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Properties of GABA-activated Channels and their Modulation by Zn^{2+} in Cerebellar Neurones

Thesis Submitted for the Degree of
Doctor Philosophiæ

CANDIDATE
Gordan Kilić

SUPERVISOR
Dr. Oscar Moran
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November 1992

TRIESTE

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This work is dedicated to the brave people of Croatia who lost their lives in the independence war.

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Notes

During accomplishment of this thesis, several papers and posters have been published:

1. Kilić G, Sciancalepore M and Cherubini E (1992) Single-channel currents of NMDA type activated by L- and D-Homocysteic acid in cerebellar granule cells in culture. *Neurosci Lett* 141:231-235.

2. Kilić G, Moran O and Cherubini E. Currents activated by GABA and their modulation by Zn^{2+} in cerebellar granule cells in culture. *Eur J Neurosci* (in press).

3. Sciancalepore M, Kilić G and Cherubini E (1992) L- and D-homocysteate activate currents of NMDA type in cerebellar granule cells in culture. 22-nd Annual Meeting Society for Neuroscience, Anaheim California.

4. Kilić G, Moran O and Cherubini E (1992) Effects of extracellular Zn^{2+} on $GABA_A$-induced currents in cerebellar granule cells in culture. 15-th Annual Meeting of European Neuroscience Association, München Germany.

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-5-isoxazolepropioanate
AP5	2-amino-5-phosphonopentanoate
AP7	2-amino-7-phosphonoheptanoate
ATP	adenosine-triphoshate
BZ	benzodiazepine
BZR	benzodiazepine receptor
$[Ca^{2+}]_i$	intracellular concentration of Ca^{2+}
cAMP	cyclic adenosine 5'-monophosphate
CNS	central nervous system
DIC	days in culture
EAA	excitatory amino acid
EC_{50}	half-maximum concentration
E_r	reversal potential
GABA	γ-aminobutryic acid
HEPES	N-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulphonic acid)
IAA	inhibitory amino acid
IC_{50}	half-blocking concentration
L-AP4	L-2-amino-4-phosphonobutanoic acid

NMDA	N-methyl-D-aspartate
n	Hill coefficient
n_i	Hill coefficient of Zn^{2+} inhibition
SD	standard deviation

Contents

Chapter 1

Introduction

The major inhibitory amino acid in central nervous system (CNS) is a γ-aminobutyric acid (GABA). One of the mechanism for inhibition is the increase of the cell permeability for anions, mainly Cl^- ions. Cl^--currents activated by GABA are negatively modulated by extracellular Zn^{2+}. Although many authors agree that Zn^{2+} blocks GABA currents in a noncompetitive way through the binding to a site which is distinct of GABA (Celentano et al. 1991; Draguhn et al. 1990; Legendre and Westbrook 1991; Smart 1992), the molecular mechanism of Zn^{2+} action is still unknown. The main idea of this work was to study the properties of GABA-activated currents and their modulation by Zn^{2+} using patch-clamp technique in cerebellar granule cells in culture.

The GABA-activated currents in cultured cerebellar granule cells have been already described (Cull-Cudy and Ogden 1985; Cull-Candy and Usowicz 1989; Vicini et al. 1986). In this study I have undertaken electrophysiological characterization of GABA-activated currents and their modulation by extracellular Zn^{2+}. I studied the properties such as the channel

kinetics, voltage dependence and desensitization.

Cerebellar achitecture and function

The cerebellum (*latin*, little brain) is located in the dorsal part of hindbrain and it constitutes only 10% of the brain, yet it contains more than half of all the neurones. These neurones are arranged in a highly regular manner. Despite its structural regularity the cerebellum is divided into several distinct regions, each of which makes connections with different areas of the brain. The cerebellar cortex is a simple structure consisting of three layers that contain only five types of neurones: stellate, basket, Purkinje, Golgi and granule cells (see Figure 1-1).

The outermost or *molecular layer* is composed primarily of the axons of granule cells, known as *parallel fibers*, that run parallel to the long axis of a folium. It also contains scattered stellate and basket cells, that function as interneurones, as well as dendrites of the underlying Purkinje neurones. Beneath the molecular layer is the *Purkinje cell layer*. It contains the large (50-80 μm) cell bodies of the Purkinje neurones that are arranged side by side in a single layer. The Purkinje neurones have extensive dendritic trees that extend up into molecular layer in a single plane peripendicular to the main axis of a folium. Purkinje neurones send their axons down into the underlying white matter. They are sole output of the cerebellar cortex. All Purkinje neurones are inhibitory and they use GABA as their neurotransmitter.

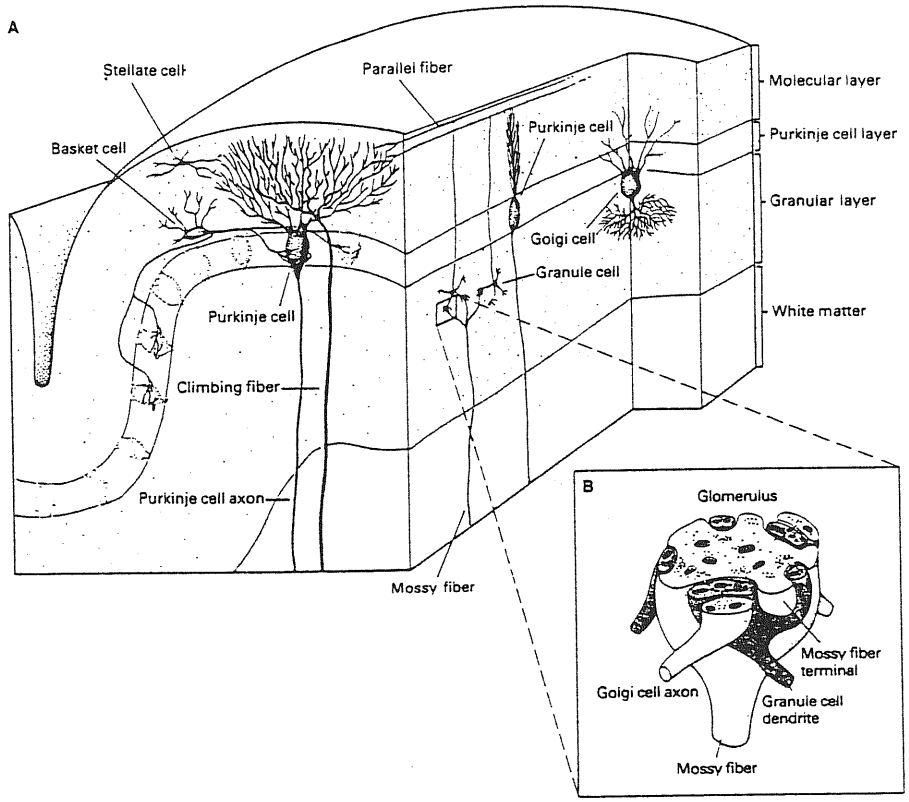


Fig 1-1. (A) This vertical section of a single cerebellar folium, in both longitudinal and transverse planes, illustrates the general organization of the cerebellar cortex. (B) Detailed structure of a cerebellar glomerulus in the granular layer. A glomerulus is a clear space where bulbous terminal of a mossy fiber makes synaptic conctact with Golgi and granule cell axons. (From Kandel et al. 1992.)

The innermost or *granular layer* contains a vast number of densely packed small neurones, mostly small granule cells. Their number about 10^{11}, exceeds the total in the cerebral cortex. A lager Golgi cells are found at the outer border. The granule layer contains small clear spaces called *cerebellar glomeruli*, where cells in the granular layer form complex synaptic contacts with the bulbous expansions of afferent fibres (see Figure 1-1B).

The axons entering cerebellum from other brain regions such as the spinal cord, the vestibular nuclei, the pons and elsewhere, tend to give off

collaterals to the deep cerebellar nuclei. The activity of the Purkinje cells, the only output from the cerebellar cortex is determined by two excitatory afferent inputs: *mossy fibres* and *climbing fibres* (Figure 1-1A). The mossy fibres arise from the pontine nuclei, as well as several other other sites, and terminate on granule cell's dendrites (see Figure 1-1B). The second class of fibres which arise mainly from the inferior olivary nucleus, literally climbs over the Purkinje cell dendritic tree and make synapses onto it (see Figure 1-1A). Even though the cerebellum contains both sensory and motor components, its complete removal does not impair either sensory perception or muscle strenght. It is known that the cerebellum regulates movement and posture indirectly by adjusting the output of the major descending motor systems of the brain. Furthermore, lesions of the cerebellum disrupt coordination of limb and eye movements, impar balance, and decrease muscle tone. Finally, the cerebellum plays a role in motor learning and it seems to partecipate in the process of perception.

Granule cells in culture

Most of cerebellar development occurs postnatally whereas only Purkinje and Golgi cells are formed in the cerebellar nucleus in embryo. Granule cells are created together with stellate and basket cells in the external granular layer of the rat cerebellum from about 3 to 20 days, postnatally. The granule cells are the most abundant cell type in the cerebellar cortex comprising about 95% of its total cell population.

Primary cultures of granule cells are obtained from 7 to 9 postnatal days old rat cerebella. This culture consists of 85% excitatory granule cells, 6%

inhibitory internurones (stellate or basket) cells, 5-8% astrocytes, 0.2% oligodendrocytes and 0.4% fibroblast (Balazs et al. 1982). The cultured neurones behave very much like what is to be expected from neurones: they send out nerve fibres, form synapses, release transmitters and express transmitter receptors (Figure 1-2).

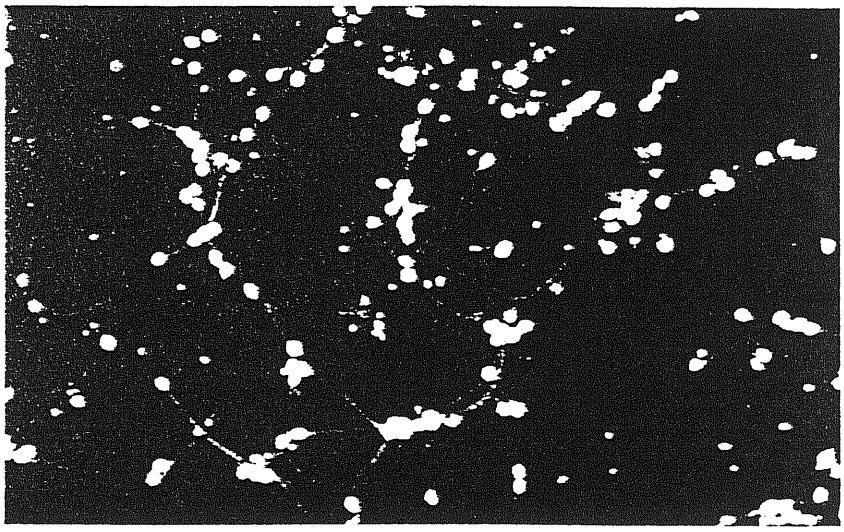


Fig 1-2. View under the microscope of the one week old primary cultures of cerebellar granule cells which are stained with fluorescent substances. Notice that neurones at this stage already possess connections which appear as a grey net in this photograph.

However, it should be noted that these neurones are kept under artificial conditions, lacking the interaction with astrocytes and other types of brain cells. Although the development of neurones in culture is far from what is happening *in vivo*, cultured granule cells can be used to study some basic mechanisms of development and certain functions of neurone.

Ionic channels

Ionic channels are membrane proteins that form water filled pores through which ions can flow passively down their electrochemical gradient (Figure 1-3). This passive flow of ions so called diffusion is governed by independent gradients of electric field and concentration. The electrochemical potential of ions which are unevenly distributed in two compartments separated by a membrane is:

$$E_r = \frac{RT}{zF} \ln \frac{[A]_o}{[A]_i} \qquad (1\text{-}1)$$

where R is the gas constant (8.315 V C K^{-1} mol^{-1})

F is the Faraday's constant (9.648 10^4 C mol^{-1})

T is the absolute temperature (in K)

$[A]_o$ is concentration of ion in the extracellular space

$[A]_i$ is concentration of ion in the cytosol

z is the valence of ion

This relation is usually called Nernst equation.

Two functional parts of ionic channels can distinguished: the pore and the gating mechanism (see Figure 1-3). The structure of the pore confers to the channel its permeability characteristics. The flow of ion along a channel depends on the type of charge present at the mouth or at the wall of the pore (for example, a net positive charge forbids cation permeation). The flow also depends on the geometry of the pore structure through which selection based upon the dimension of the hydrated ion is made, and on the ion binding properties of amino acid residues present in the wall. If ion is bound very

strong, the ion will not pass through the channel.

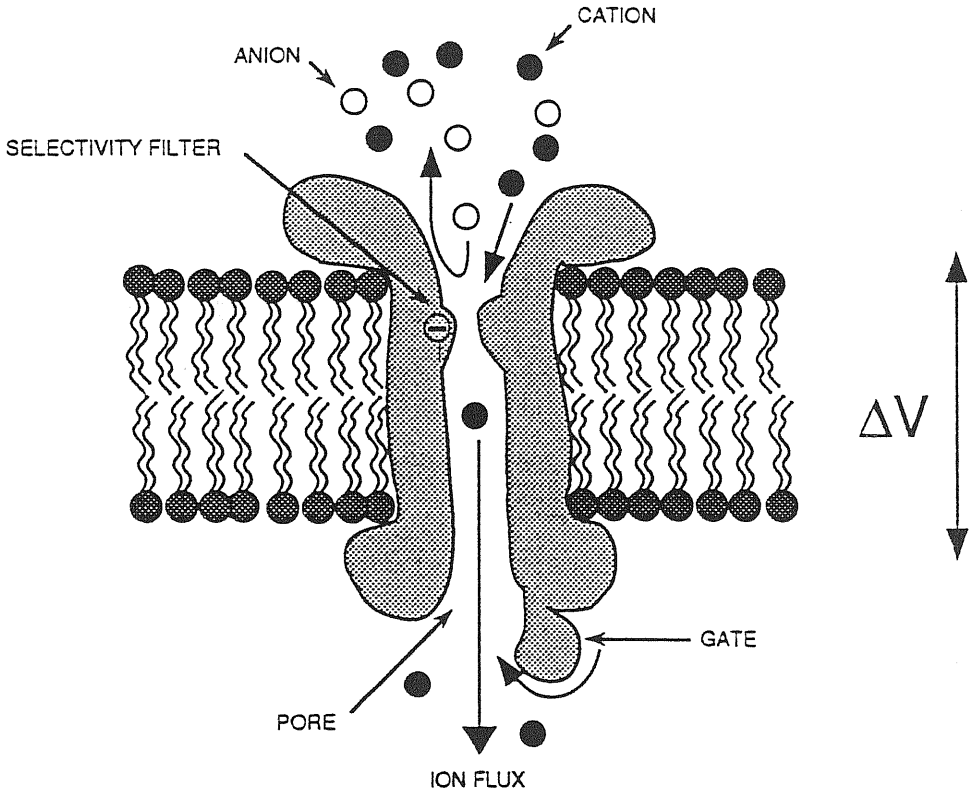


Fig 1-3. Drawing represents typical voltage-gated ionic channel immersed in the lipid bilayer. The gate is located on the cytosolic part of the membrane and it feels the voltage change ΔV, in this way regulating the flux of ions. The selectivity filter located close to the mouth of channel on extracellular side, allows the flux of only certain type of ions according to their charge or size.

The mathematical description of the permeation of ions through the ionic channels is given by Goldman-Hodgkin-Katz (GHK) current equation. The current which crosses membrane is:

$$I_A = P_A z^2 \frac{EF^2}{RT} \frac{[A]_i - [A]_o \exp(-zFE/RT)}{1 - \exp(-zFE/RT)} \qquad (1\text{-}2)$$

where E is the electrical potential along the membrane, P_A is the membrane permeability to ionic specie A and other variables are the same as defined in equation (1-1). If more than one ion can pass through the channel, the total current is a sum of the currents produced by each ionic specie, as a consequence of the independence of ionic fluxes. Assuming that Na^+, K^+ and Cl^- are permeant ions, the reversal potential where the total current is zero is given by GHK voltage equation:

$$E_r = \frac{RT}{F} \ln \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o} \qquad (1\text{-}3)$$

where $[K]_o$, $[Na]_o$ and $[Cl]_o$ are concentrations of ions in extracellular space

$[K]_i$, $[Na]_i$ and $[Cl]_i$ are concentrations of ions in the cytosol

P_K, P_{Na} and P_{Cl} are permeabilities of ions for considered channel

The other parameters are defined previously. This equation allows one to calculate permeability ratios from measurements of reversal potential if ionic concentrations are known. Once the permeability ratios are determined it is possible to make theoretical prediction of the current voltage-relation for the

situation in which there is only single ionic specie on the both sides of membrane. If the experimental current-voltage relation coincides with theoretical prediction, a given ion passively diffuses through the channel pore. On the other hand if the slope of experimental I-V relation is smaller than one calculated theoretically, the pore probably contains a binding site for that ion. This is valid only if the binding site is located somewhere in the pore and not at the mouth of the channel. The physical description of an ion binding in the pore is analogous to enzyme-substrate reaction which follow Michaelis-Menten kinetics (see Hille 1975).

The gating mechanism confers to a channel the property to be in a permeable (open) or impermeable (closed) conformation (see Figure 1-4). The probability of transition from one state to the other will depend on the difference in free energy of the two states and on the energy barrier to be overcome in the transition process (Figure 1-4). These transitions are stochastic processes since the free energy of the protein depends on its thermal energy variations under a given set of parameters. To explain the physical basis for the behavior assumed by the channel open and closed states, several mechanisms acting alone or in a concerted manner have been postulated: the movement of a part of the protein which reversibly blocks the pore; a change in the distribution of charges inside the pore with dramatical effects on the protein conformation; the existence of the pore in only certain configurations. In any case, the probability of the channel to go in the open or closed state can be greatly influenced by external factors such as the magnitude of the electric field (voltage-gated channels), the binding of effector (ligand-gated channels), or the surface tension (strech-activated

channels), which strongly modify the energy profile of the open-close transition (Figure 1-4B).

