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Real Physiological Neuronal Responses Revealed by Gramicidin Perforated Patch Recording

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In order to understand the phenomenon in a living cell correctly, it has been required to obtain intact responses from the cell membrane without disrupting the cytoplasmic circumstances. Gramicidin perforated patch configuration allows the electrical access to the whole cell with a minimal dialysis of cytoplasm and preventing the loss of native intracellular constituents, such as Cl^-. Here, we would like to show the background of this method and the actual application of the gramicidin perforated patch recording mode on the dissociated neurons.

Key Words: Gramicidin perforated patch recording mode, Dissociated CNS neuron, Intracellular Cl^- concentration, Intracellular Cl^- regulation

INTRODUCTION

Recent developments and improvements in the conventional whole cell patch technique made it easier to study the membrane physiology (Lee et al, 1980; Neher, 1992): i.e., voltage dependent (sensitive) ion channel, ligand-gated ion channel, electrogenic Na^+/K^+ pump, Na^+/Ca^{2+} exchanger and so on. While this technique allows one to control the intracellular milieu with artificial pipette solutions, the dialysis of the intracellular side ablates the cytoplasmic biochemicals, which are required for channel and receptor activities (Harata et al, 1997), second messenger mediated responses (Kakehata et al, 1993) and intracellular Ca^{2+} buffering systems (Roberts, 1994). Although these substances could be arranged beforehand by adding them into the patch pipette solution, it is impossible to compensate the materials of which the concentration and the function could change, and unknown other factors.

These disadvantages of the conventional whole cell patch recording configuration were overcome by the development of perforated patch recording configurations using antibiotics, such as nystatin (Horn & Marty, 1988; Akaike & Harata, 1994), amphotericin B (Rae et al, 1991) and gramicidin (Hladky & Haydon, 1972; Akaike, 1997). The nystatin forms small pores (diameter; 4Å) on the cell membrane that only passes small monovalent cations and anions such as K^+, Na^+ and Cl^-, and allows the electrical access to the cell with preserving large intracellular molecules such as Ca^{2+}, cAMP and ATP (Fig. 1B). Therefore, the nystatin perforated patch recording mode could be applied on a wide variety of cells to obtain native responses that would be affected by intracellular mechanisms (Akaike & Harata, 1994). However, since the nystatin pore can pass the monovalent anions, the intracellular Cl^- concentration ($[Cl^-]_i$) depends on the Cl^- concentration in the patch pipette solution. Consequently, the native cell $[Cl^-]_i$ is disturbed by using nystatin method. Cl^- is one of the major and important constituents in the living cells and extracellular space, which is involved in regulating the cell volume and pH (Hoffmannetal, 1988), salt secretion and absorption (Deisz & Lux, 1982), G-protein coupled signal transduction (Higashijima et al, 1987) and modulation of membrane excitability via γ-aminobutyric acid (GABA) and/or glycine channels (Kaila, 1994). Considering these significance of Cl^-, the electrophysiological observa-

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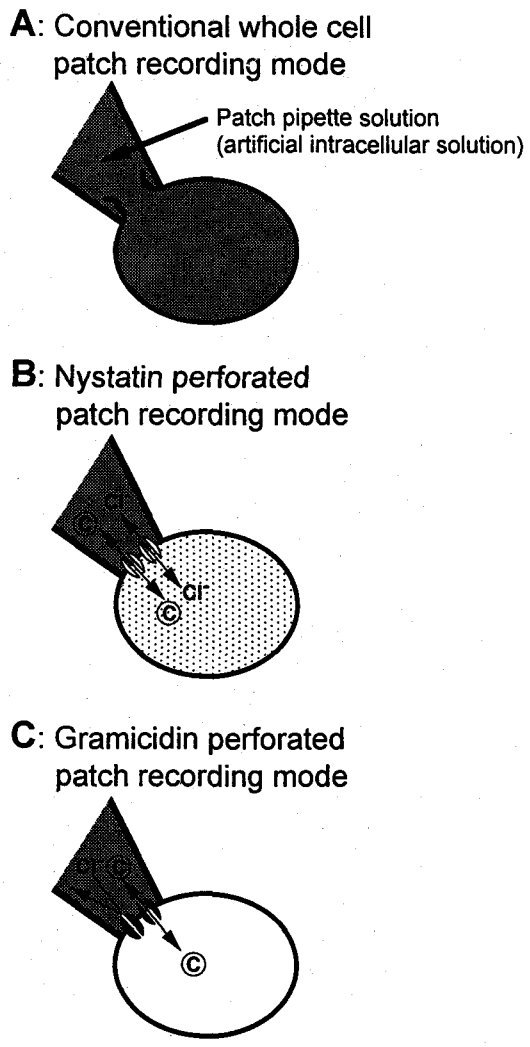

