ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

SPONTANEOUS ACTIVITY OF NEURONS IN CULTURE

Thesis Submitted for the Degree of
Magister Philosophiae

Candidate:
Gordan Kilić

Supervisors:
Dr. Oscar Moran
Prof. Antonino Borsellino

Academic Year 1989/90

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Chapter 1: Introduction

One among the most developed system of a single biological organism is the nervous system. Neurons, or nervous cells, are building blocks of the brain, which is the central organ of the nervous system. They have the same genes and the same biochemical apparatus as other cells, but they also have unique features that make the brain function in a very different manner from, for example, the kidney. The important feature of a neuron, due to its membrane excitable properties, is its capability of generating nerve impulses, transmitting, processing and receiving signals.

The human brain is thought to consist of 10^{11} neurons. Their forms generally fall into only a few broad categories, and most neurons share certain structural features that make it possible to distinguish three regions of the cell: the cell body, the dendrites and the axon, as shown in Fig. 1.1.

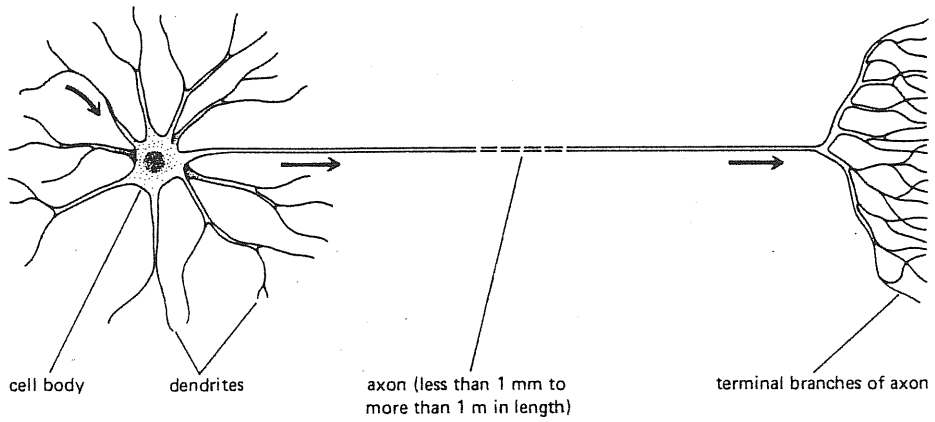

Fig. 1.1 Schematic diagram of a typical neurone of a vertebrate. The arrows indicate the direction in which signals are conveyed (from Watson 1989).

The cell body contains the nucleus of the neuron and the biochemical machinery for synthesizing enzymes and other molecules essential to the life of the cell. Usually,

the cell body is roughly spherical or pyramid–shaped. The dendrites are delicate tube like extensions that tend to branch repeatedly and form a bushy tree around the cell body. They provide the main physical surface on which the neuron receives incoming signals. The axon extends away from the cell body for long distances to other parts of the nervous system.

The axon differs from the dendrites both in structure and in the properties of its membrane. Most axons are longer and thiner than dendrites and exhibit a different branching pattern: whereas the branches of dendrites tend to cluster near the cell body, the branches of the axon tend to arise at the end of a fiber where the axon communicates with other neurons.

Although neurons are the building blocks of the brain, they are not the only kind of cell in it. For example, oxygen and nutrients are supplied by a dense network of blood vessels. There is also a need for connective tissue, particularly at the surface of the brain. A major class of cells in the central nervous system is the glial cells, or glia. The glia occupy essentially all the space in the nervous system not taken up by the neurons themselves. Although the function of glia is not fully understood, they provide structural and metabolic support for the delicate meshwork of the neurons.

Every cell has membrane proteins that perform different functions. The membrane proteins of all cells fall into five classes: pumps, channels, receptor, enzymes and structural proteins. Pumps expend metabolic energy to move ions and other molecules against concentration gradients, in order to mantain appropriate concentrations of these molecules within the cell. Because charged molecules do not pass through the lipid bilayer itself, cells have evolved channels that provide selective pathways through which specific ions can diffuse. Cell membranes must recognize and attach many types of molecules. Receptor proteins fulfill these functions by providing binding sites with great specificity and high affinity. Enzymes are placed in or on the membrane to facilitate chemical reaction at the membrane surface. Finally, structural proteins interconnect cells to form organs and also help to maintain subcellular structure. These five classes of membrane proteins are not necessarily mutually exclusive. For example, a particular protein might simultaneously be a receptor, an enzyme and a pump. Therefore, membrane proteins are the key to understand neuron functions and also brain functions.

A membrane which separates two media of different ionic concentration and allows ions to cross it, can be a good model of general biological membrane. On Fig. 1.2, ions will pass from one side to another until the sum of electrochemical gradients

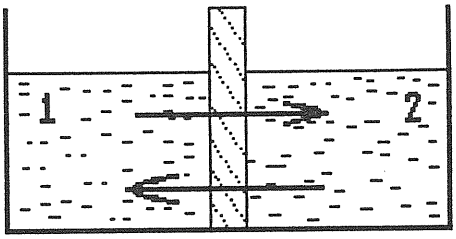

Fig. 1.2 A permeable membrane allows ions to cross along their electrochemical gradients.

of one side becomes equal to the sum of the other one.

Generally, the potential between any two media with a different concentration of for example sodium, potassium and chloride ions, separated by a membrane whose permeabilities for sodium, potassium and chloride are P_{Na}, P_K and P_{Cl} respectively, is the Goldman–Hodgkin–Katz equation

$$E_{rev} = \frac{RT}{F} ln \frac{P_K[K]_1 + P_{Na}[Na]_1 + P_{Cl}[Cl]_2}{P_K[K]_2 + P_{Na}[Na]_2 + P_{Cl}[Cl]_1} \tag{1.5}$$

where $R = 8.314 VCK^{-1}mol^{-1}$ (gas constant),
$F = 9.64810^{-19}mol^{-1}C$ (Faraday's constant),
$[K]_1$, $[Na]_1$ and $[Cl]_1$ are concentrations of these ions in region 1
$[K]_2$, $[Na]_2$ and $[Cl]_2$ are concentrations of these ions in region 2

NERVOUS COMMUNICATION

The voltage difference across a cell membrane, so called membrane potential, depends on the distribution of electric charge. Charge is carried back and forth across the nerve cell membrane by small inorganic ions, chiefly Na^+, K^+, Cl^- and Ca^{2+}. These ions can traverse the lipid bilayer only by passing through special protein channels. When the ion channels open or close, the charge distribution shifts, and the membrane potential changes. Neuronal signaling thus depends on, so called gated channels, whose permeability to ions is regulated. There are two classes of gated channels. Channels, which get opened or closed, by a sudden change of membrane

potential, are called voltage–gated channels. They play the key role in the explosions of electrical activity, by which action potentials are propagated. Ligand–gated channels, which convert extracellular chemical signals into electrical signals, play a central role in the operation of synapses.

Membrane proteins that are essential for nerve impulse propagation along the axon are, in fact, voltage–dependent ionic channels (Fig. 1.3). The transmembrane gradients of sodium and potassium ions enable the neuron, to propagate nerve impulses. Since the concentration of sodium and potassium ions on one side of the cell membrane differs from that on the other side, the interior of the axon is about 70 millivolts negative with respect to the exterior.

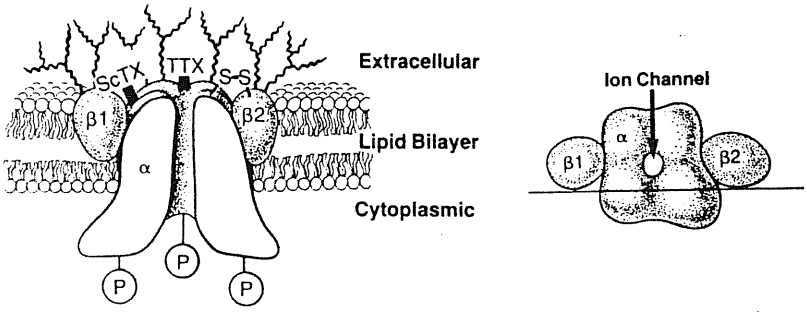

Fig. 1.3 The sodium channel protein from brain is illustrated (left) as associated with the phospholipid bilayer as inferred from biochemical and molecular biological experiments. Sites of glycosylation, phosphorylation (P), and neurotoxin (ScTx, Scorpion toxin; TTX, tetrodotoxin) binding are illustrated. The transmembrane pore of the sodium channel is illustrated (right) in *en face* view as formed in the center of the four homologous transmembrane domains of the α –subunit (from Catterall 1988).

When a nerve impulse starts at the origin of the axon, having been triggered in most cases by the cell body in response to dendritic synapses, the voltage difference across the axon membrane is locally lowered. Immediately ahead of the electrically altered region (in the direction in which the nerve impulse is propagated), channels in the membrane open and let sodium ions pour into the axon.

The process is self–reinforcing: the flow of sodium ions through the membrane opens more channels and makes it easier for other ions to follow. The sodium ions that enter change the internal potential of the membrane from negative to positive. Soon after the sodium channels open they close, and another group of channels opens and lets potassium ions flow out. This outflow restores the voltage inside the axon to its resting value of -70 mV. However, this value depends on the cell. The sharp positive and then negative charge, which shows up as a spike on an oscilloscope, is known as the action potential and is the electrical manifestation of the nerve impulse. The wave of voltage sweeps along until it reaches the end of the axon (Fig. 1.4).

This brief description of the nerve impulse illustrates the importance of channels for the electrical activity of neurons and underscores two fundamental properties of channels: selectivity and gating. Channels are selectively permeable and selectivities vary widely. For example, one type of channel lets sodium ions pass through and largely excludes potassium ions, whereas another type of channel does the reverse. The selectivity, however, is seldom absolute.

The sodium ion is about 30 percent smaller than the potassium ion. The exact molecular structure that enables the larger ion to pass through the cell membrane more readily than the smaller one is not known (Stevens 1979). The general principles that underlie the discrimination, however, are understood. They involve interactions between ions and parts of the channel structure in conjunction with a particular ordering of water molecules within a pore (Stevens 1979).

Another fundamental property, the gating, was studied in the giant axon of squid (Hodgkin and Huxley 1952 a,b,c,d; Hodgkin et al. 1949; Hodgkin and Katz 1949). The authors demonstrated that, the propagation of the nerve impulse coincides with sudden changes in the permeability of the axon membrane to sodium and potassium ions (Fig. 1.5).

To explain time course of sodium and potassium currents thus action potential (Fig. 1.6) Hodgkin and Huxley proposed simple kinetic model. Hypothetical gating particles make independent first–order transitions between permissive and nonpermissive positions to control the channel. Three "m" particles control activation and one "h" particle, inactivation, for sodium channels. On the other hand, four "n" particles control activation of potassium channels. Using close–open first–order kinetics

$$\mathrm{C}_k \rightleftharpoons \mathrm{O}_k \tag{1.1}$$

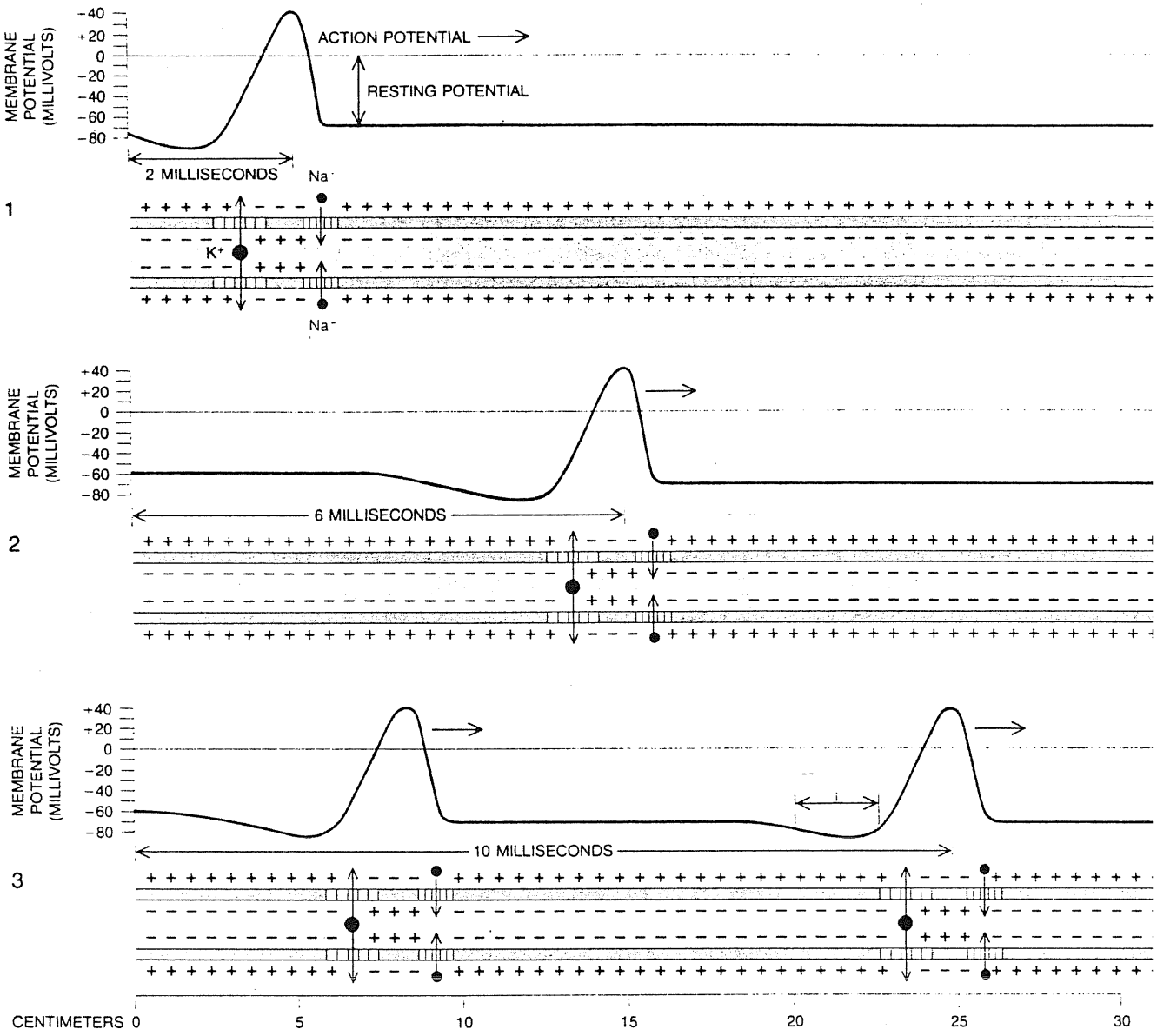

Fig. 1.4 Propagation of the nerve impulse along the axon. The electrical event that sends a nerve impulse travelling down the axon normally originates in the cell body.The impulse begins with a slight depolarization, or reduction in the negative potential, across the membrane of the axon where it leaves the cell body (from Stevens 1979).

we can get an equation for kinetic parameters k (k= m, n or h)

$$\frac{dk}{dt} = \alpha_k (1 - k) - \beta_k k \tag{1.2}$$

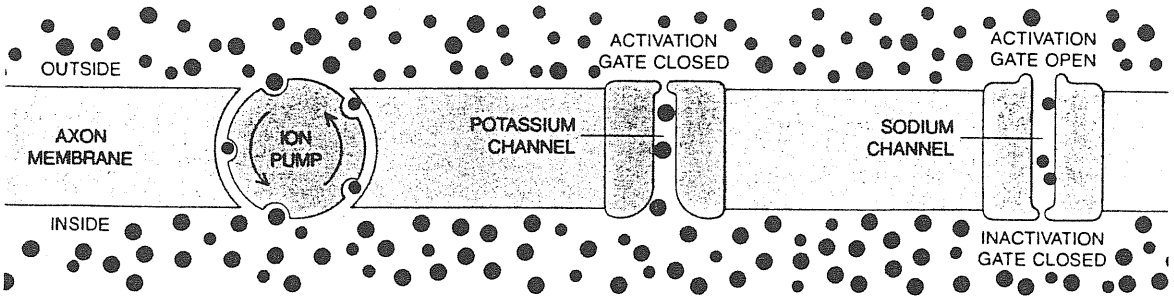

Fig. 1.5 Axon membrane separates fluids that differ greatly in their content of sodium and potassium ions. In the resting state, when no nerve impulse is being transmitted, the two types of channel are closed and the ion pump maintains the ionic disequilibrium by pumping out sodium ions in exchange for potassium ions. Sodium channel in this illustration is in the active state (from Stevens 1979).

where α_k and β_k are voltage dependent rate constants. Each kinetic parameter represents the probability that, a hypothetical particle will make change between close and open states (1.4).