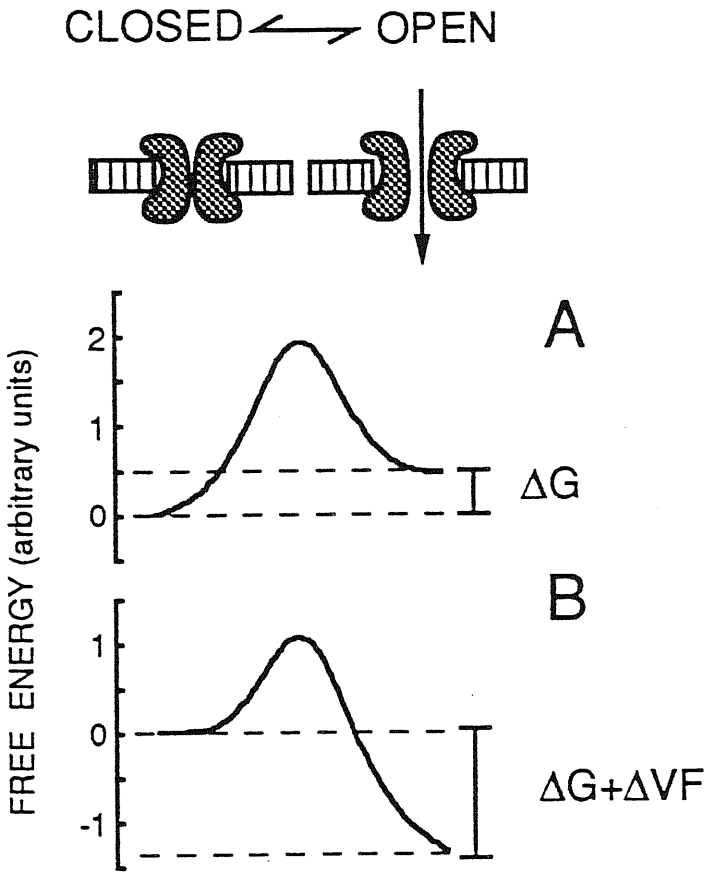


Fig 1-4. Drawing shows the ionic channel in closed and open states. Assuming that the ionic channel can exist in only these states the energy profile of closed (A) and open states (B) are plotted. In both graphs the left side represents closed and the right side the open conformation of ionic channel.

Usually the ionic channels are divided in two major groups: voltage-gated and ligand-gated channels. Voltage-gated channels are activated by a sudden depolarization or hyperpolarization of the cell membrane potential

in the process called activation. After this activation some channels tend to close in a process of inactivation, whereas some remain activated during the duration of the voltage change. Activation and inactivation are not completely independent and they differ very much in their time course depending on the potential change or channel type. A good example of voltage-gated channels is the sodium channel which is important for the cell excitability. The properties of activation and inactivation of the sodium channel are well described by Hodgkin and Huxley (1952). In their model the processes of activation and inactivation are completely independent and controlled by hypothetical gating particles that feel changes in the membrane potential. Recent advances in molecular biology and electrophysiology rendered more information about the structure and the function of this complex membrane protein (for a review see Guy and Conti 1990).

Ligand-gated channels are ionic channels that are activated directly or indirectly by chemical substances such as neurotransmitters acting on the extracellular part of the protein immersed in the membrane of nervous cell. Usually neurotransmitters are released by other neurones. A common name for directly activated channels is fast ligand-gated channels. They have intrinsic sensor that upon the binding of agonist to the ionotropic receptor, activates a gate which in turn allows ions to pass through the channel pore (Figure 1-5A). During prolonged action of some neurotransmitters, the ionic channel tends to deactivate in the process of desensitization, which resembles in the time course to the inactivation of voltage-gated channels. The desensitization shuts channels during sustained stimulation and it was explained by the existence of additional closed state which becomes

populated only in the presence of agonist (Katz and Thesleff 1957). Although desensitization was described over 40 years ago, its physiological significance and underlying molecular mechanisms remain poorly understood (Mayer et al. 1991).

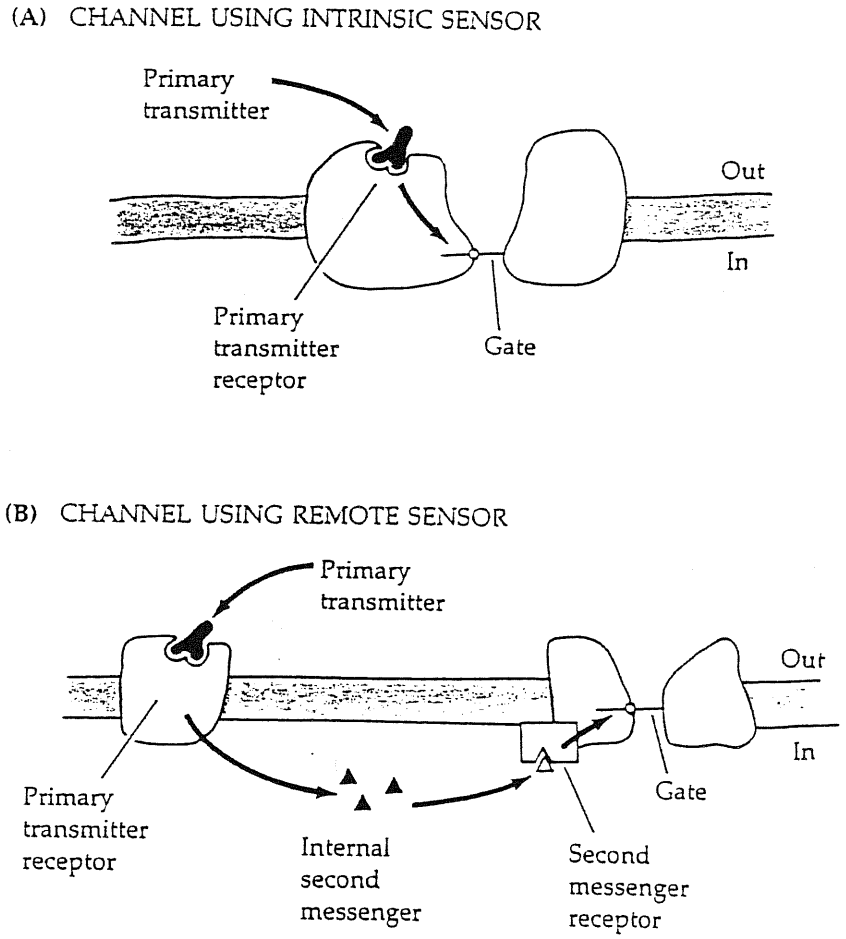


Fig 1-5. (A) General scheme for the activation of fast ligand-gated channels in which primary transmitter binds to the specific region of the channel protein, and this binding directly affect the gating function. (B) Slow ligand-gated channel channels are activated by the intracellular second messenger molecules. These molecules are products of the complex chemical reactions in the cell membrane which are induced by the binding of primary transmitter to its specific receptor. (From Hille 1992.)

The channels that are activated indirectly by agonist are slow ligand-gated channels (see Figure 1-5B). Neurotransmitter activates primary receptor in membrane (metabotropic receptor) which is separated from the ionic channel and it communicates with the channel protein through diffusible, intracellular second-messenger molecules. One example of primary receptor is the G-protein. The activation of G-protein evokes the second-messenger pathway which as final consequence has the modulation of ionic current. An example is the pathway which through cAMP-dependent protein kinase A phosphorylate the Ca^{2+}-channels, and in this way positively modulates them (for a review see Tsien 1983).

Nerve cell can be depolarized by the action of excitatory amino acid (EAA) agonists. There are two pharmacologically distinct EAA receptors, NMDA and non-NMDA types (Watkins et al. 1991). Some of EAA agonists are listed in Table 1-1. NMDA-receptor channels are ionotropic channels permeable to K^+, Na^+ and Ca^{2+}, and can be blocked by competitive NMDA antagonists such as 2-amino-5-phosphonopentanoate (AP5) and 2-amino-7-phosphonoheptanoate (AP7). Non-NMDA receptors are generally divided in KA and QA receptors subypes (Watkins et al. 1991). Quisqualate was found to activate both, ionotropic and metabotropic receptors whereas its structural analogue α-amino-3-hydroxy-5-methyl-5-isoxazolepropioanate (AMPA) activate only ionotropic-receptor channels (Krogsgaard-Larsen et al. 1980). Recent investigations indicate that KA and AMPA share the same receptor-channel complex (Zorumski and Yang 1988).

	Transmitter	Receptor activation	Type
Excitatory	L-Glu	NMDA + non-NMDA	IT + MT
	L-Asp	NMDA + non-NMDA	IT + MT
	L-HC, D-HC	NMDA	IT
	NMDA	NMDA	IT
	QA	non-NMDA	IT + MT
	KA	non-NMDA	IT
	AMPA	non-NMDA	IT
	ibotenate	NMDA	IT + MT
Inhibitory	GABA	$GABA_A$ + $GABA_B$	IT + MT
	Gly	Gly	IT
	muscimol	$GABA_A$	IT
	isoguvacine	$GABA_A$	IT
	baclofen	$GABA_B$	MT

Table 1-1. Excitatory and inhibitory amino acid transmitters are listed and characterized with respect to the receptor activation. The property of being ionotropic (IT) or metabotropic (MT) is also indicated in the table.

The most common mechanism for neurone inhibition in the CNS exploits GABA or glycine as neurotransmitter (for review see Nicoll et al. 1990). The binding of glycine to specific receptors increases membrane permeability to Cl^- which in turn hyperpolarizes cytosol (Hamill et al. 1983). On the other hand, GABA activates two types of receptors, $GABA_A$ and $GABA_B$ that can be distinguished pharmacologically. $GABA_A$-receptor protein is ionotropic channel permeable to Cl^- and it shares many

biophysical properties with channels activated by glycine (Bormann et al. 1987). Metabotropic $GABA_B$-receptors are associated with Ca^{2+} or K^+ channels (Nicoll et al. 1990). Some of inhibitory amino acid (IAA) agonists are shown in Table 1-1. More about $GABA_A$-receptor will be discussed in the next chapter.

Chapter 2

$GABA_A$-receptor Channels in CNS

Molecular biology

Recent advances in molecular biology have provided new information about the structure and function of the $GABA_A$-receptor channel complex. A complex cDNA library made from rat brain mRNA was shown to contain clones also for the family of $GABA_A$-receptor channels (Schofield et al. 1987). These clones are expressed in *Xenopus* oocytes (Sigel et al. 1990) or in human embryonic kidney cells (Shivers et al. 1989; Verdoorn et al. 1990), and reconstituted channels can be studied by electrophysiological methods.

Generally, the channels are made from homologous building blocks, whether in separate subunits or internal repeats, that assemble symmetrically to form a pore down their center. It seems likely that the native $GABA_A$-receptor is a pentamer (Stephenson 1988), formed by integral membrane proteins belonging to at least four classes α, β, γ and δ. The sequences of these proteins are markedly homologous, sharing similarity of topology with

other ligand-gated channels such as the nicotinic acetylcholine (nACh) or glycine (Gly) receptors (Schofield et al. 1987). The strong similarity suggest that these proteins arouse early in animal evolution by gene duplication from a common ancenstral ligand-gated channel.

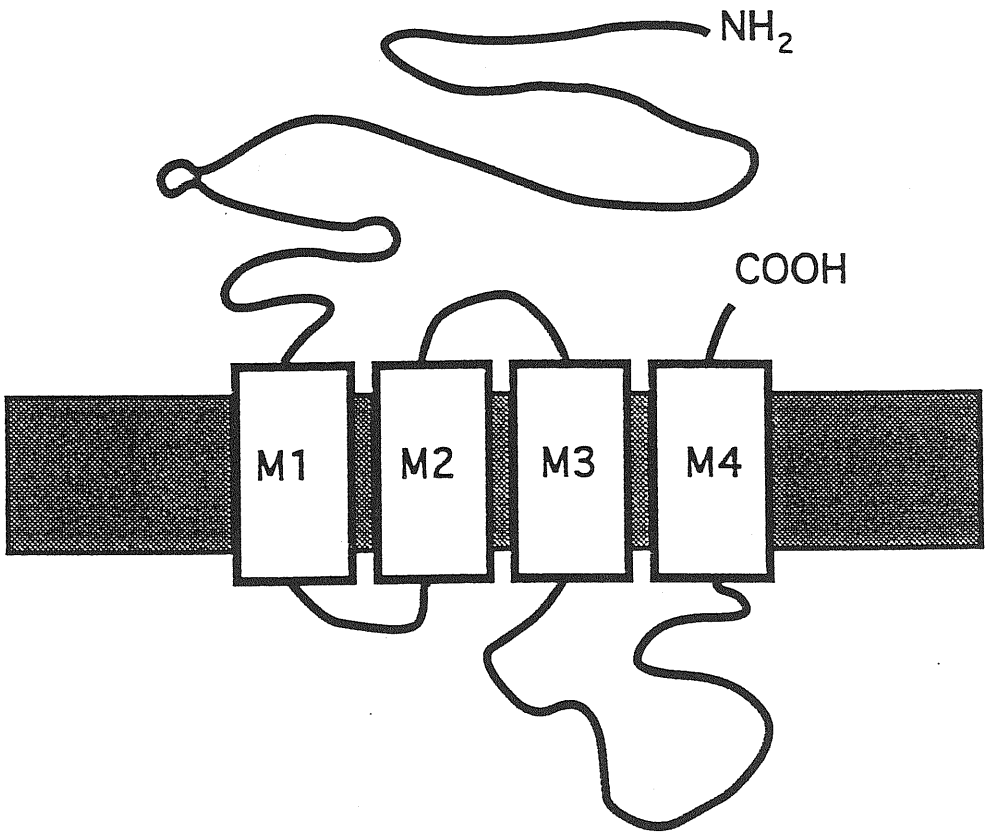


Fig 2-1. A schematic drawing represents α or β subunit of $GABA_A$-receptor. There are four proposed hydrophobic transmembrane domains. It is thought that the agonist binding site is located in the extracellular region between N-terminal and M1 region. C- and N-terminals are also indicated.

It is belived that each of the protein subtypes of $GABA_A$-receptor channel forms four hydrophobic membrane spanning regions (Barnard et al. 1987;

Schofield et al. 1987, see Figure 2-1). These regions have α-helical secondary structure and they are denoted as M1 to M4. Highly charged residues located in cytosol between M3 and M4 regions which are present only in the β-subunit, probably form a site for cAMP-dependent serine phosphorylation (Schofield et al. 1987). The precise stoichiometry of the $GABA_A$-receptor is still unknown, as well as the agonist binding site.

The properties of $GABA_A$-receptor subunits were studied by expressing different subunit combination in *Xenopus* oocytes (Khrestchatisky et al. 1989; Levitan et al. 1988; Malherbe et al. 1990; Sigel et al. 1990) or in human embryonic kidney cells (Pritchett et al. 1989; Puia et al. 1990; Shivers et al. 1989; Verdoorn et al. 1990). Following injection of only one subunit type a little or no response to the application of GABA (100 μM) was observed (Khrestchatisky et al. 1989; Sigel et al. 1990). GABA currents obtained after the injection of combinations of at least two different subunits displayed properties similar to vertebrate $GABA_A$-channels (Sigel et al. 1990). Many independent studies demonstrated that different subunits have specific properties (Draguhn et al. 1990; Möhler et al. 1989; Pritchett et al. 1989; Sigel et al. 1990). For example, the presence of at least one α-subunit was required for induction of the GABA-gated currents (Sigel et al. 1990). On the other hand, α subunits were not required for the formation of an ion channel, nor for GABA-dependent gating of the channels by compounds acting at the benzodiazepine (BZ) binding site. The presence of β-subunit is not required for the expression of GABA-gated ion currents displaying cooperativity and benzodiazepine sensitivity (Sigel et al. 1990). Formation of an anion selective, picrotoxin-sensitive channel was observed with β1

(Sigel et al. 1989) but not with β2-subunit. Another important subunit, named the γ2, is belived to be essential for the modulation of the GABA channel by drugs acting at the benzodiazepine binding site (Pritchett et al. 1989). However, Möhler and co-workers (1989) shown that GABA-gated currents mediated by α+β oligomers expressed in *Xenopus* oocytes were enhanced by benzodiazepine despite the absence of the γ2-subunit. Furthermore, it seems probable that the presence of γ2-subunit markedly reduces the sensitivity of GABA-activated currents to the blockage by Zn^{2+} (Draguhn et al. 1990; Smart et al. 1991).

Summarizing present data Sigel and co-workers (1990) proposed that the subunit combination α5β2γ2 is the minimal requirement reproducing consensus properties of the vertebrate $GABA_A$-receptor channel, including cooperativity of GABA-dependent channel gating, modulation by drugs acting at the benzodiazepine binding site, picrotoxin sensitivity and the effect of barbiturates. It should be kept in mind that expression in the *Xenopus* oocytes does not automatically assemble a channel composed of all injected subunits. Furthermore, since it is belived that the $GABA_A$-receptor channel exists as a pentameric complex of unknown stoichiometry (Seeburg et al. 1990), the minimal requirement proposed by Sigel consisting of only 3 different subunits is a matter of discussion.

Autoradiography and immunohistochemistry

One of the main function of GABA is the Cl^- mediated inhibition of neurones which possess $GABA_A$-receptors. Hence, an uneven distribution of $GABA_A$-receptors throught the mammalian brain is consequence of the

functional diversity of brain neurones. Recent improvements in biochemistry, immunology and electrophysiology rendered new powerful techniques to determine the distribution of receptor proteins at the level of a single cell.

Radioligands of benzodiazepine receptor (BZR, Richards and Möhler 1984) or monoclonal antibodies that recognize the $GABA_A$-receptor proteins (Shivers et al. 1989), were used to visualize the $GABA_A$-receptor distribution in the brain. Comparing these studies it is observed that most BZR sites are part of the $GABA_A$-receptor complex (see Figure 2-2).

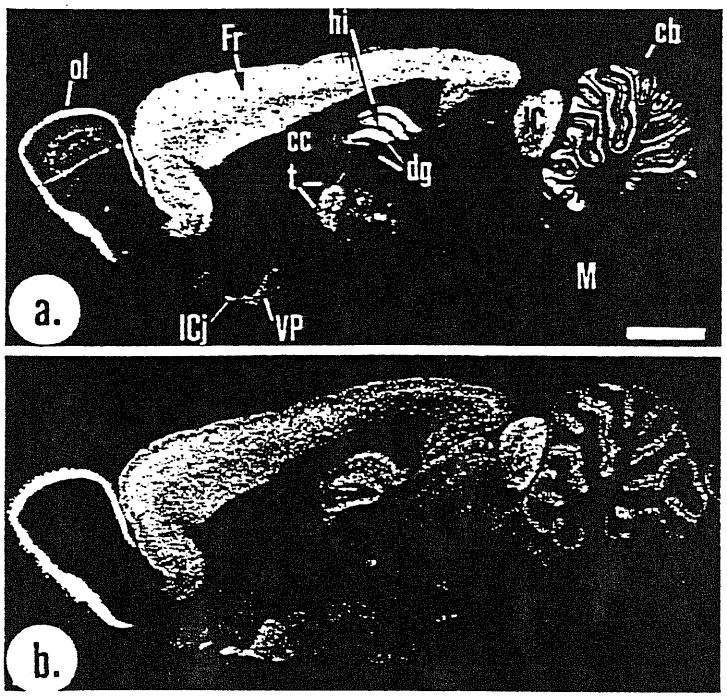


Fig 2-2. Localization of $GABA_A$-receptors in two adjacent parasagittal sections of rat brain using (A) immunohistochemical and (B) radiohistochemical methods. Notice that their distribution is very similar. Scale bar is 2 mm. (From Möhler et al. 1989.)

The intensity of BZ radiolabeling and immunostaining is found to differ only in the granular layer of the cerebellum (Möhler et al. 1989). It is not clear whether this is due to a structural or conformational heterogenity. A possible explanation could be the finding that δ-subunit exists only in the granular layer whereas γ2-subunit which is important for modulation by BZ is present in all layers of cerebellar cortex (Shivers et al. 1989).