Fig. 1. Schematic illustrations of various types of whole cell patch recording configurations. Shaded areas, ⓒ and Cl^- indicate the artificial internal patch pipette solution, a monovalent cation and Cl^- ion, respectively. While the conventional whole cell patch recording mode can not avoid washing out the inside of the cell (A), the nystatin perforated patch recording mode gives restricted permeation to the both monovalent cation and anion (B), preventing a loss of large molecules in the cell. Likewise, the gramicidin formed pores have a permeability to monovalent cation but not to anion, that allows electrical access to the cell without disturbing $[Cl^-]_i$ (C).

tions with intact $[Cl^-]_i$ has been required, and was overcome by the coming of gramicidin perforated patch recording mode. Gramicidin is a polypeptide antibiotic that forms small pores in the cell membrane as well as nystatin, and the monovalent cations can move through the pores ($H^+ > NH_4^+ > > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$; Fig. 1A & 1C), but not any anions (Hladky & Haydon, 1972; Tajima et al, 1996). Thus, we could obtain the electrical responses from the cells maintaining the native $[Cl^-]_i$ unaffected by Cl^- concentration in a patch pipette solution.

METHODS

The stock solution of gramicidin is prepared by dissolving the gramicidin D (Sigma), which is a mixture of gramicidin A, B and C, into methanol at 10 mg/ml. After ultrasonication for a few seconds, the stock solution was directly dissolved in the patch pipette solution, resulting in a final concentration 0.1 mg/ml. Since the gramicidin in the patch pipette solution loses its activity within 2 hr, the gramicidin pipette solution should be refreshed frequently.

Patch pipettes are fabricated from glass tubes (Narishige, G-1.5) in two stage vertical pipette puller. The pipette resistance is 4~6 MΩ and the internal diameter of pipette is around 2 μm. The pipette tips are initially filled with gramicidin free internal solution by immersion, and then the remainder of the pipette is back-filled with the same patch pipette solution containing gramicidin. In the dissociated rat central nervous system (CNS) neurons, the membrane perforation by gramicidin (access resistance dropping to less than 20 MΩ) requires >30 min after making GΩ seal. Once gramicidin forms ionophore on the patch cell membrane, the ion conducting channels are constantly maintained at least 3~4 hr.

APPLICATION OF GRAMICIDIN PERFORATED PATCH RECORDING MODE TO CNS NEURONS

GABA and glycine responses

Application of the gramicidin perforated patch clamp recording configuration to the neuron enables one to obtain native GABA- or glycine-induced responses (Abe et al, 1994; Ebihara et al, 1995; Kakazu et al, 1999). Both GABA and glycine are the important inhibitory neurotransmitters in the mammalian CNS neuron (Kaila, 1994). The receptors for GABA could be divided into $GABA_A$, $GABA_B$ and $GABA_C$, among which $GABA_A$ and $GABA_C$ form the receptor-Cl^- channel complex (Kaila, 1994). Fig. 2A shows the GABA-induced outward current obtained

