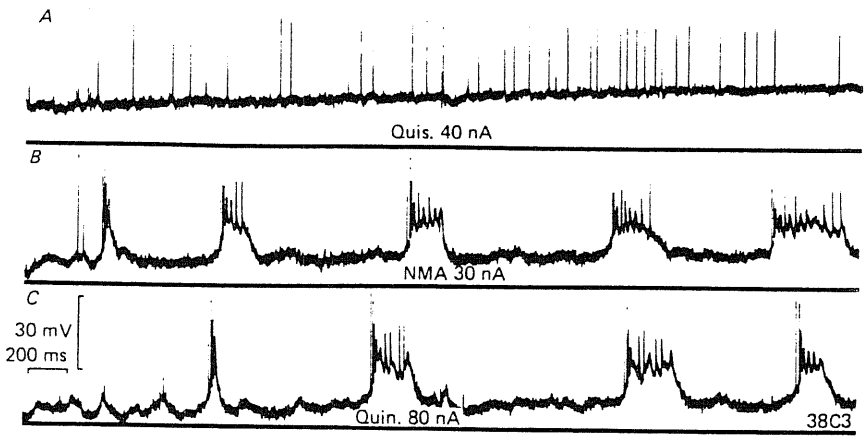


Fig. 1.2 The effects of quisqualate, N-methyl-DL-aspartate (NMA) and quinolinate on the membrane potentials of cat caudate neurones. **A** Quisqualate depolarized the membrane and elicited repetitive firing but no plateaus. **B** and **C** Both NMA and quinolinate, another NMDA receptor agonist, evoked the membrane depolarization displaying pronounced plateaus (from Herrling et al. 1983).

1.2 Role of EAA in Synaptic Transmission

L-Glutamate is the major candidate of excitatory neurotransmitter in vertebrate CNS. The synaptic release of glutamate results in excitatory postsynaptic potentials (EPSPs) that are composed of two components, the fast and the slow EPSPs (Forsythe and Westbrook 1988, Cherubini et al. 1988). Under physiological conditions and at resting membrane potential, cortically evoked EPSPs of cat caudate neurons and rat striatal or hippocampal neurons contain only the fast component (Herrling et al. 1983, Cherubini et al. 1988, Neuman et al. 1988). This fast EPSP is believed to be mediated by the action of glutamate on non-NMDA receptors since it is selectively blocked by the specific

non-NMDA receptor antagonist CNQX (Neuman et al. 1988). In experimental conditions, where spinal cord and hippocampal neurons were studied in magnesium-free solutions, the slow EPSP component was revealed in addition to the conventional fast EPSP component (Forsythe and Westbrook 1988, Lester et al. 1990). The conductance mechanism underlying the slow EPSP displays the characteristic voltage dependence and peculiar calcium permeability of the NMDA receptor-channel complex. It is also blocked by the selective NMDA receptor antagonist AP5 (Forsythe and Westbrook 1988). Thus it is believed that the slow EPSPs are mediated by NMDA receptors.

L-HCA has been proposed as an endogenous ligand with a strong preference for NMDA receptors (Herrling et al. 1989, Knopfel et al. 1987, Mayer and Westbrook 1984, Olverman et al. 1988, Pullan et al. 1987, Schwarz et al. 1990). In vertebrate CNS, L-HCA is released in a calcium-dependent manner by depolarizing stimuli (Do et al. 1986a, b) and is taken up by a specific low-affinity uptake system (Cox et al. 1977). When the L-HCA uptake system is blocked by the selective blocker, ß-p-chlorophenylglutamate (chlorpheg) (Davies et al. 1985, Zeise et al. 1988), L-HCA- but not L-glutamate-induced current is potentiated and the synaptic responses evoked in CA1 hippocampal neurons by Schaffer collaterals stimulation increase considerably (Ito et al. 1991) indicating that L-HCA can also mediate EPSPs.

1.3 Receptor Subtypes

The existence of EAA receptor subtypes was first mooted on the basis of differential sensitivity analysis, where the prototypic agonists were found to have different relative potencies on different neuronal types (McCulloch et al. 1974), and then became plainly evident with the discovery of selective antagonists. It has been found that NMDA induced responses were more susceptible than other prototypic agonists induced

responses to a range of organic substances or divalent cations, *e.g.* D-α-aminoadipate (DAA, Biscoe et al. 1977, Davies and Watkins 1979) and Mg^{2+} (Davies and Watkins 1977, Evans et al. 1977, Mayer and Westbrook 1987b). A logical deduction from these phenomena is that the sites, where NMDA acts on, are not the same as those where KA and AMPA act on. Consequently the former sites were termed as NMDA receptor (Watkins 1978), and the sites that were activated by other prototypic agonists and were insensitive to specific NMDA antagonists were called non-NMDA receptors (Davies et al. 1979).

Non-NMDA receptors were soon divided into separate KA and QA receptors. Also this separation resulted from the evidences similar to the above two lines, *i.e.* the differences in relative potencies and pharmacological properties. For instance, there were marked differences in the relative potency of KA and QA at the insect and crustacean neuromuscular junctions (Shinozaki 1978) and mammalian C fibres (Davies et al. 1979). KA and QA have shown different sensitivity to a range of weaker and less reliable antagonists such as γ-D-glutamylglycine (γDGG, Davies and Watkins 1981) and glutamate diethylester (GDEE, Hicks et al. 1978). To sum up all these observations, it has been suggested in the early 1980s that distinct EAA receptor subtypes exist and are selectively activated by and named after the prototypic agonists NMDA, KA and QA (Watkins and Evans 1981, McLennan 1983).

This EAA receptor classification has been revised recently (Watkins et al. 1991). The update is based on three points. First, quisqualate has been found to have less specificity than its structural analogue, AMPA, for the so called QA receptor and the latter can evoke similar electrophysiological responses as the former (Krogsgaard-Larsen et al. 1980). Second, quisqualate, ibotenate and other EAA have been found to activate also a metabotropic receptor coupled to inositol 1,4,5-trisphosphate (IP_3) and

diacylglycerol turnover (Sugiyama et al. 1987). Third, there are evidences that L-2-amino-4-phosphonobutanoic acid (L-AP4), a ω-phosphono analogue of glutamate, is a potent and selective synaptic antagonist at several CNS pathways where EAA functions as neurotransmitter (Koerner and Cotman 1981, Davies and Watkins 1982, Evans et al. 1982, Yamamoto et al. 1983, Monaghan et al. 1989). However, L-AP4 does not antagonize the postsynaptic excitations in these pathways induced by exogenously application of EAA (Davies and Watkins 1982, Evans et al. 1982, Ganong and Cotman 1982, Yamamoto et al. 1983), which indicates that L-AP4 acting site is probably located presynaptically (Cotman et al. 1986). So in the new recommended nomenclature, QA receptor is replaced by AMPA receptor, and a glutamate metabotropic receptor and a L-AP4 receptor are added (Watkins et al. 1991).

1.4 Pharmacology of EAA

The development of selective agonists and antagonists is one of the two major reasons for the attractive study of EAA receptors from early 1980s. Most of our knowledge about EAA, especially the classification of EAA receptor subtypes, has accumulated from the studies of pharmacology.

NMDA receptor. NMDA receptor is the best studied EAA receptor subtype. It is coupled to an ion channel that is permeable to monovalent cations as well as Ca^{2+} (Jahr and Stevens 1987, MacDermott et al. 1986, Mayer and Westbrook 1985, 1987a). In physiological system, this ligand-gated ion channel presents a peculiar voltage sensitivity. In 1984, Nowak and Ascher discovered that the voltage dependence of NMDA-linked conductances results from a voltage-dependent Mg^{2+} block of the channel (Nowak et al. 1984). The NMDA evoked single channel currents were found to be chopped in burst by Mg^{2+} (concentration below the physiological level) at negative

potential and the channel open probability is reduced (Nowak et al. 1984), which implies that Mg^{2+} acts as a channel blocker. This mechanism underlies the NMDA channels' functional dependence on membrane potential (Lodge and Johnson 1991). Other divalent cations including Ni^{2+}, Mn^{2+} and Co^{2+} have been found to have similar effects (Ascher and Nowak 1988b, Ault et al. 1980, Collingridge and Lester 1989, Mayer and Westbrook 1987b).

The divalent cation Zn^{2+} has also been found to inhibit NMDA induced currents selectively (Peters et al. 1987, Westbrook and Mayer 1987). But, in contrast to Mg^{2+}, Zn^{2+} blocks the currents in a somewhat voltage-independent way. It is believed that zinc acts on a different site from magnesium.

Besides divalent cations, other compounds can also modulate NMDA current in both a competitive and a noncompetitive fashion. A very well studied phenomenon is that the NMDA action in cell culture is facilitated by nanomolar concentrations of glycine (Johnson and Ascher 1987, Kushner et al. 1988). This is due to the action of glycine on a novel strychnine-insensitive binding site with an anatomical distribution identical to that of NMDA receptors (Bristow et al. 1986, Monaghan and Cotman 1986, Mayer et al. 1989, Lodge and Johnson 1991), suggesting the existence of a glycine binding site in NMDA receptor channel complex (Mayer et al. 1989). In single-channel recording experiments, glycine was found to increase the channel open frequency but did not change the mean open time and channel conductances (Johnson and Ascher 1987, Kemp et al. 1988). Furthermore, in rat brain mRNA injected oocytes, glycine is absolutely required for the activation of NMDA receptors (Kleckner and Dingledine 1988). In intact preparations, glycine has minor effects on NMDA receptor-mediated events, which implies that *in vivo* the glycine modulatory site is nearly saturated by the glycine present in the extracellular compartment (Lodge and Johnson 1991). However,

the enhancement of NMDA receptor-mediated EPSPs in slices of adult neocotex by focal application of EAA and glycine argues this point (Thomson et al. 1989). By acting on glycine modulatory site, chlorokynurenate, 1-hydroxy-3-amino-pyrrolidin-2-one (HA966) and other compounds act as noncompetitive NMDA receptor antagonists (*e.g.* Kemp et al. 1988).

Another class of chemical compounds such as phencyclidine (PCP), ketamine and (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK801) also antagonize the NMDA receptor noncompetitively (MacDonald et al. 1987, Wroblewski et al. 1987, Huettner and Bean 1988, Javitt and Zukin 1989). These PCP-like substances can reduce the NMDA currents at both positive and negative membrane potentials. The blockade is both use- and voltage-dependent (Wong et al. 1986, MacDonald et al. 1987). The binding and unbinding of these antagonists seems to be possible only if the channel is in the open state. Mg^{2+} inhibits the blockade by MK801 at negative membrane potentials. The single-channel recording shows that PCP and MK801 reduce the channel open time. The conclusion from all observations mentioned above is that PCP-like compounds act on a site within the NMDA ion channel which differs from Mg^{2+} binding site (Bertolino et al. 1988, Lodge and Johnson 1991).

All of the above mentioned modulations occur at some modulatory sites different from the transmitter recognition site. At NMDA recognition site, 2-amino-5-phosphonopentanoate (AP5) and 2-amino-7-phosphonoheptanoate (AP7) act as specific competitive NMDA receptor antagonists (Davies and Watkin 1982, Evans et al. 1982, Davies et al. 1981).

Non-NMDA receptors Compared with our knowledge about NMDA receptors, the pharmacology of non-NMDA receptors is still in its infancy. The difficulties arise from the lack of potent and selective antagonists for these receptors. Usually γDGG,

kynurenic acid and γ-D-glutamylamino-methylsulphonate (GAMS) were used to antagonize non-NMDA receptors (Francis et al. 1980, Robinson et al. 1984, Jones et al. 1984). These substances are weak and non-selective antagonists. Actually γDGG and kynurenic acid are more potent against NMDA receptor.

Recently two quinoxalinediones, 6,7-dinitroquinoxaline-2,3-dione (DNQX or FG9041) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX or FG9065), have been described as more potent and selective non-NMDA receptor antagonists (Drejer and Honore 1988, Honore et al. 1988, Yamada et al. 1989, Monaghan et al. 1989). It was found that CNQX blocks selectively the non-NMDA receptor mediated EPSP evoked in CA1 region by Schaffer collaterals stimulation (Blake et al. 1988) or the CA3 region by the mossy fiber stimulation (Neuman et al. 1988). CNQX is over 500 times more potent than GAMS, which was the most potent non-NMDA antagonist previously available, when examined at KA receptor subtype (Yamada et al. 1989). Quinoxalinediones antagonize AMPA and QA to a greater extent than they do to KA (Blake et al. 1988), and the antagonism is competitive (Drejer and Honore 1988). CNQX has also been found to act as an antagonist at the glycine site of the NMDA receptor. But the inhibitory effects of CNQX on NMDA-induced currents are minimal (Blake et al. 1988, Yamada et al. 1989). The antagonism CNQX to NMDA responses is noncompetitive (Yamada et al. 1989).

There are evidences that some polyamine amide toxins from wasp and spider venom, *e.g.* philanthotoxin (PhTx), can selectively antagonize non-NMDA receptors, specially QA receptor subtype, present on locust muscle (Jackson and Usherwood 1988, Bruce et al. 1990) and *Xenopus* oocytes injected with vertebrate brain mRNA (Brackley et al. 1990). These results need to be confirmed in vertebrate CNS neurons.

Until now, no antagonist has been found to distinguish between the two subtypes of non-NMDA receptors except the fact that some non-NMDA receptor antagonists antagonize AMPA- and KA-induced responses with different potencies. Some new experiments exhibit a complicate interaction between AMPA and KA. The major discovery is that concomitant application of saturating concentrations of KA and AMPA or QA does not result in a sum of the two individual electrical responses (see Chapter 4). These results indicate that KA and AMPA, in some sense, share the same receptor channel complex.

Compared with the ionotropic receptors, less is known about the L-AP4 and metabotropic receptors. There are some initial indications that L-AP4 and amino-3-phosphonopropanoate (AP3) may be noncompetitive antagonists of quisqualate- and ibotenate-induced PI turnover (Schoepp and Johnson 1989). Glutamate is likely to be the endogenous agonist for the metabotropic receptor. It stimulates phosphoinositide hydrolysis with a relatively lower potency in rat brain slices than in cultured cells

Table 1.1 Agonists and antagonists of EAA receptor subtypes (from Watkins et al. 1991)

Receptor Subtypes	NMDA	AMPA	Kainate	L-AP4	Metabotropic
Selective Agonists	NMDA *trans*-2,3-PDA ACBD	AMPA, ATPA 4-AHCP, 5-HPCA 7-HPCA, Br-HIBO	-	L-AP4	1s,3R-ACPD 2s,3s,4s-CPG
Other Important Agonists	Glu, Asp Quinolinate L-HCA, L-HCSA L-CSA, Ibotenate Glycine, D-Ser	Glu, Quisqualate Kainate L-ß-ODAP Willardiine 5-Br-Willardiine	Glu Kainate Domoate Acromelic Acids A Acromelic Acids B 5-Br-Willardiine	Glu L-O-Phosphoserine	Glu Quisqualate Ibotenate
Selective Antagonists	D-AP5, CPP CGS 19755 CGP 37849 D-CPPene	-	-	-	-
Other Antagonists	Ketamine PCP, HA966 MK801 SKF 10047 7-Chlorokynurenate CNQX, DNQX, MNQX	CNQX DNQX NBQX	CNQX, DNQX Kynurenate γDGG, GAMS BBPzDA, CBPzDA	-	-

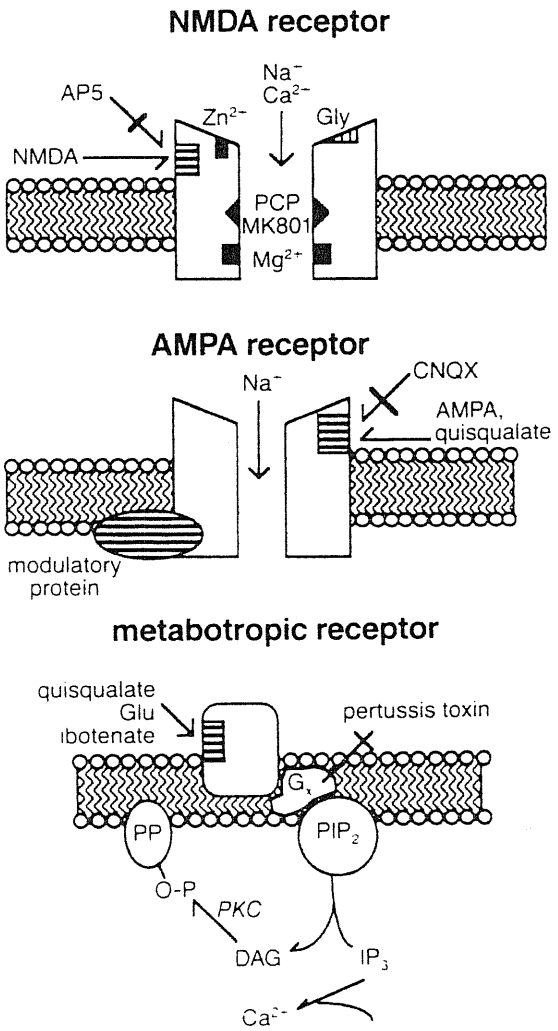
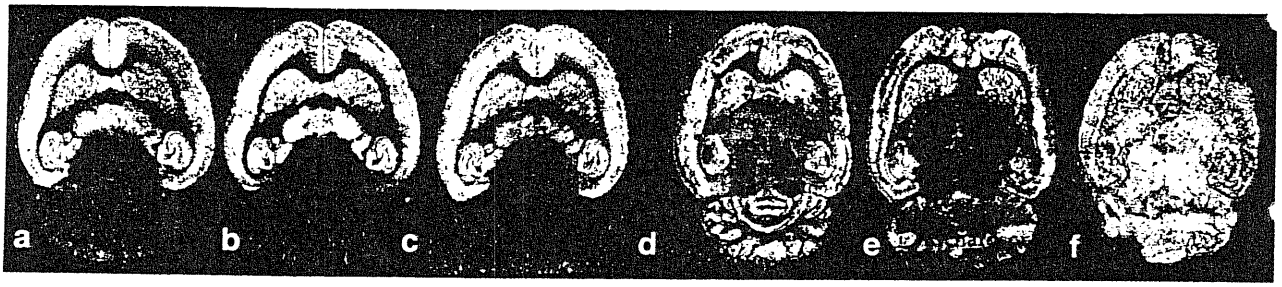


Fig.1.3 Three excitatory amino acid receptor subtypes. The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+} and is gated by Mg^{2+} in a voltage-dependent fashion; K^{+} is the counterion. The NMDA related channel is blocked by PCP and MK801 and the complex is regulated at two modulatory sites by glycine and Zn^{2+}; AP5 is a competitive antagonist acting on transmitter recognition site. The AMPA receptor gates cation through a channel that is permeable to Na^{+}; K^{+} is the counterion. The receptor appears to be influenced by a modulatory protein and chaotropic anions; AMPA and quisqualate are agonists and CNQX is an antagonist. The metabotropic receptor has a distinct but limited pharmacology and is likely to play an important role in long-term effects mediated by excitatory amino acids. Quisqualate, ibotenate and glutamate are agonists; the G protein (G_x) that is sensitive to pertussis toxin is linked with the receptor (from Young and Fagg 1991).

possibly because of the uptake of glutamate in slices (Schoepp et al. 1990). QA and ibotenate are also able to activate metabotropic receptors. But none of them are selective metabotropic receptor agonist. Recently, *cis*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD), a conformational analogue of glutamate, has been shown to be a selective agonist for metabotropic receptors (Palmer et al. 1989). Its potency is similar to ibotenate but less than QA. There are evidences that *trans*-ACPD at high concentration also activates ionotropic receptors (Scheoppe et al. 1990). Fig. 1.3 gives the diagrams of some of the EAA receptor subtypes. Pharmacological properties of EAA receptors are summarized in Table 1.1.

1.5 Membrane Binding and Receptor Autoradiographic Approaches

Membrane binding and receptor autoradiographic studies exhibit distinct anatomical distributions of high affinity binding sites for different EAA agonists (Monaghan et al. 1983, Young and Fagg 1991). Radioligand binding studies have shown that the NMDA receptors are localized, in subcellular level, mainly at the postsynapsis (Monaghan et al. 1983), and are present in high density in the cerebral cortex, hippocampus, striatum, septum and amygdala (Choi 1988, Greenamyre et al. 1985a). These kinds of experiments also provide the evidences that the PCP binding site is physically coupled to the NMDA recognition site (Young and Fagg 1991). Receptor autoradiographic investigations showed that the distribution of [^{3}H]glycine binding sites in the CNS is unlike the [^{3}H]strychnine binding sites, but very similar to the NMDA and PCP-type ligands binding sites. In membrane binding experiments, it was found that glycine does



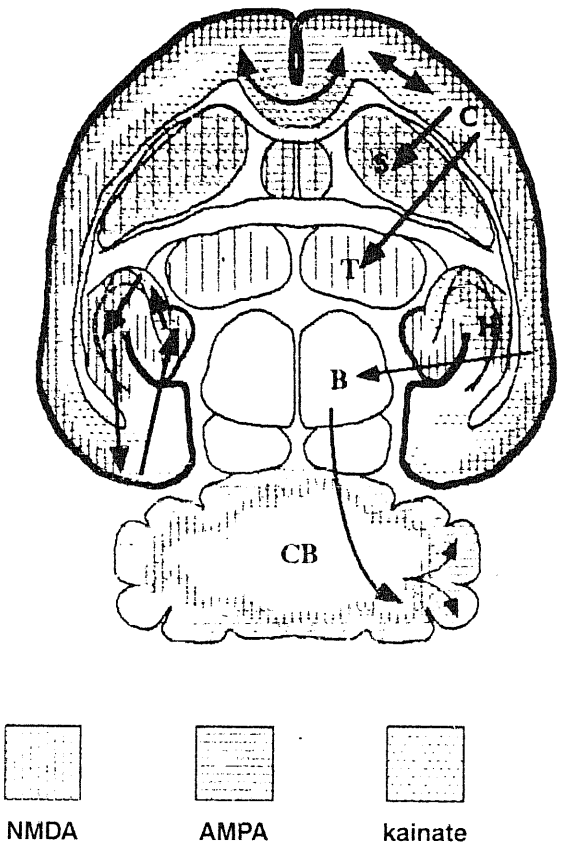


Fig.1.4 **above**: Autoradiograms showing the distributions of EAA receptor binding sites in horizontal sections of rat brain. a: Distribution of NMDA receptor recognition site as assessed using [^{3}H]glutamate; b: distrbution of glycine modulatory site as assessed using [^{3}H]glycine; c: distribution of PCP binding site using [^{3}H]MK801; d: distribution of AMPA receptor as using [^{3}H]AMPA; e: distribution of high affinity kainate site using [^{3}H]kainate; f: distribution of metabotropic receptor as using [^{3}H]glutamate.
down: Major excitatory amino acid transmitter pathways and the distribution of EAA receptor subtypes in rodent brain. The pathways are labeled by arrows. **C:** cerebral cortex; **S:** striatum; **T:** thalamus; **H:** hippocampus; **B:** brainstem; **CB:** cerebellum. (from Young and Fagg 1991)

not influence competitively the NMDA-sensitive [^{3}H]glutamate binding or [^{3}H]MK801 binding, which demonstrates that the glycine action site must be distinct from the transmitter binding site and the ion channel (Young and Fagg 1991).

AMPA receptors are localized in hippocampus, cortex, lateral septum, striatum and the molecular layer of cerebellum (Monaghan et al. 1989, Young and Fagg 1991). [^{3}H]kainate binds to two sets of sites with high and low affinities respectively. The K_d value for the high affinity site is in the range of low nanomolar concentration (Young and Fagg 1991). The regional distribution of the high affinity [^{3}H]kainate binding sites is different from that of either AMPA or NMDA receptors (Fig. 1.4), but corresponds well to those brain locations that are highly vulnerable to the neurotoxic actions of kainate (Foster and Fagg 1984, Monaghan et al. 1989, Young and Fagg 1991). Generally, a micromolar concentration, at which KA also interacts with the [^{3}H]AMPA binding site, is required to elicite an excitatory response (Mayer and Westbrook 1987a, Monaghan et al 1989, Young and Fagg 1991).

The L-AP4 binding site has not been identified. The metabotropic receptors have been represented by a subpopulation of quisqualate-sensitive, AMPA-insensitive [^{3}H]glutamate binding sites (Fig. 1.4). Figure 1.4 gives an autoradiograms of the distributions of EAA receptor binding sites in horizonal sections of rat brain and rodent brain.

1.6 Electrophysiology of EAA

The electrophysiological studies generally support the existence of distinct responses to NMDA, KA and AMPA respectively, though in most of the old works QA was used instead of AMPA. Each prototypic EAA receptor angonist has been found to elicit its

characteristic electrical response.

NMDA has been found to evoke dose-dependent desensitized macroscopic currents in both vertebrate CNS neurons and rat brain mRNA injected *Xenopus* oocytes by using whole cell patch clamp or two electrodes voltage clamp techniques (*e.g.* Lerma et al. 1989a, b, Mayer and Vyklicky Jr. 1989, Perouansky and Grantyn 1989, Shingai et al. 1990). In magnesium free solution, the NMDA response possesses a linear current-voltage (I-V) relationship (Ascher et al. 1988, Cull-Candy et al. 1988). In the presence of Mg^{2+}, the I-V relation shows a characteristic negative slope conductance at membrane potential lower than -40 mV (Nowak et al. 1984).