In this model, a total current is

$$I = m^3 h g_{Na} (E - E_{Na}) + n^4 g_K (E - E_K) + g_L (E - E_L) \tag{1.3}$$

where E is membrane potential; g_{Na}, g_K, and g_L are maximum conductances of sodium, potassium and leakage current respectively; E_{Na}, E_K, and E_L are reversal potentials for sodium, potassium, and leakage current respectively. m and h are kinetic parameters of activation and inactivation as described before.

The success of the HH model is a triumph of the classical biophysical method in answering a fundamental biological question. Voltage dependent permeability mechanism and ionic gradients are sufficient to explain electrical excitability.

A mathematical description of propagating nerve impulse is given by the cable equation in one dimension (Cole 1972)

$$\lambda \frac{\partial^2 V}{\partial x^2} - V = \tau \frac{\partial V}{\partial t} \tag{1.4}$$

where $V = V(x,t)$ is the electronic potential or deviation from the resting membrane potential. λ and τ are space and time constant which depend on the model proposed (Fig. 1.7).

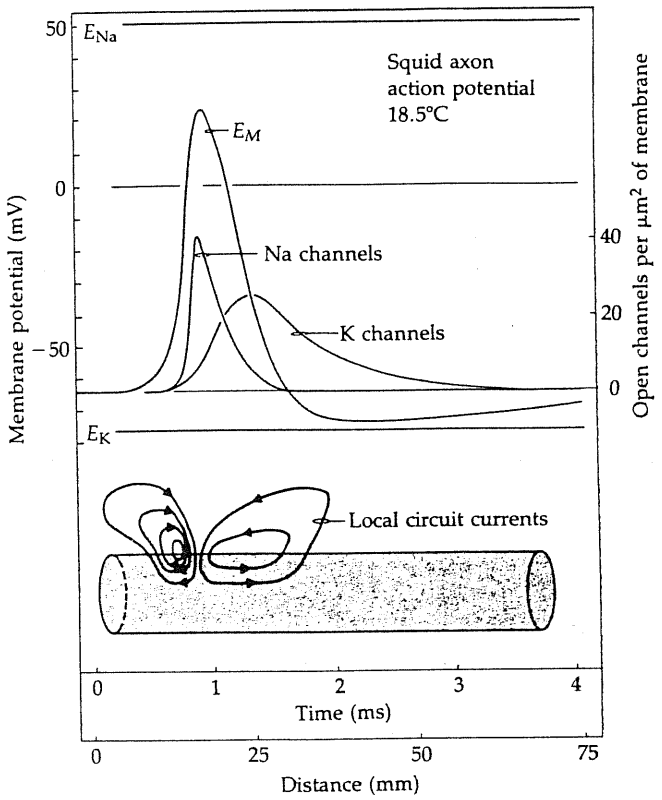

Fig. 1.6 The shape of action potential in squid axon (from Hodgkin 1952).

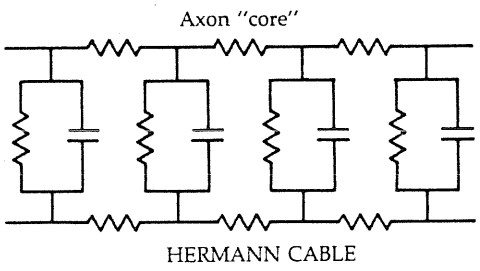

Fig. 1.7 A model used by Hermann in 1905 to describe the passive spread of potentials in axons and muscle by the theory for a " leaky " telegraph cable. Here the protoplasmic core and extracellular region are represented as chains of resistors and the region between them (now called membrane), as parallel capacitors and resistors (from Hille 1984).

Generally, they are functions of different membrane resistances and capaci-

tances. This equation is a linear differential equation whose solutions are certain exponential function of space and time coordinate. Without any permanent voltage source the magnitude of deviation of membrane potential decreases with distance and time. The equation explains so called passive transmission of an electrical signal along the axon. The transport without dissipation could be explained by soliton waves, solutions of nonlinear differential equations. An interesting property is that they do not show dispersion in space and time like a simple exponential solutions of the cable equation (Davidov 1979).

SYNAPTIC TRANSMISSION

Information is transferred from one cell to another at specialized points of contact: the synapses. Usually synapses are made between the axon of one cell and the dendrite of another. There are other kinds of synaptic junction between axon and axon, between dendrite and dendrite and between axon and cell body. The simplest way for one neuron to pass its signal to another is by direct electrical coupling through gap junction. Such electrical synapses between neurons occur at a number of sites. Electrical synapses have the virtue that transmission occurs without delay, and it is easier to find them where a fast conduction occurs. There are also chemical synapses that provide the majority of nerve cell connections.

The mechanism of chemical transmission is indirect. Since the cells are electrically isolated one from another, the only way to communicate can be by exchange of some chemical substance. A molecule, called neurotransmitter, has a property to provoke an electrical change in the cell, thus converting the chemical signal to an electric one. Most of what is known about synaptic transmission comes from experiments on a particular synapse: the neuromuscular junction that controls the contraction of muscles in the frog (Katz and Miledi 1967). The axon of the frog runs for several hundred micrometers along the surface of the muscle cell, making several hundred synaptic contacts spaced about a micrometer apart. At each presynaptic region the characteristic synaptic vesicles can be recognized readily (Fig. 1.8).

When a nerve impulse reaches a synapse it depolarizes the interior of the axon terminal of a presynaptic cell, which in turn activates Ca^{2+} voltage–gated channels, allowing Ca^{2+} ions to enter inside. Ca^{2+} ions inside help in exocytosis of synaptic vesicles, which contain the neurotransmitter. Once molecules of neurotransmitter are

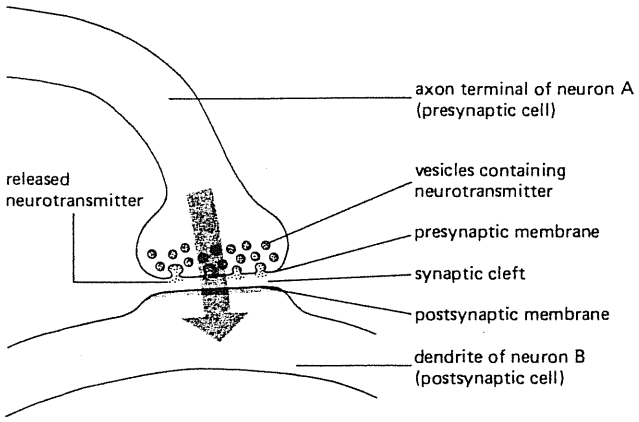

Fig. 1.8 Schematic diagram of a typical synapse. An electric signal arriving at the axon terminal of neuron A triggers the release of a chemical messenger, the neurotransmitter, which crosses the synaptic cleft and causes an electrical change in the membrane of a dendrite of neuron B. A broad arrow indicates the direction of signal transmission (from Watson 1989).

present in the synaptic cleft they diffuse to the postsynaptic terminal and bind to a special receptor molecule, which is one type of ionic channel. Binding of the neurotransmitter to receptor opens this channel, allowing ion exchange at the terminal of postsynaptic membrane, thus generating an impulse which can be transmitted to the cell body and so forth (Fig. 1.9).

Interesting details of the structure of the terminal membrane have been revealed by electron microscopy. Synaptic vesicles become attached on or near the proteins. Only these vesicles then fuse to the membrane and release their transmitter. Other vesicles seem to be held in reserve some distance away. The fusion of vesicles is a random process and occurs independently for each vesicle (Katz and Miledi 1967).

Hence, information is relayed from one neuron to another by means of transmitter. The firing of a neuron — the generation of a nerve impulse — reflects the activation of hundreds of synapses by impinging neurons. The result of the activation of synapses will depend on the permeability of the ion channel associated with the transmitter receptor. Some synapses are excitatory in that they tend to promote firing, whereas others are inhibitory and so are capable of cancelling signals that

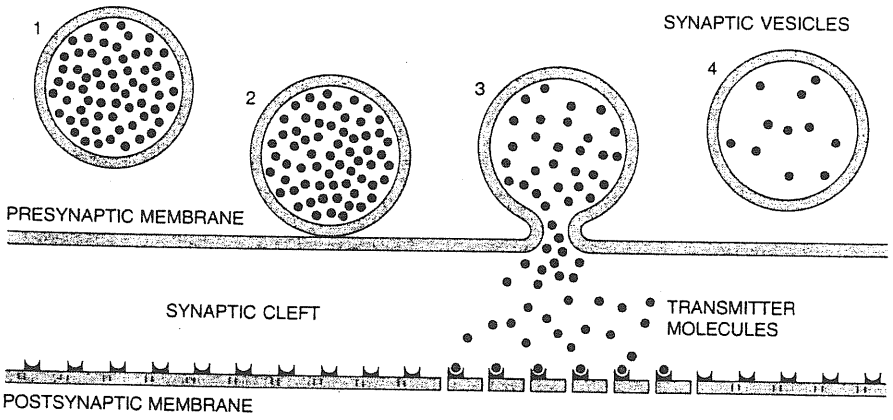

Fig. 1.9 Synaptic vesicles are clustered near the presynaptic membrane. The diagram shows the probable steps in exocytosis. Filled vesicles move up to the synaptic cleft, fuse with the membrane, discharge their contents and are reclaimed, re–formed and refilled with the transmitter (from Stevens 1979).

otherwise would excite a neuron to fire.

There are two categories of ligand–gated channels. When a chemical substance acts directly on channel's receptor, and by this action the channel opens. They are called ionotropic ligand–gated channels. If an action of substance is indirect, by using certain messenger system, we call them metabotropic.

When a synaptic transmission depends on a receptor coupled to membrane proteins, it can generate a second messenger cascade in the postsynaptic cell. In one type of synapses, binding of transmitter to the receptors activates adenylate cyclase, thereby increasing the intracellular concentration of cyclic AMP. The cyclic AMP in turn activates protein kinases that phosphorylate specific protein in the cell: for example, they phosphorylate ion channels and thus alter the cell's electrical behaviour. Indeed, cyclic AMP can, in principle, trigger changes at almost any level in the cell's control machinery, even to the extent of altering the pattern of gene expression. This second–messenger pathway is called *adenylate cyclase cascade*, and is a major transduction pathway. An another generally used pathway is the *phosphoinositide cascade*, in which the hydrolysis of a membrane phospholipid produces two intracellular messengers. An inositol trisphosphate opens calcium channels in the endoplasmic reticulum, and hence increases the intracellular calcium concentra-

tion, which in turn activates several enzymes. Moreover, another cyclic nucleotide, cyclic GMP acts as an intracellular second messenger. It primarily activates specific protein kinases, which in turn phosphorylate some membrane protein channels. As a general rule, receptor gated ion channels are responsible for actions on a time scale of millisecond to seconds, while second–messenger systems operate on a time scale of seconds, minutes, and even longer.

TRANSMITTERS AND EXCITATORY AMINO ACIDS

More than 30 different substances are known or suspected to be transmitters in the brain, and each has a characteristic excitatory and inhibitory effect on neurons. The transmitters are not randomly distributed through the brain, but are localized in specific clusters of neurons whose axons project to other highly specific brain regions. The superimposition of these diverse chemically coded systems on the neuronal circuitry endows the brain with an extra dimension of modulation and specificity. Considerable progress has been made in recent years in characterizing the various transmitter substances (although many more undoubtedly remain to be discovered), in mapping their distribution in the brain and in elucidating the molecular events of synaptic transmission (Iversen 1979).

Such research has revealed that the behavioral effects of many drugs and neurotoxins arise from their ability to disrupt or modify chemical transmission between neurons. It has also hinted that the causes of mental diseases may ultimately be traced to a defect in the functioning of a specific transmitter system in the brain. The neurotransmitter can be either a small molecule (acetylcholine, amino acids or monoamines) or neuropeptides (vasopressin, neurotensin or luteinizing–hormone releasing hormone). Depending on the type of action, inhibitory or excitatory, neurotransmitters are called respectively.

One special type of neurotransmitters are excitatory amino acids (EAA). These neurotransmitters are considered as the most widely distributed in the CNS of mammals (Watkins and Evans 1981). EAA are responsible for slow and fast synaptic transmission in the brain (Mayer et al. 1988; Marshall et al. 1980). Some of EAA as glutamate and aspartate are endogenous, whereas the others are artificial N–methyl–D–aspartate (NMDA), made in a laboratory, or obtained from natural products (domoic acid, kainic acid). They are used to distinguish among the different types of

glutamate receptors. An example of EAA are shown in Fig. 1.10.

The pharmacology of EAA receptors is under exploration using electrophysiological and biochemical techniques in a number of laboratories. The impetus for these pharmacological studies is the notion that characterization of biophysical properties of the ion channels activated by relatively selective EAA agonists would lead to functional criteria for defining EAA receptor subtypes.

EAA pharmacology has been able, so far, to distinguish between three different types of receptor to which glutamate binds, named after their specific agonists: NMDA, kainic acid (KA) and quisqualic acid (QA) (Watkins and Evans 1981). They were studied by elecrophysiology on a different kind of cells. At a microscopic level, single–channel currents have been evaluated from single–channel recordings, and the noise analysis of macroscopic currents.

NMDA activates single channel currents of maximum conductance of 50pS, and subconductance states of 15, 25, 35 and 45pS (Jahr and Stevens 1987; Cull–Candy and Usowicz 1987,1989; Ascher et al. 1988). QA activates mainly smaller conductance channels 7pS, but also rare openings of larger conductance channel (40–50pS), opened normally by NMDA. The conductances of channels activated by KA have been determined mainly by noise analysis, yielding values of 2pS to 4pS (Ascher et al. 1988). In single–channel recording Cull–Candy and Usowicz (1987) obtained conductances of 6 and 21pS, probably by activation of other receptors subtypes by KA. Recently, a conductance of 4pS on single–channel records has been observed (Zheng 1989; Sciancalepore et al. 1990). It is important to say that,all of these glutamate receptor subtypes are non–selective cationic channels (Ascher et al. 1988).