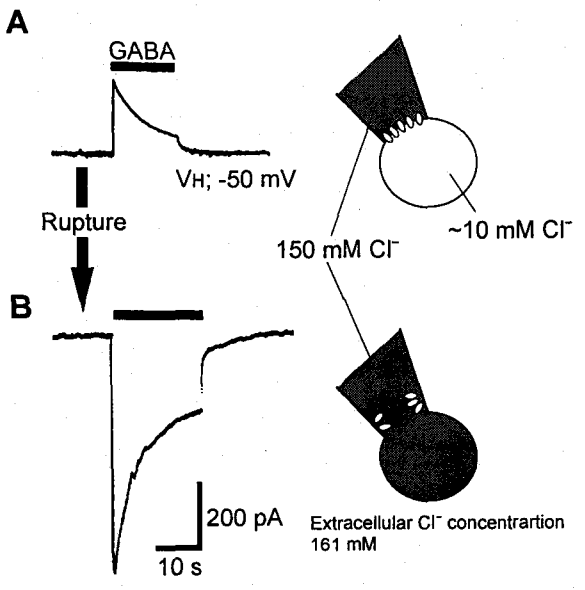

Fig. 2. Preservation of the native cell $[Cl^-]_i$ using gramicidin perforated patch recording. (A) the application of GABA induced the outward current in SNR neuron at a V_H of −50 mV. The gramicidin technique allows an electrophysiological recording with keeping intact $[Cl^-]_i$ (shown schematically at right of upper trace). (B) GABA-induced inward current was observed after the rupture of cell membrane in the same cell. Since dialysis of the neuron with patch pipette solution caused $[Cl^-]_i$ increasing, the efflux of Cl^- through $GABA_A$ receptor turned out (shown schematically at right of lower trace).

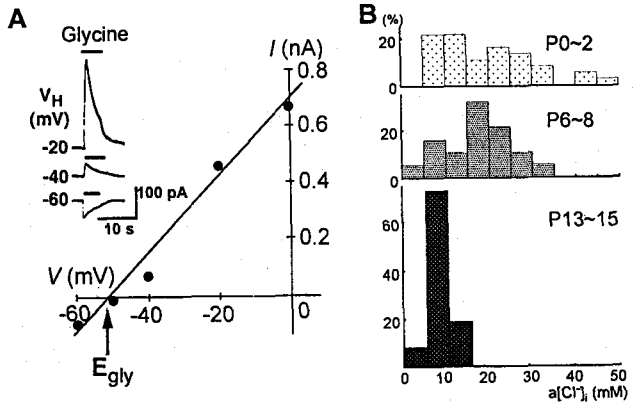

Fig. 3. Measuring the native $[Cl^-]_i$ using gramicidin perforated patch recording mode. (A) the amplitudes of glycine-induced Cl^- currents in rat VMH neuron at various V_Hs (*inset traces*) were plotted as a function of V_H, providing a reversal potential of glycine response (E_{gly}; arrow). (B) distribution of $[Cl^-]_i$ in developing rat LSO neuron was obtained by Nernst equation from $[Cl^-]_o$ and E_{gly} using gramicidin perforated patch recording mode (P; postnatal day). $[Cl^-]_i$ skewed to the low values with maturation.

from dissociated rat substantia nigra pars reticulata (SNR) neuron using gramicidin perforated patch recording mode at a holding potential (V_H) of −50 mV (Ebihara et al, 1995). Since the Cl^- is an anion, the outward current implies Cl^- flux into the cell (Cl^- influx) at that V_H. When a conventional whole cell configuration was made after rupturing the patch membrane by adding greater suction to the pipette interior, the direction of the GABA-induced current was dramatically changed to the inward (Fig. 2B). This meant that 150 mM Cl^- in the patch pipette solution intruded into the SNR neuron by the rupture of patch membrane, and the increased $[Cl^-]_i$ caused Cl^- flux out of the cell (Cl^- efflux) through $GABA_A$ receptor-Cl^- channel complex at the V_H. The series of the experiment demonstrated that Cl^- could not pass the gramicidin formed pores.

The value of intact $[Cl^-]_i$ could be drawn by making a use of native GABA or glycine responses obtained by using gramicidin perforated patch recording mode. The amplitude of glycine-induced currents in the rat ventromedial hypothalamic (VMH) neuron at postnatal day 8～12 was plotted as a function of each V_H in Fig. 3A (Abe et al, 1994). The intersection of depicted line and X-axis (voltage) provides glycine-gated Cl^- reversal potential (E_{gly}: arrow in Fig. 3A). Using both the known extracellular Cl^- concentration ($[Cl^-]_o$) and the E_{gly} value, the Nernst equation gives $[Cl^-]_i$ ($E_{gly}=58.4\times\log([Cl^-]_o/[Cl^-]_i)$).