In the single channel recording, NMDA-evoked currents present two modes of gating, a brief opening mode and a burst of the long opening mode with mean open times around 2 ms and 13 ms respectively at room temperature. The major elementary conductance measured in single channel recording or fluctuation analysis of macroscopic current is around 50 pS (Jahr and Stevens 1987, Cull-Candy et al. 1988).

The ion channel is nonselectively permeable to monovalent cations as well as Ca^{2+} (MacDermott et al. 1986, Jahr and Stevens 1987). The entrance of Ca^{2+} via NMDA channel will increase the cytoplasmic free calcium concentration (MacDermott et al. 1986) and start many important signaling processes including the activating of inositol phosphate turnover (Nahorski et al. 1986), modulating cGMP levels (Novelli et al. 1987), gene expression (Szekely et al. 1989) and arachidonate production (Lazarewicz et al. 1988). NMDA-induced cytoplasmic free calcium concentration increase has also been demonstrated by measuring the intracellular Ca^{2+} concentration using the fura analysis technique (MacDermott et al. 1986, Courtney et al. 1990).

In most of the studies, KA was found to evoke a non-desensitized macroscopic current in CNS neurons and oocytes injected with rat brain mRNA (*e.g.* Hollmann et al.

1989, Lerma et al. 1989a, Patneau and Mayer 1991). But recently it has been shown that in cerebellar granule cells and *Xenopus* oocytes expressed cDNA clone GluR-K6, isolated from rat celebellum, KA evoked currents desensitized during continuous agonist application (see **3.3** and Egebjerg et al. 1991). The I-V relation of KA response is linear in both Mg^{2+} containing and Mg^{2+} free solutions (Cull-Candy et al. 1988, Sciancalepore et al. 1990).

The single channel recording reveals that the major conductance activated by KA is about 4 pS and the mean channel open time is around 3 ms (Zheng 1989, Sciancalepore et al. 1990). Fluctuation analysis gives similar results when fitting the spectra with a single Lorentzian function (Cull-Candy et al. 1988, Sciancalepore et al. 1990). The spectra can be fitted better with two Lorentzians. In that case, two cut-off frequencies corresponding to two time constants, one smaller and one bigger than the mean open time estimated from the single Lorentzian fitting, are estimated (see **3.3** and Cull-candy et al. 1988).

The ion channel is permeable only to monovalent cations but not to Ca^{2+} (Mayer and Westbrook 1985, 1987a). However, very recently it has been found, in oocytes expressed with some combinations of EAA receptor subunits, the KA/AMPA gated conductances also have Ca^{2+} permeability (Hollmann et al. 1991).

AMPA always introduces a current with very fast desensitization (*e.g.* Mayer and Westbrook 1987a, Hollmann et al. 1989, Lerma et al. 1989a, Patneau and Mayer 1991). In some experiments only steady state currents were observed due to the lack of fast perfusion system (Huettner 1990). AMPA mainly activates conductances between 8 to 15 pS (Jahr and Stevens 1987, Cull-Candy and Usowicz 1987, Cull-Candy et al. 1988). The mean channel open time of AMPA related conductances is similar to that of KA related conductances, which is around 3 ms (Cull-Candy and Usowicz 1987). The time

constance can also be estimated from the fluctuation analysis. In some cases, the spectra density can be well fitted by two Lorentzian functions from which two time constants are obtained, one bigger and the other smaller than the time constant mentioned above (see **3.3**). As we have said before, in most of the early experiments, QA was used to study the AMPA receptor related conductances. That causes experimental difficulties since QA usually activates concomitantly the metabotropic receptors.

The endogenous excitatory neurotransmitter glutamate can activate all those receptor subtypes and evoke all of the conductances related with NMDA, KA and AMPA. In hippocampal neurons (Jahr and Stevens 1987) and cerebellar neurons (Cull-Candy and Usowicz 1987) glutamate activates at least five distinct levels of single channel currents. These five levels correspond to three conductance categories of 40-50 pS, 20-35 pS and under 15 pS. Among them, the biggest one (40-50 pS) possesses the properties similar to those of NMDA evoked currents (Ascher et al. 1988, Cull-Candy et al. 1988); the smallest one (under 15 pS) resembles the currents evoked by KA and AMPA: the conductances under 5 pS correspond to KA related conductances and others correspond to AMPA related conductances (Ascher and Nowak 1988a, Cull-Candy et al. 1988, Sciancalepore et al. 1990). The remaining one (20-35 pS) happens rarely and no details about this set of conductances have ever been reported. Jahr and Stevens (1987) and Cull-Candy and Usowicz (1987) have suggested, on the basis of their observations, that all the conductances related with the different excitatory amino acids reflect the activity of a single molecular complex but not the activity of more unique channels. But now it is clear that at least two different EAA channels exist since the non-NMDA agonists can still evoke electrical responses when the NMDA channel is blocked by the NMDA channel blocker (Lerma et al. 1989a). The separation in the receptor-channel complex does not mean that each agonist can act on only one kind of receptor. There

are evidences that KA and QA can produce high conductance (corresponding to NMDA evoked activity) openings and KA may also produces medium conductance (corresponding to QA evoked activaty) openings (Cull-Candy et al. 1988, Zheng 1989, Sciancalepore et al. 1990).

Traditionally, the electrical responses to NMDA, KA and AMPA were believed to be mediated by three different receptor subtypes named by the corresponding prototypic agonists (Watkins and Evans 1981, Mayer and Westbrook 1987a). Some experimental evidences have accumulated that support this idea. It has been demonstrated that NMDA activates a receptor-channel complex different from KA and AMPA related one (Fong et al. 1989, Hollmann et al. 1989, Lerma et al. 1989a, Nakanishi et al. 1990). Some voltage-clamp experiments showed that different sensibilities can be obtained when different EAA agonists were applied to the same site of the neuron dendrite, which indicates the different distributions of different EAA receptor subtypes (Arancio and MacDermott 1991). Recently, numerous electrophysiological and molecular biological studies suggest that, in some cases, responses to KA and AMPA occur at the same receptor(s) since the electrical responses caused by concomitant application of KA and AMPA or QA are not equal to the sum of thoes evoked by KA and AMPA or QA alone (Ishida and Neyton 1985, Zorumski and Yang 1988, Lerma et al. 1989a, Perouansky and Grantyn 1989, Patneau and Mayer 1991), and furthermore, some cDNAs isolated from rat brain cDNA library can be expressed in *Xenopus* occytes to form EAA receptor subuntis which are responsive to both KA and AMPA (Hollmann et al. 1989, Nakanishi et al. 1990, Keinänen et al. 1990). However, in other cases, some cDNAs encoding either a EAA receptor subunit, which responses only to KA but not to AMPA (Egebjerg et al. 1991), or a high affinity KA binding protein (Werner et al. 1991) have been cloned. The definite relationship

between KA and AMPA acting sites remains ambiguous.

1.7 Molecular Biology of EAA

Recently a family of cDNA clones named GluR-K1, GluR-K2 and GluR-K3 (or GluR-A to -D) have been isolated from the rat brain cDNA library (Hollmann et al. 1989, Nakanishi et al. 1990, Keinänen et al. 1990). Each of these cDNA clones encodes a single protein and, when expressed in *Xenopus* oocytes, forms a functional receptor-channel complex possessing pharmacological and electrophysiological profiles of the non-NMDA glutamate receptors. The deduced amino acid sequences of the family of these clones showed a considerable identity (Fig. 1.5). Originally, GluR-K1 was claimed to be responsible only to KA-receptor agonists (Hollmann et al. 1989). The subsequent experiments demonstrated that GluR-K1 is also responsive to AMPA (Keinänen et al. 1990, Nakanishi et al. 1990, Lambolez et al. 1990, 1991). The order of agonist potency for homomeric GluR-K1 to -K3 is QA>AMPA>Glu>KA (Nakanishi et al. 1990, Keinänen et al. 1990).

The expression of each cDNA alone in oocytes forms some oligomers possessing similar pharmacological properties but dramatically different I-V relations as those of the KA receptors observed in most of CNS neurons. The coexpression of more than two cDNA clones in oocytes improves significantly the I-V relationship of the formed receptor-channel complex and provides a system far more approximate to the properties of KA receptors in CNS neurons (Nakanishi et al. 1990). These observations indicate that the common KA/AMPA receptors are encoded by a family of genes and are likely to be composed of hetero-oligomers of at least two distinct subunits (Nakanishi et al. 1990).

Some further work shows that each receptor subunit can be expressed in

Fig.1.5 Alignment of the deduced amino acid sequences of the rat GluR-K1, GluR-K2 and GluR-K3 cDNAs with those of other two cDNAs named chick KBP (kainate binding protein) and frog KBP. (from Nakanishi et al. 1990)

different versions named Flip and Flop respectively, having different kinetic properties (Sommer et al. 1990). The sensitivity of the formed receptor-channel complex to different non-NMDA receptor agonists depends on the combination of the receptor subunits and the versions of those subunits being expressed (Keinänen et al. 1990, Nakanishi et al. 1990, Sommer et al. 1990).

Very recently, two more cDNAs have been cloned: GluR-K6 (Egebjerg et al. 1991), a member of the GluR-K family; and KA-1 (Werner et al. 1991). GluR-K6 forms a receptor subunit which can be activated by KA, QA and glutamate but not by AMPA. The apparent affinity of GluR-K6 formed homomeric receptor for KA is higher than those receptors formed by GluR-K1 to -K3 cDNAs (Egebjerg et al. 1991). The order of agonist potency for the homomeric GluR-K6 receptor is KA>QA>glutamate (Egebjerg et al. 1991). This is clearly distinct from the GluR-K1 to -K3 receptors. Furthermore, desensitization has been observed in GluR-K6 formed receptors during continous KA application (Egebjerg et al. 1991).

KA-1 forms a putative high-affinity KA receptor when expressed in hippocampal CA3 cells (Werner et al. 1991). The pharmacological profile of expressed recombinatant KA-1 determined in binding experiments with [^{3}H]kainate is different from that of cloned GluR-K receptors and similar to the mammalian high-affinity KA receptor. These results indicate that pure KA receptors exist beyond the common KA/AMPA receptors.

Chapter 2

Experimental Methods

All the results presented in this thesis were obtained from the electrophysiological experiments performed on cerebellar granule cells primary cultures. EAA activated currents were studied at both single channel and whole cell levels by using the patch-clamp technique.

2.1 Cell culture

Primary culture of cerebellar granule cells has been described previously elsewhere (Levi et al. 1984). In brief, cerebella were removed from 8-day-old Wistar rats, cleaned in physiological saline solution (contains in mM: NaCl 124, KCl 5.37, NaH_2PO_4 1.01, D-glucose 14.50, HEPES 25 and $MgSO_4$ 1.2; bovine serum albumin 3 mg/ml and phenol red 27 μM) and then minced in a chopping surface with razor under sterile condition. The connective tissue of minced cerebella was digested with 0.25 mg/ml trypsin (Sigma) at 37°C for 10 minutes. The trypsinization was stopped with 166.4 μg/ml soybean trypsin inhibitor (Sigma), and 25.6 μg/ml DNAse (Sigma) was added to degrade the DNA released from the broken cells. The granule cell pellet was further separated mechanically by a fire narrowed Pasteur pipette. The suspension was washed with calcium containing solution and resuspended in the neuron growth solution

containing: basal Eagle's medium (Gibco) plus 0.27 mg/ml L-glutamine, 20 mM KCl, 0.1 mg/ml gentamicin and one tenth volume of heat inactivated fetal calf serum (Gibco).

The granule cells were plated at a density of 1.5 million per dish in 35 mm Petri dishes (pre-covered with poly-L-lysine) and incubated at 37°C, 5% CO_2 and maximum humidity. 10 μM cytosine arabinoside furanoside was added as mitotic inhibitor after about 19 hours *in vitro*. This process allows us to get homogenous granule cell cultures, and the cells in this culture can be used for at least 3 weeks. In our experiments, cells between 5 and 15 days in culture (DIC) were used.

2.2 Patch-Clamp

The patch-clamp technique was developed by Neher and Sakmann (1976) to record the currents across single ionic channels. Later on, this technique has been improved and extended to record the membrane currents from different patch configurations, as well as from the whole cell membrane (Hamill et al. 1981).

The key point of patch-clamp is to force a heat-polished glass micropipette against a membrane to form a tight seal, with a electrical resistance up to 100 gigohms. A fine designed electrical equipment, including a current-voltage converter, is used to clamp the membrane potential at a fixed value and measure the membrane current by forming a closed electrical circuit with this piece of membrane through a electrode/pipette-solution bridge (Fig. 2.1). Under this condition, most of the currents passing through the patch of membrane will flow through the electrode and then be converted to a significant voltage signal that can be measured.

An extensive technical description of the patch-clamp technique can be found in relevent books or papers (Hamill et al. 1981, Sakmann and Neher 1983, Moran 1991).

A standard patch clamp amplifier, EPC-7 (List-Electronic, FRG), was used in the experiments. Two feedback resistors are selected alternatively according to the output gain. In low gain range a 500 MΩ resistor is selected to measure a larger current up to 25 nA. In high gain range a 50 GΩ resistor is used to measure small current up to 250 pA.

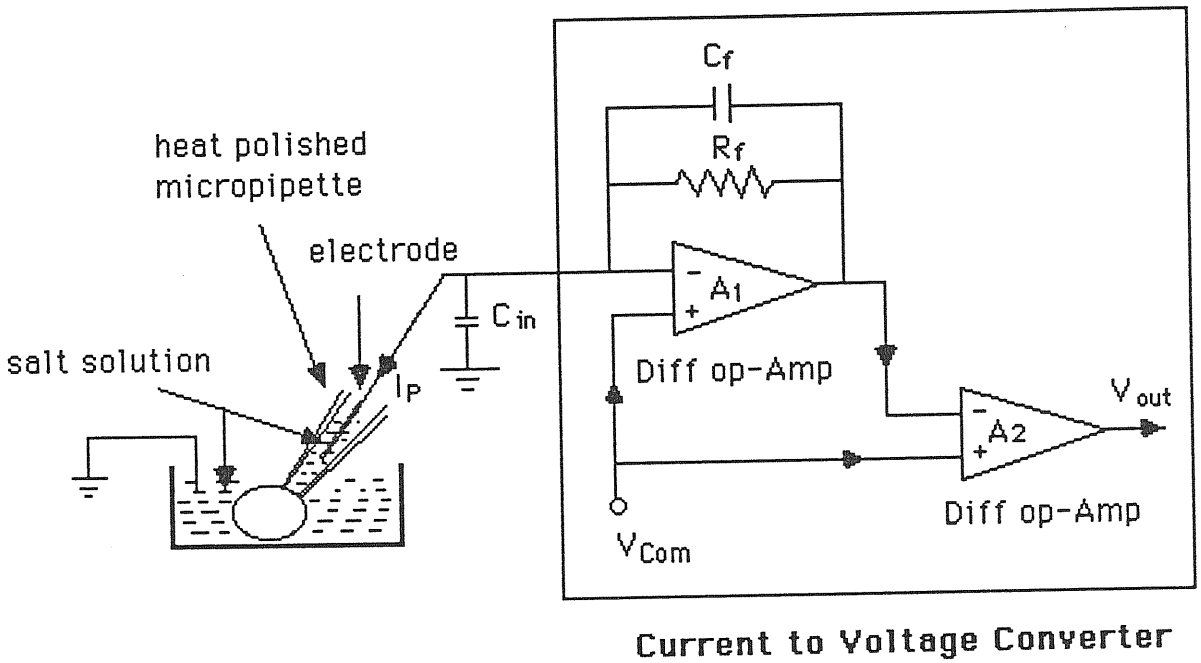


Fig. 2.1 Tight seal and current-voltage converter of the patch-clamp technique. The membrane current flows through the pipette containing salt solution to the electrode (normally a Ag/AgCl electrode) and then is passed to the current-voltage converter. The operational amplifer (op-Amp A_1), with a *gain-bandwidth product* of 10^7 s^{-1}, clamps the patch of membrane at any input command potential. This operation is done by checking and amplifying the voltage difference between membrane- and command-potential (V_{Com}). A feedback current (I_P) is injected to the membrane through R_f. The difference between the output of op-Amp A_1 and V_{Com} equals to I_PR_f, which can be measured by a second differential operational amplifer (Diff op-Amp A_2). Normally the resistance of R_f is on the order of 10 gigohms for enhancing the sensitivity.

When whole cell experiments were done, the macroscopic currents were recorded in the low gain range (500 MΩ feedback resistor) by using the voltage clamp mode of EPC-7. When currents were recorded from membrane patch, the high gain range was used (50 GΩ feedback resistor), obtaining, consequently, a lower noise feature. The cell membrane capacitance transients, the charging currents of the pipette and other stray capacitances were analogically canceled by the capacity compensation circuits of the EPC-7.

The patch pipette was manipulated under a 400x Axiovert-10 inverted microscope (Zeiss, FRG) by using a three dimensional CP-198 micromanipulator (Physik Instruments, FRG). The microscope and the micromanipulator were mounted on an antivibratory table and covered by a Faraday cage in order to minimize the mechanical and electric disturbances. All experiments were done at the room temperature (20-24°C).

2.3 Pipette Fabrication

Patch pipettes were pulled from borosilicate glass capillaries with internal filament (Hilgenberg, FRG). The inner diameter and outer diameter were 1.05 and 1.50 mm respectively. The capillaries were pulled in two steps by a vertical L/M-3P-A Patch Pipette Puller (List-Electronic, FRG).

In some cases, where the microscopic events were expected to be observed, the pipette tip was coated with Sylgard (Dow Corning, Belgium). The Sylgard was polymerized with a stream of hot air. The pipette tip was heat-polished by closing the tip to a current passing platinum filament. The pipette resistances (after polishing) were around 10 MΩ (measured in working solutions).

The double-barrel perfusion pipettes were pulled from the borosilicate glass Θ

capillaries (Hilgenberg, FRG) in the same way as making the patch pipettes. The heating currents in the two pulling steps were adjusted to get a pipette tip diameter of 2-5 μm.

2.4 Recording and Acquisition

Acquisition and electrical stimulation were performed by using a 12 bits A/D-D/A converter (M2-Lab Instrutech, USA), controlled by a microcomputer (Atari 1040ST, USA). The commercial programs RECORDER and PATCH (Instrutech, USA), written in Modula-2 by H. Affolter of Yale University, were used to control the converters, record and acquire data, and monitor the experiments.

Experimental data could be collected on-line or off-line according to the experimental protocols. The equipment arrangement and the signal pathway are illustrated in Fig. 2.2. The applied pipette potential was continuously controled by the D/A output of the converter, connected to the command input of the patch amplifier.

In the experiments where EAA evoked currents were studied, the current output of the patch amplifier was filtered at 10 kHz bandwidth by a EPC-7 built-in 3-pole Bessel filter. This current signal was fed to the input of a modified digital audio processor PCM-501ES (Sony, Japan) and then recorded by a super-ß-stereo video cassette recorder (Sony, Japan) for further analysis. The square pulse signals, which control the perfusion valves and so label the perfusion durations (see **2.5**), were also recorded by the video tape recorder. The current signal was concomitantly monitored by a digital storage oscilloscope (Hitachi VC-6041, Japan), a chart recorder (Linseis G7405) and through the A/D converter by the computer.

The current signals replayed from the video tape recorder were filtered by a Butterworth 3220 filter (Khron-Hite, USA) and sent to the A/D converter, and acquired

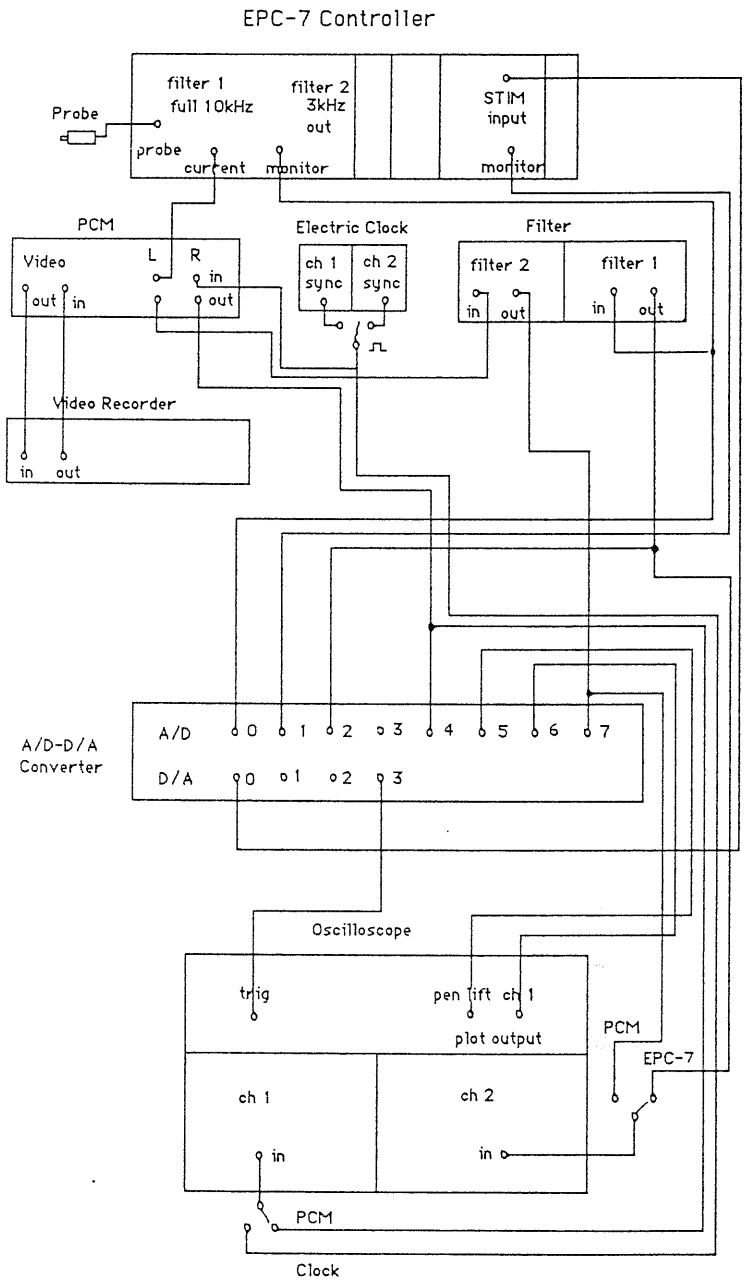


Fig. 2.2 The equipment arrangement and the pathway of the signals. The detected signals are passed to the EPC-7 controller. After a series of analogical processes, the signals are sent out from two outputs. One output is filtered by a built-in 10 kHz 3-pole Bessel filter and directly sent to a PCM device/video recorder combination. The other output can be either further filtered or not by a built-in 3 kHz 3-pole Bessel filter. The signals from this output are sent to the oscilloscope, chart recorder and personal computer for monitoring or/and recording. The video recorder recorded signals are filtered and sent to the oscilloscope, chart recorder and personal computer for further analysis. A 12 bit digital/analogue and analogue/digital converter is used to interface the signals between the computer and other instruments.

by the computer at a appropriate given sampling time. In general, the sampling frequency was decided to be fivefold of the upper frequence of the signal (Shannon and Weaver 1964).

2.5 Neurotransmitter Application

Two perfusion systems were used to apply neurotransmitters: the pressure ejection system and the multibarrel gravity perfusion system. The pressure ejection system was faster, but could be used only to perfuse maximum of two solutions. The gravity perfusion, although slower, allowed us to apply more solutions during the experiments.

In the pressure ejection system, double-barrel glass pipettes were used. Tubes from a double channel pneumatic pressure system (Medical System PPS-2, USA) were well connected to the perfusion pipette. The perfusion pipette was located at about 100 μm far from the cell under patch clamp. By giving pressure pulses of about 0.2 bar, the solutions contained in the perfusion pipette were ejected to the cell under patch, and the consequent electrical responses were observed within 30 milliseconds. The experimental chamber was continuously superfused by bath solution in order to avoid the accumulation of the pressure ejected perfusate. Furthmore, other agonists or antagonists could be applied through the bath perfusion, which results in several application combinations.