NMDA channels exhibit complex channel kinetics. The openings which occur in bursts, and latter in clusters are very frequent in NMDA response. Neither mean open time nor mean burst duration are appreciably affected by membrane potential (Collingridge and Lester 1989). NMDA receptor channel complex has different ligand–binding domains, for different substances. An agonist binding site for which structural analogues of L–glutamate have been developed as competitive antagonist, like APV. Also, there is a divalent cation–binding site within the ion channel pore, at which Mg^{2+} binds to produce voltage–dependent block (Mayer et al. 1989). The blocking effect of Mg^{2+} is seen as a high frequency flicker, at negative holding potentials (Ascher and Nowak 1988). In addition, there is an anaesthetic binding site within the pore, at which ketamine, phencyclidine, MK–801 and desipramine bind with high affinity to produce voltage–dependent ion channel block of long duration (Mayer et

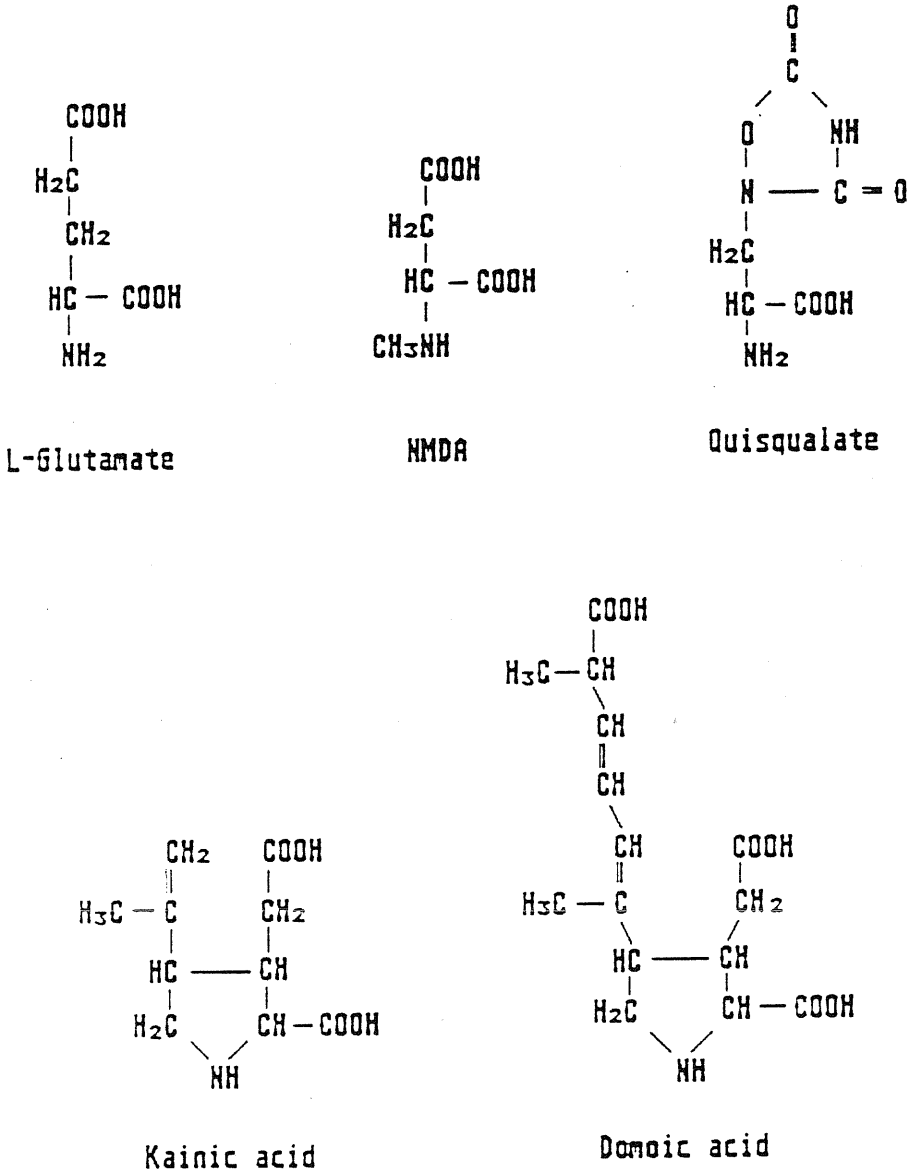

Fig. 1.10 Chemical formulas of L-glutamate, NMDA, quisqualic acid, kainic acid and domoic acid.

al. 1989). A remarkable property of the response to NMDA is that is greatly potentiated by low concentration of glycine (Johnson and Ascher 1987). The effect of glycine was detectable at 10 nM, and was near saturation at 1 μM (Collingridge and Lester 1989). Moreover, the effect was insensitive to the glycine receptor antagonisy, strychinine. During a constant application of NMDA, its current decreases slowly in time, in a process called desensitization (Mayer et al. 1989).

Pharmacological and kinetical properties of QA and KA receptors are quite diverse than those of NMDA receptor. QA current is comprised of two phases: fast and slow (Collingridge and Lester 1989). QA and KA currents are blocked by kynurenic acid and CNQX in voltage–independent way. An interesting property is that, kainate response do not desensitize, even at concentration of 500 μM, whereas QA response displays dose dependent desensitization (Mayer and Westbrook 1987).

At the present time, there are three proposed models to account for the observation that multiple conductance states are activated by agonists from the different EAA subclasses. Jahr and Stevens (1987) suggested the most general hypothesis, which is that NMDA and non–NMDA receptors are part of a complicated molecular entity that includes one type of cationic channel associated with all of the EAA receptor subtypes. This model is consistent with their observation that outside–out patches from hippocampal neurons in culture responded about equally to several agonists including NMDA, QA, and KA, but with different proportions of small, medium, and large conductance states evoked depending on the agonist applied. They suggested that each agonist interacts with one or more of the EAA receptor subtypes, all linked to a common ion channel, to produce a number of different conductance states according to the affinity of the agonist. The " main conductance state " for a particular receptor–agonist combination would be the one containing the majority of the current events. A somewhat similar model proposed by Cull–Candy and Usowicz (1987), with the major difference being that all of the EAA receptor subtypes were not constrained to a case where all of the receptor subtypes are clustered around the same ion channel protein molecule simultaneously.

From another point of view, EAA receptors and ion channels can be appropriately described by a model in which pharmacologically distinct EAA receptor subtypes are associated with different ion channels (probably at least four in number), each of which may infrequently assume substates (Ascher and Nowak 1988).

New techiques of molecular biology and genetics have provided powerful methods in studying of EAA receptors. An injection of rat brain messenger RNA (mRNA)

in *Xenopus* oocytes expresses EAA receptors, which can be studied by electrophysiological methods. Hollmann et al. (1989) isolated a complementary DNA clone of rat brain cDNA libary for expression in *Xenopus* oocyte. After expression, they obtained a functional ion channel, with the electrophysiological and pharmacological properties of the kainate receptor subtype.

Recent studies have implicated NMDA receptors as an essential part of the cellular mechanism that underlie certain forms of long–term potentiation (LTP) (Cotman et al. 1988). LTP is a long–lasting increase (potentiation) of synaptic efficacy that is induced by a train of high frequency stimulation. These studies suggest that NMDA reeptors may be an important component of the processes involved in learning.

PREVIOUS OBSERVATION OF SPONTANEOUS ACTIVITY

Spontaneous activity has been noticed in 1950 (Fatt and Katz 1950) at the neuromuscular junction, like small changes in potential. They are called miniature end–plate potentials (MEPP) that are about 0.5 mV in amplitude and occur at random moments at an average frequency of about one per second. An interesting fact is that the peaks of an end–plate potential amplitude distribution occur at 1, 2, 3 and 4 times the mean amplitude of spontaneous MEPP. The experiments with a blocking agent showed that these channels were of the Ach type. The discrete potential changes can only arise from a synchronous action of a packet of Ach containing a large number of molecules. In today's terminology these packets are synaptic vesicles. The muscle end–plate merely serves as a sensitive detector for a process of Ach secretion, which originates in the motor ending.

Spontaneous activity seems to be a common phenomenon in excitable tissue. In the presence of Mg^{2+}–free medium, the spontaneous activity bursts were observed in hippocampal slices (Neuman et al. 1987). NMDA antagonists, like APV, blocked this type of spontaneous activity, supposed to be mediated by EAA receptors. In the same preparation, they found another type of spontaneous activity, which was not blocked with NMDA antagonist (Neuman et al. 1988). This kind of spontaneous activity was probably induced by other mechanisms.

Cultured hippocampal pyramidal neurons displayed similar behaviour (Bekkers and Stevens 1989). They observed small spontaneous currents that were blocked by APV, same as NMDA evoked currents. Moreover, the linear addition of a large

number of small synaptic currents had the same form as an evoked excitatory postsynaptic current (e.p.s.c.). These synaptic currents make a background noise in study of LTP, in hippocampal cultures. This is the reason why they used to add APV to the culture medium of hippocampal neurons (Bekkers and Stevens 1990).

Spontaneous activity has been also revealed in thin slices of cerebellar granule cells, by addition of glycine (D'Angelo et al. 1990). The activity was not observable without glycine. Moreover, this spontaneous activity was abolished by APV. They suggested that, these synaptic currents were produced by spontaneous release of quanta of transmitters from presynaptic terminals.

Doing patch clamp experiments on large cerebellar neurons of the rat in culture, Cull–Candy and Usowicz (1989) observed spontaneous channels with a reversal potential close to 0 mV. They showed in a whole–cell configuration, that the spontaneous synaptic current can be blocked by $10\mu M$ bicuculline, and considerably reduced in the presence of 3 μM TTX. Moreover, reducing the pipette Cl^- concentration, a negative shift in the reversal potential has been observed. They concluded that, these spontaneous channels were mediated by $GABA_A$ receptor. Very likely GABA was already present in the culture due to the spontaneous release from the cerebellar neurons.

Many other authors claim the existence of spontaneous activity, sometimes called background channels, in outside–out patches of cerebellar neurons of the rat (Sciancalepore et al. 1989, 1990, and many personal communications from our laboratory). These patches were not of use for EAA electrophysiology, and they were thrown away (Zheng 1989). In our laboratory we were observing much of spontaneous activity, which interfered with channels activated by EAA.

In this work we attempted to determine the nature of spontaneous activity in outside–out membrane patches, in the cerebelar granule cells. From biophysical and pharmacological characteristics of these channels, we concluded that, they are glutamate activated channels, probably due to the spontaneous release of glutamate from the cells.

Chapter 2: Methods

CELL CULTURE

Cells were dissociated and cultured from 8–day old Wistar rats by update procedure (Levi et al. 1984). Cerebella were removed under sterile conditions from the rat's pups and placed in solution 1 (Appendix 1). The meninges and blood vessels were peeled off and preliminary minced on a chopping surface with a sterile razor.

The minced tissue was suspended in solution 1 and centrifuged at a speed of 1000 RPM for 1 minute. The cell pellet was resuspended in trypsin containing solution 2 (Appendix 2), and suspension was shaken for 10 minutes at 37^0C. Trypsin served to digest the connective tissue. After digestion, solution 3 (Appendix 3), containing trypsin inhibitor and DNase was added into suspension, which was immediately centrifuged at 1000 RPM. Then the cell pellet, resuspended in solution 4 (Appendix 4), underwent a mechanical separation by sucking up and ejecting in a fire polished Pasteur pipette. After this separation solution 5 (Appendix 5) was added to suspension and recentrifuged at 1000 RPM for 5 minutes. The cell pellet was resuspended in a culture medium (Appendix 6) and diluted to 0.75 million cells per milliliter.

An approximate 2 ml volume of cell suspension was added to each 35 mm Petri dish, which had been precovered by poly–L–lysine (Sigma 5 μg/ml), in order to obtain a cell density of 1.5 million per dish. The plated neurons were placed into a 37^0C, 5% CO_2, saturated humidity incubator (Heraeus B 5061 EK–O2). 10 μM cytosine arabinoside furanoside (Sigma) as a mitotic inhibitor was added in each dish after about 19 hours *in vitro*, to inhibit the growth of non–neuronal cells. The culture contained about 90% of glutamatergic granule cells and the window for use of neurons was about two weeks after start of incubation (Sciancalepore et al. 1989; Galdizcki et al. 1990). Usually, glucose is added in the culture, after seven days, to provide the energy source for the cells as well as to restore the loose of water. In same cases we did not add glucose in the culture, in order to have high spontaneous activity.

SOLUTIONS

The solutions used in experiments are listed in Tables 2.1 and 2.2. 140 mM Cs^+ and 10mM tetraethylammonium (TEA) were used in intracellular and extracellular solution respectively to block the voltage–gated potassium channels. The TTX has not been used because sodium channels, if they open, they get closed in a few milliseconds. Since our recording was much longer, openings of sodium channels can be disregarded. The osmomolarities of external and internal solutions were about the same value, maintaning the osmotic pressure very low, which was important factor in stability of the patches.

The reagents used here were purchased from Sigma or Prolabo. Drugs and pharmacological substances were from Sigma.

Substance	Concentration	Comments
CsCl	140 mM	pH=7.4 adjusted with KOH
EGTA	1 mM	
HEPES	10 mM	
Glucose·H_2O	6 mM	

Table 2.1 INTERNAL SOLUTION

Substance	Concentration	Comments
KCl	3mM	pH=7.4 adjusted with NaOH
$CaCl_2 \cdot 2H_2O$	1.5mM	
HEPES	10mM	
NaCl	130mM	
Glucose·H_2O	6mM	

Table 2.2 EXTERNAL SOLUTION

PATCH CLAMP TECHNIQUE

A patch–clamp technique was first used by Neher and Sakmann (1976) to resolve currents through single Ach activated channels in cell–attached patches of membrane of frog skeletal muscle. Single–channel recording yields information about unitary conductance and kinetic behaviour of ionic channels already investigated by classical voltage–clamp recording and by noise analysis; it is also leading to the discovery of new classes of ionic channels. Patch–clamp techniques also permit investigation of the physiological role of ionic channels in different type of cell, either excitable and not excitable, and also in subcellular membranes.

The principle of the method is to electrically isolate a patch of membrane from the external solution and to record current flowing into the patch. This is achieved by pressing a fire polished glass pipette, which has been filled with a suitable electrolyte solution, against the surface of a cell and applying light suction. Providing both glass pipette and cell membrane are clean, a seal whose electrical resistance is more than 10 GΩ is formed. Under such conditions, the glass pipette and the cell membrane will be less than 1 nm apart (Fig. 2.1).

A high seal resistance is needed for two reasons. Firstly, the higher the seal resistance, the more complete the electrical isolation of the membrane patch. Secondly, a high seal resistance reduces the current noise of recording, permitting good time resolution of single–channel currents whose amplitude is in the order of 1pA (see below).