The lateral superior olive (LSO) neurons in rat brainstem, one of the central auditory nucleus, receives the auditory information from both the contralateral side as the inhibitory glycinergic inputs and the ipsilateral side as the excitatory glutamatergic inputs, and gives cues for determination of sound localization (Sanes & Rubel, 1988). In the immature LSO neurons (postnatal day 0～2), the depolarization is induced by the stimulation of glycinergic inputs or the application of glycine (Kandler & Friauf, 1995; Kakazu et al, 1999). However, the depolarizing response turns into the well-known hyperpolarizing one with maturation. Then, using gramicidin perforated patch recording mode, the native $[Cl^-]_i$ in the LSO neuron at each developmental stage was determined by the method mentioned above (Fig. 3B) (Ehrhich et al, 1999; Kakazu et al, 1999). The results indicate that the depolarization induced by glycine in the early developmental stage of LSO neuron was due to the high $[Cl^-]_i$. Subsequently, the $[Cl^-]_i$ in the LSO neuron gradually declined with maturation, and the

hyperpolarization was induced by glycine because of the low $[Cl^-]_i$. Besides this case in developing neurons, the depolarization induced by GABA or glycine have been also reported in the traumatic neuron by excess heat, neurite transection (von den Pol et al, 1996) or epileptiform activity (McCarren & Alger, 1985). In these cases, the gramicidin perforated patch recording mode revealed native excitatory GABA or glycine responses, which is caused by increased $[Cl^-]_i$.

Regulation of intracellular Cl^- concentration

Using gramicidin perforated patch recording configuration, the exogenous application of GABA or glycine generates steady currents as long as the interval duration of the ligand application is enough. The occurrence of Cl^- currents represents influx or efflux of Cl^- through Cl^- channels, and should lead the change of $[Cl^-]_i$. Since Cl^- can not pass the gramicidin pores, the constancy of GABA- or glycine-induced currents reflects the existence of $[Cl^-]_i$ regulator which maintains a stable $[Cl^-]_i$. The amplitude of glycine-induced Cl^- currents, which were well maintained at certain intervals of each glycine application without furosemide, were gradually decreased every glycine application in the presence of furosemide (Fig. 4A, Thompson & Ghwiler, 1989; Kakazu et al, 1999). When the $[Cl^-]_i$ was monitored, the value of $[Cl^-]_i$ shifted to the passive $[Cl^-]_i$ determined by V_H and $[Cl^-]_o$ (Fig. 4B). These results indicate that the furosemide- sensitive $[Cl^-]_i$ regulator pumping Cl^- out of the cell plays an pivotal role in maintaining $[Cl^-]_i$. The furosemide-sensitive $[Cl^-]_i$ regulator is considered as the co-transport system, which is integrated in cell membrane and carries the same number of monovalent cation (Na^+, K^+) and Cl^- to the same direction (Kaila, 1994; Payne et al, 1996). The electrical neutral nature of co-transport does not allow direct observation by electrophysiological techniques. However, the advent of the gramicidin perforated patch method enable us to evaluate the contribution of co-transport by measuring the $[Cl^-]_i$, because the $[Cl^-]_i$ is thought to reflect the amount of Cl^- carried in or out by co-transport and the gramicidin technique makes it possible to monitor a intact $[Cl^-]_i$ in a real time manner. Actually, when the concentration of extracellular K^+, which is co-transported with Cl^-, was increased, $[Cl^-]_i$ also changed gradually and finally