In multibarrel gravity perfusion, the multibarrel perfusion pipette was laboratory-made, with a tip size of around 500 μm. Perfusion fluxes were gated by pinch solenoid valves (Sirai S104, Italy). The valves were controlled by a laboratory designed TTL circuit. This circuit maintains the opening of one valve, which controls the normal bath solution, until another valve opens. In this way the applied neurotransmitter could be washed away immediatly by the bath solution when the application was stopped. The

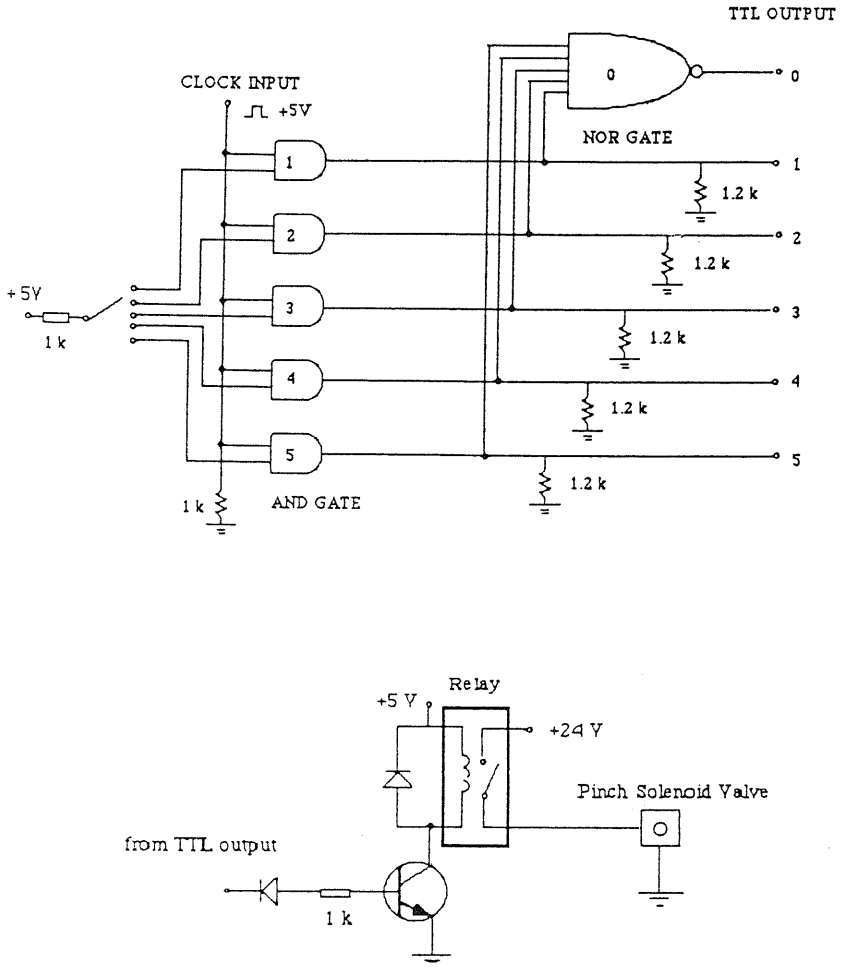


Fig 2.3 A The logical working principle of the TTL circuit used to controll the pinch solenoid valves in multiplebarrel gravity perfusion. The TTL circuit is composed mainly of five positive AND gates and one positive NOR gate. A +5 V signal is input through a selector to one of the AND gate. When a +5 V square pulse is input to the AND gate, a positive square pulse will be got from the corresponding output, and all other outputs are zero. In this case, the corresponding valve will open and the others remain close. When the outputs from all the five AND gates are zero, a positive output will be generated in the NOR gate and consequently the valve controlled by TTL output 0 will open. **B** The circuit used to amplify the TTL outputs and gate the valves through relays.

open duration of the valves which gate the flux of neurotransmitter containing solutions, could be set by using any electronic clock. Perfusion tip was located 3-5 mm far from the cell under patch. Electrical responses could be detected within the order of hundreds of milliseconds upon the onset of trigger signal, depending on the height of the perfusion solution column.

2.6 Solutions for Electrophysiology

All salts for solutions were obtained from Prolabo (Paris), Carlo Erba (Milan) or Sigma (ST Louis). Pharmacological products and neurotransmitters were obtained from Sigma and Tocris (Essex). The compositions of solutions are listed below.

Bath Solution

Substances	Concentration (mM)
KCl	3
$CaCl_2$	1.5
HEPES	10
NaCl	130
TEA	10
glucose	11

pH=7.3 adjusted by NaOH

Pipette Solution

Substances	Concentration (mM)
CsCl	145
EGTA	1
HEPES	10
glucose	5

pH=7.3 adjusted by KOH

Chapter 3

Excitatory Amino Acids Evoked Currents in Cerebellar Granule Cells

Cerebellar neurons of the granular layer are typical glutamergic neurons (Gallo et al. 1987a, b). As it has been recognized, cerebellar granule cells constitute an excellent model to study the electrical responses of excitatory amino acids (Wroblewski et al. 1985, Drejer et al. 1986, Cull-Candy et al. 1988, Howe et al. 1988, McCaslin and Smith 1988, Holopainen et al. 1989, Sciancalepore et al. 1989, 1990, Traynelis and Cull-Candy 1990). The characteristics of the electrophysiological responses of granule cells to some of excitatory amino acids obtained in our laboratory are presented in this chapter. Glutamate, NMDA, kainate, domoate and quisqualate have been used to stimulate the cerebellar granule cells in primary cultures. The solutions used for electrophysiological measurements have been described in Chapter 2.

3.1 NMDA Evoked Current

NMDA evoked currents were studied through the whole cell patch clamp technique. The agonist, at concentration used from 5 μM to 1 mM, evoked inward currents at a membrane potential of -70 mV. These currents reached a peak after about 2 sec of 20 μM NMDA application, under our multibarrel gravity perfusion condition. The current

amplitude did not remain throughout the NMDA application, and it slowly declined toward a plateau with a time constant in the order of several seconds (Fig. 3.1). This pattern of response revealed the desensitization character of the NMDA receptors described by different groups (Lerma et al. 1989a, b, Mayer and Vykicky 1989, Perouansky and Grantyn 1989, Shingai et al. 1990).

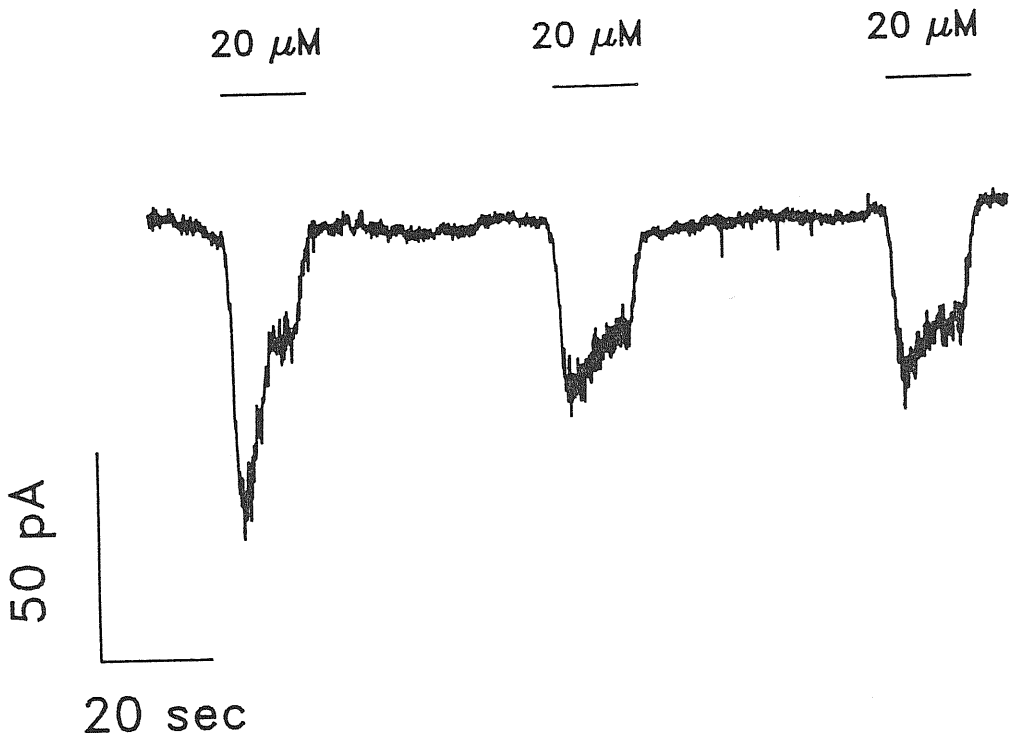


Fig. 3.1 Whole cell currents evoked by repetitive applications of 20 μM NMDA. 7 DIC granule cells were clamped at a membrane potential of -70 mV. NMDA was applied through multibarrel gravity perfusion during the period labelled above the traces. 1 μM glycine was applied together with NMDA to enhance the effects. During the application of NMDA, the membrane current first increase to a peak value and then decay to a plateau, showing a desensitization. Furthermore, the desensitization is reflected by the progressive decrease in the peak currents evoked by repetitive application of the agonist. Note that the first application of NMDA evoked a big peak current of 79 pA while the following applications (with interval 1 min) evoked only about 46 pA peak current. The steady state currents, however, remained almost constant.

The desensitization of NMDA receptors manifested itself also in two other experimental phenomena observed in our measurements. One of them is illustrated in Fig. 3.1. When the granule cell was patch clamped at -70 mV membrane potential and stimulated by successive 20 sec NMDA (20 μM) applications (the interval between two stimili was 1 min), the steady-state currents of the successive responses remained constant, but the peak currents decreased progressively toward the steady-state currents. The other desensitization phenomenon is shown on the right side of Fig. 3.2. As it is illustrated in the figure, in the low concentration (< 200 μM), the current amplitude increases with the NMDA dose; however, when the NMDA concentration is progressively increased to higher than 200 μM, both the peak and the steady-state current decrease. Similar observation has been reported previously (Lerma et al. 1989a, b). A reasonable explanation is that the high concentration NMDA application leads part of the receptors into a desensitized state, thus, less response can be evoked by the following stimulation. The bell-shaped dose-response relation (Fig.3.2) reflects this property.

In conclusion, the desensitization of NMDA activated currents has the following characteristics: i) the desensitization is not complete, since a steady-state current is always observed (Fig. 3.1, 3.2); ii) the desensitization is agonist concentration dependent (Fig. 3.2); and iii) the time constant of the onset of desensitization has an order of seconds.

Dose-response relationship was constructed by plotting the current amplitude against the agonist concentration (in logarithm scale). The lower part of Fig. 3.2 shows this dose-response relation corresponding to the steady-state current shown in the upper part of the figure. The steady-state responses are used because the desensitization of the peak currents produced at high agonist concentration (some times faster than the time

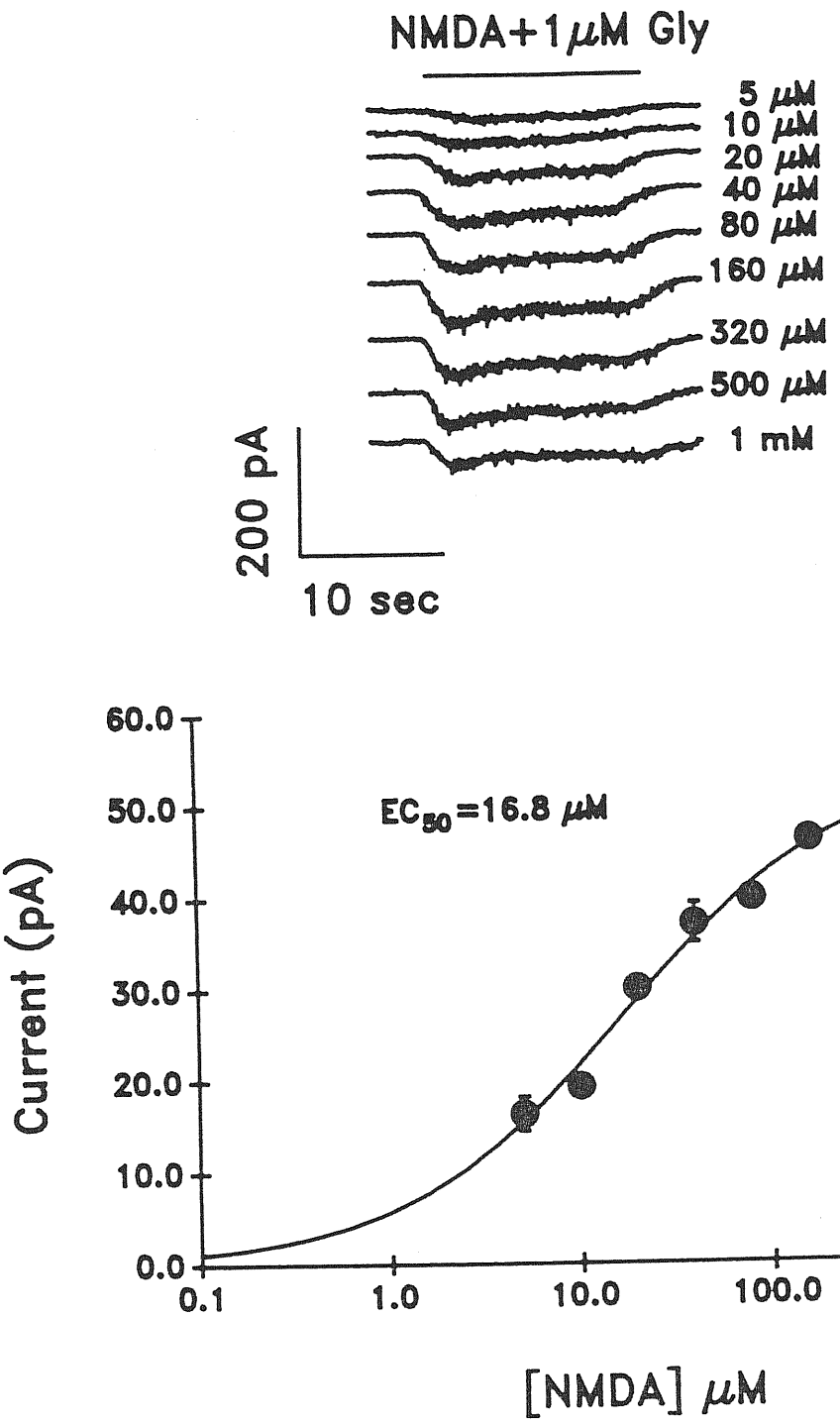


Fig. 3.2 Dose-response relation of NMDA activated currents measured from 7 DIC granule cells. Membrane potential was kept at -70 mV. NMDA, together with 1 μM glycine, was applied through multibarrel gravity perfusion during the period labelled above the traces. The NMDA concentrations are indicated on the right of the traces. When the NMDA concentration was too high, both the peak and the plateau decreased, resulting in a bell-shaped dose-response curve. The data in the figure present the mean of three observations. The curve is obtained by fitting the mean value of steady state currents with Equation (1). EC_{50}=16.8±6.8 and h=0.8±0.1 (mean ± S.D) were estimated from the above equation.

constant of the agonist application) forbided an accurate evaluation. The curve shown on the left side of Fig. 3.2 was the fitting of meaned experimental data with the Hill equation (Walter 1966):

$$I=I_{Max}/[1+(EC_{50}/[C])^{h}] \qquad (1)$$

where $[C]$ is the agonist concentration, I_{Max} is the maximum current evoked by the agonist, EC_{50} is the agonist concentration where half of the maximal response is obtained, and h is the Hill coefficient that is related with the cooperativity of the system (Walter 1966). The currents evoked by 320, 500 and 1000 μM NMDA were not used for the fitting since at those concentrations the receptors were insensitive to the agonist. The half maximum response concentration EC_{50} and the Hill coefficient h, estimated from the dose-response curve are 16.8±6.8 μM and 0.8±0.1 (mean ± S.D.) respectively. These values are in the range of those reported previously in different preparations (Lerma et al. 1989a, b, Patneau and Mayer 1990).

NMDA receptor possesses an allosteric facilitatory site activated by submicromolar concentrations of glycine (Johnson and Ascher 1987). This site is insensitive to strychnine and therefore different from the classical strychnine-sensitive glycine site (*e.g.* Betz 1987) which is associated with a Cl^- channel and present mainly in hindbrain region. To test the modulatory effect of glycine in cerebellar granule cells, NMDA responses were measured in the absence and presence of glycine. The results showed that in the nominal glycine free condition, 60 μM NMDA still evoked measureable current (see Fig. 3.3). We can not rule out the possibility that the NMDA responses were already facilitated by the endogeneous glycine present in the culture medium. When glycine (up to 10 μM) was applied alone, no electrical response was observed (data not shown). When glycine (1-5 μM) was concomitantly applied with NMDA (60 μM), the peak amplitude of NMDA evoked current was significantly

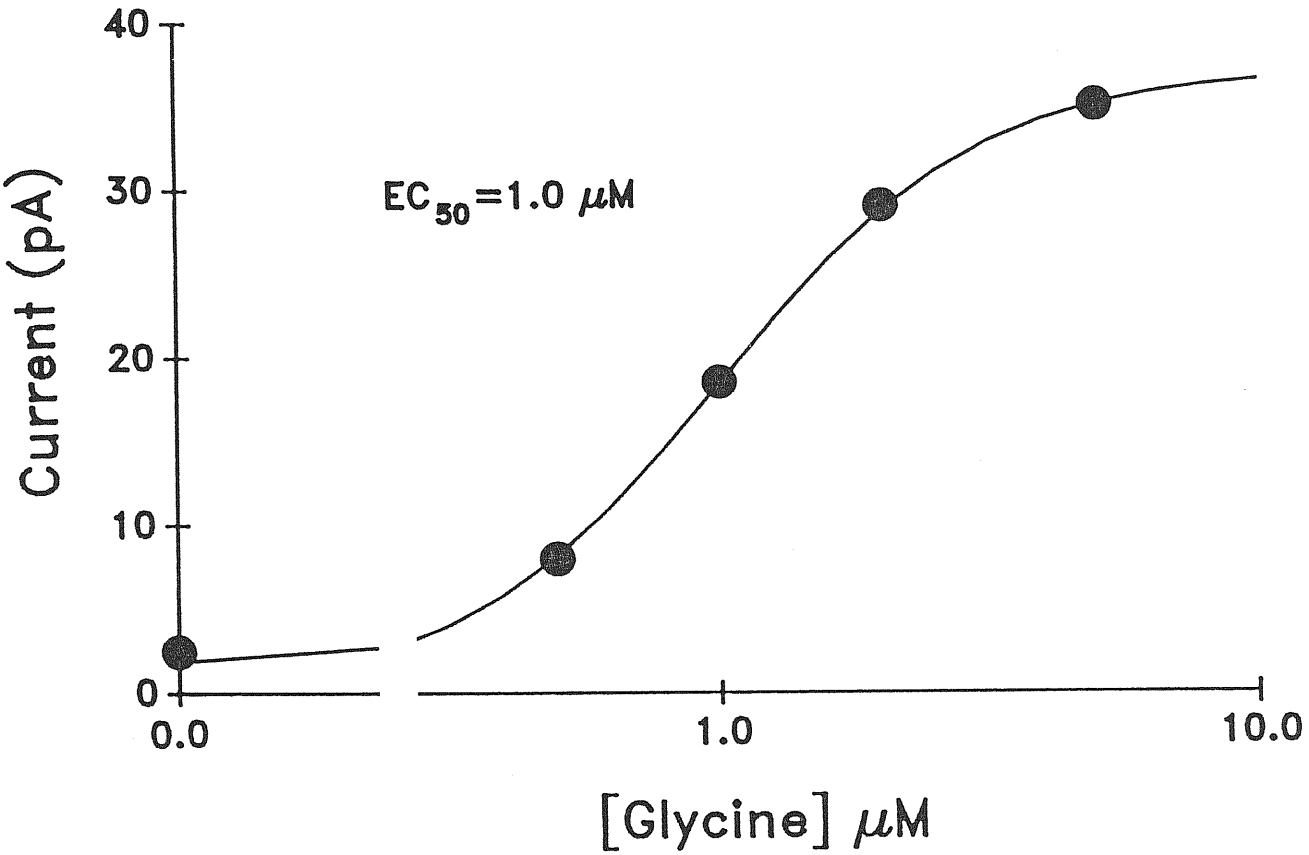


Fig. 3.3 Dose-effect curve of glycine on enhancing 60 μM NMDA evoked currents. Granule cell was 7 DIC. Membrane potential was kept at -70 mV. The nominal concentrations of glycine used in the experiment were from 0 to 5 μM. NMDA plus different concentration of glycine was applied through multibarrel gravity perfusion. The figure represents the data from one experiment. The peak currents are used to construct this dose-effect relation. The dose-response relation is fitted with Equation (1). Values of EC_{50}=1.0±0.3 and h=1.8±0.1 (mean ± S.D.) are estimated from the dose-response curve.

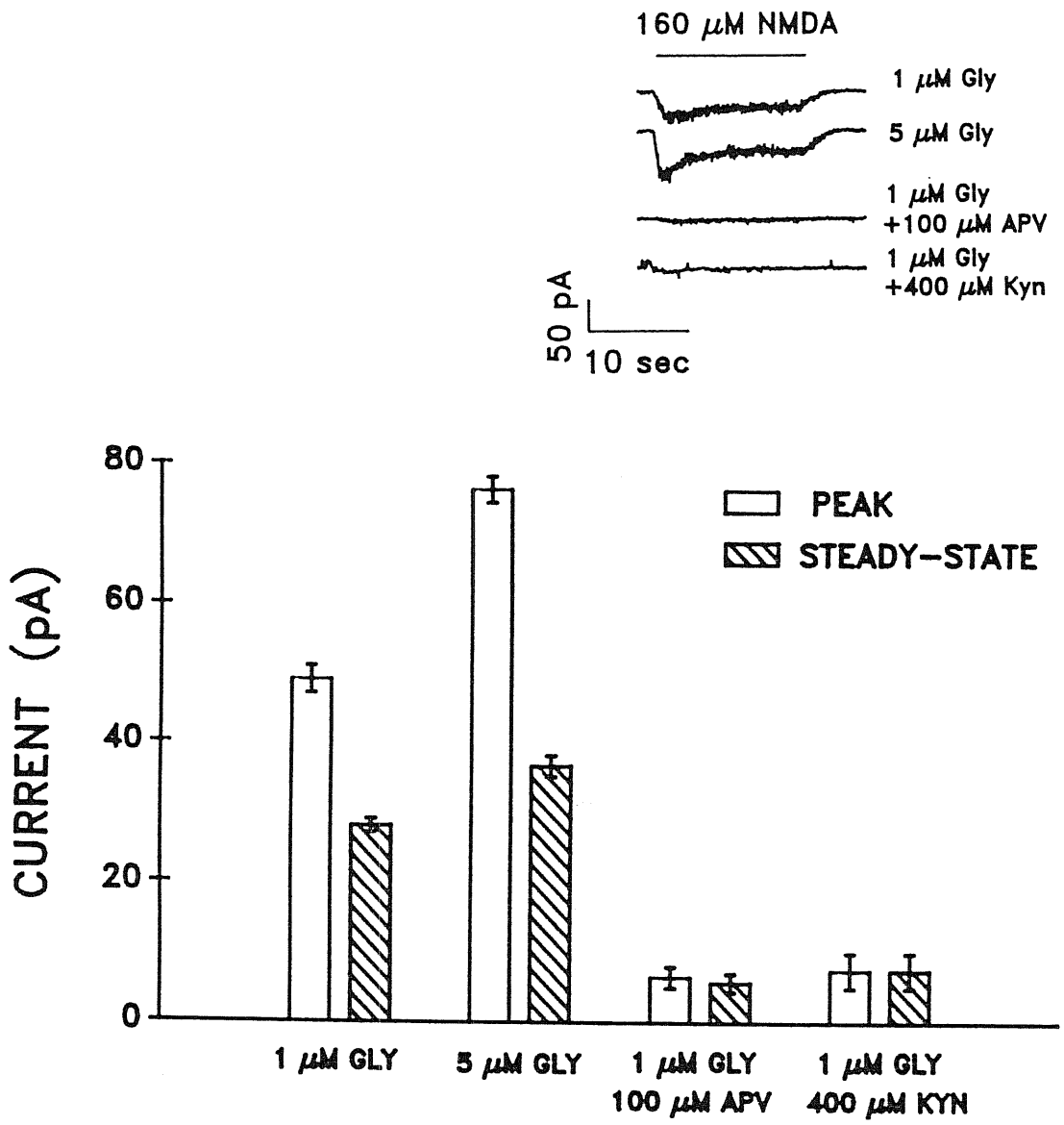


Fig. 3.4 The whole cell currents evoked by 160 μM NMDA respectively in the presence of 1 and 5 μM glycine, 100 μM APV plus 1 μM glycine and 400 μM kynurenic acid plus 1 μM glycine. 7 DIC granule cell was clamped at a membrane potential of -70 mV. Agonists and antagonists were applied through multibarrel gravity perfusion during the period labelled above the traces. The substances and their corresponding concentrations are indicated on the right of the traces. APV and kynurenic acid, at concentration of 100 and 400 μM respectively, can block almost all the currents evoked by 160 μM NMDA. The peak currents evoked by 160 μM NMDA in the presence of 1 and 5 μM glycine, 100 μM APV plus 1 μM glycine and 400 μM kynurenic acid plus 1 μM glycine are 49±2, 76.5±1.9, 6.5±1.5 and 7.5±2.5 pA (mean±std, n=2) respectively. The steady state currents are 28±1, 36.5±1.5, 5.75±1.3 and 7.5±2.5 pA (mean±std, n=2) respectively.

enhanced and it increased with the glycine dose (Fig. 3.3, 3.4). It has been reported that glycine could regulate the desensitization of NMDA receptor. With the fast perfusion system (see Johnson and Ascher 1987, Mayer et al. 1989), NMDA evoked current showed a peak followed by a plateau of enhanced amplitude in the presence of glycine (Mayer et al. 1989). The reason for this enhancement in the plateau is that in fast NMDA application, the peak current almost does not show any desensitization, however, the plateau is the result of desensitization. In our measurments, the peak current was more enhanced than the plateau by the same concentration of glycine. This is probably due to the fact that the perfusion in our experiments was slower than the one used by Johnson and Mayer, and that the time interval between the two stimulations was not long enough, thus the peak current observed in control condition had already desensitized. When glycine was applied together with NMDA, the desensitization of the peak current was diminished, therefore a predominant increase in the peak current amplitude was observed.