The binding studies usually use $GABA_A$ antagonist muscimol which has an advantage of a low affinity for uptake sites (Snodgrass 1983). At least two classes of binding sites for [^{3}H]GABA can be demonstrated, with half-maximum concentration values for muscimol of 21 nM and 365 nM for high- and low-affinity sites, respectively (Yang and Olsen 1987). Autoradiographic binding studies in explant cultures of rat cerebellum have shown that the granule cells reveal binding of [^{3}H]GABA and labeled $GABA_A$ agonists and antagonists (Hösli et al. 1985), whereas immunostaining study demonstrated that the astrocytes in explant cultures of rat CNS did not show significant reactivity (Hansen et al. 1991). Furthermore, by looking the immunostains of cerebellar granule cells in culture under the electronic microscope, Hansen and co-workers (1991) observed that $GABA_A$-receptors are evenly distributed along the membrane of both cell bodies and processes. However, a more intense labeling of $GABA_A$-receptors were found in the membrane of cell bodies as compared to the processes. The granule cells cultured in standard culture media are enriched in $GABA_A$-receptors but exhibit only one binding site for GABA which is of the high affinity type (Meier and Schousboe 1982). This is in contrast to cerebellum as well as other brain areas *in vivo* where two or

more $GABA_A$-binding sites with different affinities for GABA were found (Meier and Schousboe 1982; Wang et al. 1979). An addition of GABA to cultured cerebellar granule neurones has been shown to induce the formation of low affinity $GABA_A$-receptors and thus mimic *in vivo* situation (Meier et al. 1984).

Biophysical properties

A functional characterization of the $GABA_A$-receptor complex on the molecular level is achieved by patch-clamp recording (Bormann et al. 1987; Bormann and Clapham 1985; Hamill et al 1983) and intracellular electrode measurements (Barker et al. 1982; Eccles et al. 1977).

Early studies on the inhibitory postsynaptic potentials (IPSPs) in spinal synapses demonstrated that the postsynaptic ionic channels do conduct not only Cl^- but also several other small inorganic and organic anions up to the size of formate (3.4 Å) (Coombs et al. 1955). These observations were interpreted in terms of membrane channels that act as ionic sieves allowing a passage of hydrated ions which size is less than the diameter of channel pore. The pore diameter of $GABA_A$-receptor channel estimated in this way was 5.6 Å (Bormann et al. 1987). The selectivity sequence obtained either from whole-cell or outside-out data is $SCN^- > I^- > Br^- > Cl^- > F^-$ (Bormann et al. 1987). Furthermore, the same authors measured single-channel currents with equal anion concentrations on the both membrane faces. The slope of I-V curves did not coincide with the theoretical prediction of GHK current equation (2-2). This equation was constructed using the permeability ratios obtained from whole-cell or outside-out measurements with a mixture of Cl^-

and other monovalent anions. Such inverse sequence indicates that permeating ions bind to the channel pore.

Both, single-channel measurements and the noise analysis of the whole-cell currents have been used to determine conductance levels of $GABA_A$-channels (Cull-Candy and Ogden 1985; Cull-Candy and Usowicz 1989). The single-channel conductance displayed a variability depending on the type of the cell and patch configuration. In most studies the presence of multiple conductance states has been described (Bormann et al. 1987; Hamill et al. 1983) with main levels in the region of 20 and 27-31 pS. In the outside-out patches of rat astrocytes in culture GABA activates conductances of 12, 21, 29 and 43 pS (Bormann and Kettenmann 1988). In rat cerebellar neurones in culture GABA conductance was estimated to 16 pS using the noise-analysis (Cull-Candy and Ogden 1985) indicating a prevalence of the smaller conductance in the whole-cell current. The same observation in all single-channel studies is that the most frequently occurring state is around 30 pS (Bormann et al .1987; Bormann and Clapham 1985; Hamill et al. 1983; MacDonald et al. 1989).

From the electrophysiological and binding studies several kinetic models of operation of $GABA_A$-receptor channel have been proposed (Bormann and Clapham 1985; MacDonald et al. 1989; Sakmann et al. 1983). It is belived that two GABA molecules are needed for the activation of $GABA_A$-receptor (Bormann and Clapham 1985). However, the general scheme of the gating of $GABA_A$-channel is still a matter of discussion. Kinetic analysis is usually done for the main conductance state of 30 pS. The values for mean open and closed times differ considerably, depending mainly on the cell type. In large

cerebellar cells in culture Cull-Candy and Usowicz (1987) using noise-analysis found two open time constants of 1.9 and 23.6 ms, whereas in mouse spinal cord neurones in culture the mean open times were 1, 3.7 and 11.3 ms (MacDonald et al. 1989). The occurrence of several open and closed time constants and the regular burst duration between the groups of openings indicates that the main level of $GABA_A$-receptor channel can exist in several open and closed configurations. The effects of different concentrations and voltage dependence of kinetic constants have been also studied (MacDonald et al. 1989; Newland et al. 1991; Weiss 1988). It was observed that the change in the membrane voltage has no effect on open time constants (Weiss 1988), whereas the increase of GABA concentration from 0.5 μM to 5 μM produced a slight prolongation of the mean open time from 3.7 ms to 5.7 ms (MacDonald et al. 1989). Nevertheless, it seems likely that the concentration change has no significant effect on the open time constants (MacDonald et al. 1989). The same authors found that the longest closed time constant decreased and mean burst duration increased with augmented GABA concentrations.

$GABA_A$-receptor possesses another important property. During prolonged application of GABA, the response to GABA decreases in a process called desensitization (Bormann and Clapham 1985; Cull-Candy and Ogden 1985; Cull-Candy and Usowicz 1989). The desensitization of $GABA_A$-receptor was found to be dose-dependent (Akaike et al. 1986; Legendre and Westbrook 1991). For higher GABA concentrations the onset of desensitization is more rapid. As a consequence of desensitization, very long closed time constants (~1s) can be found in outside-out patches during application of high GABA

concentration (Newlannd et al. 1991).

Pharmacology

The pharmacological properties of $GABA_A$-receptor have been revealed by electrophysiology and the binding studies which use radioligands (Table 2-1, for a review see Sivilotti and Nistri 1991). Until 1988 it was belived that the binding site for GABA is located on the β-subunit (Bormann 1988). Interestingly, GABA was found to activate Cl^--currents in homomeric $GABA_A$-receptors formed of single subunits other than β (Blair et al. 1988). This finding indicates that each subunit type bears a site that can function as the agonist site. The binding site for GABA is also recognized by structural analogues of GABA, such as muscimol, isoguvacine, THIP (4,5,6,7-tetrahyroisoxazolo [5,4-c] pyridin-3-ol) and DAVA (δ-aminovaleric acid) (Table 2-1, Barker and Mathers 1981). Functional studies of the GABA receptor in the mammalian CNS showed the existence of significant cooperativity and variable half-maximum concentration (EC_{50}) depending on $GABA_A$-agonist (Krogsgaard-Larsen et al. 1984; Sigel and Barnard 1984; Yang and Olsen 1987; Zukin et al. 1974). In the majority of studies muscimol was found to be the most potent agonist. The antagonists of $GABA_A$-receptors have been developed using as initial model the chemical structure of GABA itself or muscimol (Krogsgaard-Larsen et al. 1984). The most important antagonist acting on the $GABA_A$- recognition site is bicuculline (Table 2-1, Curtis et al. 1970). The half-blocking concentration (IC_{50}) of bicuculline was 5 μM in the rat CNS (Zukin et al. 1974). The $GABA_A$-antagonist acting on the recognition site is also pitrazepin. In rat

hippocampus and hypothalamus the IC_{50} of pitrazepin was 240 nM (Gähwiler et al. 1984).

$GABA_A$-receptor channel complex possesses another important site for drugs such as benzodiazepines (Table 2-1). As already mentioned in the previous sections, the binding site for BZ seems to be located on the γ2-subunit. BZs modify $GABA_A$-receptor allosterically and during this action they enhance GABA response. The effect of BZ is probably accomplished by increasing the frequency of channel opening (Study and Barker 1981). The mean open time of GABA currents does not change in the presence of BZ. An interesting property of BZR is the positive or negative modulation of GABA-activated currents depending on the potency or so called intrinsic efficacy of the ligand for BZ binding site (Haefely 1989). Diazepam is an example of very potent BZ agonist, which enhances current response to GABA. The intrinsic efficacy of diazepam is +1. Another class of compounds such as Ro 19-4603, named the inverse agonists of BZR, reduces GABA currents. The potency of these drugs is -1 (Haefely 1989). Different substances that upon the binding to BZR leaves GABA-induced currents intact, and do not change kinetics of $GABA_A$-channels are named competitive antagonists, and they have intrinsic eficacy 0. An example of competitive antagonist is the anticolvusant drug flumazenil (Haefely 1985). There are many BZ ligands whose intrinsic efficacy ranges between -1 (inverse agonist) and 1 (agonist) producing a broad spectrum of pharmacological effects.

Site of action	Ligand	Effect on $I(Cl^-)$
$GABA_A$ agonist site	GABA	
	muscimol	
	isoguvacine	
	THIP	
	DAVA	
	bicuculline	–
	pitrazepin	–
	SR 42641	–
	SR 95531	–
benzodiazepine receptor	diazepam	+
	midazolam	+
	Ro 19-4603	–
	DMCM	–
Cl^- channel	picrotoxin	–
	TBPS	–
unknown	pentbarbitone	+
	alphaxalone	+
	penicillin G	–
	intracellular Ca^{2+}	–
	extracellular Zn^{2+}	–

Table 2-1. Pharmacologically important binding sites of $GABA_A$-receptor channel are listed together with the corresponding ligands. These substances positively (+) or negatively (-) modulate $GABA_A$-activated currents. Question mark stands for unknown binding sites for steroids, pentobarbiturates, intracellular Ca^{2+} and extracellular Zn^{2+}.

Another pharmacologically important site on $GABA_A$-receptor complex is the site that mediates the antagonistic effect of picrotoxin and TBPS (t-butyl-bicyclophosphorothionate, see Table 2-1). These convulsant drugs are belived to block Cl^--channels either directly or following binding to a closely located site (Barker et al. 1983). The latter view seems more likely as TBPS does not possess a net charge at physiological pH and its blocking action is independent of the direction of Cl^- flow.

The $GABA_A$-receptor channel complex is the target of many other modulators with less defined actions (see Table 2-1). Barbiturates like pentobarbitone prolong $GABA_A$-receptor channel burst duration, at low concentrations, without affecting conductance (Study and Barker 1982). At higher concentrations (~50 μM) pentobarbitone directly activates Cl^- channels. Barbiturates and BZs bind to the distinct but allosterically linked sites (Johnston and Willow 1982).

The neurotransmission mediated by GABA is potentiated by some synthetic and naturally occurring steroids. Similarly to barbiturates, the steroids such as alphaxalone directly activate $GABA_A$-receptors and this effect can be blocked by $GABA_A$ antagonist bicuculline (Table 2-1). The extracellular recognition site on $GABA_A$-receptor protein for steroids is still unknown (Lambert and Peters 1989).

The divalent cation Zn^{2+} applied extracellulary depresses $GABA_A$-evoked currents (Smart and Constanti 1982; 1983, see Table 2-1). This effect was found to be non-competitive, reversible (Smart and Constanti 1990) and voltage-independent (Legendre and Westbrook 1991). Other divalent cations such as Cd^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+} inhibit GABA-evoked currents with

different potency, whereas Ca^{2+}, Mg^{2+} or Ba^{2+} do not produce any effect (Celentano et al. 1991). The blocking effect of Zn^{2+} seems to be mediated by the binding of this divalent cation to a novel modulatory site on $GABA_A$-receptor which is thought to be located on or near the extracellular hydrophobic zone of the putative transmembrane domain M2 (Smart 1992).

$GABA_A$-receptor channel can be modulated also from cytosolic side. The agents regulating the functionality of $GABA_A$-receptor protein from the intracellular side are ATP, Mg^{2+} and Ca^{2+}. Decreasing the concentration of either intracellular ATP or Mg^{2+} it is observed a reduction in the affinity of the $GABA_A$-receptor for GABA (Stelzer et al. 1988). This reduction so called " run-down " of GABA response does not show any voltage dependence (Shirasaki et al. 1992). As already mentioned, the β-subunit of $GABA_A$-receptor channel contains a cAMP-dependent kinase consensus sequence. This site could be phosphorylated by protein kinases which use ATP and Mg^{2+}. The phosphorylation of $GABA_A$-receptor seems to be important for its functionality (Stelzer et al. 1988) and may modulate GABA desensitization (Schofield et al. 1987). In fact, a recent study (Moss et al. 1992) showed that the phosphorylation of the β1 subunit by cAMP-dependent protein kinase A modulate the desensitization kinetics of $GABA_A$-receptor channels. The response of $GABA_A$-receptor to GABA is suppressed by the increasing concentrations of intracellular Ca^{2+} (Inoue et al. 1986). Ca^{2+} ions shorten the duration of the mean open time of GABA-gated Cl^- channels without changing the single-channel conductance (Behrends et al. 1988). It is belived that Ca^{2+} inhibits GABA-receptor Cl^- currents through the intracellular interaction with a specific effector, probably a binding site.

Furthermore, the action of intracellular Ca^{2+} produces a decrease in the affinity of $GABA_A$-receptor for its agonist (Inoue et al. 1986).

Chapter 3

Materials and Methods

All electrophysiological experiments in this work were made on the primary cultures of rat cerebellar granule cells. Currents activated by GABA were studied using the patch-clamp technique.

Cell preparation

Cultured granule cells were prepared using a procedure described by Levi and co-workers (1984). Initially, cerebella were dissected from 7 to 9 postnatal day old Wistar rats and minced with razor blade in a solution containing (in mM): NaCl 124, KCl 5.37, NaH_2PO_4 1.01, D-glucose 14.50, Hepes 25, $MgSO_4$ 1.2, phenol red $2.7 \cdot 10^{-3}$ and bovine serum albumin 3 mg/ml. The minced tissue was centrifuged at 1000 RPM for 1 min. After centrifugation the cell pellet was saved and treated with 0.25 mg/ml trypsin (Sigma) at 37° C for 10 min in order to digest connective tissue. The action of trypsin was blocked by administration of 166.4 μg/ml Soybean trypsin inhibitor (Sigma). DNA released from the badly injured cells was degraded by 25.6 μg/ml DNAse. Cells were further mechanically dissociated using fire

polished Pasteur pipettes of different diameter in order to dissolve cell clumps. Separated cells were washed with solution containg Ca^{2+} and resuspended in Basal Eagle's medium (Gibco) supplemented with 0.27 mg/ml L-Glutamine, 20 mM KCl, 0.1 mg/ml gentamicine, and one tenth volume of a heat inactivated fetal calf serum (Gibco).

Cerebellar granule cells were plated on 35 mm Petri dishes, each containing about 2 ml of cell suspension at a density of 10^6 per ml of suspension. Petri dishes had been previously covered with poly L-lysine (Sigma). The cells were kept in the incubator (Heraeus B5061 EK-02) at 37° C and 5% CO_2. To inhibit the growth of non-neuronal cells 10 μM of the mitotic inhibitor cytosine arabinoside furanoside (Sigma) was added per dish at about 24 hours after plating. In order to provide food for the cells 100 μM D-glucose was added per dish after 7 days in culture (DIC). In these conditions the cerebellar granule cells were maintained in culture for about 2 weeks and they were available for experiments starting from the 1 DIC.

Patch-clamp

The patch-clamp technique emerged about 15 years ago as a variation of the classical voltage-clamp method (Neher and Sakmann 1976). The principle of the method is to electrically isolate a patch of the membrane and to record the current flowing through the patch. During recording the electric potential across the patch is controlled. To measure small currents of magnitude of 10^{-12} A, the simple way is to monitor the voltage drop across a large resistor. This is achieved by the operational amplifier having very high feedback resistance (~50 GΩ) which is constructed of field-effect

transistors. Higher resistance of the feedback implies higher gain of the operational amplifier. On the other hand, the feedback resistance coupled in a shunt with stray capacitance of amplifier form a complex RC-circuit which increases the noise of the measurement. Therefore, an optimization of the circuit must be achieved by choosing appropriate electronic elements.

Hamill and co-workers (1981) developed method to seal glass pipettes against the membrane of a living cell. The equivalent elctrical circuit of the patch-clamp configuration is shown in Figure 3-1. In order to obtain a good control of patch potential it is important to make a gigaseal in which the resistance of seal is much higher than the resistance of membrane patch ($R_s >> R_m$, see Figure 3-1). With a 10 GΩ recording resistance provided by a gigaseal and a 2 kHz bandwidth, the standard deviation of the current record could be made as small as 0.1 pA. Therefore, it is possible to record the membrane currents produced by opening and closing of the single ionic channel.

The noise of measurement is comprised of four contributions coming from different sources (Moran 1992). One is an inevitable membrane noise which is a shot noise produced by ions that cross the membrane by leakage or ionic pumps. Another background noise is due to the random thermal movement of electrical charges in the junction between the pipette and membrane. Since spectral density of this noise is inversely proportional to the seal resistance, the gigaseal formation decreases noise level. The third component is the pipette noise due to RC-circuit composed of pipette resistance and the tip capacity. This type of noise can be reduced by decreasing capacitance of the pipette. A very hydrophobic substance such as

Sylgard when coated around the pipette prevents the formation of a solution film and thickness of the coating reduces capacitance. Finally, the fourth type of noise, as already mentioned, comes from electronic instruments including the operational amplifier. The spectral density of this noise is inversely proportional to the input impedance of the amplifier.

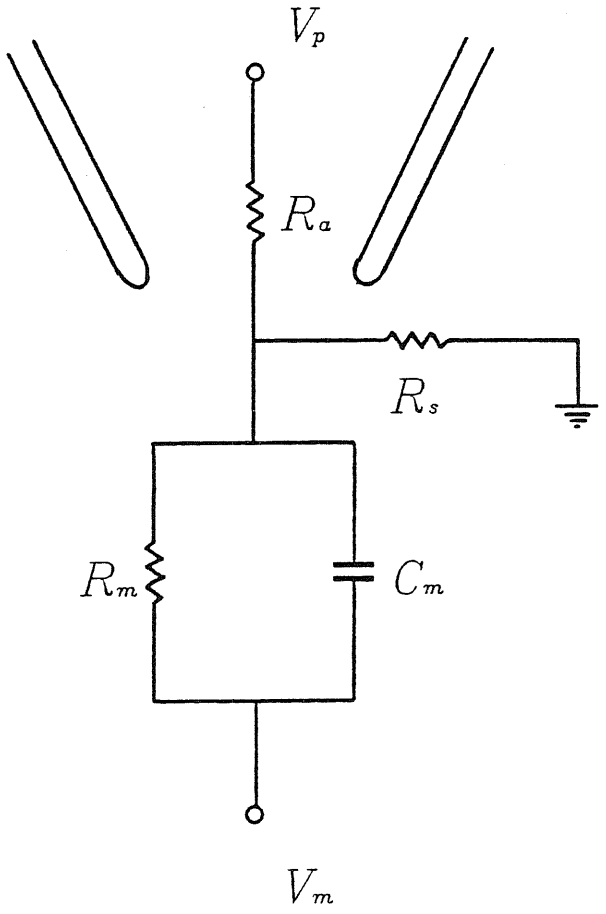


Fig 3-1. A drawing represents an equivalent circuit of the patch-clamp pipette attached to the cell membrane. Measurement of single-channel events can be obtained if the current passing from a pipette interior to the external solution is considerably small in comparison with the current produced by opening of ionic channels in the membrane patch.