In patch–clamp recording, the background noise arises from sources in the electronic circuitry, from the pipette and holder assembly, and from the tight seal and membrane itself. In the best recording situations, the contributions from each of these noise sources are roughly equal. The noise in the electronic circuitry arises primarily from the current measuring resistor, the amplifier in the I–V converter, and from the input impendance of the I–V converter. Generally speaking, at equilibrium any passive electrical network produces a noise whose spectral density can be expresed as (Sigworth 1983)

$$S_I = 4kTRe\{Y(f)\} \tag{2.1}$$

where Y(f) is the frequency dependent admittance of the network. In the patch clamp configuration (2.1) becomes

$$S_I = 4kT\frac{4\pi^2 RC^2}{1+4\pi^2 f^2 R^2 C^2} \tag{2.2}$$

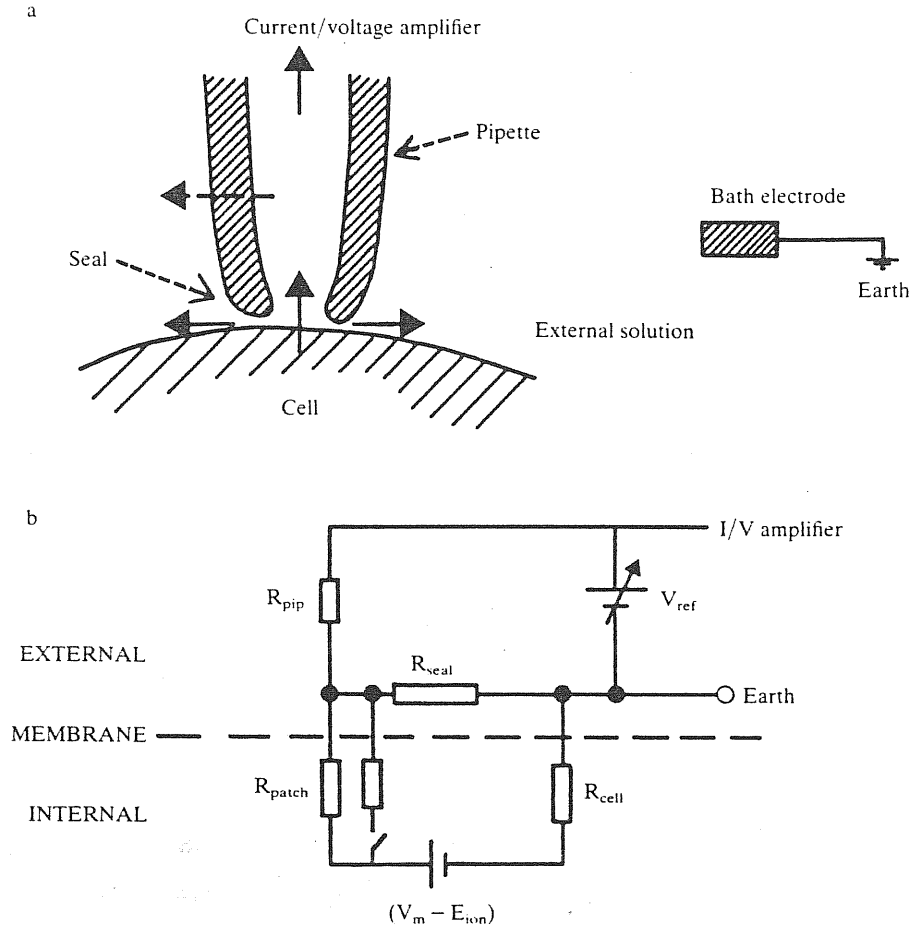

Fig. 2.1 The relation of pipette to cell and equivalent electrical circuit during patch clamp recording. The opening of an ion channel is represented as the closing of the switch in b (from Ogden 1987).

of parallel RC–circuit. So increasing R and decreasing C the noise is reduced; in other words, making a high resistance seal and using a Sylgard coating applied to the pipettes. The hydrophobic surface of Sylgard prevents the formation of a solution film and the thickness of the coating reduces the capacitance. Moreover, there are two more types of noise: a shot noise due to noncontinuous quantal current of passing ions and the 1/f noise (Conti 1984). A simplified electronic diagram is shown in Fig. 2.2.

Fig. 2.3 summarizes the main configurations of gigaseal recording. Much work

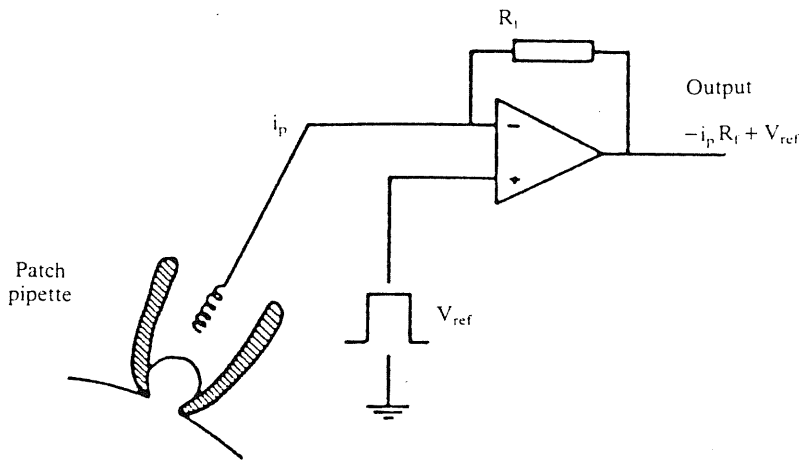

Fig. 2.2 Schematic diagram of the headstage current/voltage amplifier. The gain (V_0/i_p, mV/pA) is set by the feedback resistor R_f, $V_0 = -R_f i_p + V_{ref}$. V_{ref} is comprised of the sum of V_{hold}, V_{null} and $V_{command}$, and is subtracted from the output at a later stage (from Ogden 1987).

is done using patches in cell–attach mode, but the rest potential of the cell is not known and neither intra– nor extracellular ionic concentration can be changed easily. For these reasons, it is sometimes essential to work using a cell free mode, with excised or ripped off patches.

There are two kinds of excised patches. An inside–out made by pulling the membrane patch of the cell into the bath solution. An outside–out made by applying suction to destroy the membrane isolated by the patch pipette, and pulling the pipette away from the cell. The membrane should reseal to give a patch of membrane whose intracellular face is in contact with the pipette solution. Whole–cell recording is achieved by destroying the membrane patch using solution so that the cell, whose interior then comes into contact with the solution in the pipette, may be voltage or current clamped. The cell contents equilibrate over time with the solution within the pipette. This method allowed to voltage clamp very small cells, that cannot accomodate electrodes used by classical methods.

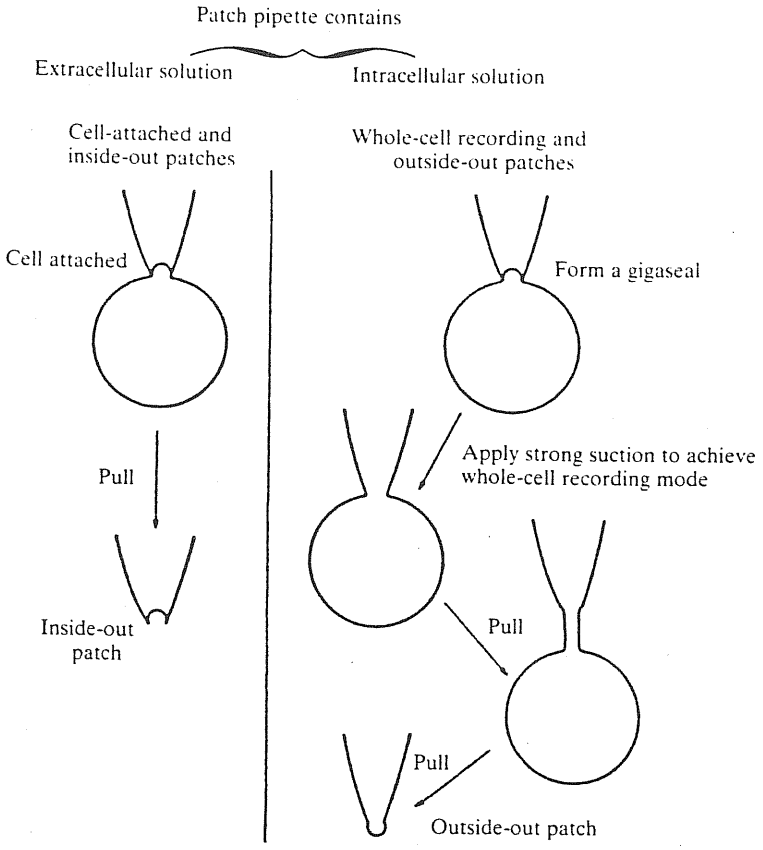

Fig. 2.3 Diagram illustrating the methods of making cell–attached and inside–out patches (left hand panel) and whole–cell and outside–out patches (right hand panel) (from Ogden 1987).

EXPERIMENTS

Single channel currents measurement was performed using mostly the outside–out configuration of the patch clamp technique. Patch pipettes made of borosilicate glass (Hilgemberg), were coated with Sylgard (Corning), and fire polished to obtain tip resistance of about 10 MΩ.

Culture dishes with the cells, were observed under inverted microscope of 320X (Zeiss IM–35), with Nomarski optics. Single–channel currents were measured with a standard patch–clamp amplifier (EPC–7 List). The I–V converter of the patch–clamp amplifier, was mounted on a three dimensional CP–198 micromanipulator (Physik In-

sruments). Before experiments the dishes were washed with external solution. Perfusions of the dishes with different solutions were applied by gravity. The miscroscope and micromanipulators were placed on an antivibratory table, and covered Faraday's cage. In this way, a very sensitive equipment for patch clamp measurement has been protected from an external electromagnetic noise, and undesired movement of the table.

The output of the patch–clamp amplifier was filtered by a built–in 10 kHz 3–pole Bessel filter. One output fed to the pulse code modulator (Instrutech VR–10), and then to the video tape recorder (VT–530E Hitachi). A second output of the patch–clamp amplifier was filtered by a 4 pole–Bessel filter (Ithaco 4320) at a cut–off frequency from 1 to 5 kHz, and proceeded to both a storage oscilloscope (Textronix 5111) for monitor, and a 12 bit A/D and D/A converter (M2–LAB Instrutech) which was connected to a microcomputer. Schematic illustration of the recording equipment used in experiments, is shown in Fig. 2.4.

The microcomputer (Atari 1040 ST) was used to control the recording during the experiments and for all data analysis. Data were recorded at a sampling time of 0.2 msec. The control of the potential were done using Recorder program (Instrutech), with a constant holding potential, and Ramp program (Instrutech), which produces a voltage stimulus with a change of potential in time. The Ramp protocol starts with potential of –80 mV, and finishes after 500 msec with potential of 80 mV.

Experiments were performed at room temperature (22–24^{0}C).

ANALYSIS

The recorded signal was taken from the tape recorder and digitalized by the A/D converter, and transferred to the microcomputer. Aquisition was done using a Recorder program. The sampling theorem states that the upper frequency limitation has to be greater than or equal to the reciprocial value of twice the interval of resolution imposed (Shannon and Weaver 1964).

$$f_c \geq \frac{1}{2\Delta} \tag{2.3}$$

The sampling time used was 0.2 msec and the limit frequency was 5 kHz, even though according to the sampling theorem 2.5 kHz could have been used. In other words, the events whose time duration was shorter than 0.2 msec were invisible for analysis.

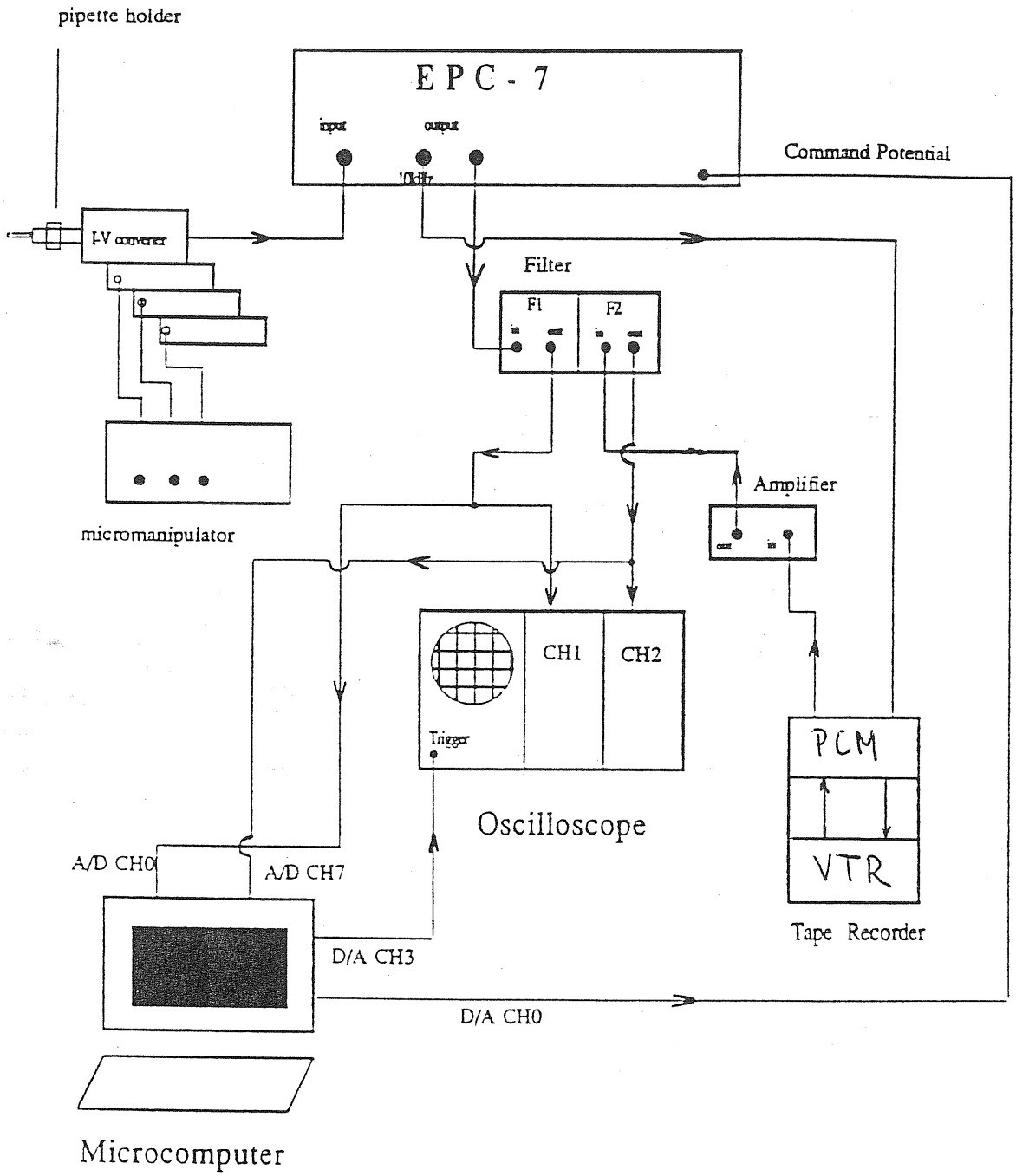

Fig. 2.4 The diagram of recording equipment.

A 1 kHz cut–off frequency of a 4–pole Bessel filter (Ithaco 4302) was used to decrease the level of noise of the recorded signal, during replaying the data from tapes.

The acquired data files have been analyzed by a TAC program (Instrutech), which is descended from the program THAC (Colquhoun and Sigworth 1983). This program provides histograms of current amplitudes and dwell times, from the tables of analyzed data.

The probability of opening is the total dwell time in open states divided by the total recorded time. From close–open kinetics and probability theory of random events the probability density function pdf has form (Sigworth and Sine 1987)

$$f(t) = \frac{1}{\tau} exp(-t\tau) \tag{2.4}$$

where τ is a mean dwell time and appropriate probability distribution is

$$F(t) = 1 - exp\left(-\frac{t}{\tau}\right) \tag{2.5}$$

and its first derivative is pdf.

In order to find τ by a fitting procedure it is convenient to introduce a new variable $x = ln(t)$ (Sigworth and Sine 1987) with new pdf

$$g(x) = exp\left[x - x_0 - exp(x - x_0)\right] \tag{2.6}$$

where $x_0 = ln(\tau)$ and defining the " generic " pdf as

$$g_0(z) = exp\left[z - exp(z)\right] \tag{2.7}$$

it is possible to write $g(x)$ simply as

$$g(x) = g_0(x - x_0) \tag{2.8}$$

The function $g(x)$ has three properties that are useful for analysis. The change in the underlying time constant τ results only in a shift of the function along the x–axis, rather than a change of scale. Then the maximum value of $g(x)$ occurs when $x = x_0$ i.e., at the logarithm of the time constant, where the value of g is $1/e$, and the maximum value is independent of the time constant, unlike pdf in the time linear histogram where the maximum value varies as $1/\tau$.