The dose-response curve of glycine was constructed by plotting the peak amplitude of NMDA evoked current against the concentration of concomitantly applied glycine (Fig. 3.3). In nominally glycine free condition, the peak amplitude of 60 μM NMDA evoked current was 2.5 pA current. In the presence of a saturating concentration of glycine, the same concentration of NMDA evoked a current with the peak amplitude of 37 pA (Fig. 3.3). The efficacy of NMDA can be enhanced about 15 fold. The real increasing proportion is probably higher than what we have mentioned before, since a real absence of glycine is experimentally difficult to be achieved. The glycine can come from either the spontaneous release of other cells or the glycine contamination of the glass material used for experiments (Johnson and Ascher 1987). The data in Fig. 3.3 are fitted with Equation (1) and EC_{50}=1.0±0.3 μM, h=1.8±0.1 (mean ± S.D.) are estimated

from the fitting. The EC_{50} estimated from our data was a little bit higher than others (Johnson and Ascher 1987, Kushner et al. 1988, Mayer et al. 1989). It is possible that in cerebellar neurons the sensitivity of NMDA evoked current to glycine is not as high as other neurons as suggested by Sekiguchi and his collegues (Sekiguchi et al. 1990).

APV and kynurenic acid are among the most extensively used NMDA receptor antagonists (Davies and Watkins 1982, Evans et al. 1982, Kemp et al. 1988, Davies et al. 1981). Their inhibitory effects on NMDA activated currents in cerebellar granule cells were tested. When 100 μM APV and 400 μM kynurenic acid were applied together with NMDA, most of the currents were blocked (Fig. 3.4). A nearly full recovery of NMDA evoked currents was observed after the wash-out of the antagonists.

3.2 Quisqualate Evoked Current

QA also evoked inward currents when it was applied to the cerebellar granule cells clamped at a membrane potential of -70 mV. The whole cell currents evoked by QA showed a transient peak then rapidly declined to a plateau, menifesting a rapid and strong desensitization (Fig. 3.5, 3.6A). The time constant of the activation was in the order of hundreds of milliseconds, which increases with the QA concentration, and the inactivation constant was in the order of seconds. The desensitization was highly concentration-dependent and lasted for a prolonged period. Usually more than 10 minutes wash was required to recover the receptor from the desensitized state. Even in this condition, it was very difficult to get identical QA responses. A typical example of QA evoked currents is shown on the upper part of Fig. 3.5. These responses could be recorded in our experimental conditions only at relatively low QA concentrations, where the densensitization is not too fast. A very fast perfusion, with the time constant in the order of tens of milliseconds, was used to observe these responses.

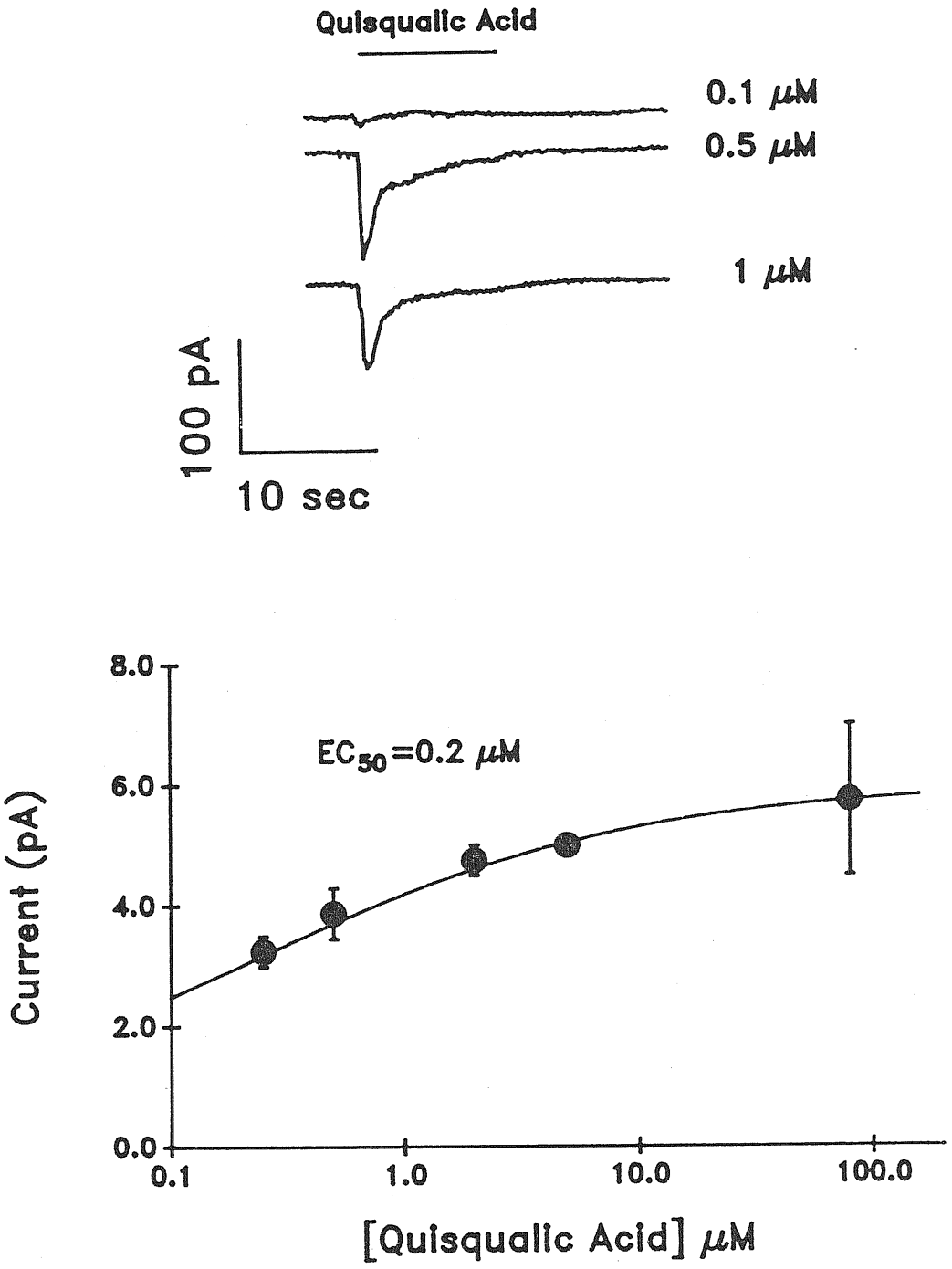


Fig. 3.5 Dose-response curve of QA evoked currents obtained from 4 DIC granule cells. Membrane potential was -70 mV. Quisqualic acid was applied through multibarrel gravity perfusion. Note that the current traces showing on the right have a big transient peak. The dose-response curve on the left is got from the steady-state currents. The data in the figure present the mean of at least 2 observations. Dose-response curve is the fitting of the experimental data with Equation (1). Values of EC_{50}=0.20±0.02 and h=0.5 are estimated from the dose-response curve.

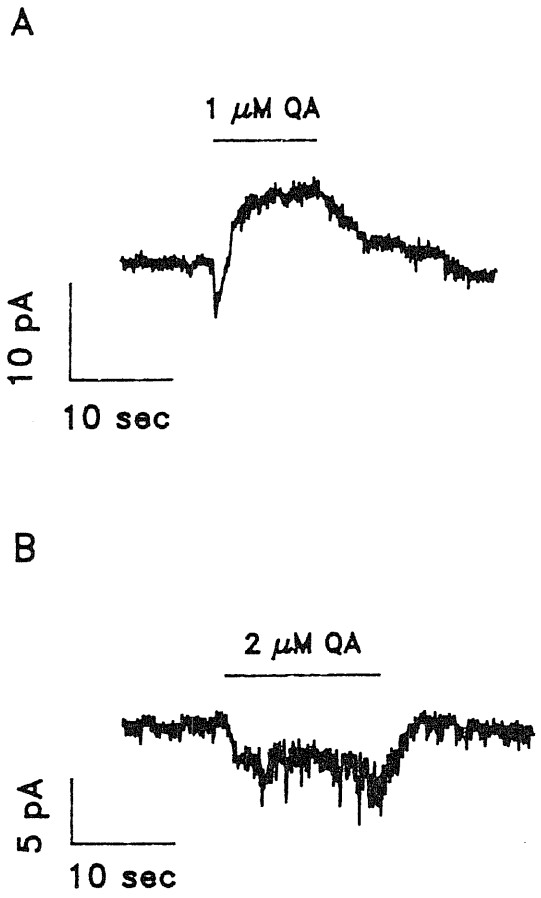


Fig. 3.6 A Whole cell current evoked by 1 μM QA in a 5 DIC granule cell. Membrane potential was kept at -70 mV. Note that an obvious outward current following the transient inward peak in this trace. **B** Desensitized whole cell current evoked by 2 μM QA in 4 DIC granule cell. Membrane potential was also kept at -70 mV. The transient inward peak does not exist any more, and only the steady-state current and the oscillations probably related to the activation of the metabotropic receptor can be observed.

At the same membrane potential, low concentration of QA (usually <0.1 μM) evoked a transient inward current followed by an outward current. In some cells that outward current could be observed obviously even in 1 μM QA evoked responses (Fig. 3.6A). The mechanism of this outward current is unclear. In most of the cases, only steady-state currents accompanying oscillations were observed (Fig. 3.6B). The reason for the missing of the transient peak could be that the activation and the inactivation of the receptor were faster than the perfusion speed of the experiments, so the receptors

were activated and then inactivated gradually but not simultaneously, consequently the transient peak was missed. Further experiments are needed to elucidate the mechanisms of the oscillations. However, it is possible that the oscillations should be due to the activation of metabotropic receptors associated with IP_3 and Ca^{2+} fluctuation (Sugiyama et al. 1987, 1989, Hirono et al. 1988).

A dose-response curve of QA evoked current was constructed by plotting the steady-state currents against the QA concentration. In the doses up to 80 μM, the steady-state current did not show a strong concentration-dependent desensitization, so all the data could be used for the fitting in this dose range. However, when the concentration is higher than 10 μM, QA usually increases the membrane leaking current and destroys the seal. This increases the difficulties in performing the experiments at high QA concentration. The left side of Fig. 3.5 shows this dose-response relation. When this relation is fitted with Equation (1), the values of EC_{50}=0.20±0.05 μM, h= 0.5±0.1 (mean ± S.D.) are estimated. The EC_{50} of QA has been reported previously elsewhere (Kiskin et al. 1986, Lerma et al. 1989a, Pin et al. 1989, Perouansky and Grantyn 1989, Zorumski et al. 1990). These values vary widely depending on the preparations. Table 3.4 listed these values.

It has been described previously that the power spectrum of the macroscopic membrane current reflects the properties of single channel events (Conti and Wanke 1975, DeFelice 1977, Conti 1984). The power spectrum can be fitted by the Lorentzian function

$$S(f)=S(0)/[1+(f/f_c)^2], \quad (2)$$

where we defined

$$S(0)=Ni^2/(2\pi f_c)^2, \quad (3)$$

N is the number of activable channel, i is the single channel current, f is the frequency and f_c is called cut-off frequency. By defining the macroscopic current $\langle I\rangle=Ni$, Equation (3) can be rewritten as

$$S(0)=\langle I\rangle i/(2\pi f_c)^2. \tag{4}$$

Therefore, the single channel current and hence the single channel conductance can be estimated as

$$i=S(0)(2\pi f_c)^2/\langle I\rangle. \tag{5}$$

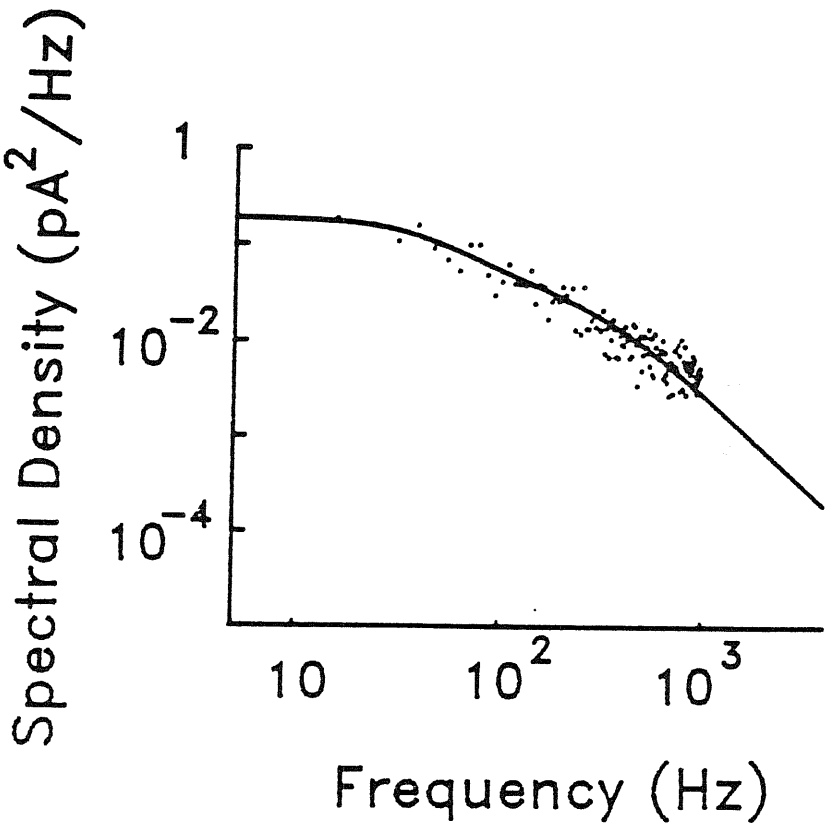


Fig. 3.7 The power spectrum of the steady-state current evoked by 10 μM QA at -70 mV membrane potential. The spectrum is fitted with two Lorentzians. The single channel conductances and mean open times estimated from the fitting are: g_1=7.9 pS, g_2=10 pS, τ_1=3.5 ms and τ_2=0.5 ms.

On the other hand, f_c corresponds to the mean frequency of the unitary events that compose the macroscopic signal (Conti and Wanke 1975, DeFelice 1977, Conti 1984). The mean duration of the events, in other words, the mean channel open time can be calculated from

$$\tau_0=1/2\pi f_c \qquad (6)$$

This fluctuation analysis has been used to study the microscopic properties of QA evoked currents. The QA evoked steady-state current, I(t), was filtered at 2 kHz by a Butterworth filter and sampled with interval of 0.5 ms. The power spectrum, $S(f)$, of these steady-state currents was obtained from the Fourier transform of the autocorrelation function of $I(t)$. Numerical calculations were done by using a Pascal program according to the fast Fourier transform algorithm (Vetterling et al. 1985).

Fig. 3.7 shows the power spectrum calculated with the above method. In our results, the spectrum could be fitted by two Lorentizan functions, which should indicate two kinds of population of single channel events evoked by QA. Three experiments were analyzed individually. The single channel conductances and the channel mean open times were calculated, and they are (mean ± S.D., n=3): g_1=7.3±0.9 pS, g_2=5.6±3.8 pS, τ_1=2.6±0.8 ms, and τ_2=0.5±0 ms. These values are close to those found in single channel recording and fluctuation analysis (Jahr and Stevens 1987, Cull-Candy and Usowicz 1987, Cull-Candy et al. 1988, Ascher and Nowak 1988a).

3.3 Kainate Evoked Current

KA evoked also inward current when the cell was patch-clamped at a membrane potential of -70 mV. Two kinds of response: one occur mainly at low KA concentration (<25 μM) and the other occur predominantly at high concentration (> 25 μM) were observed when KA was applied for a prolonged period (> 5 sec).

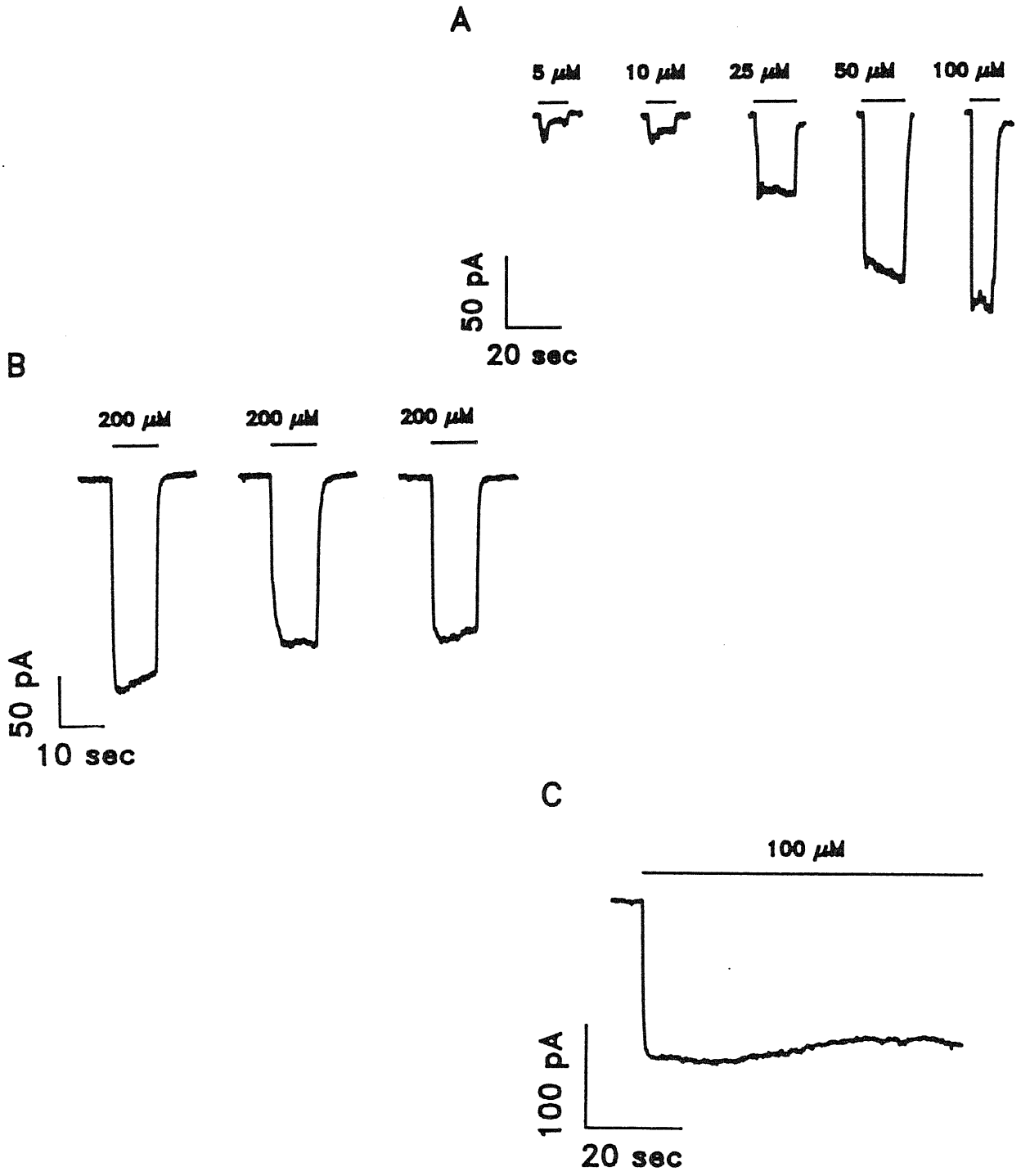


Fig. 3.8 Whole cell currents evoked by KA in cerebellar granule cells clamped at -70 mV membrane potential. **A** At low concentration (<25 μM), a current component with clear desensitization is observed. At medium concentration (25-100 μM), another relatively slow activation component is observed. **B** At high concentration (200 μM), KA evokes a current with slow desensitization and the responses to repetitive agonist application shows a progressive decrease in amplitude. **C** A prolonged bath application of KA also evokes a slow desensitizing current.

In the former condition, the amplitude of KA evoked currents increased to a peak in several hundreds of milliseconds and then declined to a minimum value in a few seconds. The amplitude did not remain at that minimal value, but slowly increased again and reached a steady state when the agonist application was maintained (Fig. 3.8A). The amplitudes of the initial peak, the minimum response and the steady state increased with the KA concentration and the speed of the increase of both the minimal response and the steady state were higher than that of the initial peak. The phenomena described above were observed in all the 22 cerebellar granule cells, where KA was applied for a period of 5-15 sec.

In the latter condition, the decline of the amplitude was observed only in a few responses (the percentage is not shown here since the total number of measurements at high concentration was not large enough for statistics) and the amplitude of the steady-state current in this case was usually higher than that of the peak. In most of the 22 cells, the initial peak could not be observed in the response when high KA concentrations were employed. Instead, a relative constant steady-state response, with small fluctuation in amplitude, was observed (Fig. 3.8A).

The decline of the amplitude observed mainly in low concentration KA evoked currents could be related to the desensitization of the receptors. However, there were two points which did not fit the phenomena observed in most of the densensitization systems: first, the current amplitudes observed in low concentration KA evoked responses did not remain at the minimal state but increased again to steady state with higher amplitude; second, the ratio between the steady-state amplitude and the initial peak amplitude increased with the KA concentration. This was contrary to what should be expected in a normal desensitization system.

In order to see the second point more clearly, we now define the desensitization ratio as

$$\text{Des. ratio}=(I_{\text{initial}}-I_{\text{minimum}})/I_{\text{initial}} \quad . \tag{7}$$

where I_{initial} is the amplitude of the initial peak observed mostly in low KA concentration, I_{minimum} is the amplitude of the minimal response measured at the end of the decline. In Fig. 3.9 this desensitization ratio is plotted against the KA concentration and a saggy-shaped profile can be observed. Data gathered from the 22 cells are used to construct this figure, but not all the cells were stimulated with all the KA concentration indicated in Fig. 3.9. Usually, the seals are easily to be destroyed by the highly concentrated KA. Thus, fewer responses were measured in high doses. The mean and the extreme values of the desensitization ratio are listed in Table 3.1.

In addition to the observations mentioned above, some other phenomana observed at high KA doses also indicated the presence of desensitization in KA evoked currents. In Fig. 3.8B, 200 μM KA was applied repeatedly to a cell clamped at -70 mV. The application process was prolonged for 10 sec each time and the cell was

Table 3.1 The desensitization ratios measured at different agonist concentrations. The maximal and minimal values obtained throughout the experiments are shown. *n* stands for the number of measurements.

Agonists (μM)	Maximum	Minimum	Mean	S.D.	*n*
[KA]=5	0.94	0.38	0.65	0.20	9
[KA]=10	1.00	0.31	0.67	0.19	27
[KA]=25	0.49	0.16	0.28	0.10	10
[KA]=30	0.19	0.15	0.17	0.02	3
[KA]=50	0.12	0.07	0.10	0.03	2
[KA]=100	0.18	0.04	0.12	0.05	8
[KA]=200	0.15	0.08	0.13	0.04	3
[DA]=1	0.33	0.33	0.33	0	1
[DA]=5	0.12	0.08	0.10	0.03	2
[DA]=10	0.07	0.07	0.07	0	1

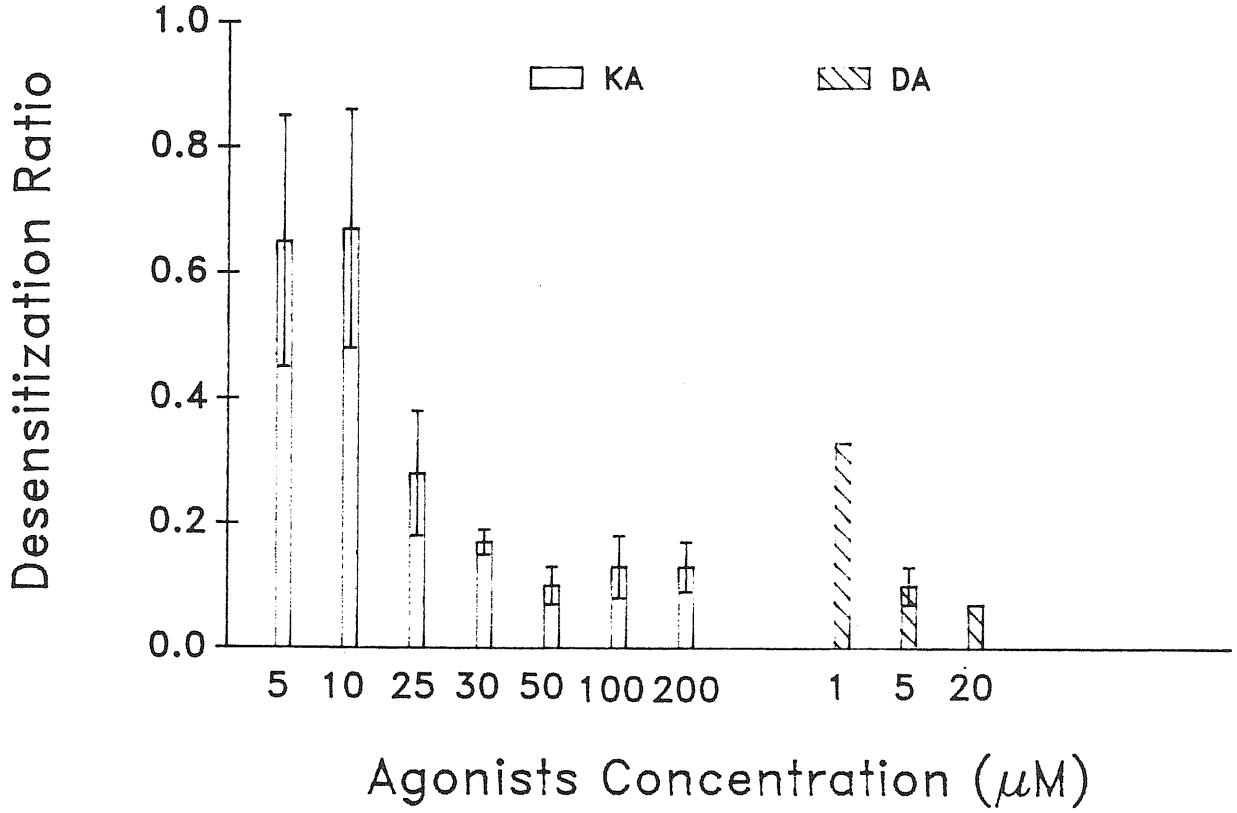


Fig. 3.9 The desensitization ratio of KA and DA evoked macroscopic currents measured at different agonist concentrations. The mean value of the densensitization ratio tends to decrease when the agonist concentration is increased. The desensitization ratio of DA evoked currents is much smaller than that of same concentration KA evoked currents. The data present the mean of the measurements from 24 cells, but not all the cells showed desensitized responses to all the agonist concentration.

washed with the normal bath solution for 3 min between two stimulations at a flux velocity same as that for the KA application. For the response to the first stimulation, primal amplitude remained at a plateau for about 3 sec and then gradually declined. It did not reach the steady state at the end of the agonist application. The responses to the subsequent stimulations were smaller than that to the first stimulation, and they almost remained at a constant level with small fluctuations. That level could correspond to the steady state of the responses. In Fig. 3.8C, 100 μM KA was applied to a cell clamped at -70 mV through bath superfusion. The amplitude of the response remains at a plateau for about 20 sec and then declines to a steady state in about 30 sec. The seal was destroyed by such a long period of KA application after more than one minute.