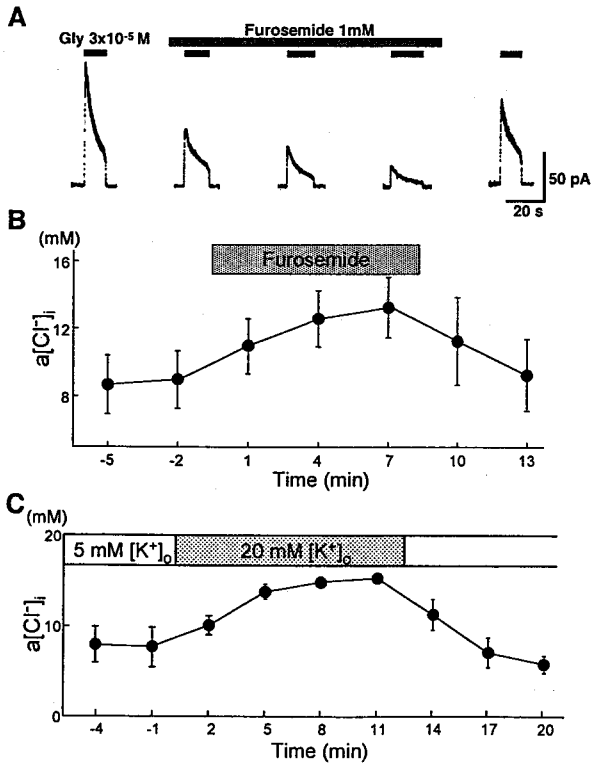

Fig. 4. Neuronal $[Cl^-]_i$ maintained by furosemide-sensitive cation coupled Cl^- cotransport. (A) In the presence of furosemide (shaded bar), the amplitude of glycine-induced outward current recorded by gramicidin perforated method in LSO neuron gradually decreased at a V_H of -50 mV. (B) $[Cl^-]_i$ were plotted as a function of time before, during and after the furosemide. The low $[Cl^-]_i$ was increased by furosemide but returned to its original value after the removal of furosemide, suggesting that the furosemide-sensitive $[Cl^-]_i$ regulator pumped Cl^- out of the cell. (C) $[Cl^-]_i$ were plotted as a function of time before, during and after 20 mM extracellular K^+ concentration.

reached to new value (Fig. 4C), demonstrating that the cation coupled co-transport acts as a major role in determining $[Cl^-]_i$. The application of this gramicidin perforated patch recording technique endows a new way of assessment to cell physiology.

There still remains a lot of questions concerning the homeostatic regulation of $[Cl^-]_i$ in living cells, such as the relationship between cation channel and Cl^- channel (Reddy et al, 1999), Cl^- pump (Inagaki et al, 1996) and the link between Cl^- and bicarbonate (Staley et al, 1995). The development of the gramicidin perforated patch technique will contribute to revealing cell physiological function and mechanism.

CONCLUSION

Recent development of whole cell patch clamp technique has contributed to the great progress in the cell physiology. However, the disturbance of the cell inside by the patch pipette solution disables us to obtain native cell responses. Perforated patch clamp technique using antibiotics (nystatin, amphotericin B and gramicidin) allows accessing cell inside electrically with a minimal dialysis of the patch pipette solution. Notably, Gramicidin perforated patch clamp recording mode whose pores can not pass Cl^- enable us to observe a native GABA or glycine responses, and to obtain an intact $[Cl^-]_i$, showing that electrical neutral Cation-Cl^- co-transport system plays an major role in determining neuronal $[Cl^-]_i$. Its broad applicability will provide important information relevant to the physiological conditions of cells.

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REFERENCES

Abe Y, Furukawa K, Itoyama Y, Akaike N. Glycine response in acutely dissociated ventromedial hypothalamic neuron of the rat. *J Neurophysiol* 72: 1530−1537, 1994

Akaike N, Harata N. Nystatin perforated patch recording and its applications to analyses of intracellular mechanisms. *Jpn J Physiol* 44: 433−473, 1994

Akaike N. Gramicidin perforated patch recording and intracellular chloride activity in excitable cells. *Prog Biophys Molec Biol* 65: 251−264, 1997

Deisz RA, Lux HD. The role of intracellular chloride in hyperpolarizing post-synaptic inhibition of crayfish stretch receptor neurones. *J Physiol (Lond)* 326: 123−138, 1982