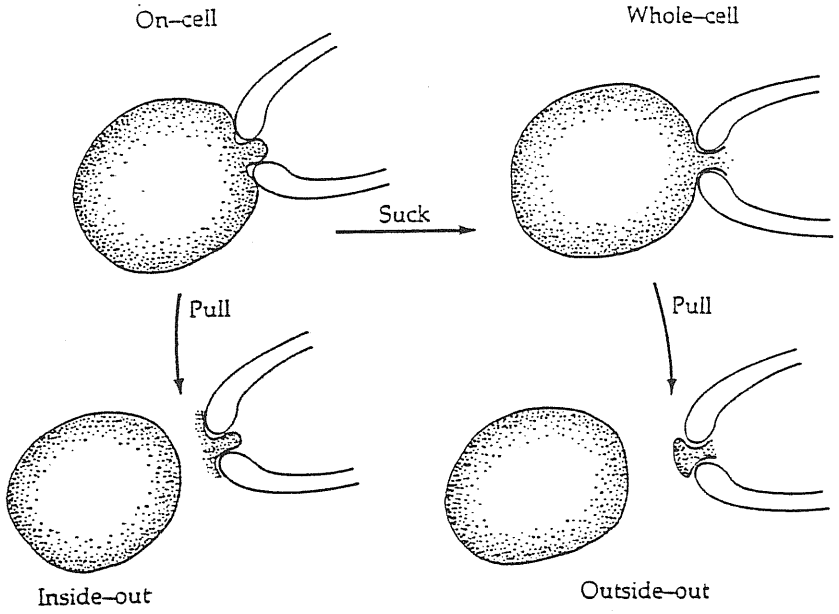


Fig 3-2. All methods starts with a clean pipette pressed against an intact cell to form a gigaohm seal between the pipette and the membrane it touches. Channels can be recorded in this on-cell mode as minute currents passing between the pipette solution and cytoplasm. Additional manipulations permit the same pipette to be used to voltage clamp a whole-cell or to excise a patch of membrane for recording in inside-out or outside-out configurations. (Adapted from Hamill et al. 1981.)

Main configurations of the patch-clamp method are shown in Figure 3-2. In on-cell or cell-attach configuration, it is possible to control the potential of only a small patch of membrane. By slight suction or sudden electric pulses the cell membrane under patch breaks, thus estabilishing an electrical connection between the measuring pipette and the cell. This configuration is called whole-cell. An exchange of ions and chemical substances occurs in a couple of seconds whereas big molecules and proteins need between few minutes to tens of minutes, to diffuse from the cytosol to pipette (Neher

1992). In whole-cell mode it is possible to measure the total current which is a sum of the openings of many channels. When the pipette in on-cell or whole-cell configurations is pulled, new configurations are formed, an inside-out or an outside-out, respectively (see Figure 3-2). These configurations so called excised patches, are useful to study some channels, when there is a need to control the solutions from both sides of the membrane or when an intracellular integrity is not important in functioning of these channels.

An outstanding property of electric measurement with gigaseal patches is its high sensitivity. This can be used not only to record currents, but also to measure the membrane electrical capacitance, which is an estimate of the cell surface area. With this method a very fine processes such as the fusion of synaptic vesicle with membrane or the exocytosis can be observed (Augustine and Neher 1992).

Experimental procedure

The whole-cell and the outside-out configurations of patch-clamp technique were used to study the electrophysiological properties of GABA-activated currents and their modulation by extracellular Zn^{2+}. In outside-out configuration most of the patches contained more than one channel. In order to avoid the simultaneous openings of several channels and a low probability of opening caused by desensitization, I used a low concentration of GABA (0.5 μM). Borosilicate glass pipettes with internal filament (Hilgenberg) were coated with Sylgard (Corning) and then fire polished to get a tip resistance of 5-20 MΩ (measured in working solutions).

The currents were measured using a standard patch-clamp amplifier (EPC-7, List Medical Instruments). The acquisition and electrical stimulation were done using a microcomputer (Atari 1040ST) and 16 bits A/D-D/A converter (ITC-16, Instrutech).

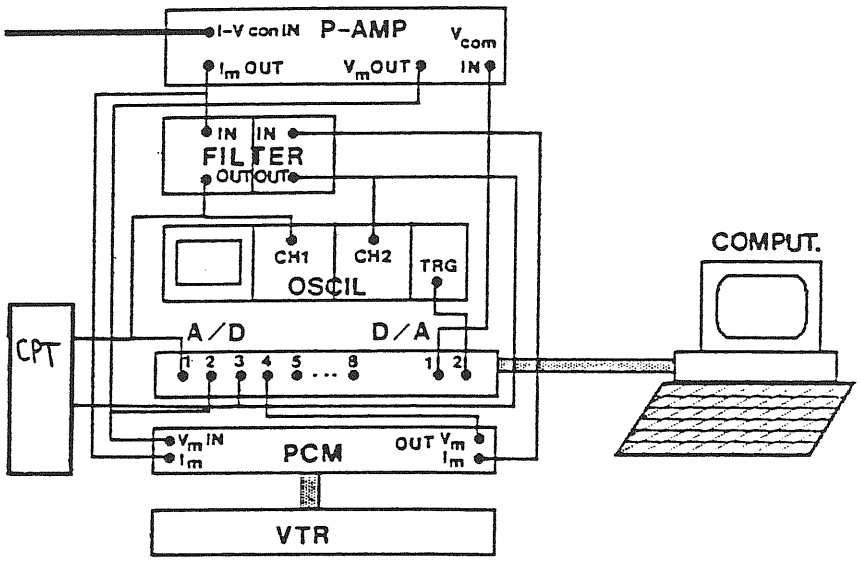


Fig 3-3. Wiring of the patch-clamp equipment. Current signal coming from the patch-clamp amplifier (P-AMP) passes through filter and goes to a channel 1 (CH1) of oscilloscope, chart plotter (CTP) and to the computer via A/D converter. The same signal is stored on the tape by video tape recorder (VTR) via Pulse-code modulator (PCM) device. When recorded data are replayed from VTR, they pass to PCM and then to the filter. The recorded signal from output of filter can pass at the same time to a channel 2 (CH2) of an oscilloscope, a chart plotter and through an A/D converter to the computer. (From Moran 1992.)

The operation of converter was controlled by the microcomputer which used a commercial program ACQUIRE (Instrutech), written in Modula-2 by H. Affolter from Yale University. The current signal filtered at 10 kHz was directly recorded from one output of EPC-7 on video tape using the

combination of Pulse Code Modulator (501ES, Sony) and video recorder operating in β-system (Sony). During experiments the current signal was filtered at 1 kHz and simultaneously monitored on an oscilloscope, a chart recorder and the microcomputer. The electric connections among the instruments in the patch-clamp set-up are shown in Figure 3-3. Data stored on the tape were filtered at a cut-off frequency of 2.5kHz with Butherworth filter (Krohn-Hite 3202). Data were then digitized and transferred to the microcomputer hard disk at appropriate sampling time. For single-channel currents a sampling time was in the range from 200 to 500 μs and for whole-cell traces it was 10 ms.

The different solutions were applied by gravity through six barrels. The barrels terminated in a small glass tube (diameter less than 0.5 mm) located at a distance of about 1 mm from the patched cell. One barrel contained control solution and other five the different drugs as required. The flow of solutions through the barrels was controlled by six electrovalves which were connected with an electronic device (Zheng 1991). This device allowed the flow of solution from only one barrel at any time. During experiments cells were continuously washed with control solution. The extracellular fluid surrounding the patched cell was completely exchanged within 200 ms after the opening of the electronic valve. In some experiments Zn^{2+} (10-30 μM) was directly added to the control solution.

Experiments were performed at room temperature (22-24° C). Salts were purchased from Sigma, Merck or Prolabo. Drugs used were: GABA and bicuculline methiodide purchased from Sigma, and midazolam purchased from La Roche.

Data analysis

The whole-cell currents were analyzed with the programs developed in our laboratory in Quick Basic (Microsoft Corporation) on PC-compatible computers. The decaying phase of whole-cell currents evoked by GABA in the control condition and in the presence of external Zn^{2+} was fitted with a single exponential time constant (τ_d). The fitting was performed in Sigma Plot (Jandel Corporation) which uses Marquard procedure.

The peak amplitude of GABA-activated whole-cell current depended on applied concentration of GABA. This dependence can be mathematically described with Hill equation:

$$I = \frac{I_{max}}{1 + (K/c)^n} \qquad (3\text{-}1)$$

where I is the peak of current, I_{max} is the peak of saturating GABA current, c is concentration of GABA, K is half-maximum concentration which corresponds to EC_{50} and n is the Hill coefficient.

To study the binding of Zn^{2+} to $GABA_A$-receptor the current activated by GABA in the presence of Zn^{2+} can be represented as:

$$I_{Zn} = I_0 (1 - f_B) \qquad (3\text{-}2)$$

where I_{Zn} is the peak of GABA current in the presence of Zn^{2+}, I_0 is the peak of GABA current in the absence of Zn^{2+} and f_B is the fraction of current

blocked by Zn^{2+}. Since the binding of Zn^{2+} can be also described by Hill equation as well, it is useful to express I_{Zn} as:

$$I_{Zn} = I_0 \left[1 - \frac{1}{1 + (K_i/[Zn])^{n_i}}\right] \qquad (3\text{-}3)$$

where $[Zn]$ is the concentration of Zn^{2+}, K_i is the half-blocking concentration of Zn^{2+} which corresponds to IC_{50} and n_i is Hill coefficient of Zn^{2+} inhibition.

The receptors activated by GABA needed some time to recover from desensitization before they were able to be activated again. To study the recovery process a double pulsed protocol was used. In each train of stimuli, two identical GABA pulses separated in a time were applied to the same cell. The time between GABA pulses varied from 10 s to 1 min. Between each pair of stimuli the cells were washed with control solution for 4 to 5 minutes. The time constant of recovery (τ_r) was estimated according to the equation:

$$I_p - I = I_p \exp(-t/\tau_r) \qquad (3\text{-}4)$$

where I_p is peak current of the first GABA pulse and I is the peak current of the same GABA pulse applied after time t.

Single-channel currents were analyzed with TAC program (Instrutech, Sigworth 1985) which uses 50 % threshold criteria in the detection of

channel opening. The open and closed time histograms were fitted with exponential functions by the method of Sigworth and Sine (1987). As already mentioned most patches contained more than one channel and therefore the probability of opening is denoted by (Np) where N stands for the number of channels in the patch, and p for the probability of opening of a single channel. This is true if the openings of separated channels in the patch are independent events. Furthermore, Np was calculated as the ratio between the sum of all open times and the total observation time, taking the duration of GABA application as the total observation time. The analysis of single-channel amplitudes were performed using programs written in our laboratory in Quick Basic.

Student's t-tests were used for comparison of paired and unpaired data. The results are presented as mean ± standard deviation.

Solutions for electrophysiology

The solutions used in experiments are shown in Table 3-1. Pipette and bath solutions were the same for whole-cell and outside-out configurations. The pH of both solutions was adjusted to 7.3. The osmolarities of both solutions were about the same value, thus maintaining the difference in osmotic pressure low. This was an important factor for the stability of patch.

High concentration of Cs^+ was present in the pipette solution in order to block K^+ channels. The presence of Mg^{2+} in extracellular solution provided a blocker at the negative holding potentials of spontaneous channels which were mainly of NMDA type (Kilić et al. 1991). The blockers of voltage-gated Na^{2+} or Ca^{2+} channels were not used in this study, since all

measurements were done at the constant holding potentials. As mentioned in Chapter 2, it seems that $GABA_A$-receptors need the phosphorylation factors to maintain its functionality (Stelzer et al. 1988). Therefore, to prevent eventual run-down of GABA-activated currents ATP and Mg^{2+} were included in the pipette solution.

Bath	[mM]	Pipette	[mM]
NaCl	140	CsCl	137
KCl	3	$MgCl_2$	4
$CaCl_2$	1.5	EGTA	5
$MgCl_2$	2	ATP-Na_2	2
Hepes-NaOH	10	Hepes-KOH	10
D-glucose	10		

Table 3-1. Extracellular (bath) and intracellular (pipette) solutions for patch-clamp recording are listed.

Chapter 4

GABA-activated whole-cell Currents

As it was mentioned in Chapter 2, the majority of $GABA_A$-receptors in cultured cerebellar granule cells are evenly distributed along the cell bodies whereas the cell processes contain much smaller number of receptors (Hansen et al. 1991). This characteristic of the granule cells make them useful for electrophysiological study.

In this work I have studied the properties of currents evoked by GABA and their modulation by Zn^{2+} in primary cultures of cerebellar granule cells.

GABA currents

An application of GABA (10 μM) for 5 s, at a holding potential of -50 mV, evoked an inward current which reached a peak in about 80 ms and then slowly declined to steady state value (see Figure 4-1A). In order to see what type of channels are activated by GABA, the $GABA_A$-antagonist bicuculline was used as a criteria. It was found that GABA-evoked currents are

reversibly blocked by bicuculline (10 μM) indicating that GABA activated $GABA_A$-receptors (see Figure 4-1A).

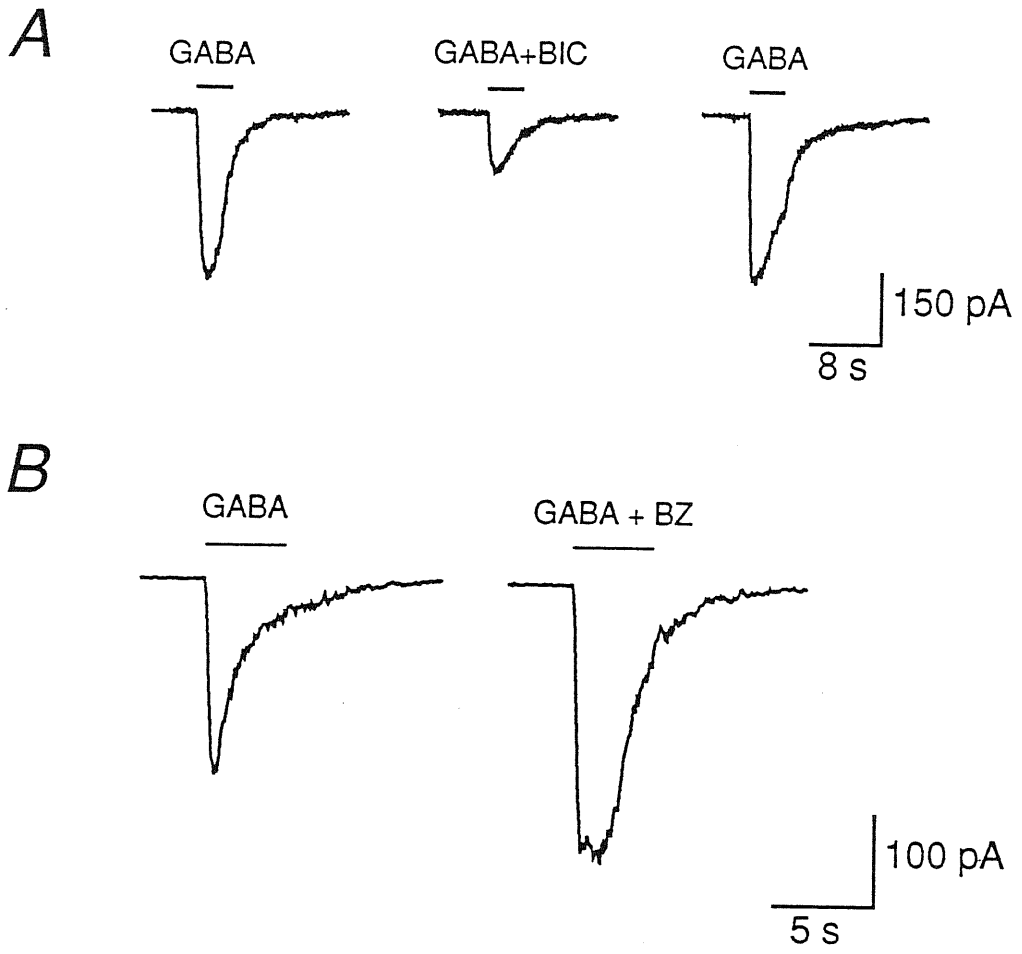


Fig 4-1. (A) Whole-cell current evoked by GABA (10 μM) is reduced in a reversible manner by bicuculline (10 μM). Notice that GABA current recovers after washing. Holding potential -50 mV. (B) Whole-cell currents evoked by GABA (10 μM) and by co-application with midazolam (0.1 μM). Notice that midazolam enhances the peak amplitude and duration of the maximum current.

The potentiating effect of benzodiazepines on GABA currents is belived to be due to the presence of γ_2 subunit (Pritchett et al. 1989). I therefore tested a BZ, midazolam, on GABA-evoked whole-cell currents in cerebellar

granule cells. Midazolam (100 nM) enhanced the response to GABA (10 μM) in a reversible manner to 153 ± 12 % (n=6, see Figure 4-1B).

Dose-response

The affinity and stoichiometry of GABA binding to its receptors can be studied using electrophysiological means by measuring the peak amplitudes of GABA-activated whole-cell currents as a function of GABA concentration. Figure 4-2 shows an example of GABA dose-response curve.

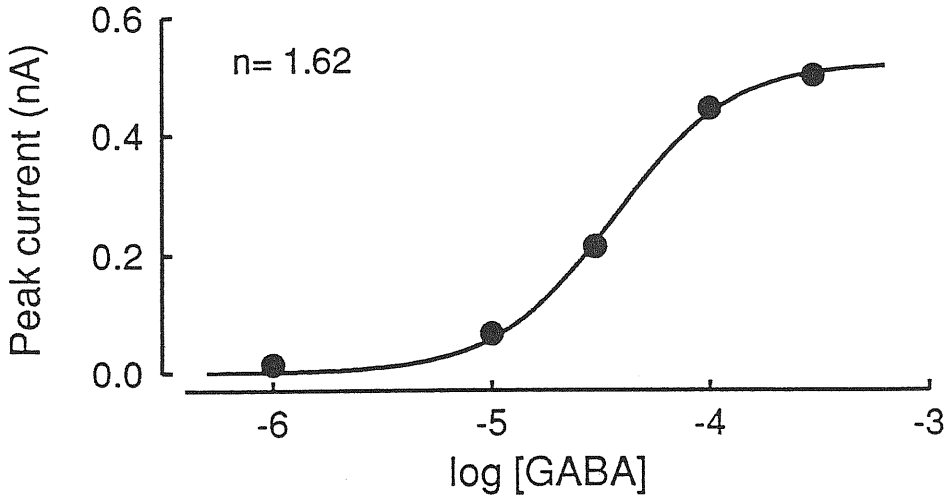


Fig 4-2. Dose-response curve of GABA in control condition. Experimental points are fitted with Hill equation. The half-maximum concentration is 36.21 μM, the maximum current is 0.52 nA and Hill coeficient is indicated.