The phenomena observed at high KA concentration (> 100 μM) suggest that there is a slow and weak desensitization process occuring during the long period (> 5 sec) of high-dose KA application. The mechanism of this slow and weak desensitization could be different from the fast desensitization observed at low concentration KA stimulation.

Domoic acid has been described as a potent agonist of KA receptors (Biscoe et al. 1975). In some of our experiments DA was used to compare with KA. An inward current was observed when DA was applied to the cell clamped at -70 mV. Also in DA evoked responses, we found the desensitization. The decline of the amplitude from the initial peak was observed in both of the two cells where DA was applied for more than 5 sec. We found that the desensitization ratio of DA evoked responses was smaller than that of KA evoked response if the agonist concentrations were the same. However, the concentration-dependence of the desensitizations were the same for KA and DA. The figure and the numerical results are shown in Fig. 3.9 and Table 3.1.

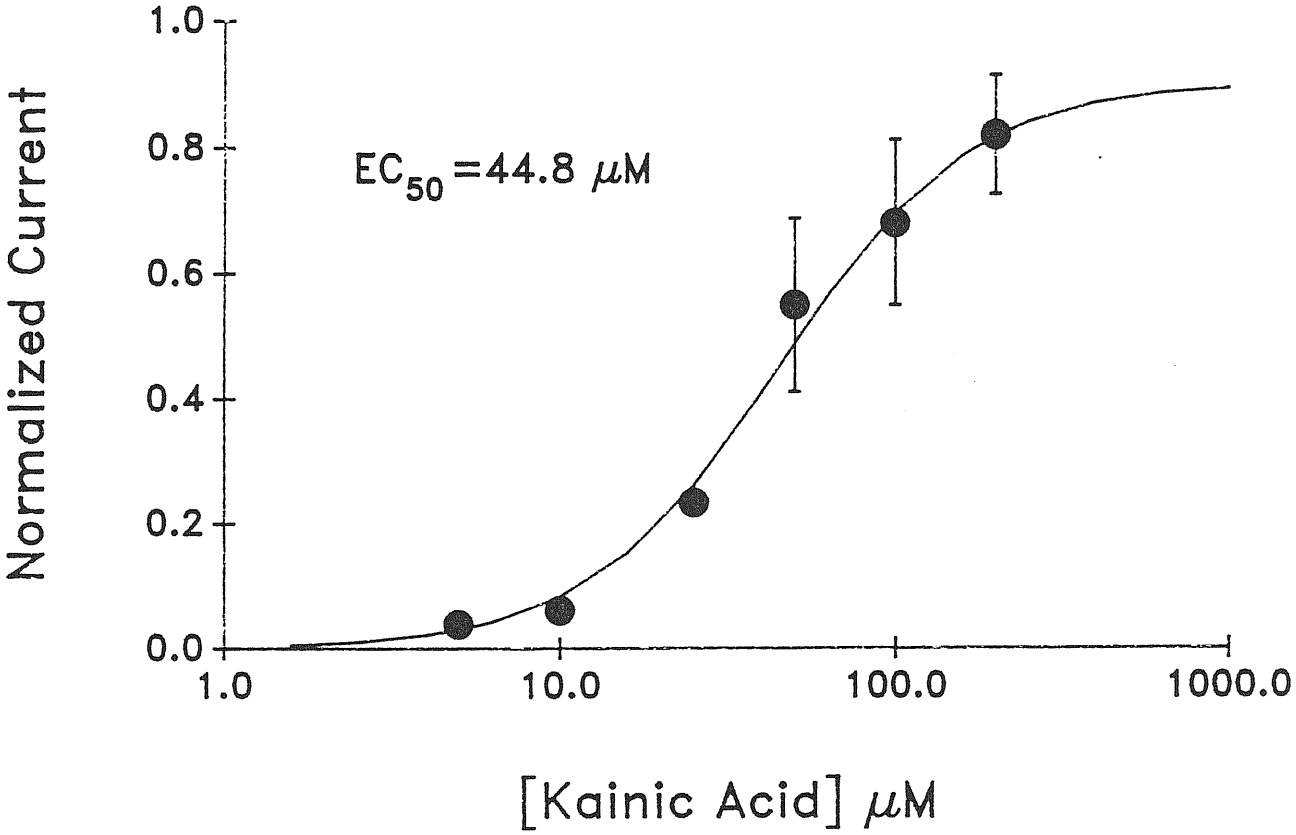


Fig. 3.10 Dose-response relation of the steady-state currents evoked by KA. Membrane potential was -70 mV. KA was applied through multibarrel gravity perfusion. The data in the figure present the mean of five experiments. The curve results from the fitting of the mean values with Equation (1). The estimated parameters are (mean ± S.D.): EC_{50}=44.8±18.1 μM, h=1.5±0.1.

The dose-response relation of KA evoked current was evaluated by plotting the steady-state current amplitudes against the KA concentrations. At each concentration, several stimulations were repeated and the stable responses (as the second and the third responses shown in Fig. 3.8B) were used for dose-response evaluation. The data obtained from five cells were calculated individually and the EC_{50} and h (mean ± S.D., n=5) were: 53.0±19.6 μM and 1.7±0.7 respectively. Fig. 3.10 shows the dose-response curve obtained from the mean of the three experiments. The EC_{50} and h estimated from our experimental data are in the range of those reported by others (Lerma et al. 1989a,

Pin et al. 1989, Perouansky and Grantyn 1989). But the EC_{50}s measured in rat hippocampal and chick spinal cord neurons are usually bigger than what our result reveals (Kiskin et al. 1986, Vlachova et al. 1987, Zorumski et al. 1990). The difference can result from the uptake system of those preparation or from the prevent of the cells from the agonists application.

The dose-response relation of DA evoked currents was also evaluated. As has been mentioned before, the desensitization of DA evoked currents was smaller than that of KA evoked currents, and, consequently, the current amplitudes remained relatively constant throughout the DA application (see the upper part of Fig. 3.11). These stable amplitudes were plotted against the DA concentration to construct the dose-response relation. Two experiments were each fitted with Equation (1), and the mean values of EC_{50} and h are (mean ± S.D., n=2) 5.8±0.7 μM and 1.2±0.1 respectively. The dose-response curve is illustrated in Fig. 3.11. From the EC_{50} we can see that DA is about 10 fold as potent as KA. A double-fold value of EC_{50} has been reported in hippocampal neurons (Patneau and Mayer 1990) wherein the relation between the two EC_{50}s, corresponding to DA and KA, is also about one to ten.

The KA receptor activated currents, either evoked by KA or DA, were blocked by kynurenic acid. The currents evoked by 25 μM KA were blocked 40% and 60% respectively by 100 and 300 μM kynurenic acid (8 observations for each). The currents evoked by 30 μM DA were blocked 57% by 400 μM kynurenic acid (2 observations). The relative current amplitudes are shown in Fig. 3.12. It has been reported that in the absence of NMDA component, kynurenate could selectively block KA evoked currents completely and the blockade capacity was kynurenate concentration dependent (Perouansky and Grantyn 1989). In type-2 cerebellar astrocytes, kynurenate blocked 70% of kainate (30-100 μM) evoked currents (Usowicz et al. 1989).

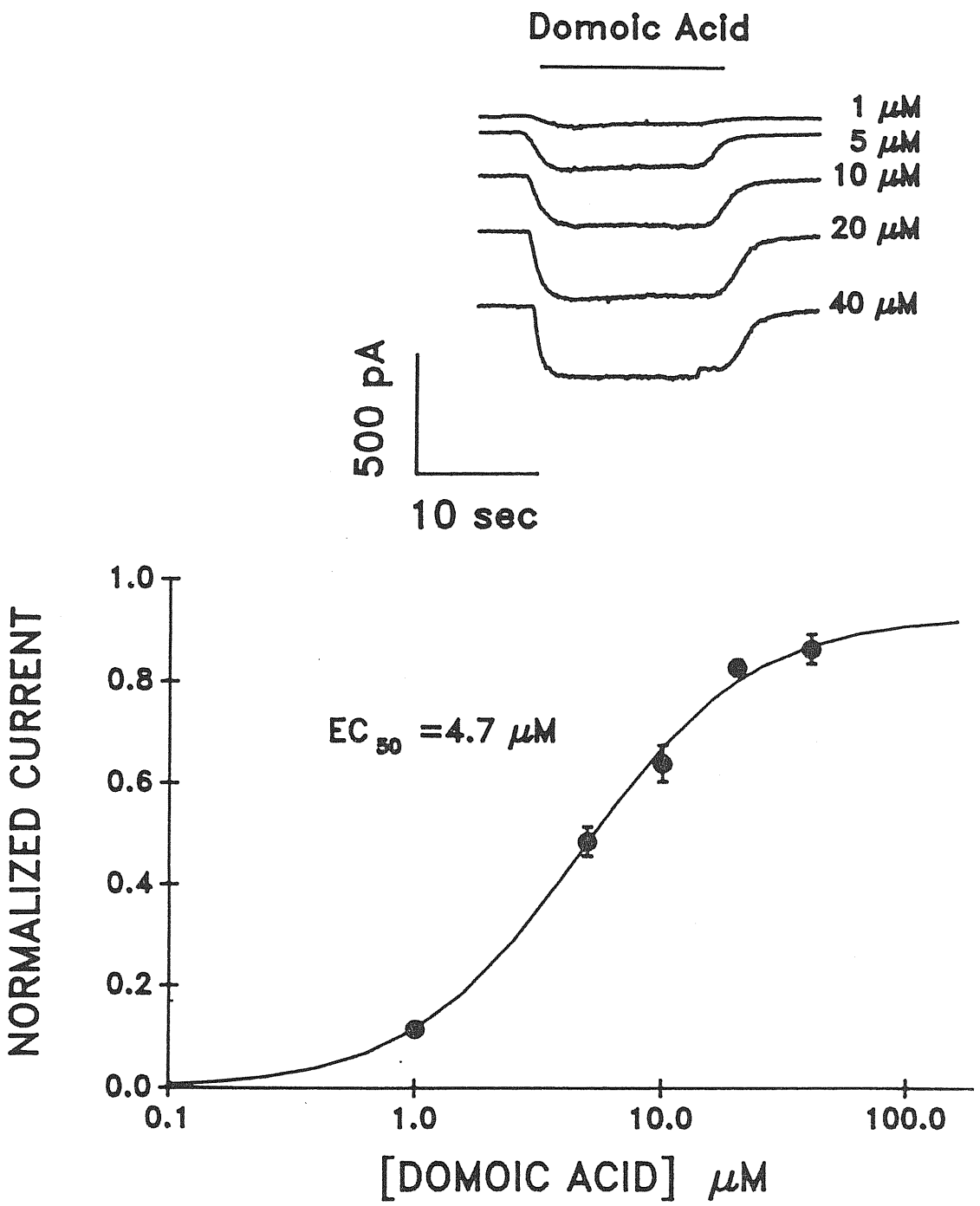


Fig. 3.11 Dose-response relation of DA evoked currents measured from a 7 DIC granule cell. Membrane potential was -70 mV. DA was applied through multibarrel gravity perfusion. The duration of DA application is labelled above the traces. DA concentrations are indicated on the right of the traces. The data represent the mean of 2 experiments. Curve comes from the fitting of the mean values with Equation (1). Values of EC_{50}=4.7±2.4 μM and h=1.3±0.1 (mean ± S.D.) are estimated from the curve. Note that the values present here come from the fitting of the mean values, and the values present in the text come from the mean of each individual fitting.

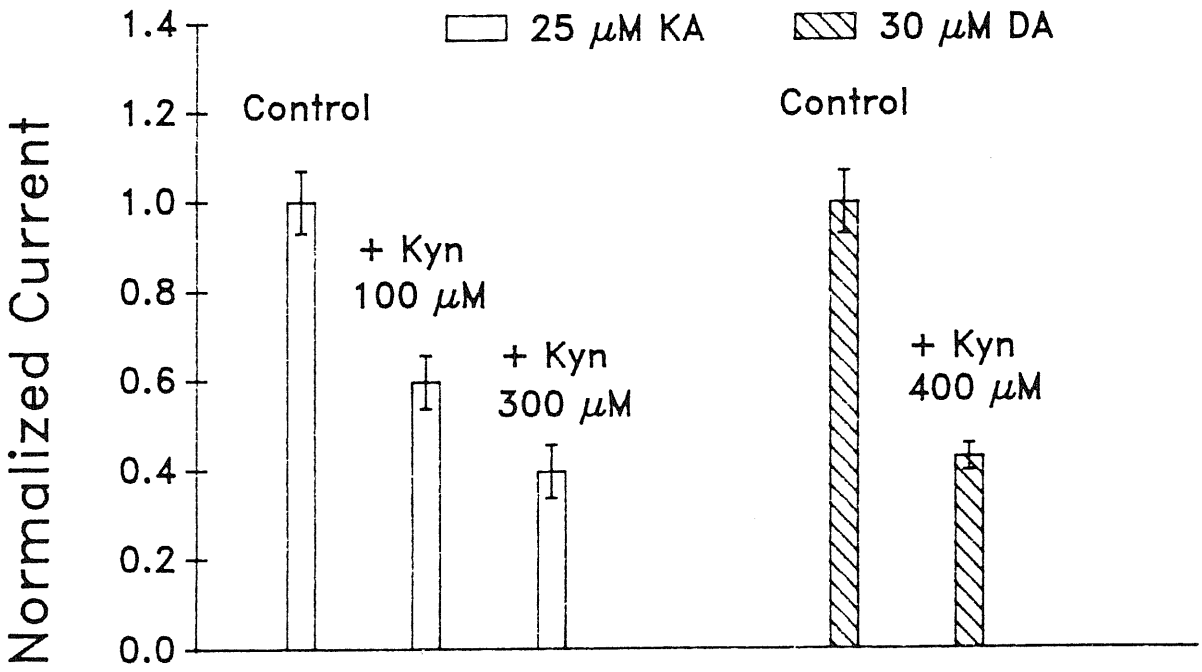


Fig. 3.12 The inhibition of KA receptor activated currents by kynurenic acid on 7 DIC granule cell. Membrane potential was -70 mV. Multibarrel gravity perfusion was used. The currents are normalized to the control value (without kynurenic acid). The data for KA are the mean of 8 observations and that for DA are the mean of at least 2 observations. The currents evoked by 25 μM KA were blocked 40% and 60% respectively by 100 and 300 μM kynurenic acid. The currents evoked by 30 μM DA were blocked 57% by 400 μM kynurenic acid.

KA receptor activated single channel currents have been extensively studied in our previous work by means of single channel recording (Zheng 1989, Sciancalepore et al. 1990). In most of our single channel records, DA was used to evoke the currents, since DA is a more potent agonist of KA receptors. The DA concentration used for single channel experiments was 10 μM. Some traces of 10 μM DA evoked single channel currents measured at different membrane potentials are shown in Fig. 3.13. The single channel conductances measured from 4 patches are (mean ± S.D., n=4) 4.4±0.3 pS (about 80%) and 2.4±0.3 pS (about 20%). The mean channel open time was (mean ± S.D., n=4) 2.9±1.3 ms. The details about the single channel recording can be referred to our previous works (Zheng 1989, Sciancalepore et al. 1990).

The single channel properties of KA and DA evoked currents were carefully studied again with fluctuation analysis. The procedure of the fluctuation analysis was the same as that described for QA evoked currents. The power spectrum of KA and DA evoked currents are shown in Fig. 3.14. Fig. 3.14A shows the spectrum of 80 μM KA evoked steady-state current fitted with two Lorentzian functions; Fig. B and C show the spectra of 25 μM KA and 25 μM DA evoked currents fitted with one Lorentzian function. The results are presented in Table 3.2.

From Table 3.2, it can be seen very clearly that the single channel properties of KA and DA evoked currents, estimated from one Lorentzian function, are exactely the same. This finding demonstrates, from the kinetic characteristics and the single channel conductances, that KA and DA do activate the same type of receptors. It also confirms our results obtained with single channel recording in the previous works (Zheng 1989, Sciancalepore et al. 1990). When the spectra were fitted with two Lorentzian functions, two time constants were estimated, one bigger and the other smaller than that estimated from the one Lorentzian fitting.

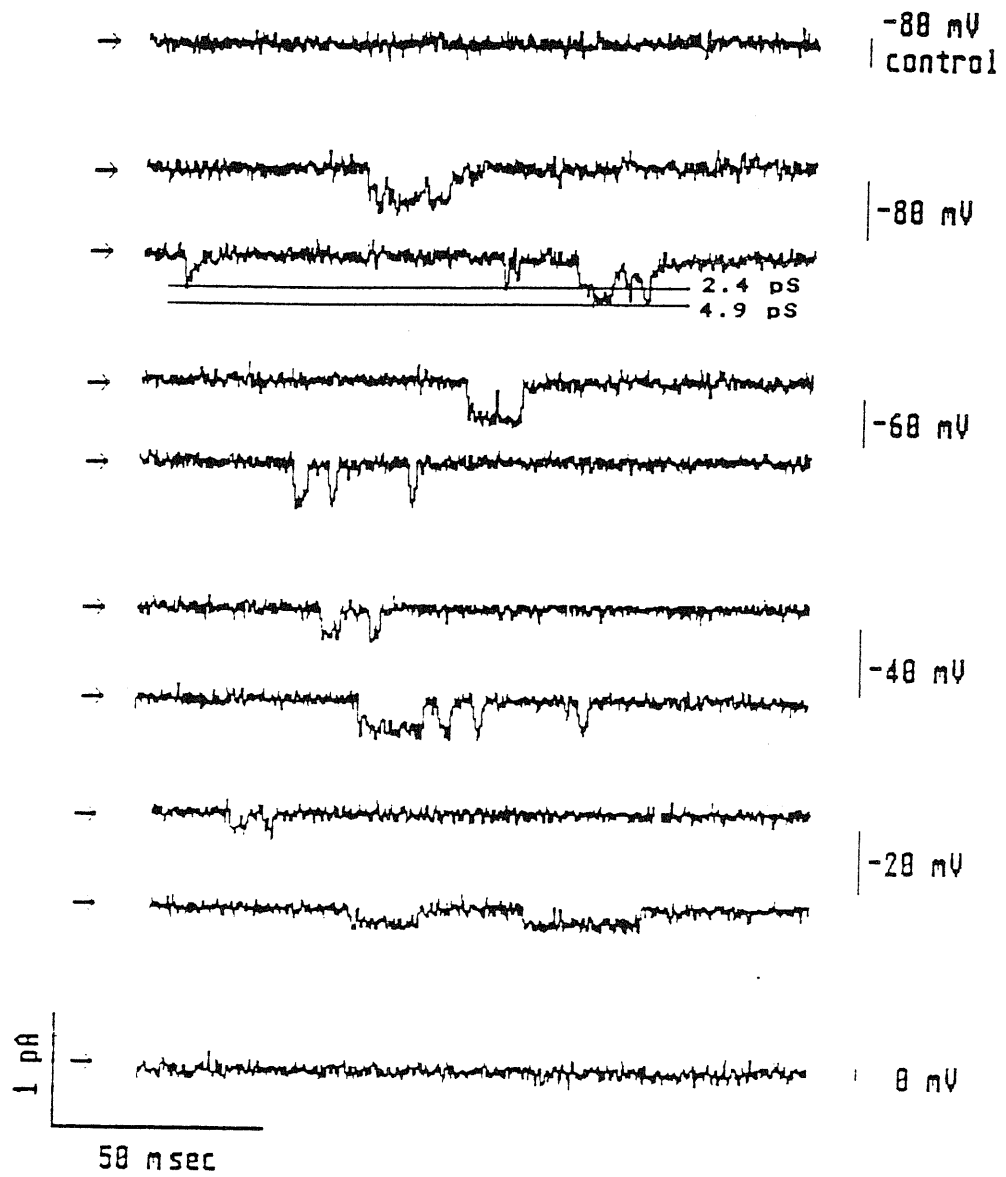


Fig. 3.13 Single channel current evoked by 10 μM domoic acid in an outside-out patch, at membrane potential from -80 to 0 mV, 20 mV in step. Domoic was applied through bath perfusate. The upper trace is the control obtained at -80 mV membrane potential. A main single channel conductance of 4.9 pS was obtained in that patch. A subconductance of 2.4 pS also appeared in that patch.

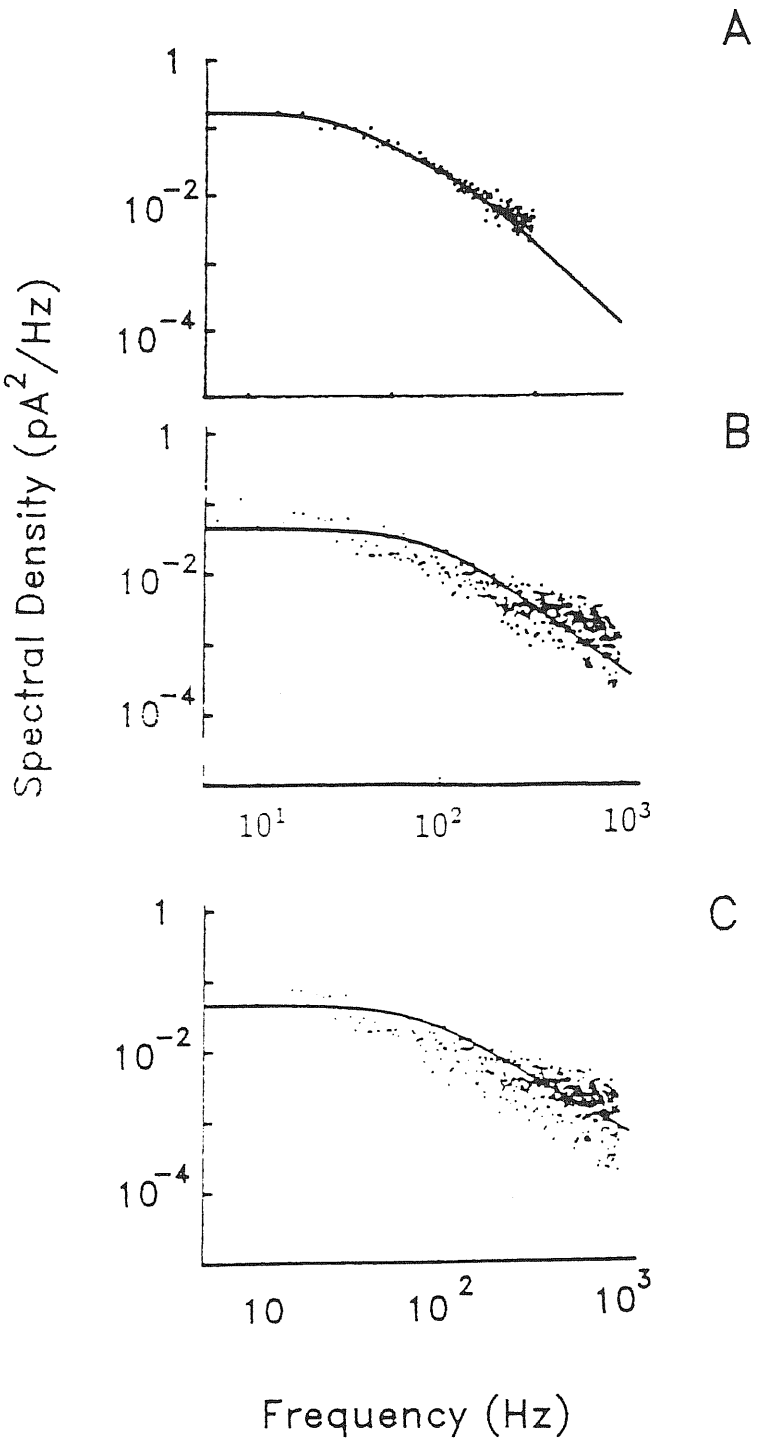


Fig. 3.14 A The power spectrum of the steady-state macroscopic current evoked by 80 μM KA at -70 mV membrane potential. The spectrum is fitted with two Lorentzians. The single channel conductances and mean channel open times estimated from this spectrum are g_1=3.8 pS, g_2=3.3 pS, τ_1=3.1 ms and τ_2=0.6 ms. B The power spectrum of 25 μM KA evoked current. Membrane potential was kept at -70 mV. From one Lorentzian fitting, the single channel conductance and mean channel open time are estimated as: 2.9 pS and 1.7 ms. C The power spectrum of 25 μM DA evoked current. Membrane potential was -70 mV. From one Lorentzian fitting, the single channel conductance and mean channel open time are estimated as: 2.5 pS and 1.7 ms respectively.