The fitting of experimental sets of dwell time measurements is typically done by maximizing the logarithm of the likelihood with respect to the set of fitting parameters, denoted by θ. In this case θ represents the set of time constants and coefficient of exponential components . The likelihood is equal to the probability of obtaining a particular set of observed dwell times t_j, given the form of the distribution and the parameters, and is proportional to the product over N observations:

$$Lik = \prod_{j=1}^{N} f(t_j, \theta) \tag{2.9}$$

where $f(t_j, \theta)$ is the probability density function evaluated at t_j with a particular set of parameters θ. Since likelihood takes on very small values, numerical evaluation of its logarithm is preferable. The log–likelihood with correction is given by (Colquhoun and Sigworth 1983):

$$L(\theta) = \sum_{j=1}^{N} lnf(t_j, \theta)/p(t_{min}, t_{max}, \theta) \tag{2.10}$$

where t_j and the N are experimentally observed dwell times and $p(t_{min}, t_{max}, \theta)$ is the probability that dwell times fall within the range of experimentally measurable times characterized by t_{min} and t_{max}, computed from the probability distribution with parameters θ. Maximum likelihood fitting of the binned data then consists of finding the set of parameters θ that maximize $L(\theta)$. Usually the programs use the simplex method.

The calculation of conductances was performed by measurements of the channel current amplitudes at different potentials. They were then fitted by the least mean square method, using the program written in the laboratory. The reversal potential of the current was evaluated from the I–V relations, or by measuring the potential at which the current was zero directly from records, obtained with Ramp protocols.

Chapter 3: Results

CONDUCTANCES AND SELECTIVITY

Spontaneous channel activity was studied on membrane patches from cerebellar granule cells in culture, in nominally Mg^{2+}–free solutions. Except when indicated, experiments were done in excised outside–out configuration. We noticed that, the spontaneous activity was much higher without injection of glucose into the dishes with cells. In order to get high spontaneous activity, we did not treat the culture cells with glucose. This spontaneous activity was present at any day in culture (DIC), from 1 DIC to 14 DIC, as shown in Fig. 3.1.

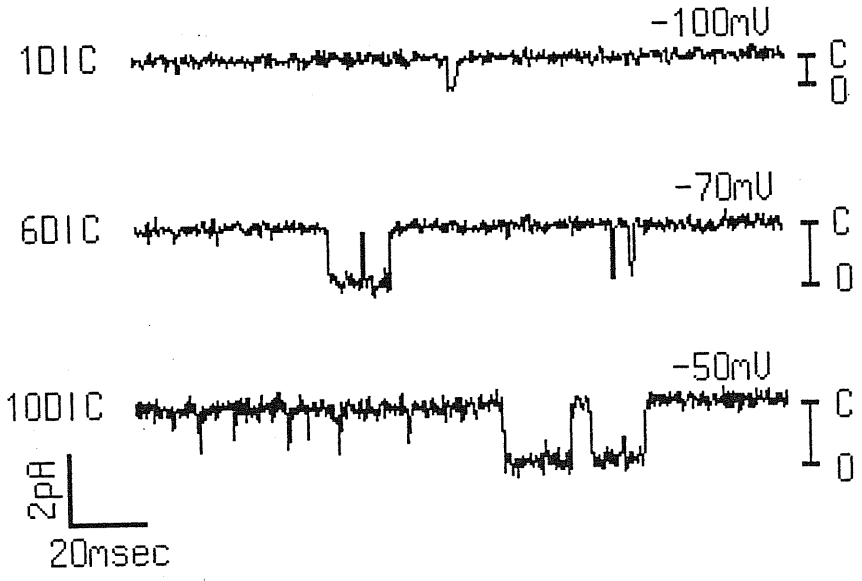

Fig. 3.1 Spontaneous channels recorded on membrane patches in outside–out configuration, at different days in culture (DIC). The holding potentials are indicated above each trace. BW=1 kHz.

Spontaneous channel activity events were of many different conductances, as

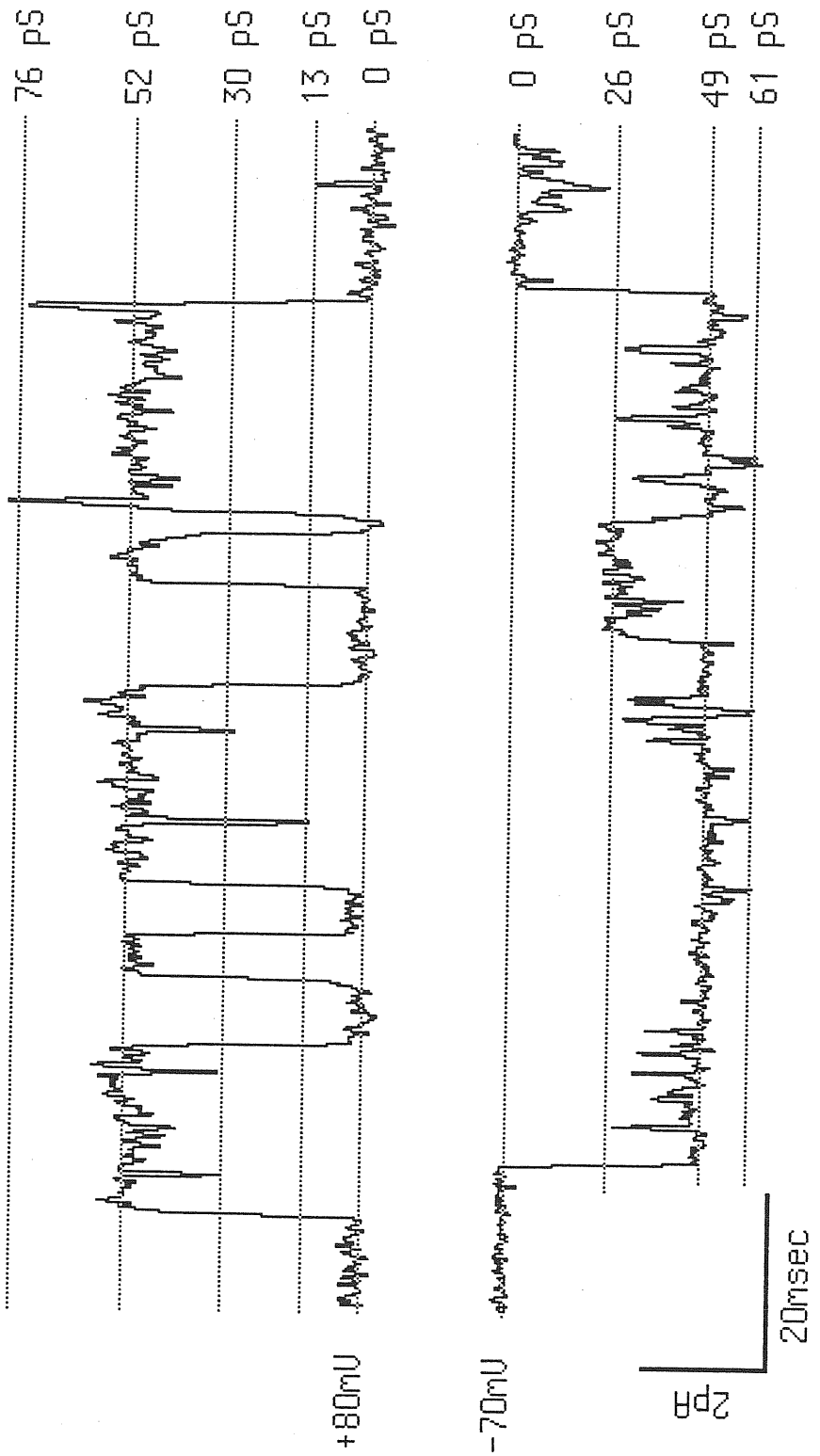

Fig. 3.2 Conductance levels of spontaneous channels observed at positive and negative potentials (6DIC). BW=1 kHz.

a)

Conductances (pS)
10
14
17
22
29
38
45

b)

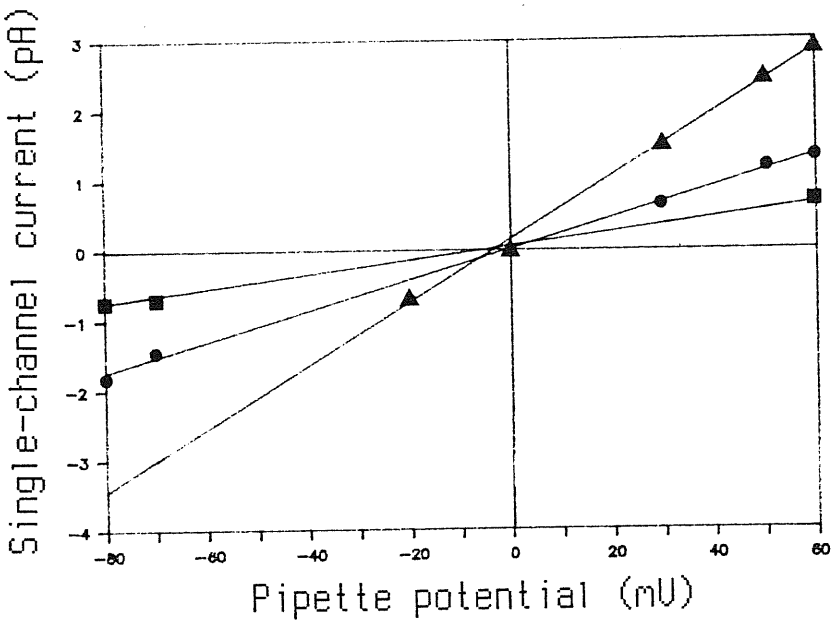

Fig. 3.3 a) Conductances of spontaneous channels. The standard deviation are omitted because they were negligible (6 DIC). b) A graph representing an example of fitting of some single–channel conductances. In all cases reversal potential was close to 0 mV. Single-channel currents for 10 pS are represented with (■), for 22 pS with (●), and for 45 pS with (▲).

shown in Fig. 3.1. At more than 5 days in culture, different conductance levels of spontaneous channels have been observed (Fig. 3.2). We used the mean least square method to fit conductances, whose values are presented in Fig. 3.3. An interesting property of spontaneous channels was that, the maximum value conductance increased with days in culture (Fig. 3.4). Same observation was made for NMDA activated channels by Sciancalepore et al. (1989), in the same culture. Moreover, the

conductances seen at previous days in culture were still present in following days.

To approximately determine the reversal potential, we used Ramp pulse protocol, described in previous chapter. We revealed that, the reversal potential of spontaneous channels was about 0 mV, at any day in culture. (Fig. 3.5). In the same figure, we can see that even subconductance levels have reversal potential aroun 0 mV.

Investigation of selectivity was performed by substitution of NaCl with Na–gluconate, keeping osmomolarities equal. Reducing the outside concentration of Cl^- from 140 mM to 40 mM, a shift in reversal potential has not been observed. (Fig. 3.6). With this condition, the balance of cations in the intracellular side (pipette) and in the extracellular side (bath), was approximatelly equal. Therefore, we concluded that, the spontaneous events correspond to non selective cationic channels.

KINETICS

Open and close time distributions were analyzed by measurements of durations of the spontaneous events, with upper limit frequency of 2.5 kHz. The analysis was performed at different membrane potentials. Results are shown in Table 3.1. The activity of spontaneous channels occured in bursts, followed by long period of silence. We can notice that, at each potential, there are more than one, both, close and open time constants.

An example of fitting of open and close time histograms is illustrated in Fig. 3.7. Probability of opening showed a big individual variability, depending very much on a patch. Anyway, not obvious voltage dependence of the probability of opening or dwell times, was observed.

PHARMACOLOGY

In order to determine the nature of the spontaneous channels, a small pharmacological profile was attempted. Chemicals and drugs were applied by a slow gravity perfusion, at a flow speed of 0.1 ml/s. The solutions containing pharmacological substances had been perfusing until a dish content changed by 5 to 10 times.

The first set of experiments was performed, using some of the typical blockers of

voltage–gated channels. When Tetrodotoxin (TTX) at concentration of 1 μM, known as a very potent blocker of sodium channels (Narahashi et al. 1964) was applied, no effect on spontaneous channels in cerebellar granule cells were observed (Fig. 3.8). TEA 10 μM and Cs^{+}(140 mM) were already used, in preparation of extracellular and intracellular solutions respectively. Their presence blocks potassium channels (Rudy 1988). Spontaneous channel activity has been observed always in their presence, at any day in culture. A perfusion with 2.5 mM Co^{2+}, as a Ca^{2+} channel blocker (Hagiwara and Takahashi 1967), did not affect spontaneous activity.

At this point, we turned to, the use of drugs that act on neurotransmitter activated channels. The dish was perfused with 100 μM bicuculline. Bicuculline is widely used to block $GABA_A$ receptor activated channels (Curtis and Johnston 1974). The presence of bicuculline in the bath solution has not affected spontaneous activity (Fig. 3.9).

From now on, we used only chemicals that affect EAA receptors. This strategy was choosen, since the high density of EAA receptors in cerebellar granule cells has been already reported (Cull–Candy and Usowicz 1987,1989; Levi et al. 1984).

The perfusion with 200 μM kynurenic acid lowered the activity of spontaneous channel. Moreover, in the presence of kynurenic acid, the maximum conductance channel open time became longer. This effect was observed both, at negative and positive potentials (Fig. 3.10).

The application of glycine, which potentiates NMDA response (Johnson and Ascher 1987), has not produced any noticable effect on spontaneous activity, when it was perfused at concentration of 3 μM (Fig. 3.11).

When 2–amino–5–phosphonovaleric acid (APV) and MK–801, the most potent blockers of NMDA receptor–channel complex and channel pore, respectively (Ascher et al. 1988; Mayer et al. 1989), applied at concentration of 100 μM, almost complete blockage of big conductance of spontaneous channels was observed (Fig. 3.13). The blockage was voltage independent way.

The perfusion of the bath solution with 5 mM Mg^{2+} blocked spontaneous channels in voltage dependent way (Fig. 3.14). In the presence of Mg^{2+}, the spontaneous channels disappeared at negative potentials, while they were still present at positive potentials.

All pharmacological experiments performed, were reproducible at any day in culture. The effects were more evident for the older cells, since their spontaneous activity was higher than of the younger cells.

OTHER OBSERVATIONS

The activity of spontaneous channels depends on the position of the patch pipette with respect to the rest of the cells, and on the extent of external perfusion. The spontaneous activity lowered, if the dish was extensively washed with the standard external solution (Fig. 3.15). By putting the pipette near the bottom of dish, spontaneous activity was higher than, when the pipette was placed near the surface of external solution, thus away from the bottom, where the rest of the cells are attached.

Glutamate at a concentration of 20 μM, was applied externally to previously washed dish. Glutamate activated some channels in outside–out membrane patches with reversal potential of about 0 mV. The characteristics of glutamate activated channels were similar to spontaneous activity channels (Fig. 3.16).

An attempt to activate glutamate channels in symmetrical solutions was done in inside–out configuration, with pipette filled with the external solution plus 30 μM glutamate. We observed some channels with reversal potential about 0 mV, although a low activity was recorded (Fig. 3.12).

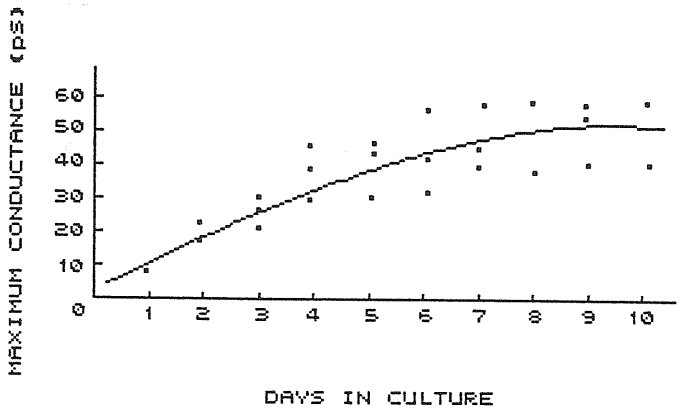

Fig. 3.4 Single–channel maximum conductances of spontaneous channels versus days in culture. A curve was fitted by hand.