The range of GABA concentrations was from 1 to 300 μM. Peak values of currents evoked by different concentrations of GABA were fitted with the Hill equation (3-1) (see Figure 4-2). I have found that for some cells the Hill coefficient was 0.94 ± 0.04 (n=6) whereas for others it was 1.48 ± 0.10

(n=4). These two values were statistically different ($P < 0.05$). The half-maximum concentration displayed more variability ($K = 20.65 \pm 17.56$ μM, n=10). No correlation between values of n and K has been found.

In order to compare the two populations of neurones having different Hill coefficients with their size, I calculated the membrane surface area from the compesation of the capacitance transient. The membrane capacitance was 2.4 ± 0.5 pF (n=6) for the group of cells having Hill coefficient 0.94 and 2.7 ± 0.7 pF (n=4) for the cells with Hill coefficient of 1.48. Assuming that the membrane specific capacity is 1 pF/100 μm^2 (Hille 1992) the cell surface area of these two populations was calculated. The surface area of the first population was 240 ± 50 μm^2 and that of second group was 270 ± 70 μm^2 (n=4). This finding suggests that the cells possessing two different Hill coefficients were about the same size.

Current-voltage relation

The peak amplitudes of whole-cell currents were measured at different holding potentials. Figure 4-3 shows peaks of whole-cell currents evoked by GABA (10 μM) at different holding potentials. The I-V relation was found to be linear in the potential range from -70 to +50 mV (see Figure 4-3). It is known that the currents produced by activation of $GABA_A$-receptor are mediated by Cl^-. The reversal potential (E_r) for GABA responses was 4.35 ± 2.71 mV (n=5). This value was very close to the Cl^- equilibrium potential (-0.86 mV), calculated from Nernst equation (1-1).

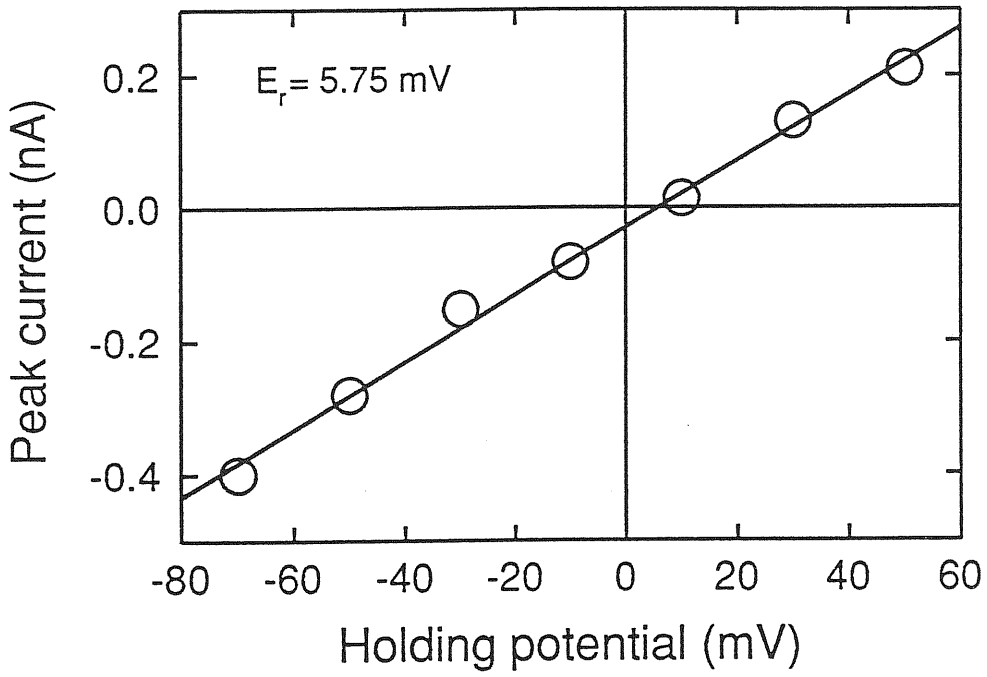


Fig 4-3. Maximum amplitudes of whole-cell currents activated by GABA (10 μM) are plotted versus holding potential. The line is obtained by linear fit of the peaks. Reversal potential is also indicated.

Desensitization and recovery

As already mentioned, the amplitude of GABA-activated current decreases after reaching the peak. To quantify this decay the time constant of desensitization τ_d was measured for different GABA concentrations. The desensitization time constant of GABA-evoked whole-cell currents was found to depend on GABA concentrations (Figure 4-4). In fact with a relatively low concentration of GABA (1 μM) the current decline developed slowly (Figure 4-4A,B) whereas with a high dose of GABA (300 μM) the

decline progressed very rapidly. Furthermore, the desensitization time constants were measured for two groups of cells having different Hill coefficients. I have found that τ_d is the same for both groups of cells. It should be noted that during the patch recording in whole-cell configuration the desensitization kinetics did not change.

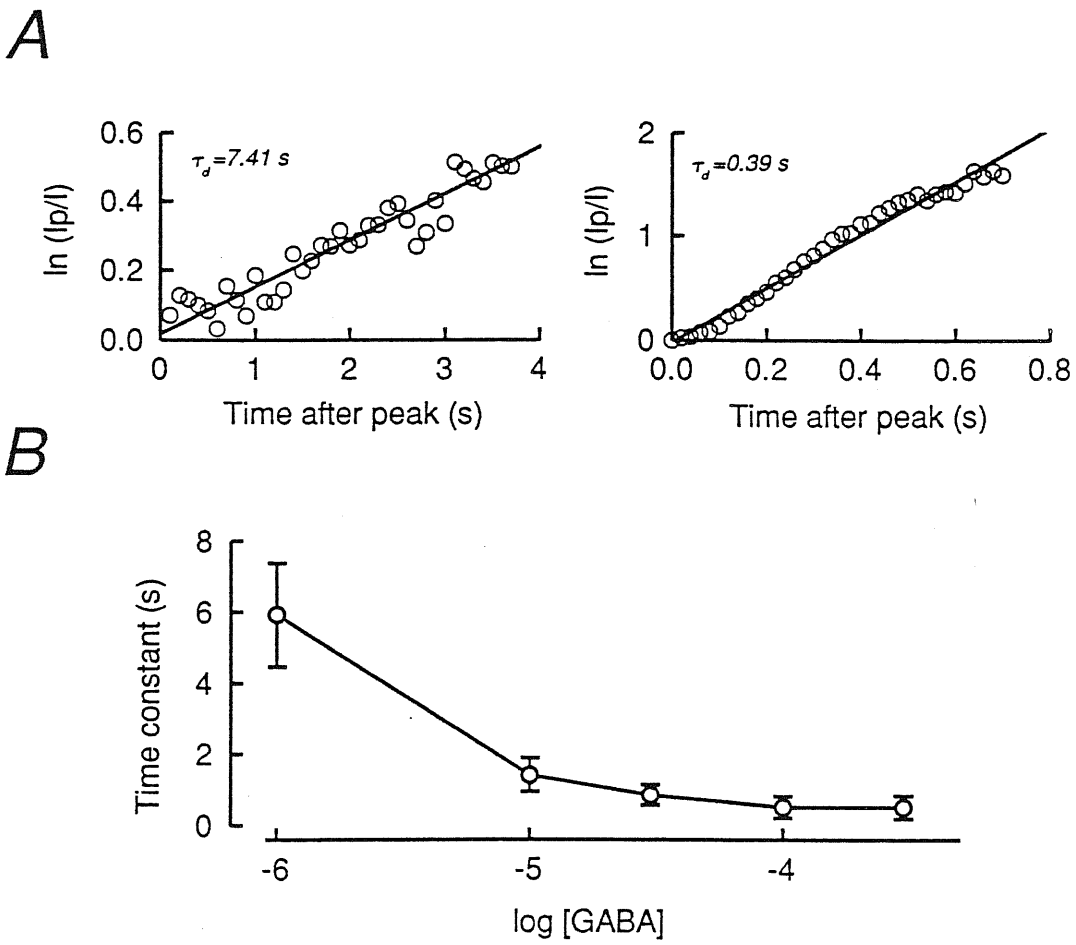


Fig 4-4. (**A**) Graphs show the fitting of experimental points (ln I_p/I, where I_p is the peak current and I is the current amplitude after the peak is reached) as a function of time after the current peak. Left and right graphs are made for 1 and 100 μM GABA, respectively. (**B**) Time constants τ_d are plotted as a function of GABA concentration in the control condition (open circle). Each point is the mean of 8 experiments. Bars represent SD.

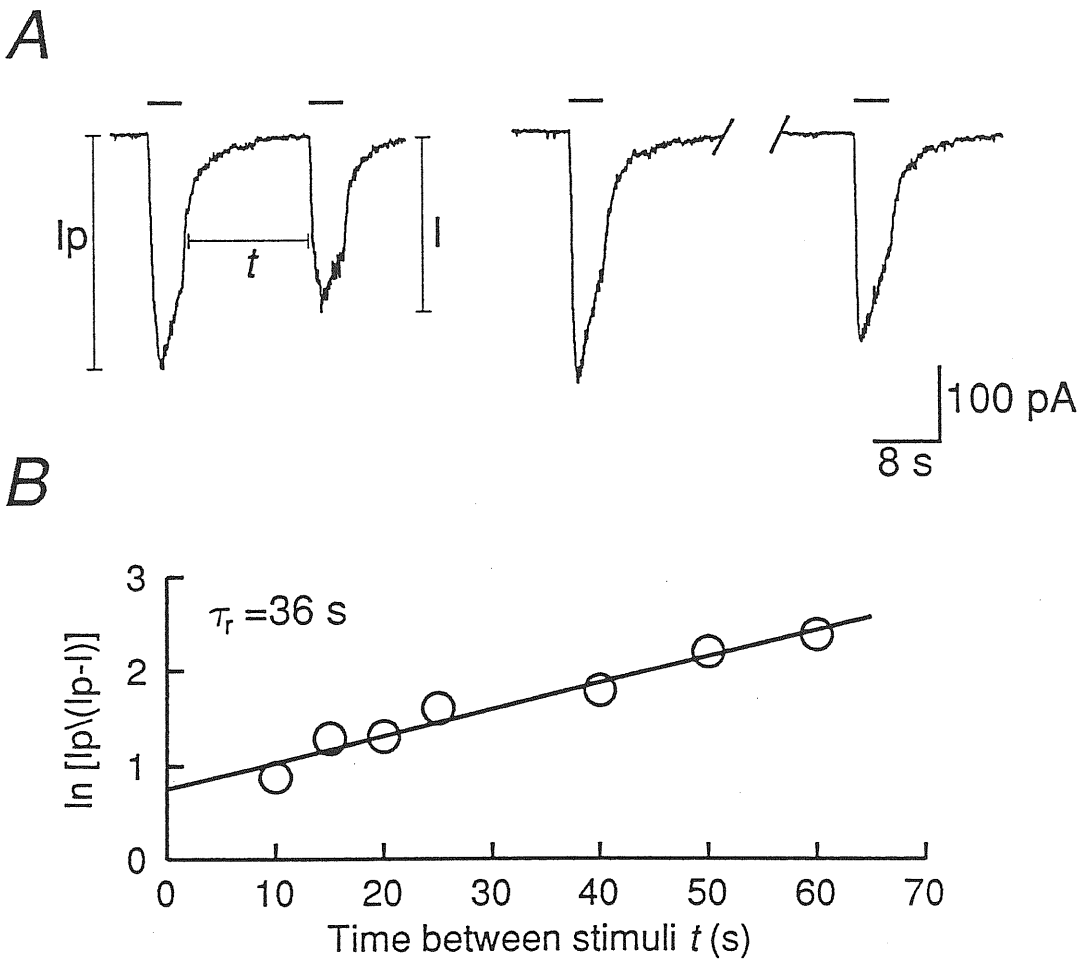


Fig 4-5. (A) Pairs of GABA pulses (10 μM, bars), separated in time by 15 s (left) and 40 s (right). GABA pulses are applied to the same cell. Notice that the amplitude of peak current of the second pulse depends on the interval between pulses. (B) Fitting of recovery time constant in control condition. The values for ln $[I_p/(I_p-I)]$ are plotted versus time between the train of stimuli.

The time constant of recovery τ_r was calculated using equation (3-4, see methods). In control condition and at holding potential of -50 mV, τ_r was 36.0 ± 1.2 s for 10 μM GABA (n=5, Figure 4-5A,B). Consequently, because

of this long τ_r, the cells required 4 to 5 min washing after each application of GABA in order to gain a complete recovery. For different concentrations of GABA τ_r was in the same range, indicating that the process of recovery from desensitization does not depend on GABA concentration.

Zn^{2+} effect

Zn^{2+} reduced the amplitude of GABA-activated currents. Initially, Zn^{2+} was applied in two different ways. In a first set of experiments, Zn^{2+} (30 μM) was continously superfused through the bathing solution. It reduced the peak of GABA (10 μM) currents to 51 ± 7 % (n=12, Figure 4-6). A full recovery was observed 10 min after reintroduction of the control solution. In a second set of experiments Zn^{2+} (30 μM) was co-applied with GABA. In this case, the peak of GABA currents decreased to 61 ± 16 % (n=12, Figure 4-6). The same behaviour was found for higher concentrations of GABA (up to 100 μM). Since there was no significant difference in the effects of Zn^{2+} applied via the bathing solution or topically, in later experiments Zn^{2+} was superfused in the bath. The concentrations of Zn^{2+} used here are comparable with those employed by other authors (Draguhn et al. 1990; Legendre and Westbrook 1991; Westbrook and Mayer 1987). Furthermore, higher concentrations of Zn^{2+} (up to 300 μM, c.f. Smart and Constanti 1990), were found to produce instability of the patch recording and therefore could not be used.

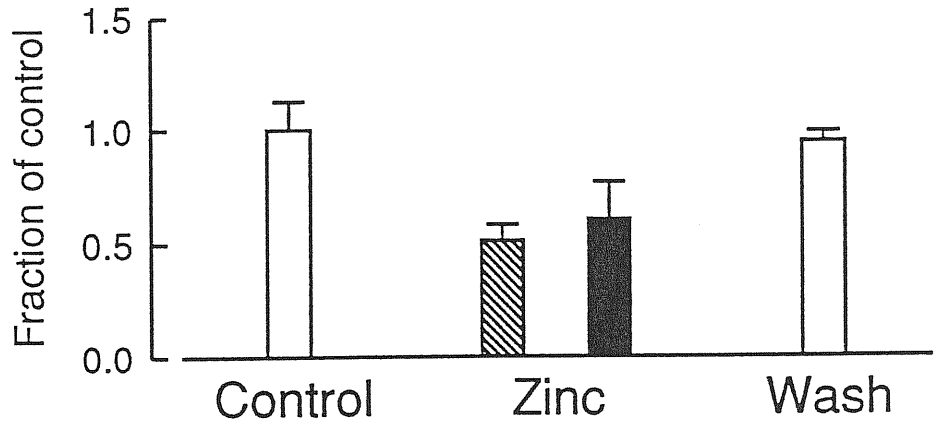


Fig 4-6. Normalized peaks of GABA currents in control condition, during superfusion (stripped rectangle) and co-application (solid rectangle) of Zn^{2+} (30 μM). Notice that after washing GABA current recovers.

It is belived that Zn^{2+} depresses GABA responses in a noncompetitive manner (Kaneko and Tachibana 1986; Legendre and Westbrook 1991). To verify this hypothesis I have measured the peaks of the whole-cell currents evoked by different GABA concentrations in the presence of a constant concentration of Zn^{2+}. Figure 4-7A shows whole-cell currents induced by increasing concentrations of GABA (from 10 to 100 μM) in control condition or in the presence of Zn^{2+} (30 μM). Interestingly, bath application of Zn^{2+} (30 μM) had no significant effect on the Hill coefficient and half-maximum concentration (see Figure 4-7). In the presence of Zn^{2+}, Hill coefficients were 0.97 ± 0.02 (n=4) and 1.38 ± 0.09 (n=3), ($P < 0.05$). Similarly to control, the half-maximum concentration was $K = 21.35 \pm 15.42$ μM (n=7). This finding indicates that external Zn^{2+} blocked $GABA_A$-receptors in a noncompetitive way without significant change in the

stoichiometry for agonist binding.

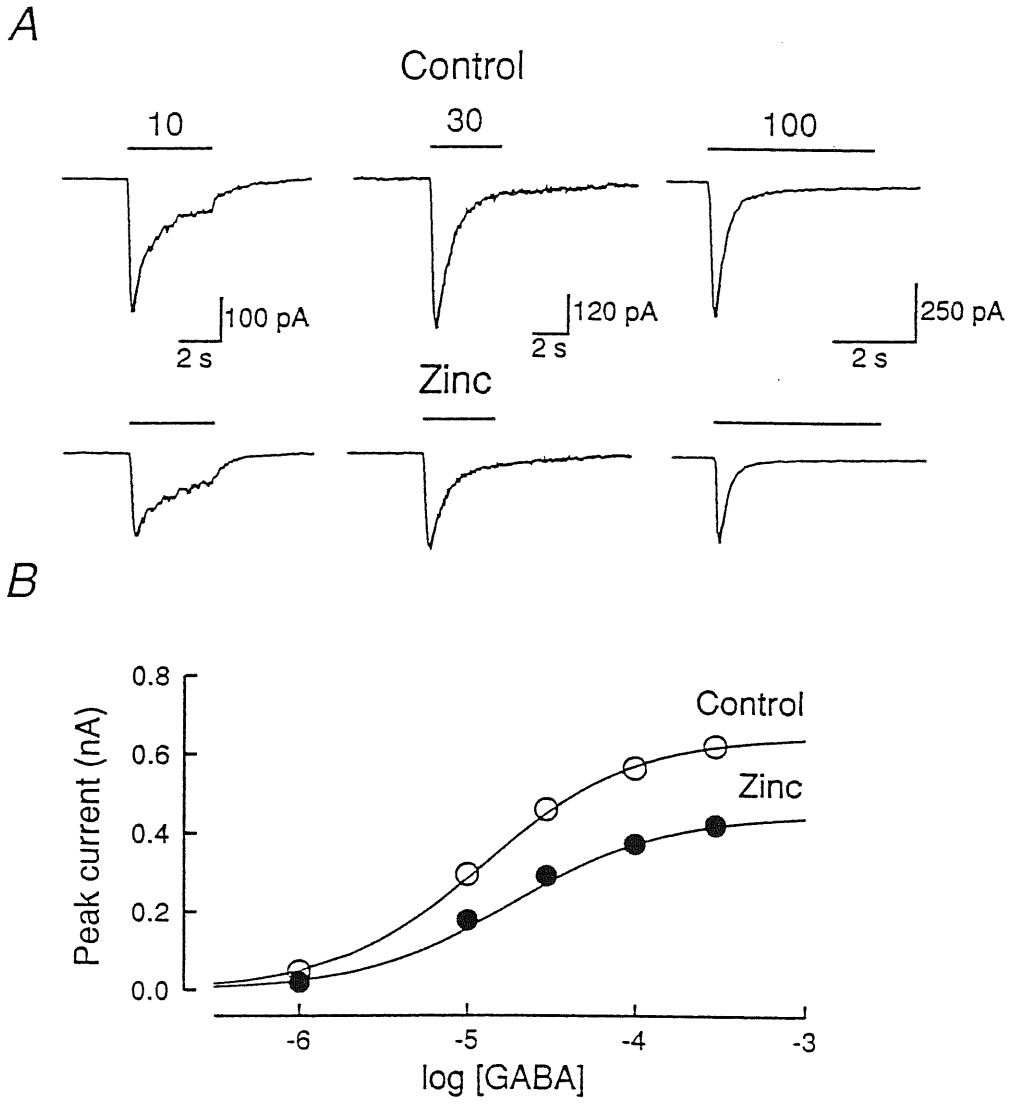


Fig 4-7. (A) Whole-cell currents induced by 10, 30 or 100 μM of GABA (indicated by bars) in control condition or during bath application of Zn^{2+} (30 μM). Holding potential -50 mV. All traces are from the same cell. Note the different calibration bars for different concentrations of GABA. (B) Dose-response curves of GABA-activated currents shown in A, in control condition (open circle) and in the presence of Zn^{2+} (filled circle). Zn^{2+} blocks GABA currents in a noncompetitive way. In control condition n = 0.99, K = 12.10 μM and I_{max} = 0.640 nA. In the presence of Zn^{2+} n = 0.99, K = 15.17 μM and I_{max} = 0.436 nA.