Table 3.2 The single channel properties of KA-, DA-evoked currents. The values show mean ± S.D.

Agonists	g_1 (pS)	g_2 (pS)	τ_1 (ms)	τ_2 (ms)	n
KA	4.4±2.0	3.8±1.6	3.2±0.7	0.6±0.1	10
KA	3.5±0.9	-	1.3±0.6	-	5
DA	3.5±0.8	-	1.8±0.4	-	10

3.4 Glutamate Evoked Current

The endogeneous excitatory neurotransmitter, glutamate, evoked also inward currents in cerebellar granule cells clamped at -70 mV. The behaviour of these currents was very complicated, due to the combined contribution of all glutamate receptor subtypes described above. A very strong desensitization was observed in glutamate evoked currents. Both the peak and the steady-state currents varied widely and wildely when the agonist stimulations were repeated. It seems as if the increase of the intervals between two stimuli would reduce the desensitization.

Glutamate evoked current in the presence and the absence of glycine were measured. The glutamate receptor antagonists, APV and kynurenic acid were used for block the glutamate evoked current. The current amplitudes of 160 μM glutamate evoked, together with the corresponding experimental conditions, are presented in Table 3.3.

Comparing the glutamate evoked current in the absence and the presence of glycine, it was clear that the peak amplitude was enhanced about 7-fold by 1 μM glycine, probably by acting on the NMDA receptor (Johnson and Ascher 1987, Kushner et al. 1988, Mayer et al. 1989). The total glutamate evoked currents (in the presence of 1 μM glycine) were blocked 40% by 100 μM APV and 88% by 400 μM kynurenic

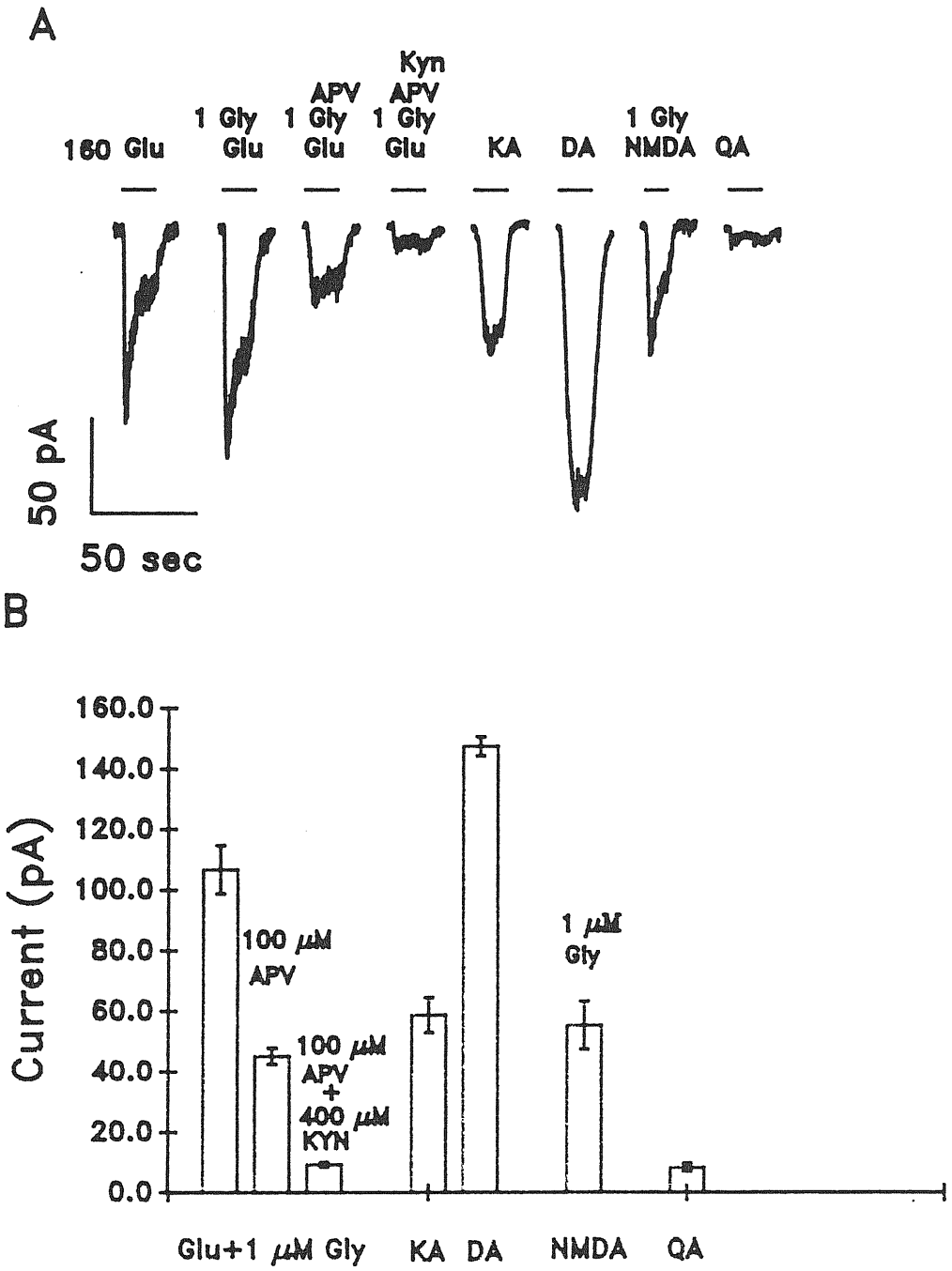


Fig. 3.15 The whole cell currents evoked by different agonists in a single 7 DIC granule cell. Membrane potential was -70 mV. Multibarrel gravity perfusion was used. 30 μM Glutamate, NMDA, KA, DA and QA were used to stimulate a same cell. The substances, their concentrations and the application durations are indicated above the traces. The first trace is the response of the cell to 160 μM glutamate in the absence of glycine. In other traces concerning the responses of NMDA and glutamate, 1 μM glycine always exist. The values in the lower figure were (mean ± S.D., n=3, from left to right): 106.8±8.0, 45.3±2.7, 9.3±0.9, 58.8±5.8, 147.3±3.1, 55.3±8.0 and 8.3±1.3 pA respectively.

acid. In the presence of 400 μM kynurenic acid, no more sufficient blocking effects were found when 100 μM APV was added (Table 3.3).

Fig. 3.15 shows the current traces evoked by 30 μM of glutamate, NMDA, kainate, domoate and quisqualate in a same cell. The peak amplitudes (mean ± S.D.) are shown below the traces. The first trace in Fig. 3.15 is the response to 160 μM glutamate alone. Compared with the second trace, which is the response to 30 μM glutamate plus 1 μM glycine, we can see that the amplitude of glutamate evoked current is significantly enhanced by glycine. The order of the current amplitude is DA>Glu>KA>NMDA>QA.

Table 3.3 Glutamate (160 μM) evoked currents are facilitated by glycine and antagonized by APV and kynurenic acid. Values are mean ± S.D..

Substance(s) applied concomitant with Glu (μM)	Currents (pA)	*n*
-	14.5±0.8	2
Glycine 1	103.2±8.5	6
Glycine 1 + APV 100	61.5±5.2	4
Glycine 1 + kynurenic acid 400	12.5±2.0	4
Glycine 1 + APV 100 + kynurenic acid 400	11.0±1.5	2

Chapter 4

Interaction Between Kainate and Quisqualate on Non-NMDA Receptors

The capacity of quisqualate and glutamate to antagonize the electrical responses evoked by kainate was first noted in goldfish retinal horizontal-cells by Ishida and Neyton (1985). Such a phenomenon was then observed in hippocampal neurons (Kiskin et al. 1986, 1990, Mayer and Vyklicky 1989, Patneau and Mayer 1991), embryonic chick motoneurons (O'Brien and Fischbach 1986), superior colliculus cells (Perouansky and Grantyn 1989) and *Xenopus* oocytes injected with rat brain mRNA (Lerma et al. 1989a). Lately, AMPA, L-HCA and D-HCA have also been reported of possessing the capacity (Patneau and Mayer 1991).

Several hypotheses have been put forward, but the mechanism of this interaction still remains controversial. In the last two years, the results from molecular biology favour the model of a common KA/AMPA receptor. A family of cDNAs coding for four glutamate receptor subunits have been isolated from the rat brain cDNA library and cloned. When they were expressed either alone or in combination in *Xenopus* oocytes, the resulting receptors respond to AMPA, QA, glutamate as well as KA (Keinänen et al. 1990, Nakanishi et al. 1990, Lambolez et al. 1991). But recently, a cDNA coding a

protein responding to KA, QA and glutamate but not to AMPA has been cloned (Egebjerg et al 1991). Furthermore, the success in cloning a cDNA coding for the high affinity KA binding protein has been reported (Werner et al. 1991).

In the previous chapter, it has been shown that all three prototypic glutamate receptor agonists evoke electrical responses in the primary cultures of cerebellar granule cells. The receptor autoradiographic experiments have shown that, in contrast to the hippocampus, the cerebellum has a plenty of AMPA binding sites, but less numerous of KA binding sites (see Chapter 1). So the action of QA and AMPA should be more effective in this region than in other regions of the brain. In this aspect, this preparation provides an ideal system for the study of the interaction between KA and QA (or AMPA). The results concerning the interaction of KA and QA on the non-NMDA receptors of the cerebellar granule cells are presented in this chapter.

4.1 Interaction Between Kainate and Quisqualate

The electrical responses evoked by concomitant application of KA and QA were measured by using two experimental protocols: i) QA was applied through bath superfusate and KA was applied through pressure ejection; ii) both QA and KA were applied through pressure ejection.

In the first experimental protocol, QA (at concentrations up to 5 μM) was periodically applied through bath superfusion. During each QA application the QA containing bath solution was perfused for a volume of at least 10 times of that of the experimental chamber. A homogeneous QA distribution could be obtained in the preceding of KA application by using this protocol. Brief pulses (1 sec duration) of KA were focally applied to the cell through pressure ejection. The KA of 25 and 50 μM concentration were used in the experiments.

At a membrane potential of -70 mV, QA (0.5-5 μM) applied in the bath increased the noise of the baseline current without significantly changing the baseline current (11 cells). The reason was probably that the continuous application of QA led most of the receptors, which mediated QA evoked electrical responses, into the desensitized state. Furthermore, the perfusion speed was too low to show the peak current, which should be observed right at the beginning of the QA application if the perfusion speed is sufficiently high. At concentration higher than what was used, QA induced a fluctuating baseline current and hence disturbed the accurate amplitude measurements.

The brief pressure ejection of KA evoked a spike-shaped inward current at -70 mV membrane potential. In each experiment, the amplitude of KA evoked responses showed a run-down at first stage, then it stabilized after several stimuli. These stable responses were used as the control of the following experiments. In the presence of QA (0.5 μM was ample), the amplitude of these currents (evoked by 25 and 50 μM KA) was significantly reduced in comparision to that evoked before the bath application of QA. The inhibitory effect was enhanced in the presence of high QA concentration (up to 5 μM). A typical example is shown in Fig. 4.1. The upper part of the figure shows the 25 μM KA pulses (1 sec) evoked current traces in the absence and the presence of different QA concentrations. QA at the concentrations indicated in the figure was superfused for a period longer than 10 minutes. The periods, during which QA distributed itself homogeneously, were indicated by the bars below the traces. Under the control condition, KA evoked a current current of 42 pA amplitude. The same stimulus evoked only a current of 5 pA amplitude in the presence of 5 μM QA. The effect of QA on KA evoked currents was concentration-dependent. A concentration of 0.5 μM QA reduced 60% of the KA evoked currents and 5 μM QA reduced 90% of the

currents. An almost complete recovery was achieved after wash QA away. The current amplitudes used in the analysis were measured from the baseline to the peak.

In several experiments, only partial recovery of KA evoked currents could be achieved when the cell was returned back to the control solution. This could be due to: i) the partial wash of QA; ii) some changes in the cell properties because of a long exposure to QA; iii) the desensitization produced by repetitive KA application. In order to avoid the experimental error from this non-complete recovery, KA evoked currents were measured as control before each QA application. The current evoked by KA in the presence of QA was normalized to the control measured before that QA application.

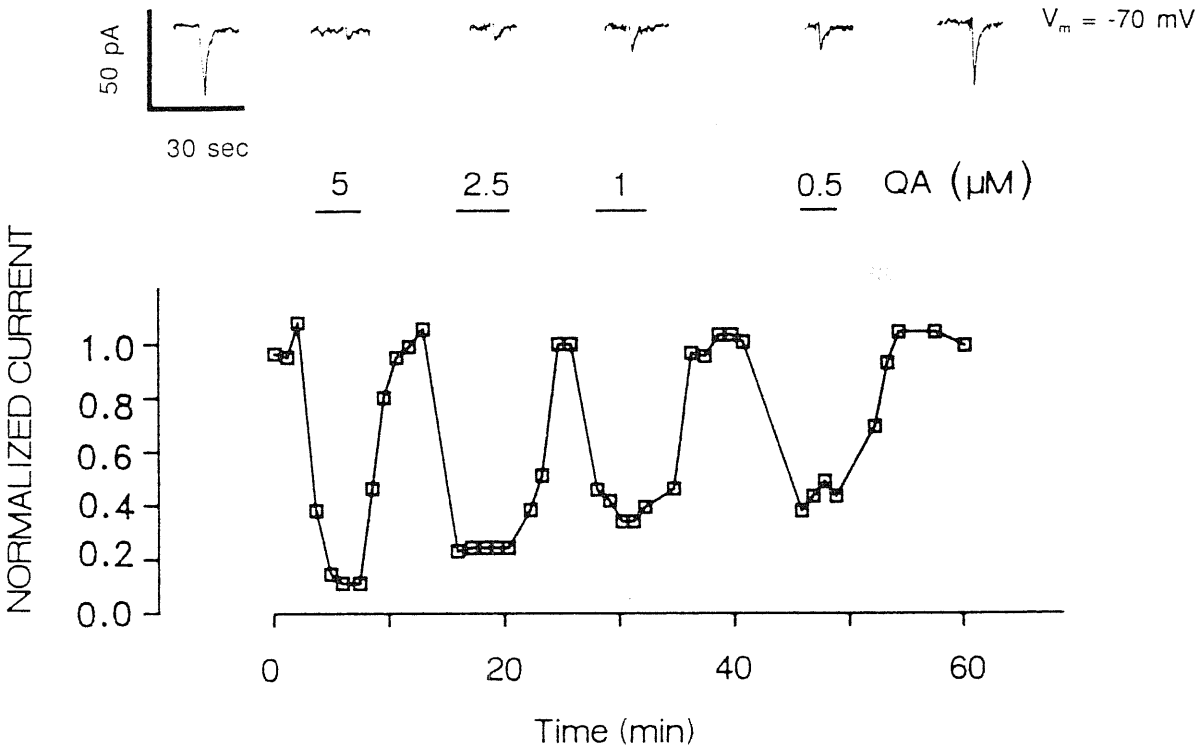


Fig. 4.1 Whole cell currents evoked by 1 sec pressure ejection of 25 μM KA in 8 DIC cerebellar granule cell. Membrane potential was kept at -70 mV. QA was applied through bath solution. The QA concentration and corresponding application duration were (bars and the values above them). The data correspond to the current amplitudes normalized to the mean value of the controls measured before the application QA.

Similar results were achieved by using 25 μM DA instead of KA. The statistics of KA (25 and 50 μM) and DA (25 μM) evoked current in the presence of different QA concentrations, which are obtained from 11 cells, are shown in Fig. 4.2 and Table 4.1.

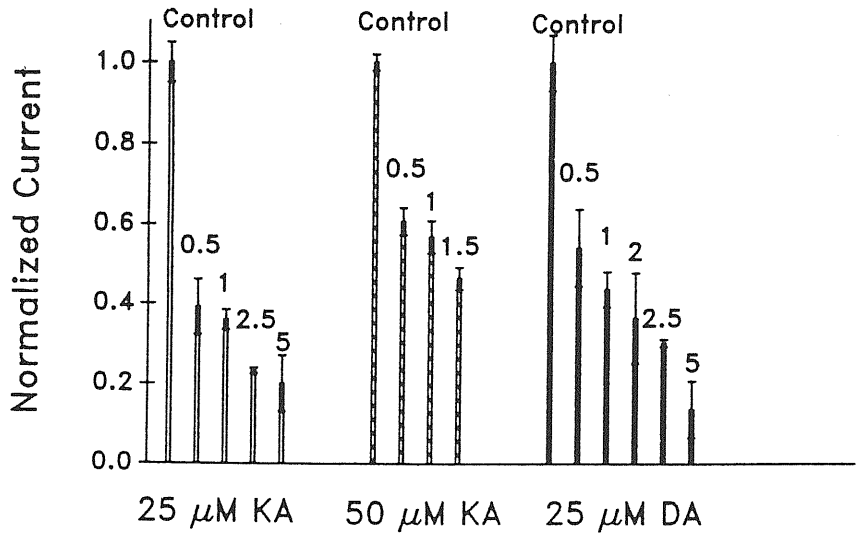


Fig. 4.2 The normalized currents evoked by 25, 50 μM KA and 25 μM DA in the presence of bath applied QA. 1 sec pulses of KA and DA were applied through pressure ejection. The data presented the mean of several measurements (indicated in Table 4.1). Each measurement was normalized to its control as explained in the text.

Table 4.1 Whole cell currents evoked by 25, 50 μM KA and 25 μM DA in the presence of bath applied QA (mean ± S.D., number of observations). Each current amplitude was normalized to the mean amplitude of the control currents measured before the corresponding QA application.

[QA] μM	25 μM KA	50 μM KA	25 μM DA
0 (Control)	1.00±0.05 (15)	1.00±0.02 (3)	1.00±0.07 (78)
0.5	0.39±0.07 (4)	0.61±0.04 (3)	0.54±0.10 (24)
1	0.36±0.03 (3)	0.57±0.05 (4)	0.44±0.05 (15)
1.5	-	0.46±0.03 (2)	-
2	-	-	0.36±0.12 (24)
2.5	0.23±0.01 (4)	-	0.30±0.01 (2)
4	-	-	0.25±0.16 (17)
5	0.20±0.07 (7)	-	0.14±0.08 (15)

In the second experimental protocol, 80 μM KA and 1 μM QA were applied separately or concomitantly through pressure ejection (last up to 1 min) using double barrel pipette positioned close to the cell. In this condition, the quick application (evoked response within 30 ms) of QA (1 μM) evoked measurable steady-state whole cell currents (Fig. 4.3A). The amplitude of the current evoked by QA (1 μM) was smaller than that of the current evoked by KA (80 μM). When they were applied concomitantly to the cell, a current with amplitude smaller than that evoked by 80 μM KA but larger than that evoked by 1 μM QA was obtained (Fig. 4.3B). In the experiment illustrated in Fig. 4.3B, KA (80 μM) was applied for 60 sec through one barrel of the perfusion pipette. 30 sec after the beginning of the KA application, QA (1 μM) was applied through a second barrel for 60 sec. In this process, the cell was sequently perfused by 30 seconds of 80 μM KA, 80 μM KA plus 1 μM QA, and 1 μM QA. Consequently, a step-shaped whole cell steady-state current was measured (Fig. 4.3B). At the end of KA application, QA evoked a current equal to the control (see Fig. 4.3A).

A similar result was obtained when 50 μM AMPA was used instead of 1 μM QA (Fig. 4.3C, D). As already described in Chapter 1, QA activates both a metabotropic receptor and an ionotropic receptor whereas AMPA activates only an ionotropic receptor. Since both QA and AMPA caused similar effects on KA evoked electrical responses, it is suggested that the antagonism of QA on KA responses is mostly mediated by the activation of the ionotropic receptor.

In this experimental protocol, only a small amount of QA was applied to the cell for a short time. Thus it was easy to wash the QA away, and the stability of the seal was not very much affected. Consequently, a faster and more complete recovery was

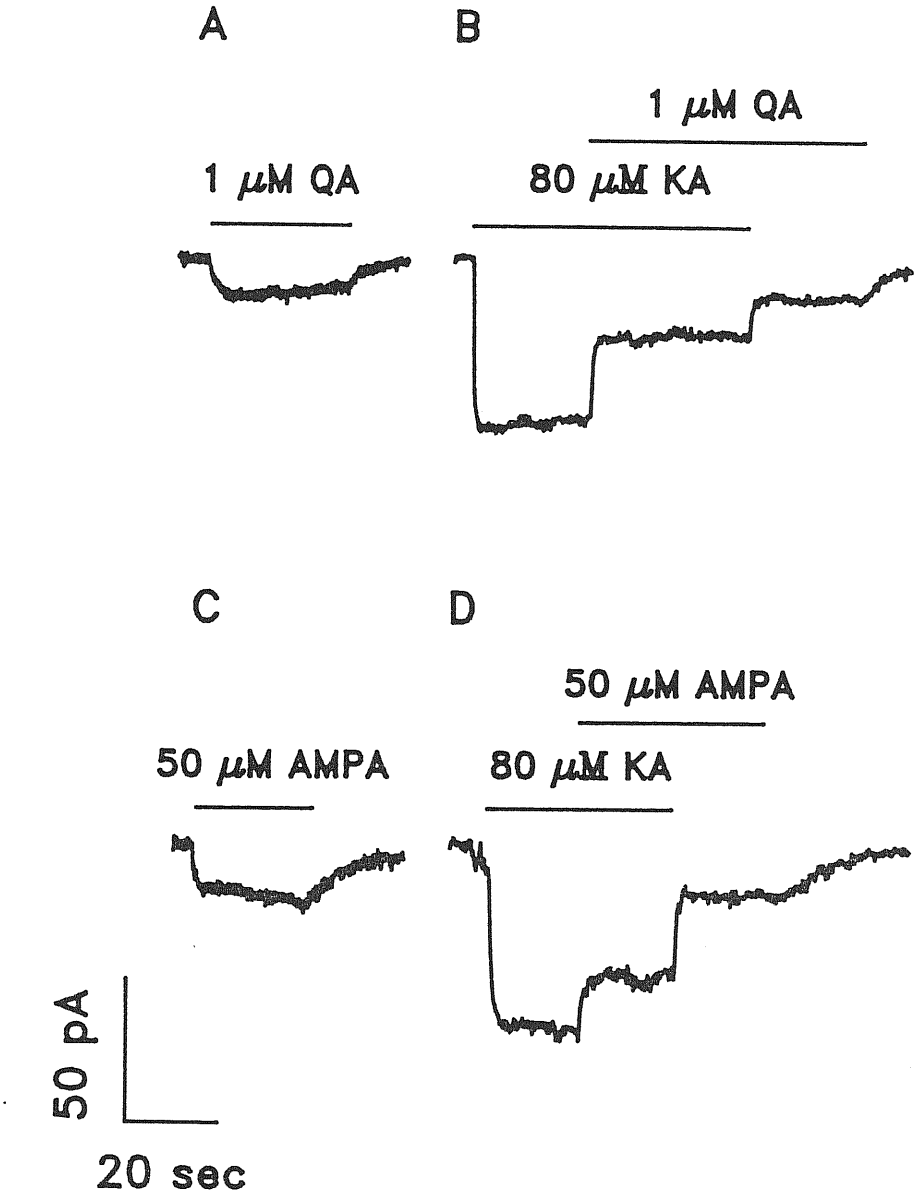


Fig. 4.3 A Whole cell current evoked by 1 μM QA in a 5 DIC cerebellar granule cell. Membrane potential was -70 mV. QA was applied through presure ejection. Note that the peak current has desensitizied. B The whole cell current evoked by 80 μM KA, 1 μM QA and the concomitant application of both of the two agonists in the same cell. Membrane potential was -70 mV. KA and QA were applied through double barrel pressure ejection. The duration of each application was indicated above the trace. The amplitude of the current evoked by the concomitant application of 80 μM KA and 1 μM QA fell between that evoked by 80 μM KA and that by 1 μM QA. C Similar to A, 50 μM AMPA was used instead of 1 μM QA. D Similar to B, 50 μM AMPA was used instead of 1 μM QA.

observed in comparision with the first experimental protocol. Fig. 4.4A shows an experiment where KA (80 μM) was applied to the cell for 40 sec. 10 sec after the beginning of KA application, QA was applied for 10 sec. The amplitude of KA evoked whole cell current was reduced to 43% during the QA application (Fig. 4.4A).

