GABA and Lioresal Actions on the Identified *Onchidium* Neuron

Nobuaki SHIMIZU, Norio AKAIKE, Yutaka OOMURA,
Juro MARUHASHI,* and Manfred R. KLEE**

Department of Physiology, Faculty of Medicine, Kyushu University,
Fukuoka, Fukuoka, 812 Japan

*Department of Biology, Daiichi College of Pharmaceutical Sciences,
Fukuoka, Fukuoka, 815 Japan

**Max-Planck Institute, Frankfurt, Germany

Abstract Responses of identified single neurons in the isolated right cerebral ganglion of *Onchidium* to bath applied γ -aminobutyric acid (GABA) and lioresal (LRS) were measured using conventional intracellular microelectrode techniques under current and voltage clamp conditions. In normal medium the neurons responded to both GABA and LRS. GABA induced two successive hyperpolarizations (phase I and II). Phase I was a short-lasting Cl^- permeability increase while phase II was a long-lasting K^+ permeability increase. LRS evoked only a long-lasting K^+ permeability increase. In Ca^{2+} -free high Mg^{2+} medium GABA produced only short-lasting hyperpolarization while LRS had no effect. GABA-induced phase I responses behaved as a simple Cl^- electrode following changes of external Cl^- concentrations. The Hill coefficient obtained from the relation between the relative change in the GABA current and the GABA concentration was almost one during the current increase phase in Ca^{2+} -free high Mg^{2+} medium. These results indicate that GABA has a direct Cl^- permeability increase action on the postsynaptic cell membrane of the identified *Onchidium* neuron while LRS has no such direct action.

Key Words: *Onchidium*, neuron, GABA, lioresal, Cl^- current.

Lioresal (LRS), the β -(*p*-chlorophenyl) derivative of γ -aminobutyric acid (GABA) has been thought to be GABA-mimetic at neuronal receptors. Recently, it has been reported that there is evidence for and against a GABA-mimetic action of LRS (NAIK *et al.*, 1976; DAVIDOFF, 1978; FOX *et al.*, 1978; POTASHNER, 1979; WADDINGTON and CROSS, 1979; BOWERY *et al.*, 1980; WADDINGTON and CROSS, 1980). In an identified *Onchidium* neuron immersed in sea water having

Received for publication January 14, 1983

清水宣明, 赤池紀扶, 大村 裕, 丸橋寿郎

half Ca^{2+} and 3 times Mg^{2+} , GABA produces hyperpolarization in two phases, *i. e.* a short-lasting Cl^- permeability increase followed by a rapid desensitization and a long-lasting K^+ permeability increase, while LRS elicits only a long-lasting hyperpolarization resulting from K^+ permeability increase (OOMURA *et al.*, 1979, 1982). In our recent preliminary experiments, however, the *Onchidium* neuron perfused with sea water containing half Ca^{2+} and 3 times Mg^{2+} could still elicit IPSPs, and the presynaptic activities could be blocked only by a continuous perfusion of Ca^{2+} -free high Mg^{2+} solution containing Co^{2+} for 20 to 30 min. The long-lasting hyperpolarization to both GABA and LRS decreased in parallel with the decrease of IPSPs amplitude in Ca^{2+} -free high Mg^{2+} solution. Therefore, the aim of the present experiments is to examine quantitatively whether GABA and LRS have a direct action on postsynaptic soma membrane of the identified *Onchidium* neuron under current and voltage clamp conditions.

MATERIALS AND METHODS

The identified neuron in the isolated right cerebral ganglion of *Onchidium* was used (Fig. 1). The experimental set up was similar to that of previous studies (OOMURA *et al.*, 1979). Briefly intracellular microelectrodes were filled with 0.7 M K_2SO_4 . Two microelectrodes were inserted into an identified single neuron, one being used to pass current and the other to record membrane potential.

The ionic composition (mM) of normal artificial sea water was the same as that in the previous report (OOMURA *et al.*, 1979): NaCl, 457.6; KCl, 9.6; CaCl_2 , 10.4; MgCl_2 , 48.5; pH 7.5 by Hepes-NaOH. Ca^{2+} -free high Mg^{2+} sea water had

Fig. 1. A schematic illustration of the dorsal view of the *Onchidium* oesophageal ganglia. The GABA-sensitive neuron, #11 (indicated by an arrow), is located in the right cerebral ganglion (RCG). LCG, left cerebral ganglion; LPG and RPG, left and right pleural ganglia; AN, MN, and PN, anterior, medial, and posterior pleural nerves; TN and VN, tentacle and visceral nerves.

the following composition (mM): NaCl, 400; KCl, 9.6; CoCl₂, 10; MgCl₂, 100; pH 7.5. The Ca²⁺-free high Mg²⁺ test solutions containing various concentrations of Cl⁻ were made by replacing Cl⁻ with an equimolar isethionate⁻ in the external solution while increase of Cl⁻ was made by adding extra NaCl to the medium. Preparations were superfused at a rate of 2 ml/min.

The ionic currents during voltage clamp were stored on a tape recorder and plotted directly by a pen-writing recorder. GABA (Tokyo Kasei) and LRS (Ciba-Geigy) were dissolved in test solution just before use, and the drugs were examined by bath application. All experiments were carried out at room temperatures of 18 to 21°C.