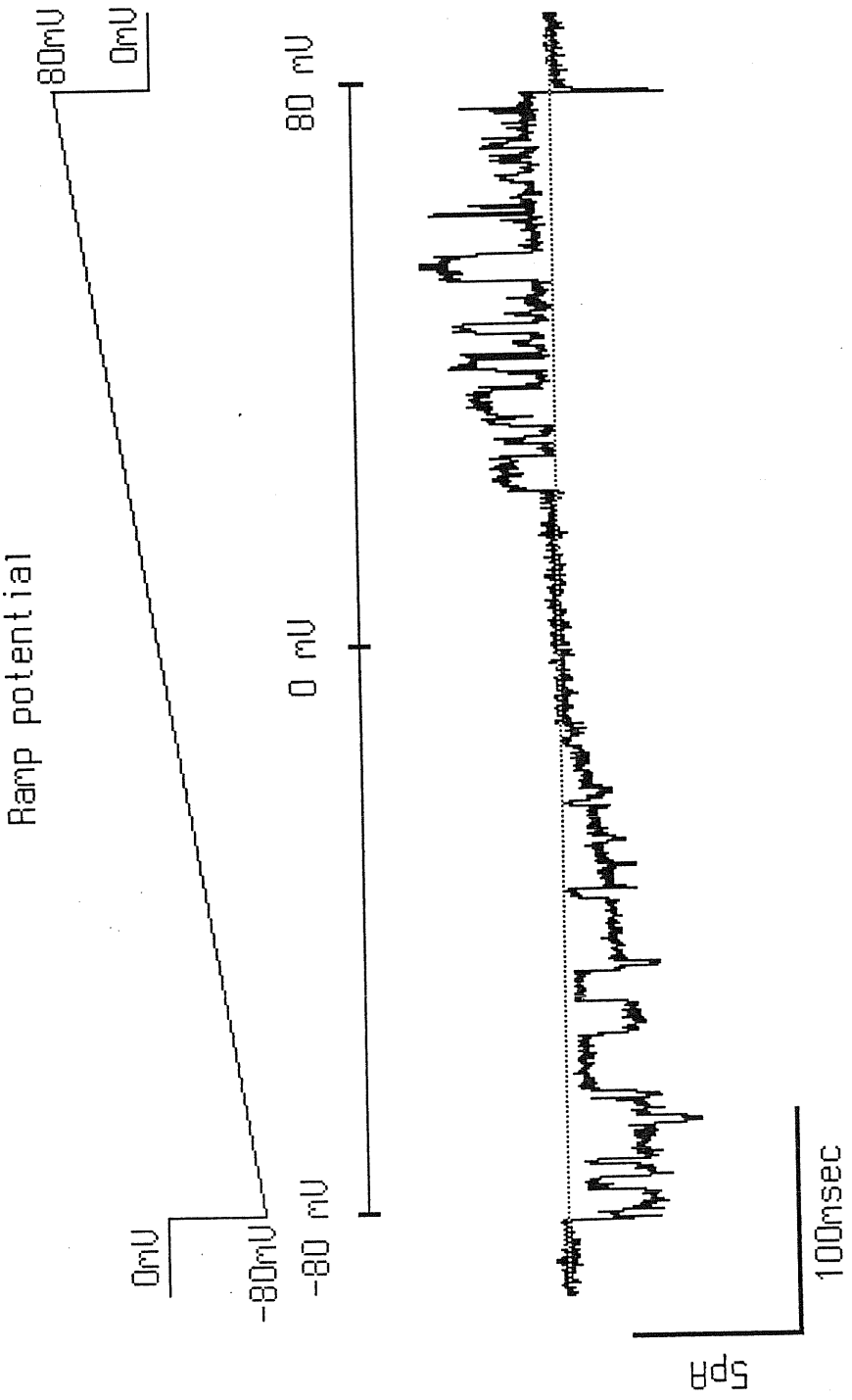

Fig. 3.5 The spontaneous activity obtained using Ramp potential. Reversal potential is close to 0 mV. BW=1 kHz, (6DIC).

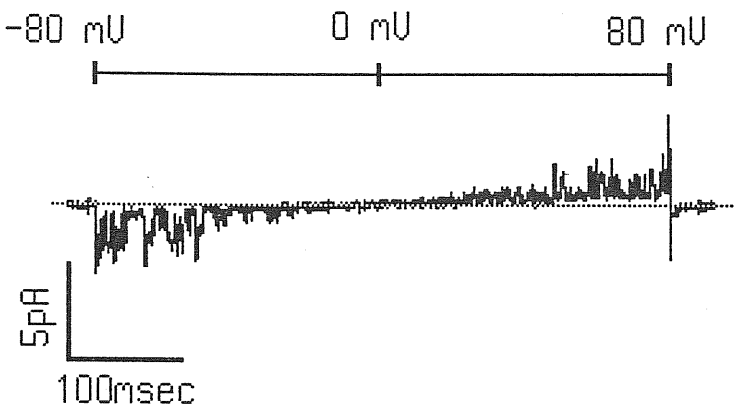

Fig. 3.6 The Ramp after reduction of Cl^- concentration in external solution by 100 mM. BW=1 kHz. Notice that reversal potential is about 0 mV.

V_h(mV)	probability of opening	mean open time (msec)	mean close time (msec)
-100	0.0173	0.571 3.48	0.496 8.57 266
-90	0.0207	0.413 3.472	0.702 55.49 8.501 188.83
-70	0.0459	0.315 2.62	0.282 4.85 27.2
-30	0.0176	1.81 11.64	0.277 9.645 488.65
30	0.0242	0.677 5.853	0.233 12.30 318.5
50	0.0329	0.753 6.033	1.090 16.101 282.8

Table 3.1 Mean open, close times and probability of opening of spontaneous channels at different potentials. The records have been made on different patches.

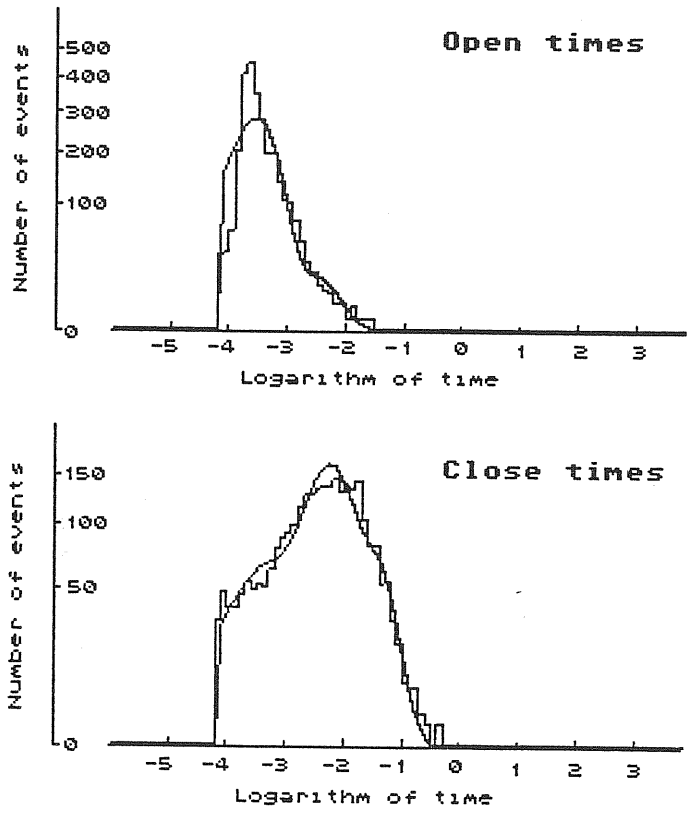

Fig. 3.7 Likelihood fitting of open and close times at potential of −70 mV, listed in Fig. 3.7. A total number of events was 2603. BW= 2.5kHz.

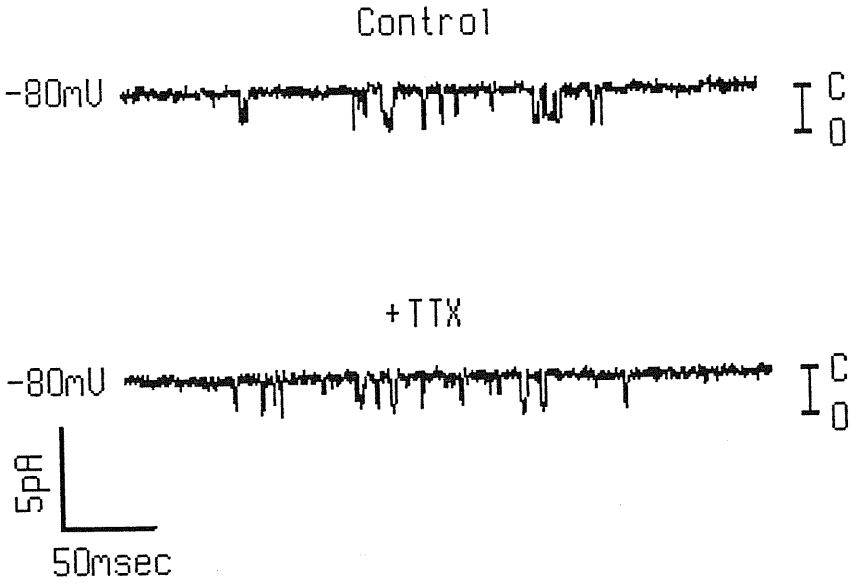

Fig. 3.8 Perfusion with 1 μM TTX has no effect on spontaneous activity. BW=1 kHz (V_{hold}=−80 mV).

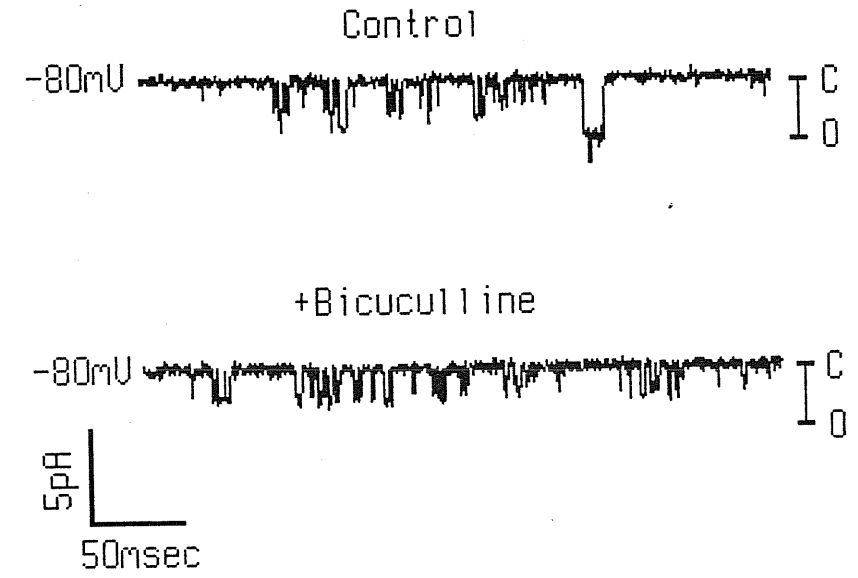

Fig. 3.9 The effect of 100 μM bicuculline on spontaneous channels at negative holding potential of −80 mV. BW=1 kHz.

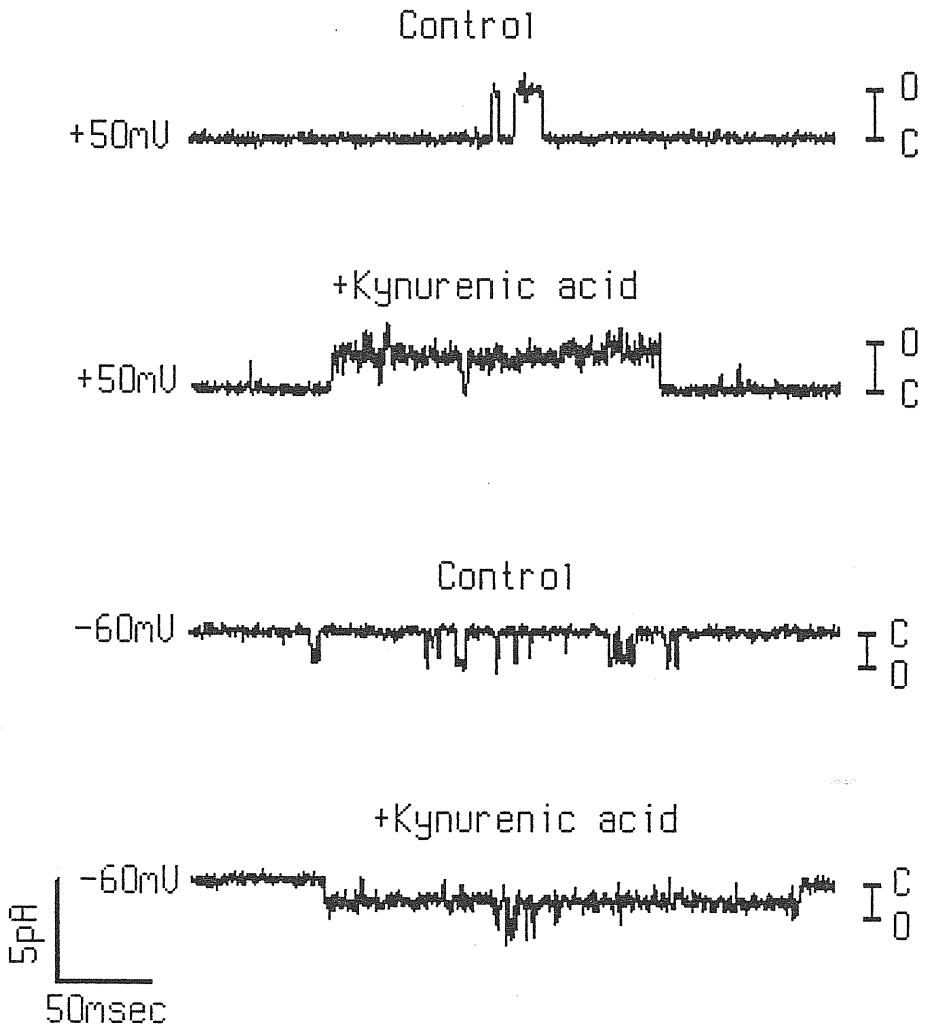

Fig. 3.10 The effect of 200 μM kynurenic acid on spontaneous channels, recorded at different potentials. BW=1 kHz.

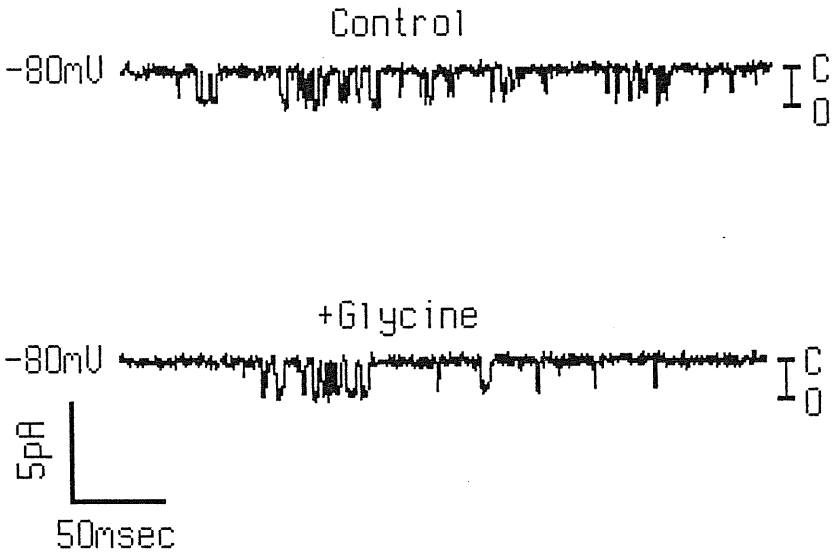

Fig. 3.11 The effect of 3 μM glycine on spontaneous activity at holding potential of –80 mV. BW=1 kHz.