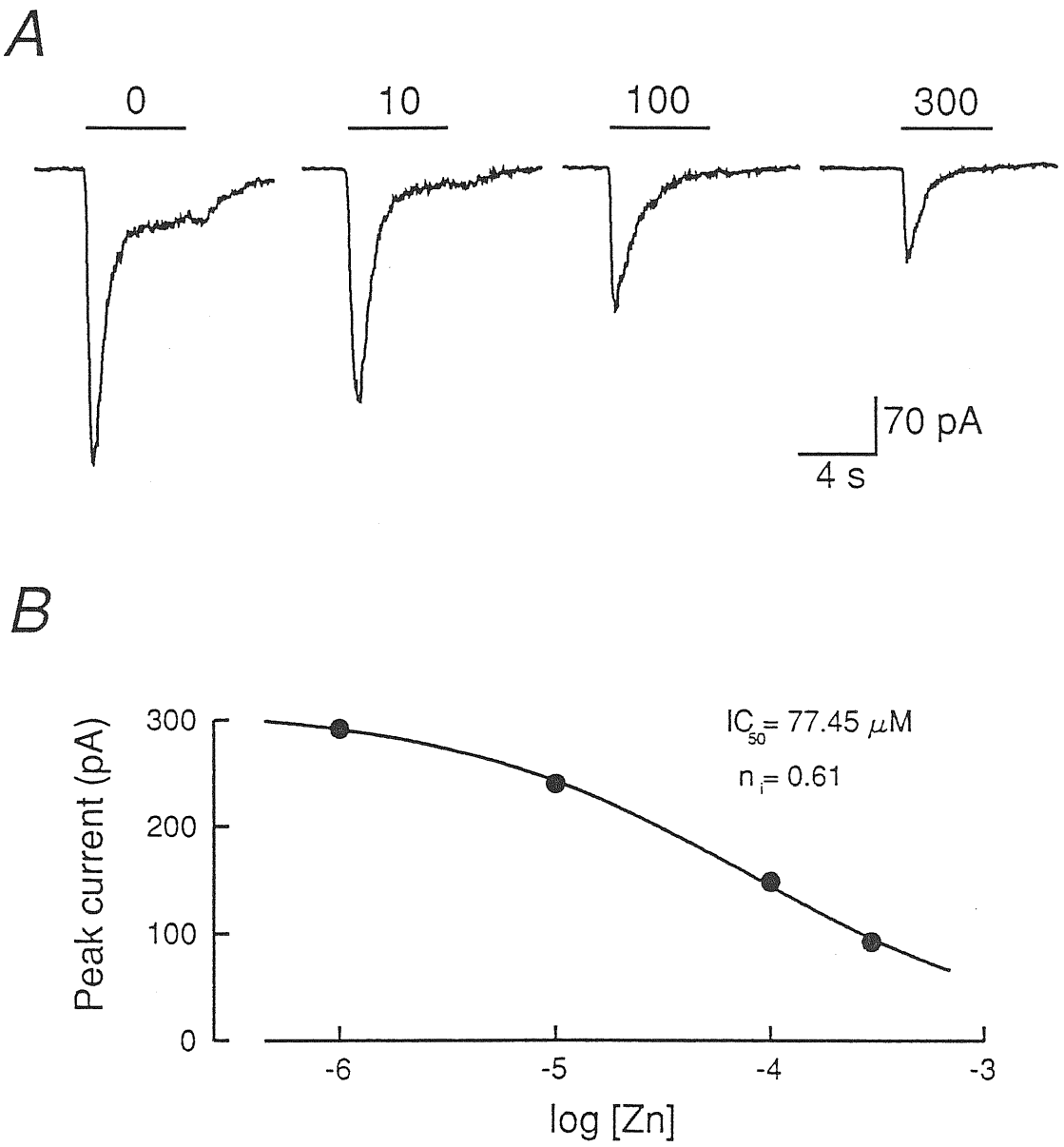


Fig 4-8. (A) Whole-cell currents activated by co-application of GABA (10 μM) and different concentrations of Zn^{2+}. The concentrations of Zn^{2+} (in μM) are indicated above bars. Holding potential -50 mV. All traces are from the same cell. (B) Dose-response curve for Zn^{2+} block constructed from currents shown in A. The peak amplitudes are shown as filled circle. The IC_{50} and n_i are also indicated. The peak current in the absence of Zn^{2+} is 312 pA.

At this point it was suspected that Zn^{2+} might bind to a site distinct of the agonist one. In order to quantify the chemical affinity of Zn^{2+} to a site on $GABA_A$-receptor protein I have performed experiments using constant concentration of GABA and different concentrations of Zn^{2+} (see Figure 4-8A). It should be noted that in these experiments GABA was co-applied with Zn^{2+}, since in the presence of high concentrations of Zn^{2+} the patch was unstable. The amplitudes of GABA-evoked whole-cell currents decreased with increasing concentrations of Zn^{2+} (see Figure 4-8B) and they were fitted with equation (3-3, see methods). When GABA (10 μM) was co-applied with different concentration of Zn^{2+} (1-300 μM) Hill coefficient was 0.63 ± 0.04 and corresponding IC_{50} was 57.20 ± 10.24 μM (n=5). These findings suggest that Zn^{2+} binds to $GABA_A$-receptor protein with slightly lower affinity and different stoichiometry than GABA itself.

In order to investigate the effect of Zn^{2+} on Cl^- permeability through $GABA_A$-channel I have compared the reversal potentials of GABA currents in the control condition and in the presence of extracellular Zn^{2+}. Figure 4-9 shows whole-cell currents activated by GABA (10 μM) at different holding potentials in control condition or in the presence of Zn^{2+}. In the presence of Zn^{2+} (30 μM) the linear voltage dependence of GABA currents persisted and the reversal potential was 5.21 ± 2.64 mV (n=4, Figure 4-9B). This value did not significantly differ for that of GABA in control condition ($P > 0.1$).

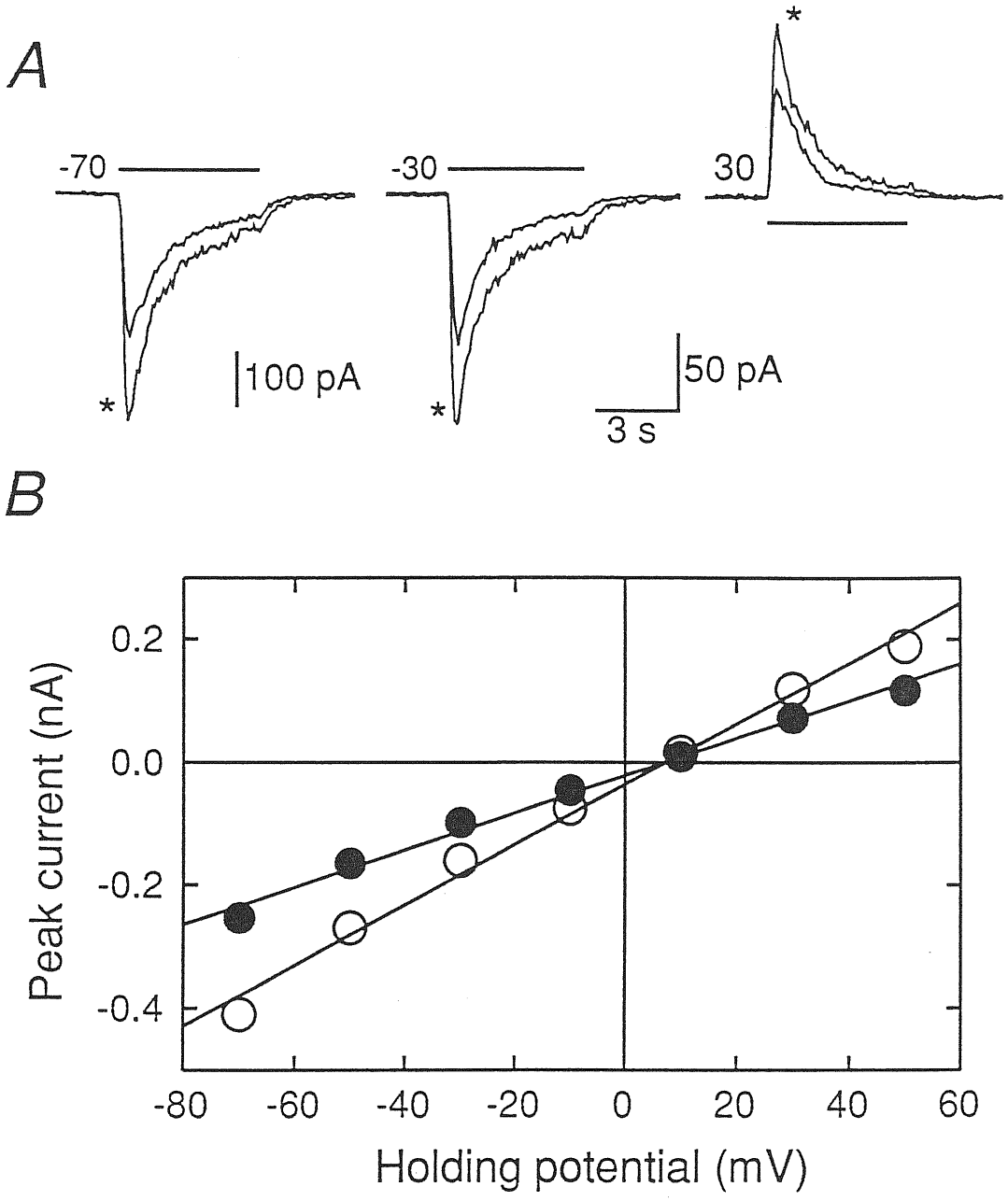


Fig 4-9. (A) Superimposed whole-cell currents elicited by GABA (10 μM, bars) at different holding potentials, in control condition (*) or in the presence of Zn^{2+} (30 μM). Holding potentials are indicated above each trace. All records are from the same cell. (B) Peak amplitudes of GABA currents shown in A, are plotted as a function of holding potential in control (open circle) or in the presence of Zn^{2+} (filled circle). The I-V relation is linear and the reversal potential is the same for both conditions. Reversal potential is 6.97 mV.

The effect of Zn^{2+} on desensitization was studied by measuring the time constant of desensitization τ_d in the presence of Zn^{2+} (30 μM). The desensitization kinetics did not change significantly when Zn^{2+} (30 μM) was applied to the bath (see Figure 4-10A,B and Table 4-1). Also, I have measured τ_d of GABA-activated currents at different holding potentials. In control condition or during Zn^{2+} superfusion τ_d remained constant (see Figure 4-9A), suggesting that the process of desensitization of the $GABA_A$-receptors is voltage-insensitive.

GABA[M]	τ_d (Control) (s)	τ_d (Zn^{2+}) (s)
1	5.92 ± 1.46	5.80 ± 1.23
300	0.52 ± 0.32	0.50 ± 0.31

Table 4-1. Desensitization time constants (τ_d) calculated for two GABA concentrations (1 and 300 μM) in control condition (n=10) and in the presence of Zn^{2+} (n=7). Holding potential -50 mV. Zn^{2+} (30 μM) does not change τ_d significantly, either at 1 μM ($P < 0.10$) or at 300 μM ($0.05 < P < 0.10$).

Similarly to the process of desensitization, Zn^{2+} did not affect the process of recovery from desensitization. In the presence of Zn^{2+} (30 μM) recovery time constant was 35.5 ± 0.9 s (n=4, Figure 4-10C) suggesting that Zn^{2+} does not influence the recovery of $GABA_A$-receptor from desensitization.

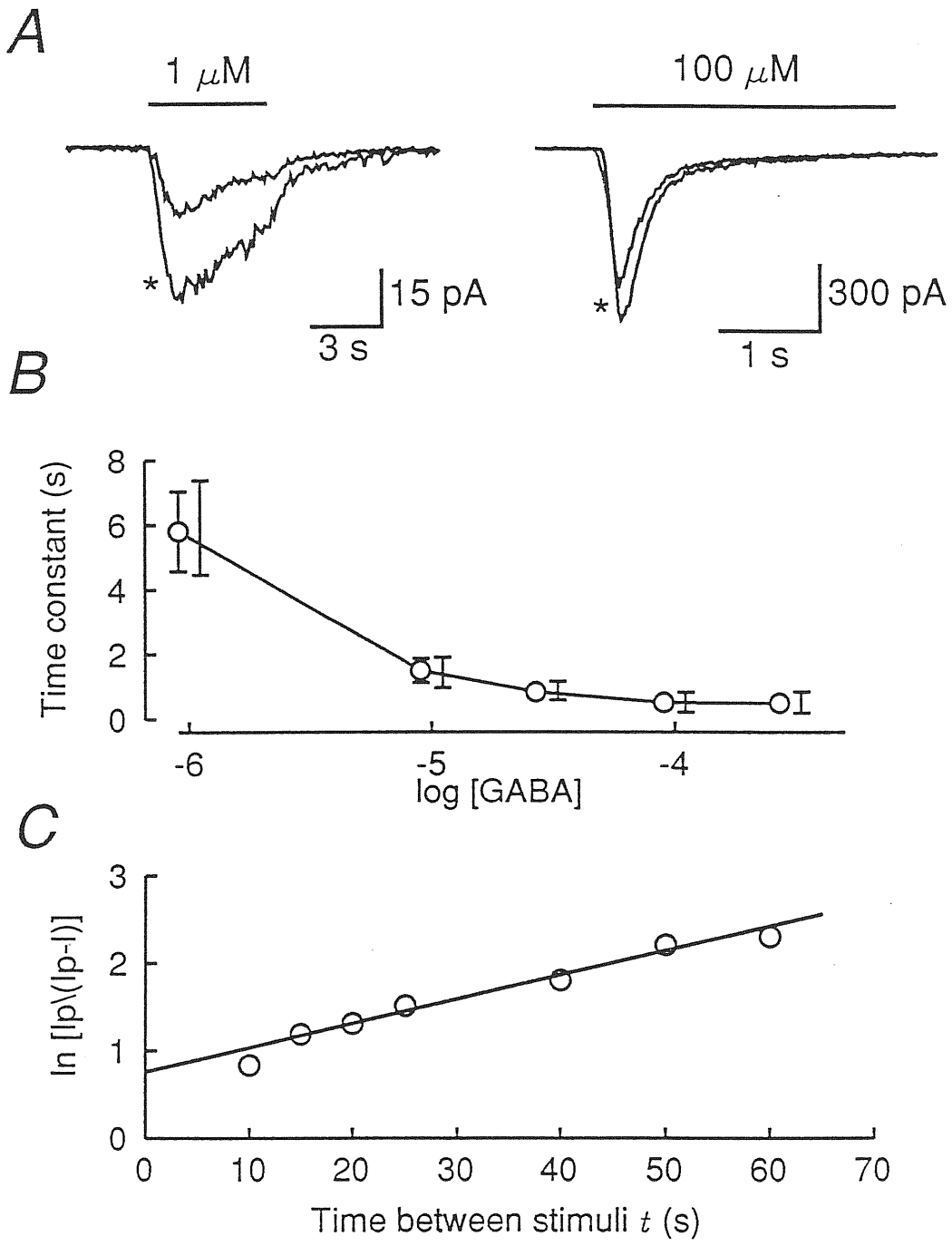


Fig 4-10. (A) Superimposed whole-cell currents activated by 1 or 100 μM GABA in control condition (*) and or the presence of 30 μM Zn^{2+}. Holding potential -50 mV. (B) Time constants τ_d are plotted as a function of GABA concentration in the presence of Zn^{2+} (open circle) and for comparison the values in control are indicated by bars. Notice that Zn^{2+} does not change τ_d.

Each point is the mean of 8 experiments. Bars represent SD. (C) Fitting of recovery time constant in the presence of 30 μM Zn^{2+} (open circle) and straight line is obtained from the fit of control values. Zn^{2+} does not affect the process of recovery from desensitization of $GABA_A$-receptors.

Sensitivity to Zn^{2+} during DIC

Zn^{2+} blocks GABA responses and this effect depends on the stage of neuronal development, being more pronounced in embryonic and young neurones than in adult ones (Smart and Constanti 1990; Smart 1992). Thus, I have performed additional experiments to study the possible developmental changes in the sensitivity of GABA-activated whole-cell currents to Zn^{2+} inhibition during the period from 1 to 15 DIC. The sensitivity of GABA currents did not change during DIC. During the first days (1-4 DIC) the amplitudes of whole-cell currents evoked by GABA (10 μM) were reduced by Zn^{2+} (30 μM) to 57 ± 10 % (n=12). From day 5 to 15 the amplitudes of GABA (10 μM) currents were reduced by Zn^{2+} (30 μM) to 59 ± 14 % (n=27). It was concluded that GABA currents do not show any variation in sensitivity to Zn^{2+} during different DIC.