Similar results were obtained with AMPA (50 μM). The reduction of KA evoked current by AMPA was voltage-independent. At a membrane potential of -70 mV, the KA evoked current was reduced to 60% by AMPA (50 μM). At +30 mV, the KA evoked outward current was reduced to the same fraction by 50 μM AMPA (Fig. 4.4B).

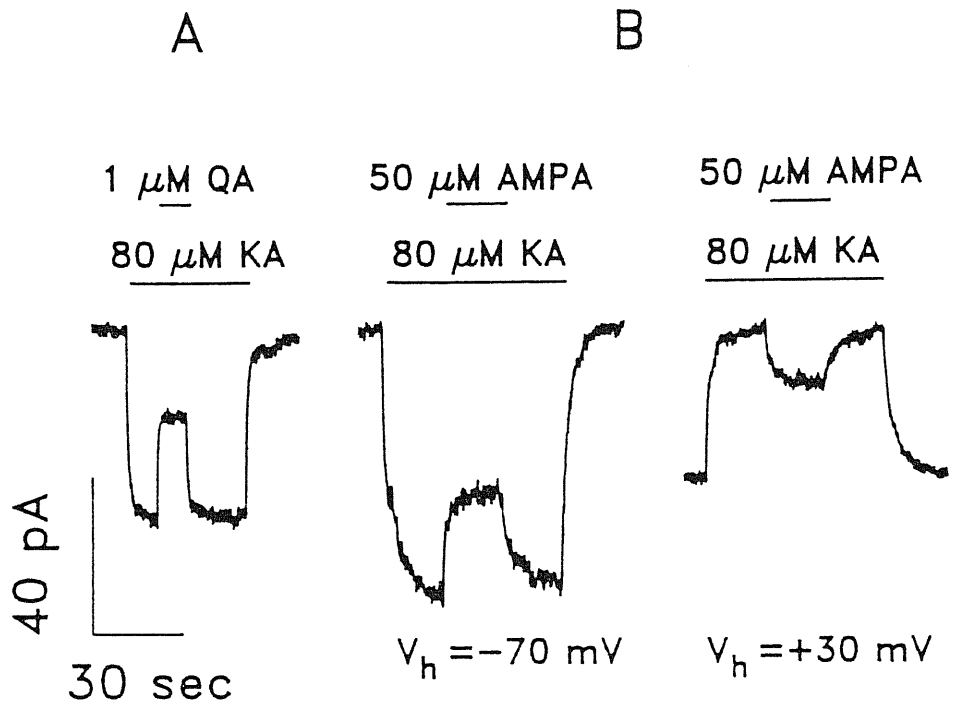


Fig. 4.4 A Whole cell current evoked by 80 μM KA in the absence and presence of 1 μM QA. Granule cell was 5 DIC. Membrane potential was kept at -70 mV. KA and QA were applied through double barrel pressure ejection perfusion. The duration of each application was indicated above the trace. When 1 μM QA was ejected to the cell together with 80 μM KA, the current amplitude reduced immediately and such a reduction was recovered as soon as QA ejection was stopped. B Similar to what described in A. 50 μM AMPA was used instead of 1 μM QA. The same experiments performed at membrane potentials of -70 and +30 mV. The results were similar to those described in A. The antagonism was voltage-independent.

This indicated that the reduction in the whole cell current amplitude is not caused by the effect of an open channel blocker functioning in an analogy as Mg^{2+} blocking NMDA channel. Another thing is noticed is that the inhibition effect of AMPA on KA evoked current is one order less than that of QA.

4.2 Competition of the Two Agonists

The dose-response curve of KA evoked currents in the absence of QA has been described in the previous chapter. In the presence of QA, the amplitude of KA evoked response was smaller than that obtained in the absence of QA. In order to get the same amplitude response, higher KA concentration was required. The dose-response relation of KA evoked steady-state currents in the presence of 1 μM QA was evaluated in the same way as we evaluated the dose-response curve of pure KA evoked currents. In the presence of QA, the dose-response curve shifted to the right. Five experiments were evaluated separately. The EC_{50} and h were estimated from each fitting and the values are (mean ± S.D., n=5) 118.4±30.1 and 1.3±0.2 respectively. Table 4.2 shows the difference between these data and those obtained in the absence of QA.

Table 4.2 The half maximum effect concentration EC_{50} and Hill coefficient h estimated from the dose-response curve of KA evoked current in the absence and the presence of 1 μM QA. Data presented are mean ± S.D. (number of measurements)

	Kainate	Kainate plus 1 μM Quisqualate
EC_{50} (μM)	53.0±19.6 (5)	118.4±30.1 (5)
h	1.7±0.7 (5)	1.3±0.2 (5)

Fig. 4.5 shows the dose-response curves obtained in the absence and the presence of 1 μM QA. Each curve was normalized to its maximum response. An increase of 65.4±49.7 μM (mean ± S.D.) was found in the EC_{50}. The h values did not show significant difference. The presence of QA induced a unparalleled shift in the dose-response curve. This result indicates that QA competed with KA in some sense, but this competition is not a regular one, otherwise, a paralleled shift of the dose-response curve should be observed.

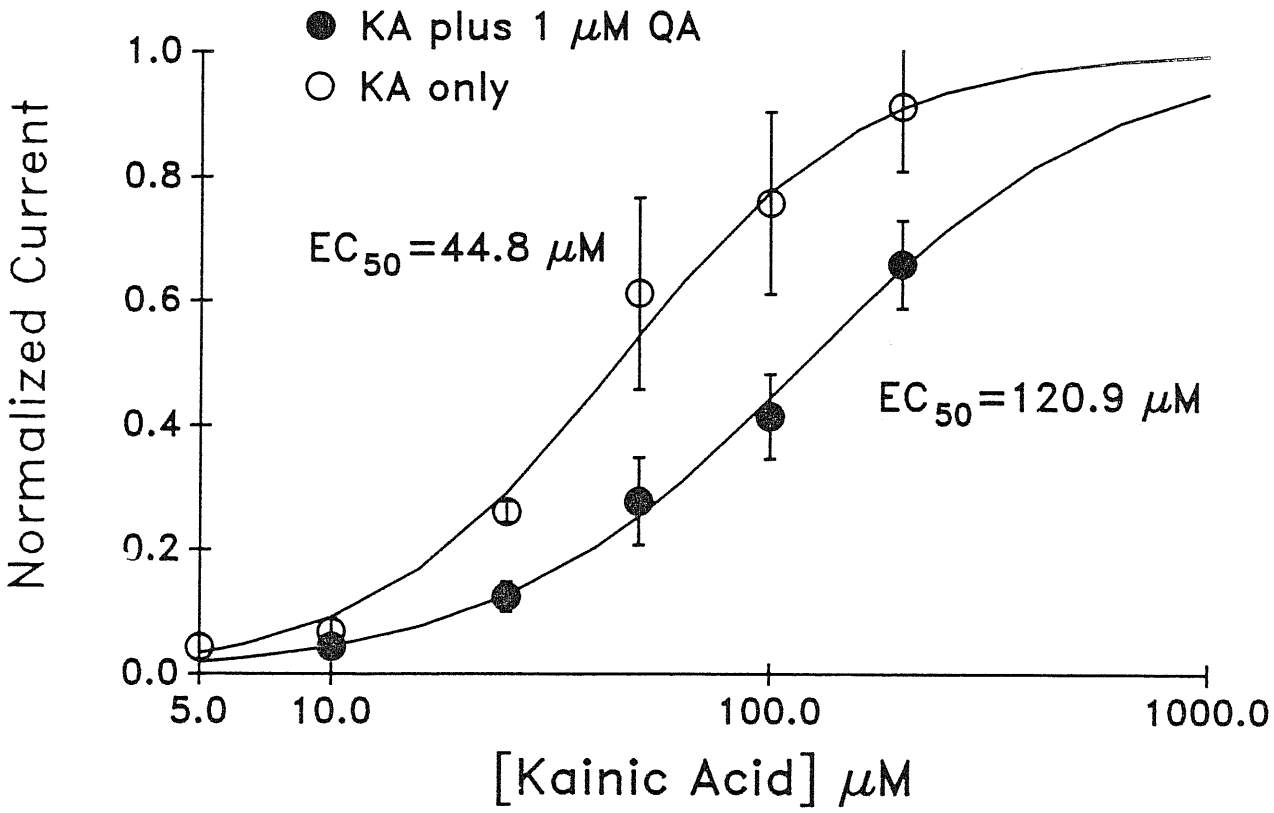


Fig. 4.5 The dose-response curves of KA evoked steady-state current in the presence and the absence of 1 μM QA. Curves were fitted with Equation (1). Values estimated from the curves are (mean ± S.D.): EC_{50}=120.9±31.8 μM, h=1.2±0.1 in the presence of QA, and EC_{50}=44.8±18.1 μM, h=1.5±0.1 in the absence of QA. The curve shifted to the right in a unparalleled way in the presence of QA.

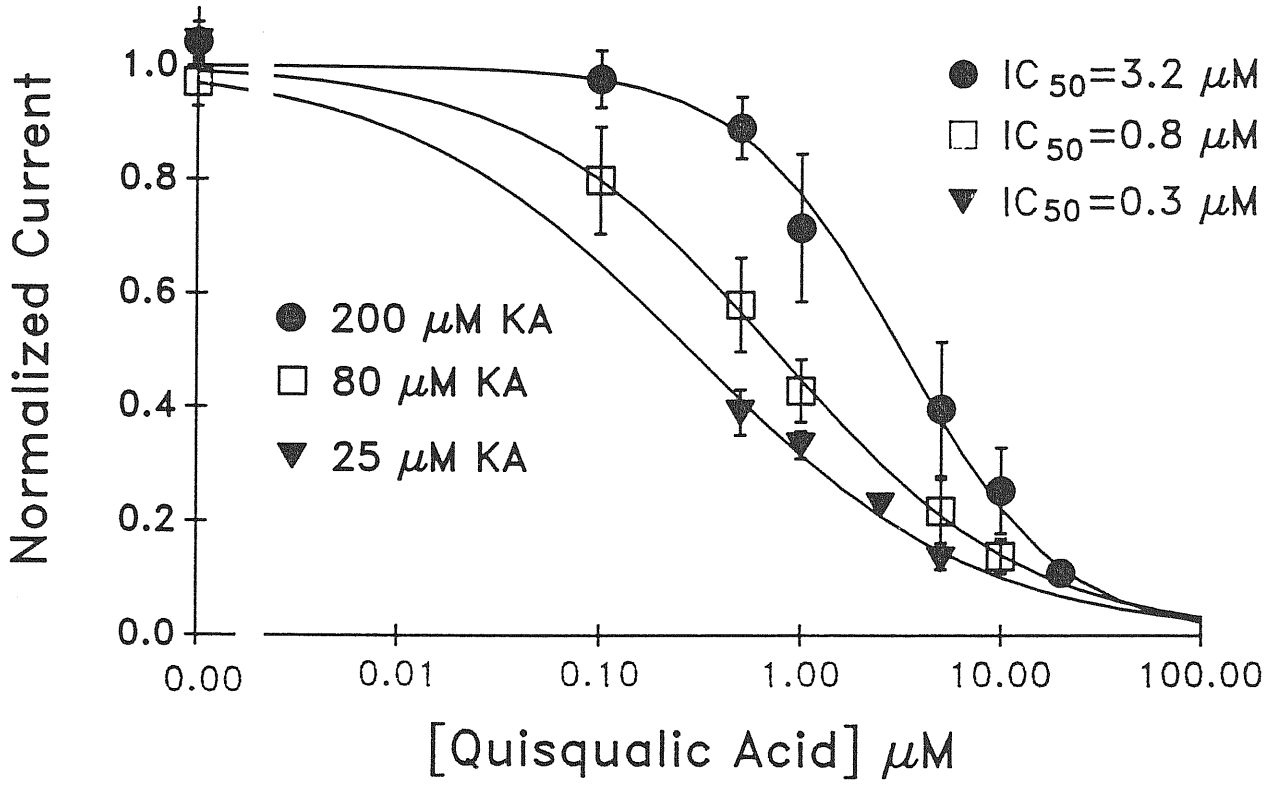


Fig. 4.6 The dose-inhibition curves of QA in the presence of 25, 80 and 200 μM KA. The IC_{50}s are (mean ± S.D.): 0.25±0.08, 0.74±0.07 and 3.16±0.79 μM in the presence of 25, 80 and 200 μM KA respectively. The curve shifted to the right in a unparalleled way in the presence of high KA concentration.

Fig. 4.6 represented the dose-response curves obtained by perfusing KA (25, 80 and 200 μM individually) together with different concentrations of QA (0.1-20 μM). Steady-state currents were used to construct these curves. The amplitude of the currents was normalized to the control obtained in the absence of QA. A shift of the dose-response curve to the right was obtained with higher KA concentration. The inhibition

concentrations IC_{50}, at which QA inhibites half of the control current, were (mean ± S.D., *n*) 0.3±0 μM (1), 0.9±0.9 μM (3) and 3.5±1.8 μM (5) for 25, 80 and 200 μM KA evoked currents respectively. About 90% of the KA evoked current was suppressed by 20 μM of QA. The shifts of the dose-response curve induced by the increase of KA concentration were also unparalleled. It indicates again that the competition of KA with QA is not complete.

4.3 Microscopic Events Evoked by the Concomitant Application of KA and QA

In order to characterize the microscopic events underlying the macroscopic current evoked by the concomitant application of KA and QA, fluctuation analysis was used to study the steady-state currents evoked by the concomitant application of the two agonists. The procedure of the fluctuation analysis was the same as that described in Chapter 3. Fig 4.7 gives one example of the power spectra obtained from the stead-state current evoked by KA together with QA at -70 mV membrane potential. The spectrum was very well fitted with two Lorentzian functions. The mean values of the single channel conductances and the mean channel open times from the 7 spectra were presented in Table 4.3, where they were compared with thoes values obtained from the spectra of KA or/and QA evoked currents.

In comparision with those obtained for QA and KA by using the fluctuation analysis, the time constant of the microscopic events evoked by concomitant application of KA and QA was statistically the same as those of the microscopic events underlying the QA and KA evoked currents. The calculated single channel conductances in the presence of KA+QA have values between those calculated in the presence of KA and QA alone. These values could be explained as the weight averages of the KA and

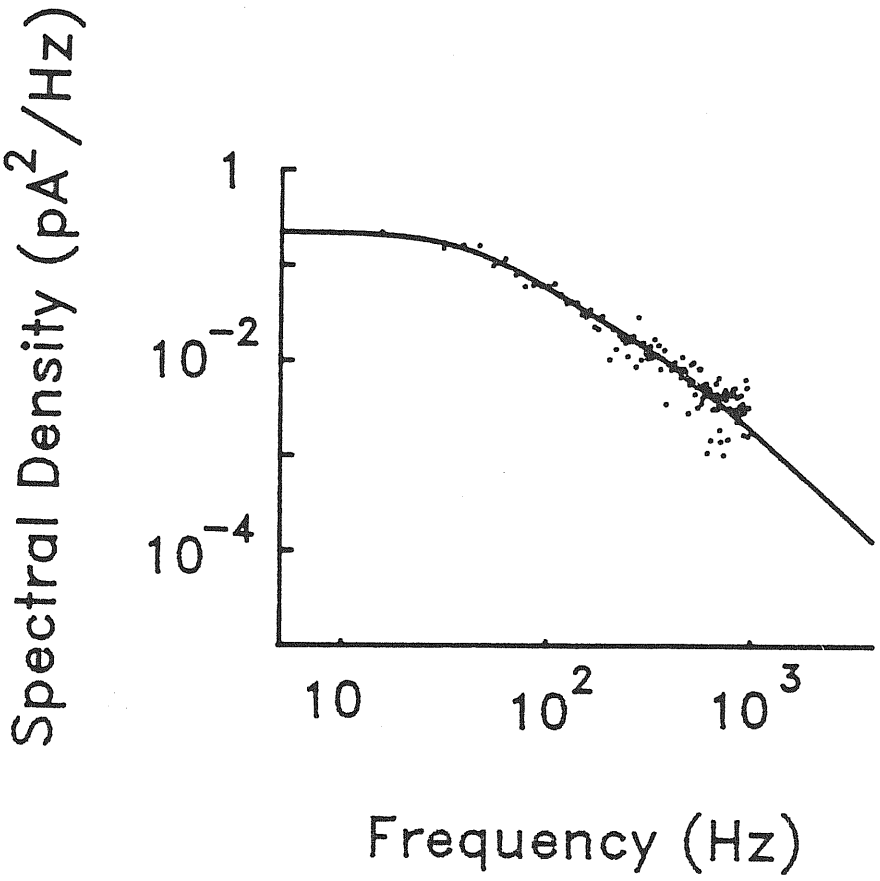


Fig. 4.7 The power spectrum of the steady-state macroscopic current evoked by the concomitant application of 80 μM KA and 10 μM QA at -70 mV membrane potential. The spectrum was fitted by two Lorentzian functions. Single channel conductances and mean channel open times estimated from the fitting are: g_1=6.3 pS, g_2=2.7 pS, τ_1=3.1 ms and τ_2=0.5 mS.

Table 4.3 Comparision of the single channel properties

Agonists	g_1	g_2	τ_1	τ_2	*n*
KA	4.4±2.0	3.8±1.6	3.2±0.7	0.6±0.1	10
QA	7.3±0.9	5.6±3.8	2.6±0.8	0.5	3
KA+QA	5.7±2.5	4.7±1.6	3.7±0.9	0.7±0.3	7

QA activated conductances. The results from the fluctuation analysis indicates that the concomitant application of KA and QA does not induce any new microscopic events beyond those induced by KA and QA alone.

To summarize, the results presented in this chapter are:

i) The amplitude of KA (25, 50 μM) and DA (25 μM) evoked currents can be reduced by bath application of QA (0.5-5 μM). The reduction is reversible and enhanced by high QA concentration.

ii) QA (1 μM) and AMPA (50 μM) can reduce KA (80 μM) evoked current when applied concomitantly with KA. The reduction is also reversible and, what's more, voltage-independent.

iii) The inhibition of KA evoked current by QA and AMPA is mainly mediated by ionotropic receptor.

iv) QA (1 μM) shifts the dose-response curve of KA evoked current to its right in an unparalleled way.

v) The dose-inhibition curve of QA is shifted to the right by high concentration of KA in an unparalleled way.

vi) Concomitant application of KA and QA does not induce new microscopic events as estimated by noise analysis.

Chapter 5

Discussion

The primary culture of cerebellar granule cell described by Levi and his collegues (Levi et al, 1984) constitutes a homogeneous culture. As we mentioned at the beginning of Chapter 3, it has been used in various electrophysiological studies. The same system has also been adopted to study biochemical responses of EAA (*e.g.* Nicoletti et al. 1986, Novelli et al. 1987). Several aspects regarding the electrophysiological properties of this cell culture have received careful examination in our laboratory (Sciancalepore et al. 1989, 1990, Zheng 1989, Lin and Moran 1990, Lin et al. 1991, Moran et al. 1991, Kilic 1990, Kilic et al. 1991, Galdzicki et al. 1991, Zheng et al. 1991).

The cerebellar granule cells in this primary culture respond to all three prototypic glutamate receptor agonists: NMDA, KA and QA (or AMPA). The electrical responses were characteristically dependent on the agonists tested. We have studied the electrical responses of this system to the agonists mentioned above in order to get systematic characteristics of this cell preparation. The macroscopic currents evoked by glutamate (Fig. 3.15), NMDA (Fig. 3.1, 3.2, 3.4) and quisqualate (Fig. 3.5) in our preparation are consistent with those described in a large number of studies (Cull-Candy et al. 1988, Lerma et al. 1989a, Mayer and Vyklicky 1989, Perouansky and Grantyn 1989, Zorumski et al. 1990). However, a desensitization of KA evoked currents has been observed in

primary cultured cerebellar granule cells. The glutamate receptors in these cells also possesses all the typical pharmacological properties: the NMDA and glutamate evoked currents could be enhanced by the exogeneous application of glycine and antagonized by the specific NMDA receptor antagonist APV as well as the wide spectrum glutamate receptor antagonist kynurenic acid. KA, DA evoked currents could be antagonized as well by kynurenic acid.

Table 5.1 A list of partial reported EC_{50} of different EAA

Agonists	EC_{50} (μM)	h	References
NMDA	16.0±1.0	-	Kushner et al. 1988
	29.3	0.93	Lerma et al. 1989a
	31.3	1.03	Lerma et al. 1989a
	34.9±9.2	1.40±0.24	Patneau and Mayer 1990
	16.8±6.8	0.8±	Our Results
Quisqualate	93	-	Kiskin et al. 1986
	0.21	2.6	Lerma et al. 1989a
	0.14±0.04	-	Pin et al. 1989
	0.5	-	Perouansky and Grantyn 1989
	3	-	Zorumski et al. 1990
	0.9±0.05	1.5±0.2	Patneau and Mayer 1990
	0.20±0.02	0.5±	Our Results
Kainate	500	-	Kiskin et al. 1986
	200	1.9	Vlachova et al. 1987
	56	2.4	Lerma et al. 1989a
	85±20	-	Pin et al. 1989
	30	-	Perouansky and Grantyn 1989
	176	-	Zorumski et al. 1990
	15	-	Huettner 1990
	120	-	Huettner 1990
	142	-	Patneau and Mayer 1990
	53.1±12.3	1.5±0.1	Our Results
Domoate	11		Patneau and Mayer 1990
	0.7		Huettner 1990
	32		Huettner 1990
	5.8±0.7	1.2±0.1	Our Results

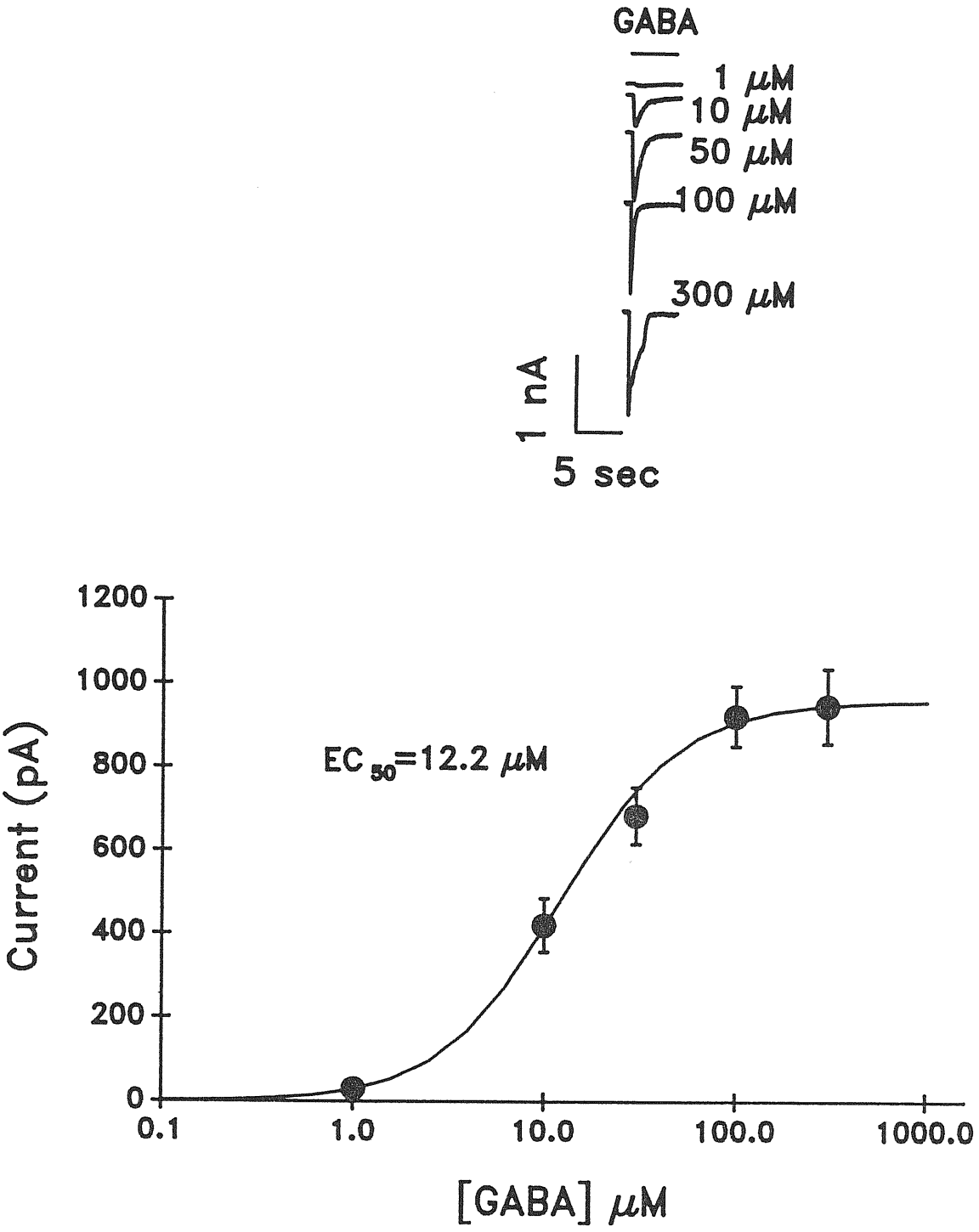


Fig. 5.1 The current traces and the dose-response relation of GABA evoked current in 2 DIC granule cells. Membrane potential was kept at -50 mV. 4 mM $MgCl_2$ was added in both bath and pipette solutions. 1mM ATP was used in pipette solution to reduce the run-down of the GABA response. GABA was applied through multibarrel gravity perfusion. The data present the mean of 2 observations. Curve is from the fitting of the mean valus with Equation (1). EC_{50}=12.2±4.0 μM and h=1.4±0.1 (mean ± S.D.) are estimated from the fitting.