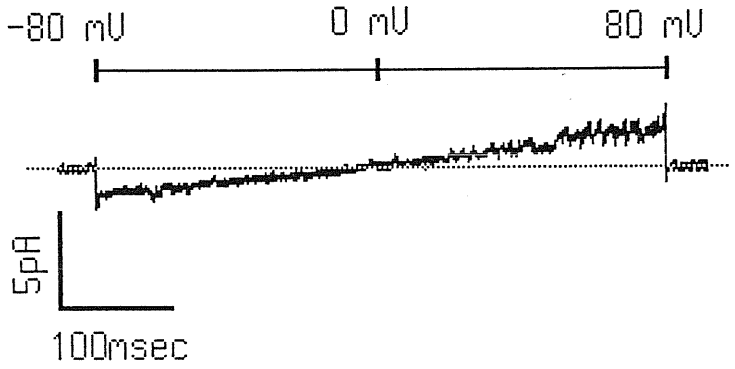

Fig. 3.12 Currents record done by Ramp potential protocol, of inside–out patch of symmetric solutions (external solution), with 30 μM glutamate present in the pipette. BW=1 kHz.

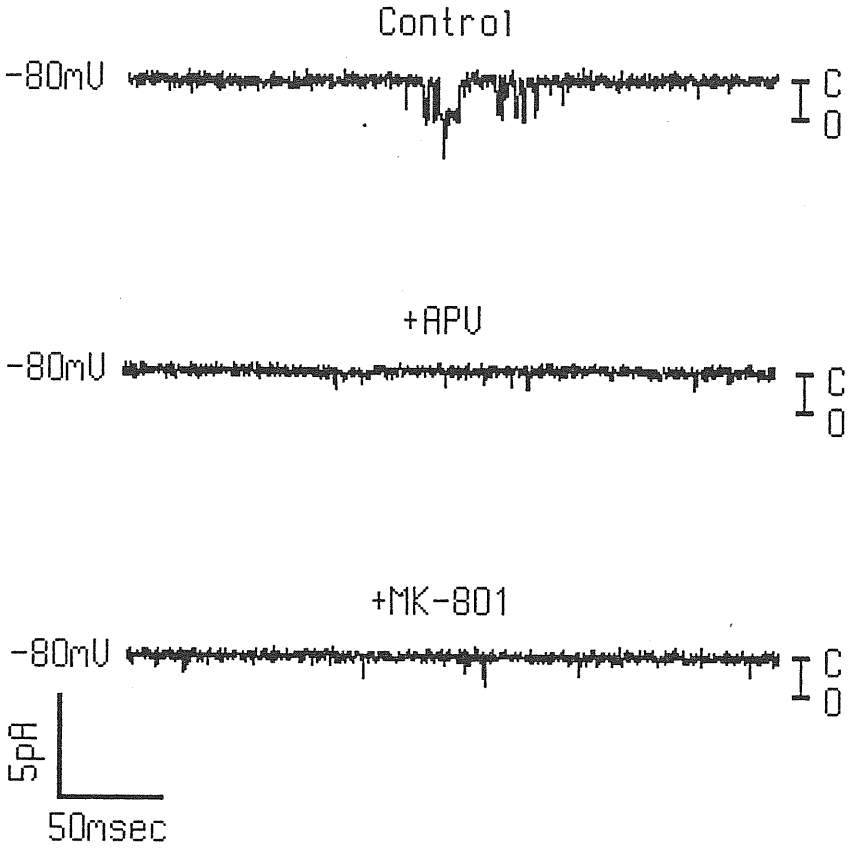

Fig. 3.13 An effect of 100μM APV and MK–801 on spontaneous activity. The same effect was noticed at any potential. BW=1 kHz.

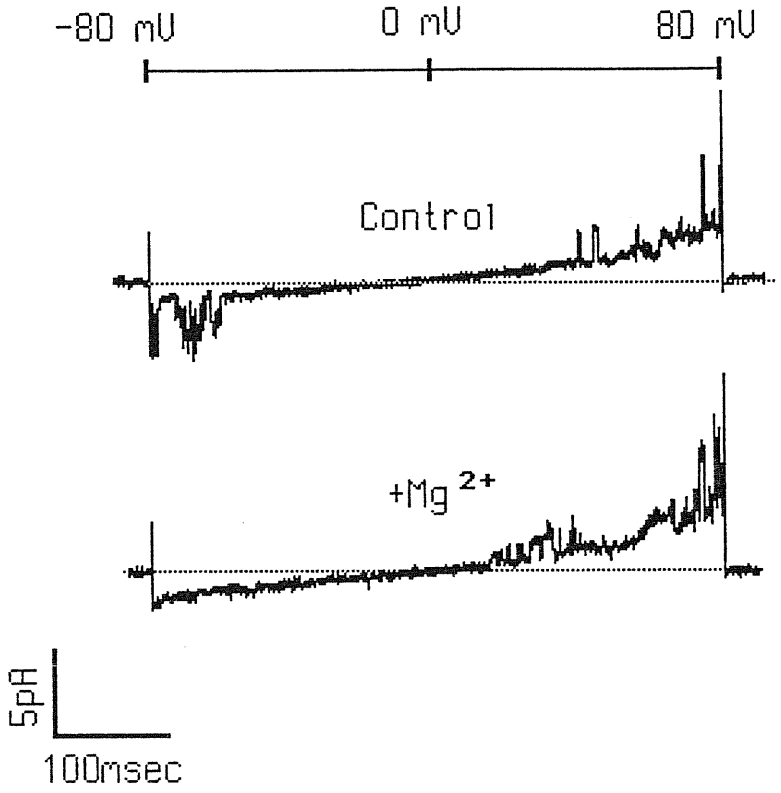

Fig. 3.14 The effect of 5 mM Mg^{2+} on spontaneous channels, in Ramp pulse protocol. BW=1 kHz.

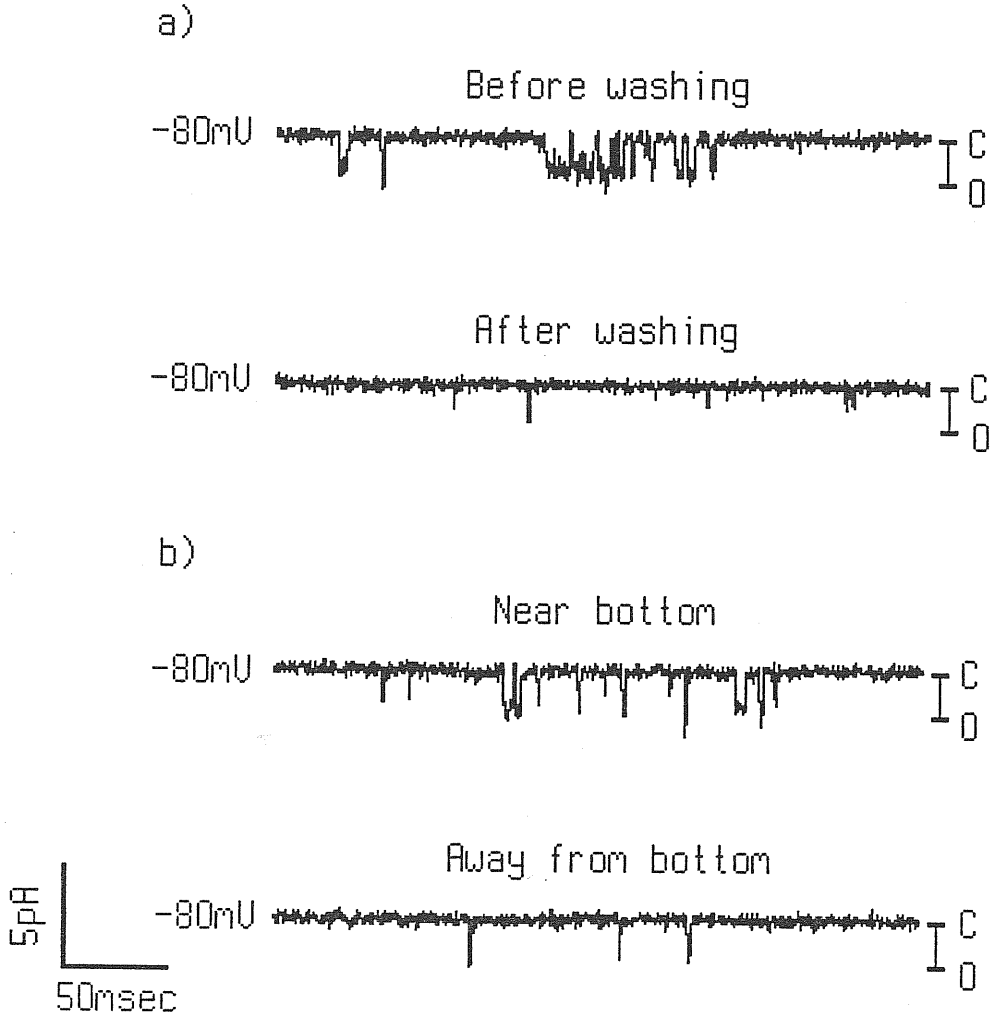

Fig. 3.15 The change of activity after a) washing, and b) moving pipette away from the bottom of a dish. Holding potential was -80 mV. BW=1 kHz.

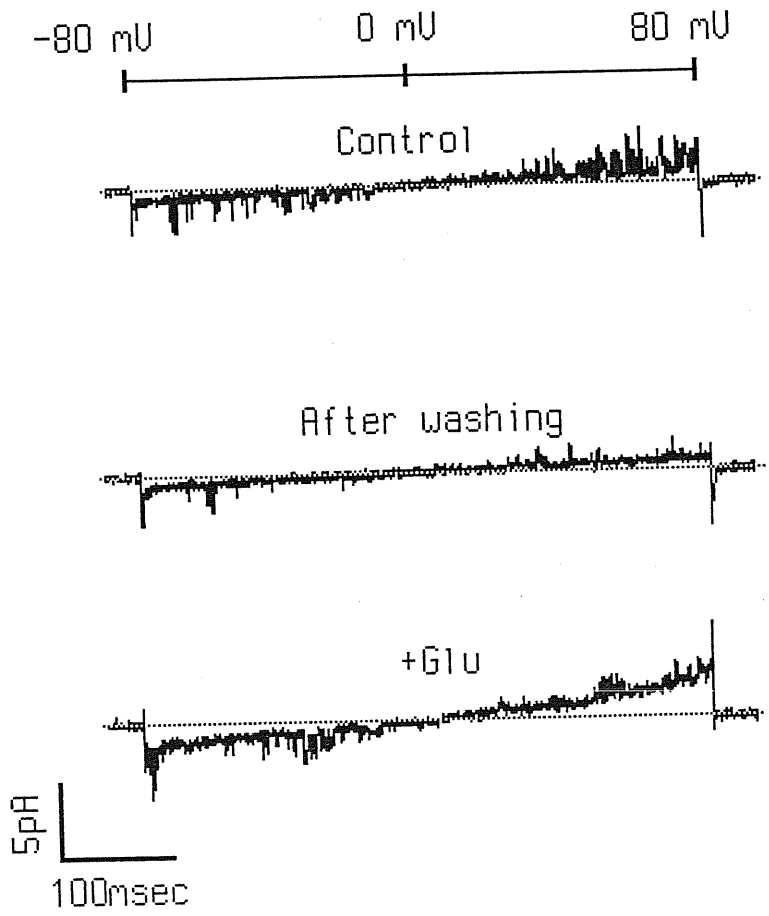

Fig. 3.16 The effect of externally applied 20 μM glutamate in Ramp pulse protocol, after washing with pure external solution. BW=1 kHz.

Chapter 4: Discussion

Considering the results obtained here, we demostrate that, biophysical and pharmacological properties of the spontaneous channels activity, observed in the culture of cerebellar neurons, correspond to an activation of glutamate receptors. It would be produced by spontaneous release of EAA (Novelli et al. 1988) from the cells, that activates the glutamate receptors on an excised membrane patch. This release is mainly composed of glutamate and aspartate.

CONDUCTANCES

The conductances of the spontaneous channels in our experiments are very similar to those observed on glutamate activated channels in hippocampal neurons (Jahr and Stevens 1987), and Glu and Asp activated channels in the cerebellar neurons (Cull–Candy and Usowicz 1987). Moreover, similar conductance levels have been reported in several neuron preparations, when NMDA was applied (Ascher et al. 1988; Cull–Candy and Usowicz 1987; Sciancalepore et al. 1989).

It is extremely unlikely that events with two conductance levels are due to the superimposition of openings of two or more independent channels. For example, the probability of opening at a patch potential of –90 mV is 0.02. From there, one can deduce that the probability of coincidence of two independent events should be an about 0.0004. It is very likely that, this low probability of simultaneous opening do not fit with the obtained results. Nevertheless, the conductances levels shown in Fig. 3.2, may arise from superimposition of two or even more independent channels, if the patch contained, at least, more than one receptor–channel complex.

The subconductance states observed in the histograms, and evaluated at different holding potentials displayed a variability. This variability was also observed in cerebellar granule cells stimulated with glutamate. The observation has been made by Cull–Candy and Usowicz (1987).

Maximum conductances at different DIC shows almost the same dependence like those of NMDA channels (Sciancalepore et al. 1989).

KINETICS

We evaluated kinetic properties of the spontaneous channels, in order to compare them, to those of already known channels. Analysis of open and close times distributions can provide useful informations about kinetics of channels.

The mean open times (0.315–11.64 msec) of spontaneous channels are in about the same range to those activated by NMDA in granule cells (Sciancalepore et al. 1989; Cull–Candy and Usowicz 1987) The open time distribution was fitted with two constants. It is reasonable to propose that, the spontaneous channels could correspond to, at least two open states of a single channel, or two different channels.

The mean close times show much more diversity, as expected, since the bursts of openings are observed. Usually, bursts account for the existence of at least, two closed states, one fast and other slow (Ascher et al. 1988). A mean close time of 27.2 msec (V_{hold}=–70 mV), which ranges out from rest of the values, may be attributed to the fact that, when the probability of opening is high, a spontaneous release is more frequent. This happens every time, when a just excised cell ejects glutamate from its internal stores. This observation is consistent with the fact that measurements made at potential of –70 mV, which were recorded at very beggining, that is, immediately after the process of pulling the pipette from the whole–cell configuration.

A possible explanation for diversity in the time constants could be that, glutamate concentration changes in vicinity of the patch, since mean open and close times vary with glutamate concentration (Kerry et al. 1988). However, this is in contraposition with the observation of Ascher et al. (1998), who reported that, in the mouse central neurons, varying glutamate concentration (0.5–50 μM) there was no change in mean open times.

I-V RELATION AND SELECTIVITY

The reversal potential of NMDA–activated channels in the mouse central neurons is near 0 mV (Ascher et al. 1988). Then, the reversal potential of channels activated by Glu, NMDA, Asp and QA in cerebellar neurons was close to –5 mV (Cull–Candy and Usowicz 1987). The reversal potential of spontaneous channels is about 0 mV (Fig. 3.5). Not only the Ramp of potential, and steady–state reads made at different applied membrane potentials, but also a long recording at zero potential

approves this statement.