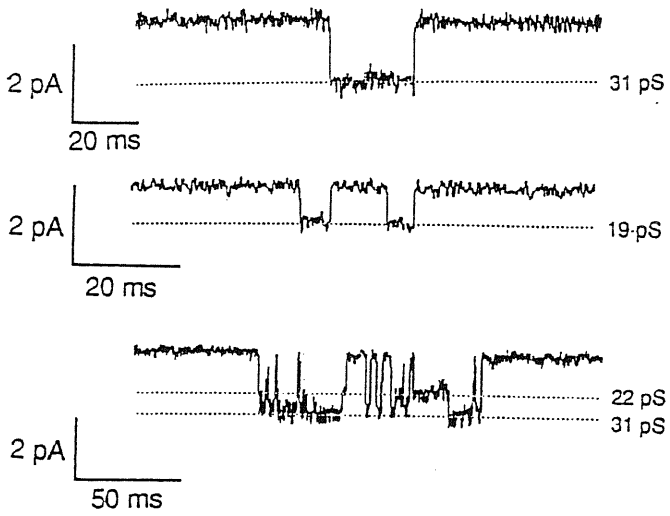
Chapter 5

GABA-activated single-channel Currents

Conductances

The microscopic properties of $GABA_A$-activated currents were studied in excised patches. Single-channel events were recorded in outside-out patches which contained a few channel units. It is known that GABA activates two main conductance states in many neurone preparations (Bormann and Clapham 1985; Bormann and Kettenmann 1988; MacDonald et al. 1989). In cultured cerebellar granule cells the application of GABA (0.5 μM) activated single-channel currents with two conductance states of 19 and 31 pS (Figure 5-1A). Occasionally, a subconductance state of 22 pS was also found (see Figure 5-1A). The most frequently occurring conductance state was 31 pS, observed as the highest peak in the amplitude histogram (Figure 5-1B).

A



B

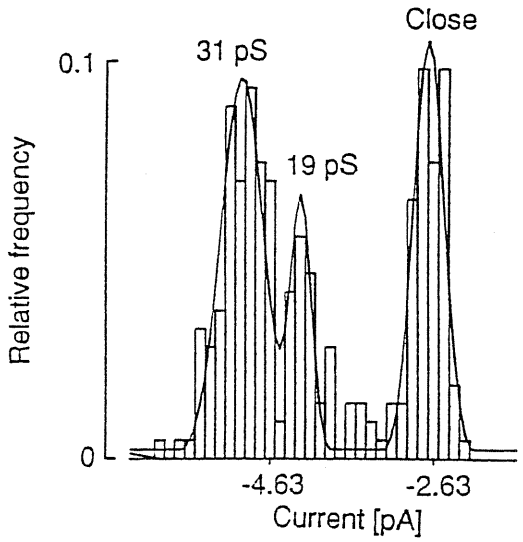


Fig 5-1. (A) Single-channel currents activated by 0.5 μM GABA in outside-out patches of cultured cerebellar granule cells. Two conductance states of 19 and 31 pS are observed. Holding potential -50 mV. The bottom trace

shows a subconductance level of 22 pS at holding potential -70 mV. All records are from the same cell. Band width is 1 kHz. (B) Amplitude histogram of GABA single-channel events at holding potential of -50 mV. Notice that 31 pS state is the most frequent. The histograms are fitted with 3 gaussian distributions.

The amplitudes of all conductance levels were measured at different holding potentials. Similarly to whole-cell currents the linear I-V relationship persisted for all conductance levels (Figure 5-2). This finding suggest that the pore of $GABA_A$-receptor channel does not change the permeability to Cl^- by a voltage. Also, the reversal potentials were in the same range as for whole-cell currents (see Figure 5-2).

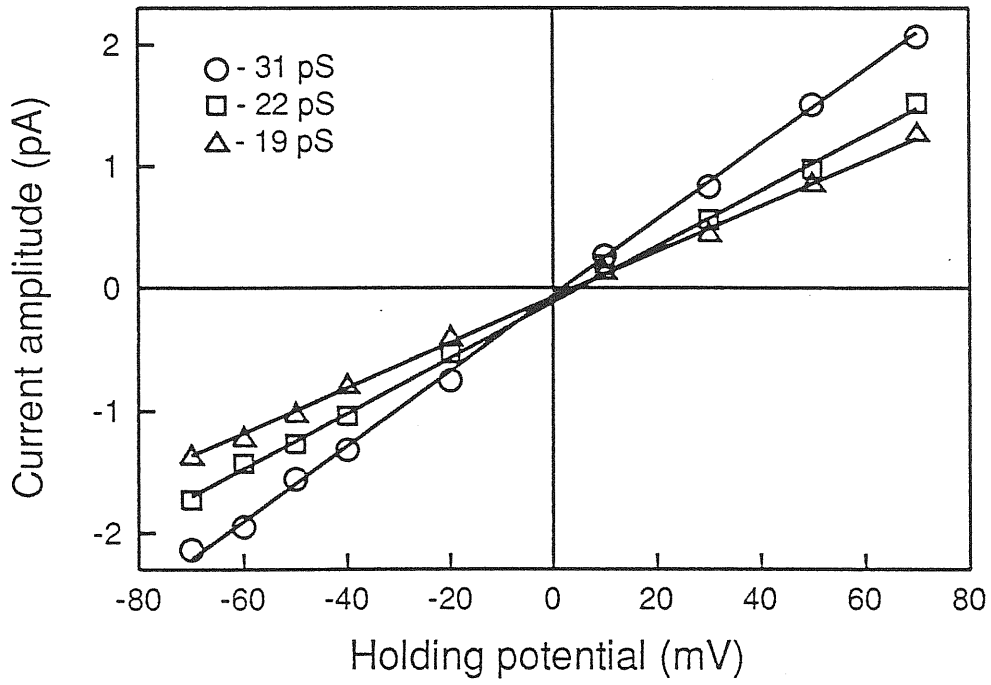


Fig 5-2. Mean amplitudes of single-channel currents are shown as a function of holding potential. Two main conductance states are denoted with triangles and circles whereas the subconductance state with squares. All measurements

are made from the same cell. Notice that the linear dependence holds for all conductance states. Reversal potentials are 3.78, 1.78 and 4.92 mV for 19, 31 and 22 pS, respectively.

When Zn^{2+} (10 μM) was co-applied with GABA (0.5 μM), the single-channel conductances and the subconductance level did not change. Further analysis was made by measuring the probability of opening in control or in the presence of Zn^{2+}. In the presence of Zn^{2+} the probability of opening decreased approximately three times (see Table 5-1 and Figure 5-3). These findings suggest that the effect of Zn^{2+} on whole-cell currents could be interpreted as a reduction in the probability of opening without any change in single-channel conductances of GABA-activated currents.

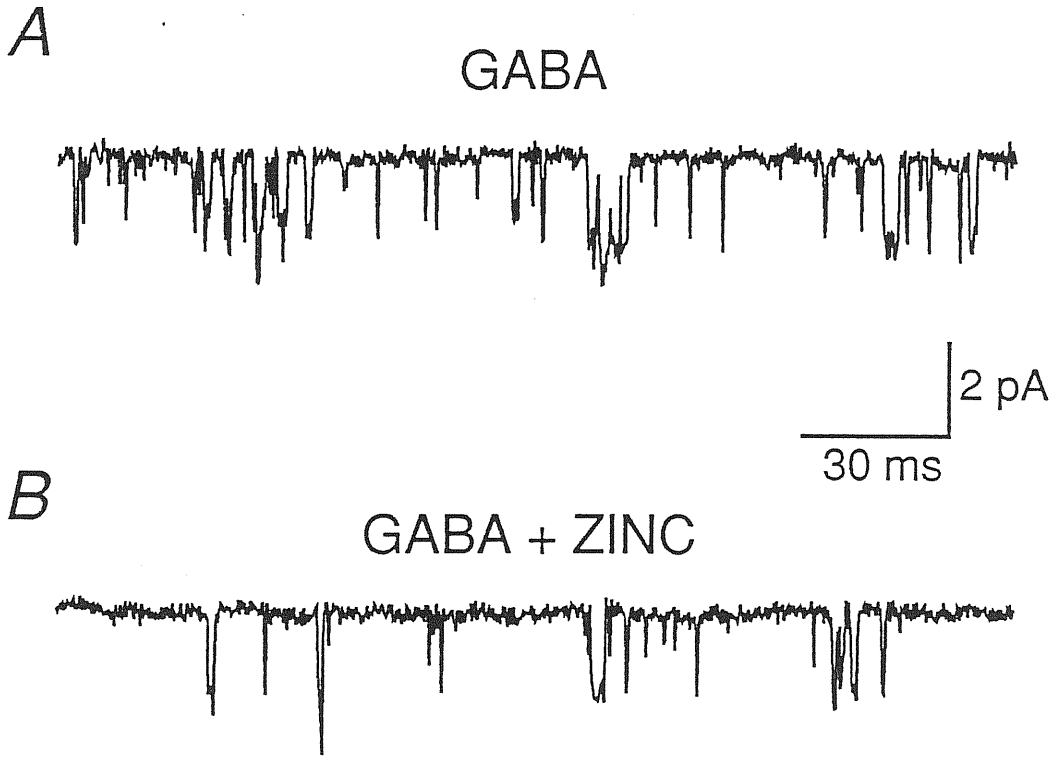


Fig 5-3. Current records obtained at holding potential of -70 mV showing single-channel events activated by GABA (0.5 μM) (A) and by the co-application of the same concentration of GABA and 10 μM Zn^{2+}. (B) The probability of opening is 0.21 in A and 0.09 in B. Zn^{2+} markedly reduces the probability of opening. Band width is 1 kHz.

Kinetic analysis

An investigation of time kinetics provided information about the molecular mechanism of the channel, through a description of the channel kinetics of conformational changes at the microscopic level. Further information about the action of Zn^{2+} at the single-channel level can be obtained by comparing the kinetics of channel in control condition and in the presence of Zn^{2+}. The time analysis of GABA-activated channels was done exclusively for the two

conductance states of 19 and 31 pS. The subconductance level of 22 pS, which occurred rarely was excluded from the present analysis. The close time histogram revealed two populations of dwell times with different time constants (see Figure 5-4A and Table 5-1). This is in agreement with the finding that GABA single channels occurred in the bursts as it is evident from the record shown in Figure 5-1A.

	Control	Zn^{2+} (10 M)
Open τ_o (ms)	3.64 ± 1.73 (7)	3.22 ± 1.51 (5)
area	1	1
Closed τ_{c1} (ms)	2.70 ± 0.76 (7)	2.53 ± 0.65 (5)
area	0.41 ± 0.09	0.32 ± 0.07
τ_{c2} (ms)	205 ± 8 (7)	209 ± 10 (5)
area	0.57 ± 0.11	0.69 ± 0.10
Np	0.17 ± 0.06 (6)	0.06 ± 0.04 (4)

Table 5-1. Open (τ_o) and closed (τ_{c1}, τ_{c2}) time constants of single-channel currents activated by GABA (0.5 μM) at holding potential of -50 mV. The areas under the fitted curves are normalized to 1. Below each time constant value the corresponding relative area contributing to the histograms is shown. The number of patches are indicated in parenthesis. Notice that co-application of Zn^{2+} does not affect these time constants but it significantly decreases the probability of opening *Np* ($P < 0.025$). Also it decreases the relative area of the shorther closed time τ_{c1} ($0.05 < P < 0.10$) and consequently increases the relative area of the longer closed time τ_{c2} ($P < 0.05$).

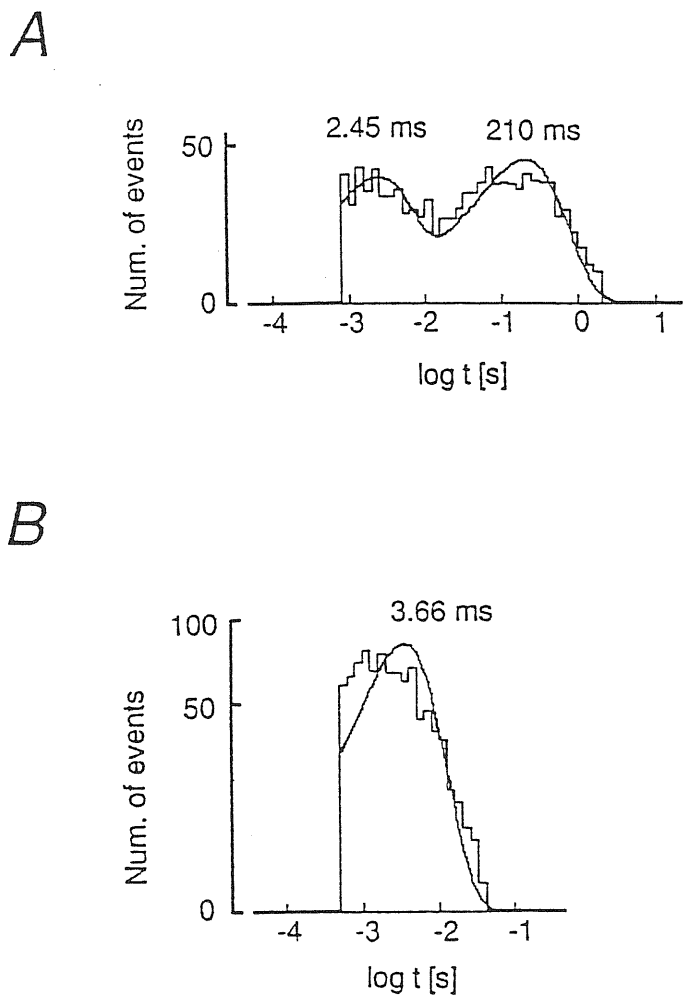


Fig 5-4. Closed (A) and open (B) time histograms. Two mean closed and one open times are used to fit these histograms. The histograms are displayed with a square-root transformed ordinates versus log transformed abscissa.

Open time histograms were fitted with a single exponential time constant (see Figure 5-4B and Table 5-1). The time constants in control or in the presence of Zn^{2+} are shown in Table 5-1. From Table 5-1 it is clear that Zn^{2+} does not change single-channel kinetics of $GABA_A$-receptors in cerebellar granule cells. Since the probability of opening *Np* decreased in the presence of Zn^{2+}, I have compared the relative contributions of the closed time

constants to histograms obtained in control condition and during Zn^{2+} co-application. Zn^{2+} (10 μM) decreased the relative area of the shorter closed time τ_{c1} as a consequence of the reduced number of openings (see Table 5-1). Furthermore, I found that the mean open and closed times calculated in the presence or abscence of Zn^{2+} were independent of holding potential.

Run-down of GABA currents

It has been suggested that the $GABA_A$-receptor channel needs to be phosphorylated in order to be functional (Stelzer et al. 1988). Although ATP and Mg^{2+} were included in the pipette solution, a substantial run-down of GABA-activated currents was observed after some time. For single-channel recording this period was about 20-30 min whereas it was about 90 min for the whole-cell configuration.

Chapter 6

Discussion

The basic findings of this study are: i.) cerebellar granule cells in culture exhibited $GABA_A$-mediated responses which were depressed by Zn^{2+} in a reversible manner. ii.) the inhibitory effect of Zn^{2+} on GABA currents was apparently due to the decrease in the open probability of single-channel events.

GABA currents

The class of neurones, namely the cerebellar granule cells, investigated here was found to posses $GABA_A$-receptors as shown by the ability of $GABA_A$ antagonist bicuculline to block GABA-activated currents. This is in agreement with previous studies on the same and different type of cerebellar neurones (Cull-Candy and Ogden 1985; Cull-Candy and Usowicz 1989; Huck and Lux 1987; Vicini et al. 1986). Further evidence in favour of existence of $GABA_A$-receptors is provided by immunohistochemical studies which have demonstrated the presence of $GABA_A$ immunoreactivity in the granule cell layer of the cerebellum (Taguchi et al. 1989).

GABA-activated currents were potentiated by midazolam. The same effect has been observed by Vicini and co-workers (1986) in the indentical preparation. Taking into account that the γ2 subunit is very likely indispensable for the positive modulation of benzodiazepines (Pritchett et al. 1989), and the fact that γ2 subunit is already present in cerebellum after the first postnatal week (Möhler et al. 1989) it seems that BZR located on γ2 subunit in cerebellar granule cells is already functional at that stage.

In whole-cell configuration the I-V relation of GABA currents observed in cerebellar granule cells was linear over the potential range between -70 and +50 mV. This finding is in contrast to other neuronal preparations in which GABA currents show a marked outward rectification (Bormann et al. 1987; Legendre and Westbrook 1991; Smart and Constanti 1990). Futhermore, the presence of the γ2 subunit might have conferred this linearity, since $GABA_A$-receptors reconstituted only from α1 and β2 subunits display a characteristic outward rectification (Verdoorn et al. 1990). The linearity of GABA currents persisted on the single-channel level in outside-out configuration. Same behaviour has been found in other neuronal preparations (Bormann and Clapham 1985; Bormann et al. 1987) as well for reconstituted $GABA_A$-channels (Puia et al. 1990; Verdoorn et al. 1990). In conclusion, the linear current to voltage relationship at microscopic or macroscopic levels when the large number of identical channels are involved in making of total current, indicates a voltage insensitivity of gating of $GABA_A$-receptor channels.

GABA responses showed marked desensitization which was concentration dependent. The desensitization was already apparent at low

concentrations of GABA (1 μM). For high GABA concentrations (30-300 μM) the current desensitized very rapidly. In this respect, the cerebellar granule cells in culture behaved like hippocampal (Legendre and Westbrook 1991), or sympathetic neurones (Newland et al. 1991), or reconstituted $GABA_A$-channels (Verdoorn et al. 1990). However, in comparison with other neuronal preparations the same GABA concentration produced a much faster desensitization in cerebellar granule cells. A different subunit composition of the native $GABA_A$-receptor may account for this dissimilarity since different subunit combinations when expressed in *Xenopus* oocytes displayed a variable time course of desensitization (Verdoorn et al. 1990). The same argument may also underlie the different recovery time from the desensitized state found in cerebellar granule cells as compared with other cells (Bormann and Clapham 1985). It should be noted that the onset of desensitization τ_d observed here is much faster than the recovery τ_r. This suggest that the desensitization state of $GABA_A$-receptor in the presence of agonist may have a characteristic of an absorbent state. In another words, once $GABA_A$-receptor enters into desensitized state it is very unlikely that a backward transition will occur. Furthermore, the transition from open state to desensitized state is very likely voltage independent since τ_d was not found to depend on the holding potential. Conversely, in cultured hippocampal neurones the desensitization was found to decrease with depolarization (Oh and Dichter 1992). Again, a different subunit combination in different preparation may explain this discrepancy.

GABA activated single-channel events of two main conductance states and rarely single subconductance state. Similar values have been found by

Huck and Lux (1987), Bormann and Kettenmann (1988) and Macdonald et al. (1989). It is interesting that the occurrence of different conductances found here are very similar to those obtained from the reconstituted $\alpha 1\gamma 2$ subunit composition (Verdoorn et al. 1990). This again confirms the hypothesis that $\gamma 2$ subunit is present in cerebellar granule cells in culture. A slightly smaller estimate for single channel conductance was obtained by Cull-Candy and Usowicz (1989) in large cerebellar neurones, using noise analysis of the whole-cell currents.