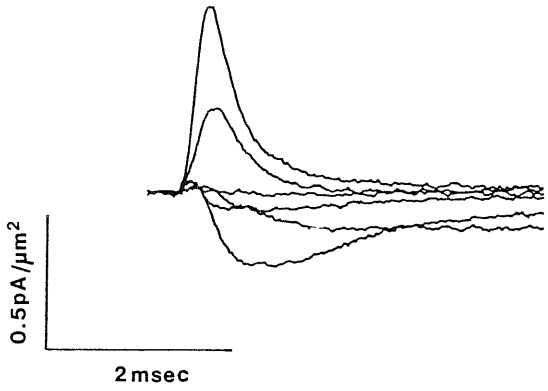


Fig. 5.2 Sodium currents obtained from whole cell patch. The holding potential was -90 mV. Voltage pulses to various test potentials from -20 to -80 mV in 20 mV steps were preceded by a 5 ms prepulse of -100 mV. Each trace is the average of 8 records after the subtraction of linear leakage and capacitance current. (from Lin et al. 1991)

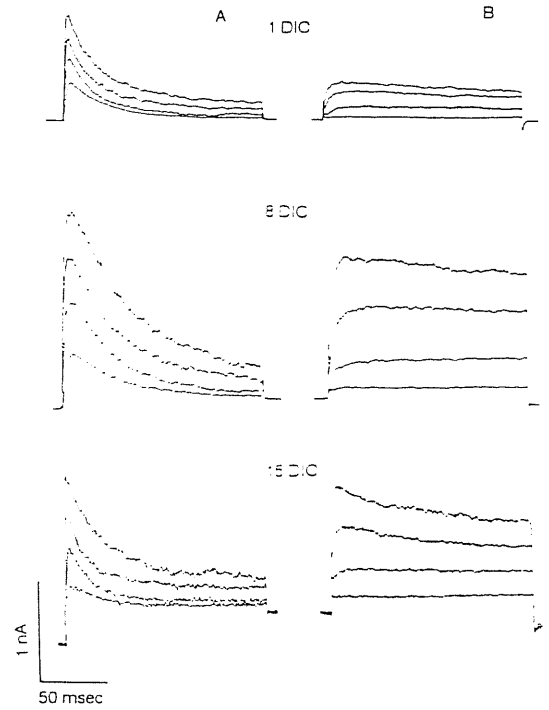


Fig. 5.3 Transient potassium currents, I_A (**A**) and delayed rectifier currents, I_K (**B**) separated according their inactivation properties. The test pulses varied from -20 to 40 mV, in 20 mV steps, preceded by 300 ms prepulses of -40 mV (**B**) and -90 mV (for calculate **A**) respectively (from Galdzicki et al. 1991).

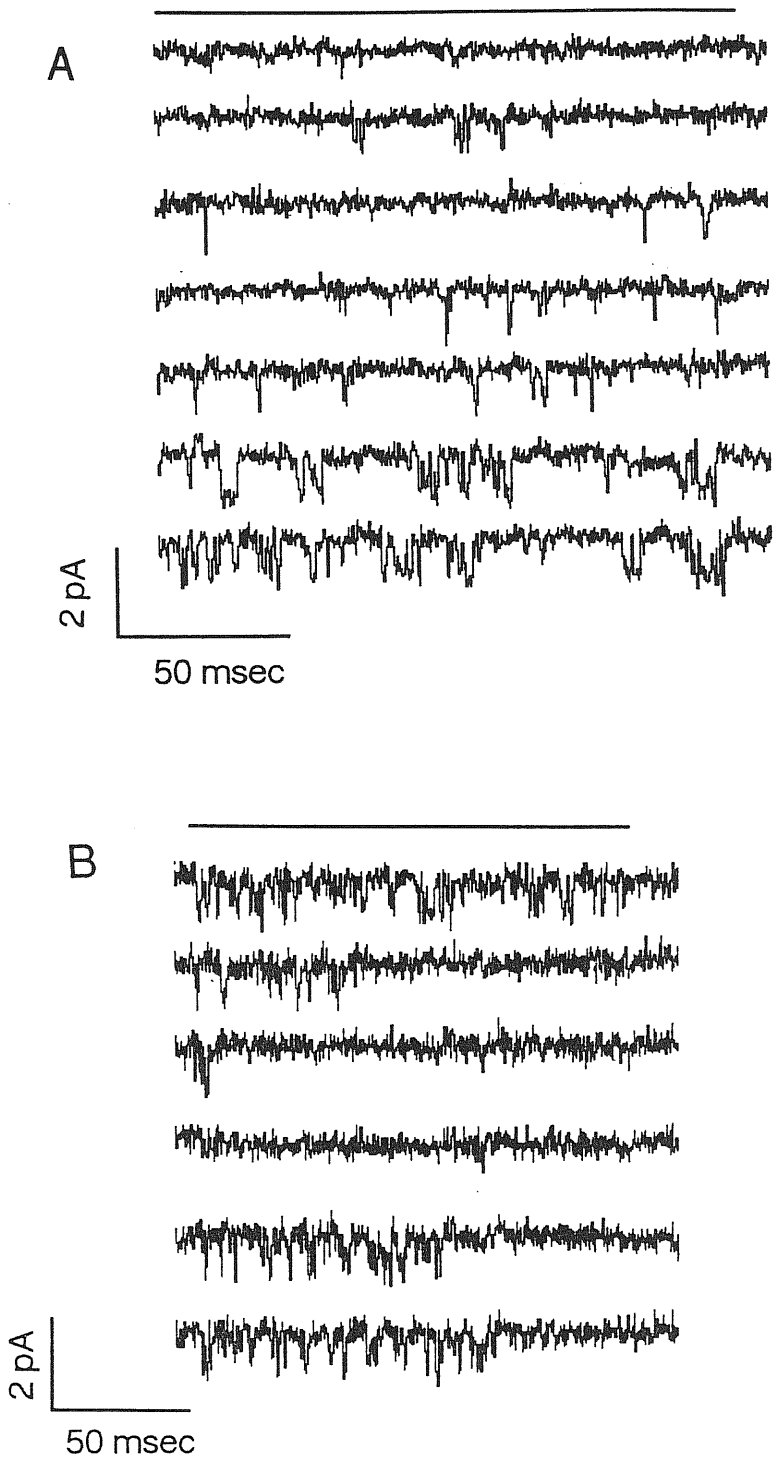


Fig. 5.4 Calcium currents obtained from cell-attached patches. Holding potential was -80 mV. Membrane potential was -10 mV. Pipette solution contained 80 mM $BaCl_2$ and 20 mM TEA; bath solution was Ca^{2+} and contained 120 mM K-gluconate. **A** Dihydropyridin-sensitive calcium currents. Single channel conductance is 18.3±1.7 pS. Half avtivation potential is 23.6 mV. It does not inactivate. **B** Dihydropyridin-insensitive calcium currents. Single channel conductance is 9.8±1.5 pS. Half avtivation potential is -65 mV. Half inactivation potential is -31.8 mV (from Moran et al. 1991).

The EC_{50} and the Hill coefficient of each agonist presented in this thesis are similar to some of those values obtained from different cell preparations. But not all the reported data are consistent with ours. Table 5.1 gives a comparison of these values.

In addition to all those EAA induced conductances presented in this thesis, the primary cultured granule cell expresses also inhibitory amino acid receptors and voltage gated sodium, potassium and calcium channels. Examples taken from the relevent works of our laboratory are provided here for the purpose of illustration (Fig. 5.1 to 5.4).

To summarize, it can be said that cerebellar granule cell in primary cultures constitute an excellent native CNS neuron model for studying the electrophysiology of both ligand-gated and voltage-gated ionic channels.

5.1 Single Channel Properties of EAA Evoked Current

The single channel conductance of NMDA evoked current in this culture has been studied in detail in other projects of our laboratory (Sciancalepore et al. 1989, Kilic 1990, Kilic et al. 1991). The maximum conductance has been found to increase during the development of the cell in culture from 15.5 pS (1-3 days) through 35.7 pS (5-8 days) to 46.8 pS (9-11 days).

The single channel conductances of QA (up to 10 μM) evoked current estimated from our fluctuation analysis are 7.3±0.9 pS and 5.6±3.8 pS. They are quite similar to the mean conductance (8.4±1.5 pS) of the small amplitute openings reported by Cull-Candy and his collegues using the same cells (Cull-Candy et al. 1988) and by Ascher and Nowak (Ascher and Nowak 1988a). In Cull-Candy and his coworkers' experiments, some higher conductances (19, 17, 41, 52 pS) have also been detected by the single channel recording. The fluctuation analysis made by Cull-Candy and his coworkers also revealed some higher conductances such as 15.2 and 30.2 pS. These high conductances

could be induced by the relatively high QA doses (10-30 μM) used in their experiments. In most of our fluctuation analyses, QA concentration was less than 10 μM. In this concentration, less higher conductances were activated. The single channel conductances obtained by Ascher and Nowak using both single channel recording (1 μM) and noise analysis (10 μM) were 8 and 4.5 pS respectively. These data are quite similar to our results. One of the time constants obtained from our experimental data is 2.6±0.8 ms, which is consistent with the τ_{noise}=2.3 reported by Cull-Candy using noise analysis, in which the power spectrum was fitted with two Lorentzian functions. But the other time constant we obtained is 0.5 ms, which is 20 times less than the results achieved by Cull-Candy (10.8 ms) and Ascher (8.9 ms). This difference could also be caused by the different agonist concentrations used in the experiments.

The results from our noise analysis of KA receptor related events fit perfectly the previous ones we obtained from direct single channel recording (Zheng 1989, Sciancalepore 1990). They are also consistent with those reported by others (Ascher and Nowak 1988a, Cull-Candy and Usowicz 1987, Cull-Candy et al. 1988). The full identity between the microscopic properties, *i.e.* the single channel conductance and the mean channel open time, calculated from KA and DA evoked macroscopic currents, demonstrates that KA and DA act on the same set of receptors. Therefore, it is valid to use the potent agonist DA to study the properties of KA receptor.

5.2 Desensitization of KA Evoked Current

In most of the published papers concerning the CNS neurons, KA evoked currents have been claimed not to desensitize (*e.g.* Kiskin et al. 1986, Hollmann et al. 1989, Lerma et al. 1989a, Mayer and Vyklicky 1989, Perouansky ang Grantyn 1989, Patneau and Mayer 1990, 1991, Zorumski et al. 1990). The current traces presented in those papers are

similar to our traces obtained with relatively high doses of KA. In most of those studies, KA was applied through bath perfusate. The low perfusion speed could inhibit the observation of the fast desensitizing current as it happened in observing QA evoked current. The fast perfusion has been used by Mayer's group. However, the KA concentration in their experiments was relative high. So the desensitization observed mainly in low doses could be ambiguous in their measurements.

In rat DRG neurons, KA evoked response has been reported to desensitize with prolonged agonist exposure (Huettner 1990). In our experiments, two different desensitizations were observed in CNS neurons. At low concentration, a significant and relatively speedy amplitude decline was observed in all the cells when KA was applied for more than 5 seconds, whereas at high concentration, a slow decrease in amplitude was observed.

The following facts possibly underlie the reports that KA induces non-desensitized current:

i) KA probably evokes different responses in different preparations. There are some evidences showing that the neurons from cerebellum have different properties than those from other regions. For instance, in cerebellar mRNA-injected *Xenopus* oocytes, the NMDA evoked current was insensitive to glycine (Sekiguchi et al. 1990). Very recently, a cDNA has been isolated from rat cerebellar cDNA library. When this cDNA clone, GluR6, was expressed in *Xenopus* oocytes, it formed a homomeric non-NMDA glutamate receptor subunit, which could be desensitized by continuous application of KA (Egebjerg et al. 1991). This observation supports our results.

ii) The doses used in their experiments were in the middle range (around EC_{50}), so the densensitization was covered.

iii) The slow decline in amplitude was neglected when compared with more obvious desensitization phenomena, such as the QA or glutamate evoked current. Indeed, in some of the papers, a slow decline in amplitude corresponding to our second kind of desensitization could be seen (*e.g.* Kiskin et al. 1986, Perouansky ang Grantyn 1989, Mayer and Vyllicky 1989, Petneau and Mayer 1991).

From our results, it seems that in cerebellar granule cells the KA evoked currents consist of two components: the fast desensitization component which can be easily observed in low concentration (< 25 μM) KA evoked responses; and the relative stable component, which activates slowlier than the first component and can be observed in high concentration (> 25 μM) KA evoked responses. The second component has a small contribution at low KA concentration, which becomes more and more important and finally predominant with the increase KA concentration. Its activation rate also appear to increase with the KA concentration. The KA evoked total response, at any concentration, should be the sum of the two components.

5.3 The Interaction Between KA and QA

A depression of KA evoked electrical response by QA observed in our experiments has been already reported in other preparations (Ishida and Neyton 1985, Kiskin et al. 1986, 1990, O'Brien and Fischbach 1986, Lerma et al. 1989a, Mayer and Vyklicky 1989, O'Dell and Christensen 1989, Perouansky and Grantyn 1989, Patneau and Mayer 1991). The dose-response curve of KA evoked steady-state current shifted to the right in the presence of QA (Fig. 4.5). Similarly, when we evaluated the ability of QA to depress KA evoked steady-state current, the dose-inhibition curve was also shifted to the right in the presence of increased concentrations of KA (Fig. 4.6). However it was clear in our experiments that the shift to the right was not via parallel way (Fig. 4.5, 4.6).

Similar observation was reported by Patneau and Mayer (1991). This result suggests that the antagonism of QA on KA evoked currents is not purely competitive.

The results of the fluctuation analyses showed that KA and QA evoked single channel events have different conductances but similar kinetic properties (Table 4.3). From the power spectra of KA+QA evoked currents, conductances between the two values corresponding to KA and QA activated conducances were estimated (Table 4.3). The time constants of KA, QA and KA+QA evoked events were undistinguishable. These middle value conductances can be explained as the weight averages of the conductances activated by KA and QA alone. In summary, the spectral analysis revealed that the microscopic events underlying the steady-state current evoked by concomitant application of QA and KA were not beyond those underlying the steady-state currents evoked by QA and KA alone. So, the concomitant agonists application did not induce any conductance or kinetic change at single channel level.

5.4 Mechanism of the Antagonism

Several controversial hypotheses have been put forward to explain the interaction between KA and QA. The most popular explanation is that both the responses to QA and KA are mediated by a common KA/AMPA receptor, which could be desensitized by glutamate, QA and AMPA but not by KA and DA. The original idea was proposed by Ishida and Neyton (1985) on the basis of their experiments which showed that: i)The noise analysis yielded an estimated single channel conductance and time constant from KA (3-30 μM), glutamate (30-300 μM) and QA (3-30 μM) evoked currents were the same; ii) glutamate and QA inhibited KA evoked currents and furthermore the currents evoked by 10 and 30 μM KA were reduced to the same amplitude by the application of 10 μM QA.

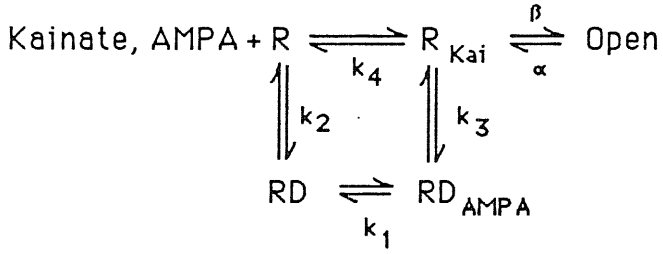
The same model was used by Kiskin and his collegues (1986, 1990). Their new evidence was that QA or/and glutamate could cross desensitize KA activated receptor, in other words, the pre-application of QA/and glutamate reduced the initial value of KA evoked current and slowed the onset of the response (Kiskin et al. 1986). They proposed that two sites exist in the common KA/AMPA receptor: one controls the activation and could be activated by both KA and QA; the other controls the receptor desensitization and can be activated only by QA.

The recent paper of Patneau and Mayer (1991) further support the single receptor model. These evidences were: i) the dose-response curve of KA evoked current shifted to the right in the presence of QA; ii) in the presence of QA, KA evoked current presented two distinct components: a rapidly activating response, similar to that recorded in the absence of QA, but of reduced amplitude; and a more slowly activating component, which was observed only in the presence of QA; iii) kainate reduced the peak amplitude of QA evoked current and slowed down its desensitization rate.

Also the molecular biology studies support in part the notion of a common KA/AMPA receptor. Four cDNAs have been isolated from rat brain cDNA library to code the glutamate receptor subunits, all of them responded to KA, QA, AMPA and glutamate when expressed alone or in combination in oocytes (Keinänen et al. 1990, Nakanishi et al. 1990). However, more recently, two separate groups succeded in cloning cDNAs coding a putative high-affinity kainate receptor and a glutamate receptor subunit activated by KA, QA and glutamate but not by AMPA (Egebjerg et al. 1991, Werner et al. 1991). This suggests the existence of separate KA and AMPA receptors.

Although the common KA/AMPA receptor model have been accepted by many groups, most of the previous experimental designs and interpretations have been challenged by Patneau and Mayer (1991). It seems that the kinetic model proposed by

Mayer is the most complete one. It was described as:



Four assumptions were made: i) both AMPA and kainate bind to the same receptor and show mutual competition for the agonist recognition site; ii) the relative affinities of individual agonists for the resting and desensitized (RD) states of the receptor differ, as do their absolute affinities for each state; iii) the initial application of either KA or AMPA produces a conformational change in the receptor that enables ion channel activation; iv) for AMPA-like agonists, $k_1>k_4$ and the majority of receptors enter the desensitized state and for KA-like agonists $k_1<k_4$ thus large number of receptors are activated. In this model, the antagonism of AMPA-like agonists on KA-like agonists was explained as that AMPA bind with high affinity to the desensitized state of receptor hence reduce the population of receptors available for activation by KA. Although this model explained the interaction of AMPA and KA as well as the cross desensitization, there are still points can not be easily explained by this model. First, why the antagonism is not purely competitive if there is only one common receptor. Second, how to explain the desensitization of KA evoked current observed at low KA concentration.

In alternative to the common KA/AMPA receptor model, it has been proposed another one based on separated AMPA and KA receptors (Perouansky and Grantyn 1989, Lerma et al. 1989a). In this model, AMPA-like agonists were supposed to act as competitive antagonists or weak partial agonists. This one direction interaction model can not explain the fact that KA-like agonists block the rapidly desensitizing responses

to AMPA-like agonists.

On the basis of our experiments, we put forward a new model based on the two distinct non-NMDA receptors with mutual interaction. We assume that: i) Two kinds of non-NMDA glutamate receptors, R1 and R2, existed. R1 mediates the relative stable response, which correspondes to the steady state of KA evoked response; R2 mediates the fast desensitizing response, which correspondes to the initial peak of KA and QA evoked currents. ii) Either KA-like agonists or AMPA-like agonists act on both R1 and R2 receptors. KA-like agonists act as full agonists on R1 receptor and partial agonists on R2 receptor whereas AMPA-like agonists act as full agonists on R2 receptor and partial agonists on R1 receptor (Fig. 5.5). iii) KA has higher binding affinity for R2 receptor than for R1 receptor. This could be due to the fact that KA is not a specific agonist for KA receptor, as indicated by pharmacological studies (see table 1.1). iiii) In comparision with KA, QA has higher binding affinity for both AMPA and KA receptors.

In this model, KA evoked current has two components which results from the activation of both R1 and R2 receptors. The fast desensitized component is mediated by R2 receptors and the relative stable component is mediated by R1 receptors. The single channel events measured by single channel recording and fluctuation analysis are mainly evoked by the activation of the R1 receptors. Occasionally, some high conductance of 7 pS, which corresponding to QA evoked events could be observed in KA evoked responses (Zheng 1989), that could be the results of not complete desensitization of the R2 receptors. The QA evoked peak current is mediated by R2 receptors while the steady-state current is mediated by both R1 receptor and the non-complete desensitized R2 receptors. That induces the small conductance in QA evoked current (Table 4.3).

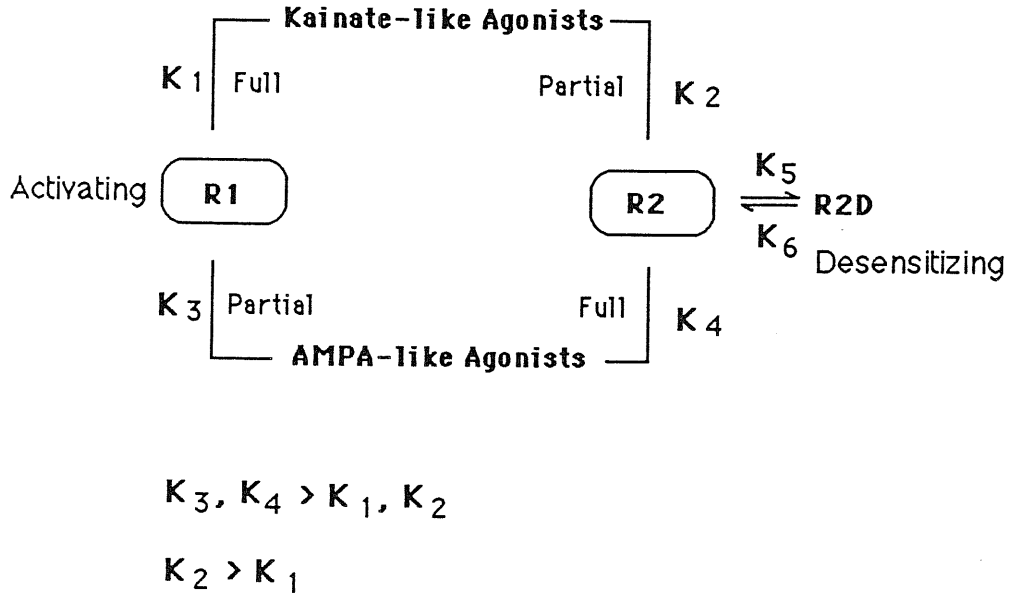


Fig. 5.5 The action of KA-like and AMPA-like agonists on the two non-NMDA glutamate receptors. Either KA-like or AMPA-like agonists act on both two non-NMDA receptors, R1 and R2. The efficacies and the binding affinities are indicated beside the lines linking the agonists and the receptors. The relations between the four binding affinities, K_1 to K_4, are: i) $K_2 > K_1$; ii) K_3, $K_4 > K_1$, K_2.

The suppression of KA-like agonists evoked steady-state current by AMPA-like agonists is due to the competition of AMPA-like agonists with KA-like agonists at the R1 receptors. Since AMPA-like agonists act as partial agonists but not antagonists, the shift of the KA dose-response curve in the presence of AMPA-like agonists will not be parallel, as it was observed in the experiments of Patneau and Mayer (1991) and ours. The unparalleled shift of the dose-inhibition curve of QA can be explained in the same way.

The block of the rapid desensitizing avtivation of AMPA-like agonists by KA-like agonists is due to the competition of KA-like agonists with AMPA-like agonists at the R2 receptors. This competition slows the onset of the activation since the receptor

can not be occupied by AMPA-like agonists until KA-like agonists dissociate from them. The amplitude of AMPA evoked initial peak current is reduced as well by this competition since KA-like agonists act as partial agonists on the R2 receptors. The increase of the activation time constant of KA-like agonists evoked responses by AMPA-like agonists can be explained in the same way since the fast activation of KA-like agonists evoked responses are mediated by the R2 receptors.

In conclusion, two distinct non-NMDA glutamate receptors exist. KA-like and AMPA-like agonists have different binding affinities and efficiencies for these two receptors. Both KA-like and AMPA-like agonists evoked currents are the contributions of these two receptors. The complicate interaction between the two kinds of agonist is due to the competition of the agonists at these two receptors.

The only point that remains unclear is wether these two binding sites are present in the same structure, probably as a part of different protein subunits, or there is the coexistence of physically separated receptors in the cell membrane. These two possibilities are indistinguible from our electrophysiological measurenments, unless the possibility of recording unambiguously the separate structure responses could be achieved. Therefore, a final response to such problem will be probably given by a more refinate molecular biology approach.

CONCLUSIONS

1. The primary cultures of cerebellar granule cells represents an excellent model for the study of the electrophysiological properties of the neurones. They expressed voltage-dependent sodium, potassium and calcium currents, as well as excitatory and inhibitory amino acids evoked currents.

2. Most of the behaviour of the EAA evoked currents in primary cultures of cerebellar granule are similar to those reported by others using the same or different preparations.

3. The noise analyses of KA and DA evoked currents shows considerable identity in both single channel conductance and mean channel open time. The results of the noise analyses also fit the results obtained by using single channel recording.

4. A desensitizing component exists in KA evoked current, which has never been described before.

5. A fast desensitizing current is observed in low concentration KA evoked current; a slow densensitizing current is observed in high concentration KA evoked current. The mechanism of these two desensitization are not the same.

6. QA suppresses KA evoked current in a concentration-dependent but voltage-independent way. The suppression is fully reversible.

7. Detailed dose-response studies shows that KA and QA compete mutually, but a not purelly competition is indicated by the changing of the slope in the dose-response curve.

8. A model based on two distinct non-NMDA receptors can explain all the experimental results.

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