Spontaneous channels seem to be voltage–independent, in nominally Mg^{2+}–free solution. In fact, long records at different potentials, where activity was observed, encourage us to claim previous statement.

A discussion about possibility that spontaneous channels are of Cl^- type is skiped because the reduction of Cl^- concentration externally by 100 mM did not produce any shift in reversal potential of these channels. The conclusion is that, the spontaneous channels are cationic selective. Cull–Candy and Usowicz (1989) reported that, a change in external concentration of Cl^- produced the shift in reversal potential of spontaneous channels in same culture. Obviously, these spontaneous channels were anionic selective, which is not the case here.

For comparison, response to EAA is produced by activation of cation–selective channels, equally permeable to Na^+ and K^+ (Mayer et al. 1989).

PHARMACOLOGY

Following discussion is made in such manner that, we discard or accept the possibility that, spontaneous channels are of certain type, by using pharmacological probes for that type of channels.

Before we did perfusion with TTX we had expected that it would not produce any effect, because kinetical properties of sodium channels are very different than those of the spontaneous channels. They are very fast, and they inactivate in a couple of milliseconds and do not open again at the same potential without repolarization. The prediction was right, since TTX did not change the spontaneous activity (Fig. 3.8). As mentioned in Chapter 2 the presence of TEA externally and Cs^+ internally prevents potassium channels to get opened (Rudy 1988). Without a perfusion, the spontaneous channels were present always, and undoubtedly they are not even potassium channels. Moreover, they are not of Ca^{2+} type, since a perfusion with Co^{2+} did not change the activity of spontaneous channels.

Bicuculline, is very potent blocker of $GABA_A$ channels. It has not shown any effect on spontaneous activity (Fig. 3.9). This is consistent with the fact that, the spontaneous channels are not permeable to Cl^-. From here, we can conclude that they are not, at least, of $GABA_A$–type, since Cl^- is permeant in these type of channels (Franciolini and Petris 1990).

The most potent blockers of NMDA channels APV and MK–801 (Mayer et al. 1989) blocked big conductances of spontaneous channels (Fig. 3.13). The effect was observed at any holding potential. Moreover, 5 mM Mg^{2+} has blocked these channels in voltage dependent way (Fig. 3.14). At positive potential Mg^{2+} has no effect, while at negative potentials, the spontaneous activity decreases. Same behaviour was observed by several authors (Mayer et al. 1984; Nowak et al. 1984), on NMDA activated channels. Then, at physiological Mg^{2+} concentration (near 1 mM), NMDA receptor channels do not conduct current if postsynaptic membrane potential is more negative than about –80 mV, but they conduct current freely if the cell is depolarized (Jahr and Stevens 1990).

These are two very strong facts, which persuaded us that, at least big conductances of spontaneous channels, correspond to NMDA–type.

Regarding all the facts up to now, we can propose hypothesis that the spontaneous channels are of glutamate type, since up to now they really have the properties of glutamate channels. But, from where does it come, since normally, glutamate should not be present outside of the cells. It is likely that the cells release glutamate into solution in a random way, like those of Ach at neuromuscular junction (Fatt and Katz 1952). An excised outside–out patch which very likely contains glutamate channels, is like tiny antenna that opens every time when glutamate molecule binds to its receptors.

The glycine, which potentiates NMDA response, even in nM concentration (Johnson and Ascher 1987), did not change spontaneous activity. One of the reasons could be a non–constant concentration of glutamate in vicinity of the patch. Since the probability of opening of glutamate channels depends very much on concentration of glutamate (Kerry et al. 1988), maybe the glycine really does change the activity of these channels. This may not be possible to notice, because the concentration of glutamate in a vicinity of patch is a random variable. The other possibility, more reasonable is that, the glycine is already present in the culture (due to its spontaneous release from the cells) (Johnson and Ascher 1987), so after perfusion with glycine, its concentration is not changed noticeably. The glycine concentration found in conditioned medium of mouse neurons, was about 0.32 μM (Johnson and Ascher 1987). When they used technique of fast perfusion, an effect of desensitization was observed. We used slow perfusion technique, where potentiating action of glycine reflects an equilibrium of nondesensitized and desensitized receptors (Mayer et al. 1989), difficult to see in a single–channel recording.

The series of experiments which are in agreement with our hypothesis, are presented in Fig. 3.12, 15 and 16. Before washing a certain concentration of glutamate, already released from the cells, is present in the dish. After a more extensive washing, with a pure external solution, the activity decreases (Fig. 3.15a), since the concentration has been lowered, and glutamate released from the cells has been washed out. Similar experiment by moving pipette away from the bottom of the dish where neurons are placed, decreases activity (Fig. 3.15b), because if the cells release glutamate, clearly higher concentration will be present in the vicinity of the cells, i.e. near the bottom of the dish.

It should be noted that, soon after every perfusion, spontaneous activity decreases. After some time it becomes as before, if the perfusion has not contained a drug which affects spontaneous activity.

A further test has been done, in order to observe glutamate channels in the same patches. A perfusion with glutamate after washing opens some channels, with similar characteristics of spontaneous channels (Fig. 3.16).

An interesting experiment was inside–out excised patch in symmetric solutions with glutamate present in the pipette, where we saw some channels with reversal potential about 0 mV. Unfortunatelly, the effect was not evident, as shown in Fig. 3.12, because almost always an EAA experiment in cell–attach and inside–out configuration gives bad record (Sciancalepore, Personal communication).

EFFECT OF KYNURENIC ACID

Kynurenic acid (Kyn) is a very known blocker of non–NMDA receptor (Perouansky and Grantyn 1989), and reflects competitive antagonism, at both NMDA–agonist site and glycine–binding site (Mayer et al. 1989). The last two effects occur with no change in mean open time or single–channel conductance, estimated from spectral analysis of agonist–activated currents, and using rapid perfusion technique (Mayer et al. 1988). Kynurenic acid also reduces slow and fast components of glutamate activated whole–cell current, for about 70 %, in rat hippocampal neurons (Trussell et al. 1988). In their experiments glutamate current was probably affected by an antagonist action of kynurenic acid on agonist receptor, and also on glycine–binding site. This action decreases the probability of opening, during a constant pulse of glutamate.

Here, we report that, the activity of spontaneous channels was lowered, and big conductance channels remained open for much longer period of time, at positive and negative potentials, in the presence of kynurenic acid (Fig. 3.10). Mean open and close times were much longer.

If kynurenic acid, really affects spontaneous channels of big conductances in this way, a possible explanation would be that, a barrier of chemical reaction Glu+Glu–(receptor for EAA) is higher, in the presence of kynurenic acid. An illustration is presented in Fig. 4.1. Notice that, the energy difference between closed and open states does not change by an action of kynurenic acid. This is in agreement with an observation made by Trussell et al. (1988). They noticed that, the time curse of glutamate response did not change in the presence of Kyn. So, when glycine is binded to its receptor, it facilitates the glutamate response. In the presence of kynurenic acid, the glycine–receptor site is blocked, and the barrier of Glu–Glu(receptor for EAA) is higher, thus increasing the mean open and close times.

THE MECHANISM OF EAA RELEASE

Here we are discussing the possible mechanism of spontaneous release of EAA, in cerebellar granule cells culture, and also some recent works dealing with the same matter.

The neurotoxicity of EAA, especially glutamate, was observed in many neurological disorders (Novelli et al. 1988). Under " ischemic " conditions (namely, lack of oxygen and glucose), endogenous aspartate, glutamate, and some free radicals increased, in culture of the rat hippocampal slices (Pellegrini–Giampietro et al. 1990). Then in type of tissue damage observed within the thalamus of PTD (pyritthiamine–induced thiamine deficiency) rats, closely resembles that observed following anoxic–ischemic insults, and suggests the involvement of excitoxic amino acids in its pathogenesis. The glutamate antagonist MK–801, injected during the late stages, resulted in a marked attenuation of necrotic damage to thalamus and periacqueductal gray matter, and a reduction in the number and size of hemorrhagic lessions (Langlais and Mair 1990). It also has a protective effect against neuronal degeneration in rat brain after intracerebellar injection of NMDA agonist (Iversen et al. 1989). The addition of high concentration of magnesium, which blocks transmitter release, largely prevents neuronal loss under ischemic condition (Choi and Rothman

1990). Choi and Rothman (1990) proposed that during hypoxic–ischemic condition in the brain there is an overstimulation of glutamate receptors.

Now, our complete hypothesis would be: When the cells are cultured in normal condition, the presence of spontaneous channels is very rare, as reported by many authors. Spontaneous activity could arise from excitatory amino acids, mainly glutamate and aspartate. The excised outside–out patch receives an EAA pulses, probably an amount escaped from cell's EAA uptake system at synaptic terminal, or even spontaneous release of EAA due to a normal metabolic processes. In these condition the spontaneous activity is very low. On the other hand, when the cells are cultured in bad conditions (lacking glucose), the drastic increase of spontaneous activity is observed. This is exactly what happens with older cells in the cerebellar granule cells culture, when all glucose is wasted.

The release of EAA is much more frequent due to an increase of intracellular calcium. A possible explanation could be: Since glucose and oxygen are essential substances for mitochondrial function, deficiency of one of them or both, affects very much the process of producing an electrochemical proton gradient. The mitochondria harnesses a proton gradient to produce ATP from ADP, and import calcium from the cytosol to its interior (Watson et al. 1989). Thus, without glucose and oxygen calcium escapes from mitochondria to cytosol, and releases EAA in uncontroled way.

If our hypothesis is correct, it would be an electophysiological confirmation of biochemical quantitative experiments, done on hippocampal slices (Pellegrini–Giampietro et al. 1990). Very likely the same process occurs in the cerebellar granule cells *in vitro*. In fact, it has been demostrated that glucose metabolism, ATP production, and functioning Na^+, K^+–ATPases are necessary to generate a resting potential sufficient to mantain the voltage–dependent Mg^{2+} block of NMDA receptor channel (Novelli et al 1988). Very recent findings, in the culture of pyramidal neurons, from fields CA1 and CA3 of the hippocampus, suggests that probably the process occurs in this region (Bekkers and Stevens 1990). They observed spontaneous bursts of action potentials. In order to remove this spontaneous activity, they used cultures previously treated with APV.

FURTHER RESEARCH

We have successfully indentified the nature of these spontaneous activity of cerebellar granule cells in culture. They are glutamate activated channels, probably being activated by the spontaneous release of glutamate from other cells. This finding open the possibility to study also, the spontaneous synaptic activity in this preparation. It also has solved a practical problem in our experiments: the presence of spontaneous activity, that is a very frequent phenomena, has been a problem when experiments were undertaken. Now, using the appropriate blockers, more efficiency can be obtained.

The lack of glucose and oxygen is present during a stroke, during which, an depleted region of the brain is dying in short period of time. Some laboratories are searching for a cure based on the glutamate antagonist (Choi and Rothman 1990).

Swelling of primary astrocyte cultures by exposing them to hypotonic media caused release of L–glutamate (Kimelberg et al. 1990). Their studies *in vitro* suggest that swollen astrocytes may be an additional source of release for L–glutamate and L–aspartate.

The study of glutamate receptor with different concentration pulses could give us more information about " glutamate communication " between the cells, both in excised outside–out, and whole–cell configuration. Moreover, NMDA receptor gate a variety of complex behaviours, including memory formation, neuronal cell death in stroke and ischemia, and electrical excitability in epilepsy (Mayer et al. 1989). It is belived that calcium influx produced by learning rapidly and irreversibly increases the number of glutamate receptor in the synaptic membrane, which in turn induce the growth of synaptic contacts. The behavioral studies of learning in vertebrates suggest that memory lasting days or weeks can be disrupted by the inhibition of protein synthesis. So an electrophysiology of glutamate receptor should collaborate with genetic studies in order to resolve the problem of the long–term memory (Goelet et al. 1986).

Whereas overactivation of NMDA would lead to neuropathology, the loss of NMDA receptor function in various neurological diseases could lead to losses in cognition. For example in Alzheimer's disease (Cotman et al. 1988).

These examples, and many others not cited here, tell us how important is a study of the glutamate receptor, to understand a function of the brain.

Conclusions

1. Spontaneous channel activity is present in the cerebellar granule cells *in vitro*, at any day in culture.

2. Spontaneous channels have different conductances, ranging from 7pS to 50pS. The maximum value of conductance increases with days in culture.

3. Reversal potential of spontaneous channels is about 0 mV. They are cationic selective with high degree of selectivity.

4. Kinetical and pharmacological properties of spontaneous channels are very similar to glutamate receptor channels.

5. The spontaneous activity, which may arise from activation of EAA receptors, is due to spontaneous release of glutamate.

Appendix

1. SOLUTION 1

Composition	Concentration	Comments
NaCl	124.00mM	(1) pH=7.4, adjusted with NaOH.
KCl	5.37mM	(2) similar with physiological
NaH_2PO_4	1.01mM	solution.
D-glucose	14.50mM	(3) to be used for washing
HEPES	25mM	cerebellar tissue.
Phenol red	27μM	
$MgSO_4$	1.2mM	
BSA*	3mg/ml	

*bovine serum albumin (Sigma, St Louis)

2. SOLUTION 2

Composition	Concentration	Comments
NaCl	124.00mM	(1) made of 50ml solution 1
KCl	5.37mM	+ 12.5mg trypsin.
NaH_2PO_4	1.01mM	(2) for digesting the connective
D-glucose	14.50mM	tissue.
HEPES	25mM	
Phenol red	27μM	
$MgSO_4$	1.2mM	
BSA	3mg/ml	
trypsin*	0.25mg/ml	

* Sigma

3. SOLUTION 3

Composition	Concentration	Comments
NaCl	124.00mM	(1) made of 17ml solution 1 +
KCl	5.37mM	8ml solution 4.
NaH_2PO_4	1.01mM	(2) for stopping trypsinization
D-glucose	14.50mM	and degradation DNA.
HEPES	25mM	
Phenol red	27μM	
$MgSO_4$	1.7mM	
BSA	3mg/ml	
DNase*	25.6μg/ml	
SBTI**	166.4μg/ml	

* Sigma

** soybean trypsin inhibitor (Sigma)

4. SOLUTION 4

Composition	Concentration	Comments
NaCl	124.00mM	(1) made of 15ml solution 1
KCl	5.37mM	+ 1.2mg DNase + 7.8mg
NaH_2PO_4	1.01mM	SBTI + 150μl $MgSO_4$
D-glucose	14.50mM	stock solution (155mM).
HEPES	25mM	
Phenol red	27μM	(2) for proceeding mechanical
$MgSO_4$	2.75mM	separation.
BSA	3mg/ml	
DNase	80μg/ml	
SBTI	0.52mg/ml	

5. SOLUTION 5

Composition	Concentration	Comments
NaCl	124.00mM	(1) made of 12.5ml solution 1 +
KCl	5.37mM	15μl $CaCl_2$ stock solution
NaH_2PO_4	1.01mM	(81.6 mM) + 100μl $MgSO_4$
D-glucose	14.50mM	stock solution(155mM).
HEPES	25mM	
Phenol red	27μM	(2) Add Ca^{2+},which can not
$MgSO_4$	2.44mM	be added before the operation
$CaCl_2$	0.1mM	of trypsin.

6. NEURON GROWTH SOLUTION

Composition	Quantity	Comments
BEM*	500ml	for neuron growth.
L-glutamine	147mg	
KCl	825mg	
gentamicin**	1ml×50mg/ml	
FCS***	50ml	

* basal Eagle's medium (Flow Laboratories,Scotland, Irvine)

** Sigma

*** fetal calf serum heat inactivated (Flow Laboratories)

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