Kinetic model of $GABA_A$-receptor channel

The time analysis on single-channel level yielded one open τ_o and two closed time constants (τ_{c1}, τ_{c2}). On the macroscopic level the time constants of desenstization τ_d and recovery τ_r have been also measured. These data provide an evidence that $GABA_A$-receptor channel in cultured cerebelar granule cells can exist in several open and closed conformations.

In the absence of GABA, the channel lives in closed nonactivated state. It is possible that there are more than one nonactivated states. The transitions among them can occur so fast that they are not detectable with the patch-clamp method. Therefore, a general closed nonactivated state is denoted with C_0. When GABA is applied, an activation of the receptor triggers transitions to activable states.

The simpliest scheme would be:

$$C_0 \underset{\beta}{\overset{\text{GABA}\ \alpha}{\rightleftharpoons}} O \qquad (6\text{-}1)$$

where O is open activated state, α and β are the rate constants. Because of very strong desensitization, an accurate study of single-channel currents activated by high concentration of GABA was not feasible here. Nevertheless, it is probable that high GABA concentrations do not change the mean open time (Newland et al. 1991). This is the reason to introduce a closed activated state C_1 between the nonactivated state C_0 and activated open state O, therefore simulating the fast transitions which do not depend on agonist concentration. A general scheme for activation of $GABA_A$-receptor could be:

$$C_0 \overset{\text{GABA}}{\rightleftharpoons} C_1 \rightleftharpoons \cdots \rightleftharpoons C_n \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \qquad (6\text{-}2)$$

where C_n are closed activated states. A number of the closed states is related to a number of the mean closed times obtained from the kinetic analysis of single-channel records. It seems that $GABA_A$-receptor channels in the granule cells have at least two closed states. In order to simplify the reaction the following assumptions can be made: a) the binding and unbinding steps

($C_0 \leftrightarrow C_1$) are too fast to be dected; b) all possible states between C_1 and C_{n-1} should be reduced to a single state C_2. Therefore, the scheme (6-2) can be written as follows:

$$C_0 \overset{\text{GABA}}{\rightleftharpoons} C_1 \underset{\mu}{\overset{\lambda}{\rightleftharpoons}} C_2 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \qquad \textbf{(6-3)}$$

where λ and μ are the rate constants.

During prolonged application of GABA, the probability of opening of single-channel unit decreases. The receptor tends to go in the closed desensitized state (D). Another property of $GABA_A$-receptor is that after removal of GABA, the receptor needs some time to recover *i.e.* to be activable again. Taking together these results a simple scheme for desensitization of the receptor may look like:

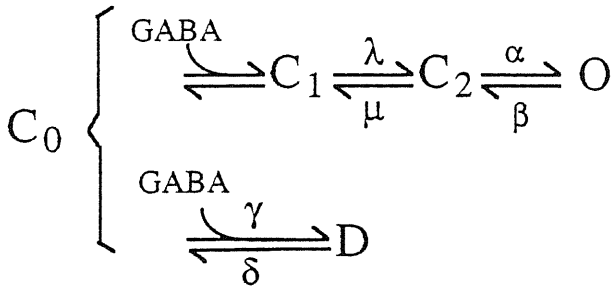
$$O \underset{\delta}{\overset{\gamma}{\rightleftharpoons}} D \qquad \textbf{(6-4)}$$

where all states are defined previously, γ and δ are the rate constants for desensitization and recovery, respectively. The rate of desensitization is much higher than the rate of recovery implying that this reaction moves an equilibrium to the right side. On the other hand, the transition rate to desensitized state depends very much on the concentration of GABA, as it

is evident from the fact that τ_d is a function of GABA concentration. Therefore, I argue the possibility of existence of specific region of $GABA_A$-receptor being responsible for desensitization. Whether it is the same GABA binding site or distinct region of $GABA_A$-receptor channel protein which interacts with GABA binding site via a cooperative effect, it feels a change in GABA concentration and accordingly changes the probability of opening of the single channel unit *p*. This change as a final result has a different time course of whole-cell currents. In agreement with this idea, Bormann and Clapham (1985) suggest that two GABA molecules bind to unique site on the receptor according to rather different kinetic scheme. Similar model with the single agonist binding site that binds two molecules of GABA has been also proposed by MacDonald et al. (1989). However, both authors did not try to explain the desensitization in terms of of kinetic states. In order to explain a concentration dependent desensitization, two kinetic schemes can be proposed:

$$C_0 \overset{\text{GABA}}{\rightleftharpoons} C_1 \underset{\mu}{\overset{\lambda}{\rightleftharpoons}} C_2 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \overset{\textit{GABA?}}{\underset{\delta}{\overset{\gamma}{\rightleftharpoons}}} D \qquad \text{(6-5)}$$

or similar one



(6-6)

where states and rate constants are defined previously. The mean lifetime of states are defined as:

$$\tau_o = \frac{1}{\beta+\gamma} \qquad \tau_{c1} = \frac{1}{\lambda} \qquad \tau_{c2} = \frac{1}{\alpha+\mu} \qquad \tau_d = \frac{1}{\gamma} \qquad \tau_r = \frac{1}{\delta} \tag{6-7}$$

for reaction (6-5), whereas for the reaction (6-6) the lifetimes are the same except $\tau_o = 1/\beta$. In the present study the time constant of desensitization τ_d is much grater than the mean open time τ_o. Therefore, it follows that $\beta >> \gamma$, and consequently for the reaction (6-5) τ_o is approximately equal $1/\beta$. Furthermore, it is possible to imagine a subset of the states C_2 and O. This subset for the low GABA concentrations would have the lifetime about $1/\mu$, considering that $\gamma << \mu$. This value may correspond to the duration of a burst. For high concentrations of GABA the desensitization rate γ increases and may reach the same range of values of λ. The γ probably never exceeds the value of λ *i.e.* it is not possible to observe the desensitization constant

τ_d shorter than the mean closed time τ_{c1}. This is true, however, only if the duration of GABA application is long enough to resolve these transitions on microscopic and macroscopic levels. In the reaction (6-5) the receptor must be activated before it goes into desensitized state. As indicated this transition could be accomplished by an interaction between activated receptor and GABA molecule. The question mark stands for an unknown mechanism of modulation of the desensitization by GABA. A right side of reaction should be favoured in the presence of GABA. When GABA is removed the receptor slowly goes back to closed state C_0 with rate $1/\tau_r$.

In the reaction (6-6) the receptor goes directly to desensitized state D without previous passage to the open state. The transition to desensitized state is also concentration dependent as indicated. During action of GABA the number of receptors that can go in the open state O decreases with an increasing GABA concentration thus simulating the whole-cell current decay. In contrast to the reaction (6-5), the receptor may as well pass directly to the closed nonactivated state C_0 from where it can be activated again. Both reactions represents working model which is likely to be incomplete. It has been shown that models containing multiple open and closed states may be equally well described by a number of indistinguishable kinetic schemes (Blatz and Magleby 1986).

Effect of Zn^{2+}

Data presented here show that GABA response can be antagonized by Zn^{2+} in a noncompetitive manner. The inhibitory effect of Zn^{2+} on GABA-mediated responses has been already reported using other neuronal

preparations (Celentano et al. 1991; Legendre and Westbrook 1991; Mayer and Vyklicky 1989; Smart et al. 1991; Smart 1992; Westbrook and Mayer 1987).

Zn^{2+} does not change single-channel conductances and linear I-V relationship of GABA currents as expected from the results obtained in different cultures (Legendre and Westbrook 1991; Smart 1992). Also, Zn^{2+} does not change the linear dependence of whole-cell GABA currents on membrane potential. Hence, the possibility that Zn^{2+} blocked the pore of $GABA_A$-channels is unlikely since the block of ion channels by cations is often voltage-dependent. It should be noted that in the present study no evidence was obtained concerning the speed of the blocking effect of Zn^{2+}. Therefore, previous statement is valid only if Zn^{2+} blocked GABA-activated currents on the time scale which was faster than the administration of Zn^{2+} in the bath.

As expected from other studies (Celentano et al. 1991; Legendre and Westbrook 1991; Smart 1992), in my preparation Zn^{2+} blocked GABA currents in a noncompetitive way without significantly changing the Hill coefficient and half-maximum concentration. Therefore, it seems likely that Zn^{2+} binds to a site different from that of GABA without a change in the stoichiometry and affinity of GABA binding. The dose-response of Zn^{2+} with a constant GABA concentration shed more light on the interaction between Zn^{2+} and $GABA_A$-receptor. Zn^{2+} blocked GABA-evoked currents in a dose-dependent way with the IC_{50} of 57.20 μM. The IC_{50} for Zn^{2+} inhibition of GABA currents induced by the activation of reconstituted $GABA_A$-receptors lacking $\gamma 2$ subunit was much lower comparing with the values obtained

here. For the combination of $\alpha 1\beta 1$ subunits the IC_{50} was 1.5 μM (Smart et al. 1991) whereas for the combination of $\alpha 1\beta 2$ the IC_{50} was 0.56 μM (Draguhn et al. 1990). It has been suggested that the sensitivity of GABA response depends on the presence of $\gamma 2$ subunit (Draguhn et al. 1990). The low sensitivity to Zn^{2+} inhibition observed here is in agreement with midazolam potentiation, and probably reflects the presence of the $\gamma 2$ subunit in cerebellar granule cells in culture.

As already reported for hippocampal neurones (Legendre and Westbrook 1991), Zn^{2+} did not modify τ_d suggesting that the process which is responsible for desensitization of the $GABA_A$-receptor is not influenced by extracellular Zn^{2+}. Like τ_d, τ_r was also unaltered by Zn^{2+}. Possible explanation could be that Zn^{2+} prevents activation of receptors and those that are activated behave in a manner identical to that seen in the absence of Zn^{2+}.

Only one exponential component was required to describe the frequency histogram for all open times, whereas the mean close time distribution was fitted by the sum of two exponentials, suggesting the existence of at least two closed states. In the present study, the mean open and closed times were not significantly changed by Zn^{2+} (Legendre and Westbrook 1991; Smart 1992). On the other hand, Smart (1992) has found an additional closed time as a consequence of the decreased probability of opening in the presence of Zn^{2+}. Although in cerebellar granule cells, the probability of opening decreased to about 30% of control during Zn^{2+} co-application, the closed times were not significantly changed. In this condition it seems that the number of single-channel events within a group of openings reduces, thus

decreasing the relative area of the shorter closed time constant τ_{c1} in the histogram. This effect could be also explained by the shortening of the burst duration and accompanying reduction of the number of openings per burst as observed by Smart (1992). The present finding together with invariable whole-cell kinetics in the presence of Zn^{2+} suggest that this divalent cation probably deactivates $GABA_A$-receptor channels, and it has no effect on these channels while are activated by GABA.

The kinetic model described in previous section still holds in the presence of Zn^{2+} or upon the change of the membrane potential. In conclusion, $GABA_A$-currents in cerebellar granule cells in culture were inhibited by Zn^{2+} in a noncompetitive and voltage-independent way suggesting that Zn^{2+} binds to a site which is distinct from that of GABA. This binding site could be located on the extracellular side of the receptor-channel protein as suggested by Smart (1992).

Molecular model of $GABA_A$-receptor channel

The granule cells responded to GABA in a dose-dependent manner. Here, because of the inevitable desensitization of GABA whole-cell currents, the Hill coefficient can probably provide only a lower limit for the stoichiometry of GABA binding. The functional properties of many different subunit compositions have been studied by expressing the corresponding mRNA combination in *Xenopus* oocytes (Sigel et al. 1990). Although it cannot be excluded that *Xenopus* oocyte carries out biosynthesis of the $GABA_A$-receptor channels in a slightly different manner than rat neurones, a comparison of Hill coefficients and half-maximum concentrations with

present results suggests the possibility for existence of at least two different subunit combinations in cultured cerebellar neurones. A Hill coefficient value of 1.5 corresponds to the combination of $\alpha1\alpha3\alpha5\beta2\gamma2$ subunits whereas a value of 1 corresponds to the combination of $\alpha1\beta1\gamma2$. The first combination containing five different subunits may exist with stoichiometry one to one for all five subunits, since $GABA_A$-receptor channel is a pentamer (Stephenson 1988). The stoichiometry of the second combination containing only three different subunits can be only speculated. Both combinations contain $\alpha5$ subunit. Recently two independent works appeared, one claiming the absence of $\alpha5$ subunit in the granule layer of rat cerebellum (Laurie et al. 1992) and other one claiming the existence of the same subunit (Bovolin et al. 1992). Moreover, Bovolin and co-workers (1992) identified the $\alpha5$ subunit in rat cerebellar granule cells in culture which is in accordance with my hypothesis. Nevertheless, both combinations contain the $\gamma2$ subunit which confers the sensitivity of GABA to BZ (Pritchett et al. 1989). This is in agreement with the positive modulation of GABA currents by midazolam observed in my experiments, and the presence of high concentration of the $\gamma2$ subunit found in young rat cerebellum by *in situ* hybridization (Malherbe et al. 1990; Shivers et al. 1989).

Two groups of cells having different Hill coefficient exhibited the same desensitization time constant τ_d. If two different subunit combinations explain different stoichiometry of GABA binding it is probable that a region of $GABA_A$-receptor responsible for desensitization process interacts with GABA in the same manner in both combinations.

Development

The development of individual neurones may be generally divided into three phases: the proliferation, the migration and the differentiation, during which they acquire their neuronal phenotype and establish interneuronal contacts. The neurones require specific thropic factors during defined developmental time windows to promote their viability. In addition to the neurotrophic factors such as the nerve-growth factor and related peptides, the neurotransmitters released from nerve terminals may also have trophic properties. Developmental processes such as creation and continuous adaptation of neuronal circuits in the mammalian brain need two complementary mechanisms for regulation. It is very likely that these mechanisms operate through the activation of EAA and IAA receptors (Meier et al. 1991).

Exogenously applied NMDA promotes cell survival in cerebellar granule cell cultures in a dose dependent manner (Balazs et al. 1988). The trophic effect of NMDA is dependent both on the degree of membrane depolarization and the developmental stage of the neurones. Ca^{2+} entry through NMDA-receptor associated channels, and/or voltage-sensitive Ca^{2+} channels (produced by depolarization in 25 mM K^+-containing medium) probably underlines the trophic effect of NMDA on granule cells, since the neuronal survival is dependent on the concentration of extracellular Ca^{2+}. Also, it has been found that EAAs regulate axonal neurite outgrowth in some neurones (Mattson et al. 1988a,b). When entorhinal neurones are co-cultured with dissociated hippocampal pyramidal cells, spontaneously released glutamate from the entorhinal neurones reduces dendritic growth in

the pyramidal neurones of hippocampal cells (Mattson et al. 1988b). In cerebellar granule cells we have found that spontaneously released transmitter mainly activates NMDA receptors (Kilić 1990; Kilić et al. 1991). This spontaneous activity may inhibit dendritic growth in the granule cell cultures thus stabilizing the synaptic connections and autoregulating the neurone survival. In general, it seems that moderate and subtoxic doses of EAAs that activate NMDA receptors, reduce a dendritic growth and stabilize the synaptic contacts, whereas high doses of these transmitters produce an overactivation of NMDA receptors which yield neuronal death.

An inhibitory amino acid GABA has complementary properties of EAAs. GABA inhibits a neuronal firing and promotes a neuronal development while glutamate hinders the development of neurones. Exposure of cultured neuroblastoma cells (Spoerri and Wolf 1981) and cultured cerebellar granule cells (Meier and Schousboe 1982) to GABA agonist during their development induces an increase in density of neurotubules, Golgi apparatus, vesicles and rough endoplasmic reticulum, whereas others, such as mitochondria and smoth endoplasmic reticulum are uneffected. The trophic effect of GABA in granule cells in culture is blocked by $GABA_A$ antagonist bicuculline suggesting that the trophic modulation is mediated by Cl^- ions (Meier 1988). Furthermore, the application of GABA to the culture of cerebellar granule cells was found to cause a small depolarization, a large conductance increase, and a persistent increase in the concentration of intracellular $[Ca^{2+}]_i$ that lasted for many minutes (Connor et al. 1987). Similarly, in the cortical slices GABA application induces depolarization and an increase in $[Ca^{2+}]_i$ (Yuste and Katz 1991). It seems

that this increase is mediated via voltage-gated Ca^{2+} channels. A mechanism by which the currents activated by GABA induce the trophic effect on neurones is still unknown.

Spontaneous inhibitory currents probably due to the spontaneous release of GABA has been found in cultured cerebellar neurones (Cull-Candy and Usowicz 1989). This spontaneous inhibition may represent a developmental signal for the cells in culture. It seems that the granule cell's maturation in culture does not affect the sensitivity of $GABA_A$-receptor to Zn^{2+}, since during the entire period of observation (from 1 DIC to 15 DIC) the granule cells maintain the same sensitivity to Zn^{2+}. This is not the case for sympathetic neurones in which the sensitivity to Zn^{2+} decreases with days in culture (Smart 1992). The sensitivity of GABA responses to Zn^{2+} appears to be more pronounced in embryonic rather than in adult neurones (Smart et al. 1991) and seems to be inversely related to the expression of the $\gamma 2$ subunit (Draguhn et al. 1990). Therefore, it is resonable to propose that the $\gamma 2$ subunit present after first postnatal week in the cerebellar granule layer (Malherbe et al. 1990) persists in cultured the cerebellar granule cells and remains functional during cell's maturation in culture.

Conclusions

1.) In cerebellar granule cells in culture GABA activates the $GABA_A$-receptor channels. These channels are blocked by bicuculline and enhanced by benzodiazepines.

2.) The current-voltage relations of whole-cell and single-channel GABA currents display linearity in the range from -70 to +50 mV. Macroscopic currents activated by GABA show a concentration dependent desensitization.

3.) The half-maximum concentration *K* for GABA binding is around 21 μM. Two populations of cells with different Hill coefficients are found. One type has *n* around 1.5 whereas the other has *n* around 1.

4.) Kinetic analysis of the single-channel and the whole-cell currents suggests that $GABA_A$-receptor can exist in several open and closed conformations. Two independent kinetic schemes are proposed to explain the present results including the dose-dependent desensitization.

5.) An extracellular Zn^{2+} blocks GABA currents in a reversible and noncompetitive manner. Zn^{2+} does not change Hill coefficient *n* or half-maximum concentration *K*.

6.) The processes of desensitization and recovery from desensitization are not modulated by Zn^{2+}. Zn^{2+} does not change single-channel conductances, subconductance state, mean open and closed states, but it reduces the probability of opening.

7.) Linear current-voltage relationship persists in the presence of Zn^{2+} indicating that this divalent cation very likely does not block the channel pore.

8.) It is suggested that Zn^{2+} binds to an extracellular region of the $GABA_A$-receptor channel complex. This action simply deactivates the receptor which cannot be activated by GABA.

9.) The $GABA_A$-receptor channels maintain the same sensitivity to Zn^{2+} inhibition during different DIC.

10.) The properties of GABA-activated currents found here, when compared with those found in reconstituted channels suggest that a minimal requirement could be satisfied with two subunit combinations. They are $\alpha1\alpha3\alpha5\beta2\gamma2$ and $\alpha1\beta1\gamma2$.

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