Ebihara S, Shirato K, Harata N, Akaike N. Gramicidin-perforated patch recording: GABA response in mammalian neurones with intact intracellular chloride. *J Physiol (Lond)* 484: 77−86, 1995

Ehrlich I, Lohrke S, Friauf E. Shift from depolarizing to hyperpolarizing glycine action in rat auditory neurones is due to age-dependent Cl^- regulation. *J Physiol (Lond)* 520: 121−137, 1999

Harata N, Wu J, Ishibashi H, Ono K, Akaike N. Run-down of the $GABA_A$ response under experimental ischaemia in acutely dissociated CA1 pyramidal neurones of the rat. *J Physiol (Lond)* 500: 673−688, 1997

Higashijima T, Ferguson KM, Sternweis PC. Regulation of hormone-sensitive GTP-dependent regulatory proteins by chloride. *J Biol Chem* 262: 3597−3602, 1987

Hladky SB, Haydon DA. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim Biophys Acta* 274: 294−312, 1972

Hoffmann EK, Lambert IH, Simonsen LO. Mechanisms in volume regulation in Ehrlich ascites tumor cells. *Ren Physiol Biochem* 11: 221−247, 1988

Horn R, Marty A. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* 92: 145−159, 1988

Inagaki C, Hara M, Zeng XT. A Cl^- pump in rat brain neurons. *J Exp Zool* 275: 262−268, 1996

Kaila K. Ionic basis of $GABA_A$ receptor channel function in the nervous system. *Prog Neurobiol* 42: 489−537, 1994

Kakazu Y, Akaike N, Komiyama S, Nabekura J. Regulation of intracellular chloride by cotransporters in developing lateral superior olive neurons. *J Neurosci* 19: 2843−2851, 1999

Kakehata S, Nakagawa T, Takasaka T, Akaike N. Cellular mechanism of acetylcholine-induced response in dissociated outer hair cells of guinea-pig cochlea. *J Physiol (Lond)* 359: 189−217, 1993

Kandler K, Friauf E. Development of glycinergic and glutamatergic synaptic transmission in the auditory brainstem of perinatal rats. *J Neurosci* 15: 6890−6904, 1995

Lee KS, Akaike N, Brown AM. The suction pipette method for internal perfusion and voltage clamp of small excitable cells. *J Neurosci Methods* 2: 51−78, 1980

McCarren M, Alger BE. Use-dependent depression of IPSPs in rat hippocampal pyramidal cells in vitro. *J Neurophysiol* 53: 557−571, 1985

Neher E. Ion channels for communication between and within cells. *Science* 256: 498−502, 1992

Payne JA, Stevenson TJ, Donaldson LF. Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem* 271: 16245−16252, 1996

Rae J, Cooper K, Gates P, Watsky M. Low access resistance perforated patch recordings using amphotericin B. *J Neurosci Methods* 37: 15−26, 1991

Reddy MM, Light MJ, Quinton PM. Activation of the epithelial Na^+ channel (ENaC) requires CFTR Cl^- channel function. *Nature* 402: 301−304, 1999

Roberts WM. Localization of calcium signals by a mobile calcium buffer in frog saccular hair cells. *J Neurosci* 14: 3246−3262, 1994

Sanes DH, Rubel EW. The ontogeny of inhibition and excitation in the gerbil lateral superior olive. *J Neurosci* 8: 682−700, 1988

Staley KJ, Soldo BL, Proctor WR. Ionic mechanisms of neuronal excitation by inhibitory $GABA_A$ receptors. *Science* 269: 977−981, 1995

Tajima Y, Ono K, Akaike N. Perforated patch-clamp recording in cardiac myocytes using cation-selective ionophore gramicidin. *Am J Physiol* 271: C524−C532, 1996

Thompson SM, Gähwiler BH. Activity-dependent disinhibition. II. Effects of extracellular potassium, furosemide, and membrane potential on E_{Cl-} in hippocampal CA3 neurons. *J Neurophysiol* 61: 512−523, 1989

von den Pol AN, Obrietan K, Chen G. Excitatory actions of GABA after neuronal trauma. *J Neurosci* 16: 4283−4292, 1996