RESULTS

1. Ionic mechanism

In normal artificial sea water, two different types of hyperpolarizing responses to 3 mM GABA were observed in this identified neuron (Fig. 2A). The present results were quite similar to those reported by OOMURA *et al.* (1979). The phase I hyperpolarization was rapid, peaking at about 30 sec, and was followed by desensitization. It resulted exclusively from an increased conductance to Cl⁻, since the responses were sensitive to external Cl⁻ concentration and picrotoxin. The results were also the same as the results of OOMURA *et al.* (1982). The GABA-induced phase II hyperpolarization increased to a maximal value in 3 to 4 min, and the increased conductance continued for an additional 10 min even after washing out GABA by normal external solution. According to OOMURA *et al.* (1982), the phase II hyperpolarization results from the increased conductance to K⁺. On the other hand, LRS produced only a continuous tonic hyperpolarization resulting from K⁺ conductance increase as shown in Fig. 2B.

Involvement of Na⁺ in GABA- or LRS-induced potential and conductance changes was tested by replacing external NaCl with an equimolar Tris Cl. Neither of the two successive hyperpolarizations nor the conductance increases produced by GABA were diminished. LRS action was also hardly affected by the Na⁺-free medium. In the same cell, the substitution of isethionate⁻ for external Cl⁻ markedly changed only the GABA-induced short-lasting hyperpolarization. The concentration changes of external K⁺ affected on GABA- and LRS-induced long-lasting hyperpolarization. The qualitative results about the relationship between GABA response and the external K⁺ or Cl⁻ concentration were already reported from our laboratory (OOMURA *et al.*, 1982).

When the bathing medium was exchanged from a normal medium to a Ca²⁺-free high Mg²⁺ medium, considerable fluctuations of resting membrane potentials and bursts of action potentials were evoked and continued for at least 20 to 30 min. Thereafter, the synaptic inputs on the postsynaptic cell membrane and the spontaneous action potentials ceased, at which time current injection could elicit a full

Fig. 2. GABA, and LRS-induced hyperpolarization in the same neuron. Perfusion of GABA or LRS was indicated by each horizontal bars. Resting membrane potential: -40 mV. Downward and upward deflections indicate the membrane input resistance produced by constant square pulses of 3 nA and 1 sec in duration given at each 10 sec intervals and the action potentials (their amplitude was distorted by pen response), respectively. Note that GABA-induced short- and long-lasting hyperpolarizations are accompanied by the increase in membrane conductance. LRS induced only a long-lasting hyperpolarization.

Fig. 3. GABA action in Ca^{2+} -free high Mg^{2+} medium. A: 3 mM GABA elicited only a short-lasting hyperpolarization. Resting membrane potential: -40 mV. Electrotonic potentials were evoked by current pulses of 1 nA and 500 msec in duration at 5 sec intervals. B: Na-spikes in the same neuron immersed in Ca^{2+} -free high Mg^{2+} medium for 6 hr. Resting membrane potential (V_r) is increased to -60 mV.

size Na-spike (Fig. 3B). This result suggests that the electrical properties of postsynaptic membrane are intact in Ca^{2+} -free high Mg^{2+} solution. In this Ca^{2+} -free high Mg^{2+} medium, GABA elicited only the phase I hyperpolarization which is sensitive to external Cl^- concentrations and picrotoxin (Fig. 3A). The GABA-

induced phase II hyperpolarization as well as the LRS-induced hyperpolarization completely disappeared in Ca²⁺-free high Mg²⁺ solution, indicating that the phase II hyperpolarization found in the presence of Ca²⁺ is probably attributed to a secondary effect by presynaptic origin or Ca²⁺-activated K⁺ current. Therefore, following voltage clamp experiments were performed in Ca²⁺-free high Mg²⁺ medium.

2. Dose-response relationship of GABA in Ca²⁺-free high Mg²⁺ medium

The ionic current of the identified neuron of *Onchidium* was evoked by adding GABA to Ca²⁺-free high Mg²⁺ external solution while the solution was flowing. The increase of ionic current depended on GABA concentrations, although the responses were followed with a more rapid desensitization at higher concentration (Fig. 4A). The dose-response curve of GABA was sigmoidal and was almost maximal at 10 mM GABA (Fig. 4B). It took a wash of about 30 min to completely reverse the GABA effect at such a high concentration.

According to HIGASHI and NISHI (1982), if it is assumed that GABA combines with the receptor and that the increase of GABA-induced current is proportional to the number of receptors occupied by GABA, then $n(\text{GABA}) + \text{Receptor} \rightleftharpoons (\text{GABA})_n(\text{Receptor}) \rightarrow \text{Current increase}$, where n is the number of GABA molecules required to activate a receptor. At equilibrium,

Fig. 4. GABA responses from the identified neuron immersed in Ca²⁺-free high Mg²⁺ external solution. A: GABA-induced outward currents. Holding potential (V_H) was -40 mV. All records were obtained from the same cell. B: dose-response curve of GABA-induced ionic current. A solid line represents a theoretical curve obtained from Eq. 1 in the text. Each filled circle and vertical bars are mean $\pm$ S.D. in 6 neurons.

$$I = I_{\max} \left(\frac{a^n}{a^n + k^n} \right) \quad (1)$$

where I is the GABA induced current, $I_{\max}$ is the maximal value of a GABA induced current, a is the GABA concentration, and k is the geometric mean of the successive microscopic binding constants (ADAMS, 1975). The curve in Fig. 4B is drawn according to Eq. 1, assuming $n=1$ and $k=5.7 \times 10^{-4}$ M. The filled circles represent the mean of 6 experiments. The theoretical curve fits the experimental results well.

3. Ionic mechanism of the GABA-induced current in Ca^{2+} -free high Mg^{2+} medium

The equilibrium potential for the current activated by 1 mM GABA (E_{GABA}) was determined by evoking the GABA responses after shifting the membrane potential from holding potential (V_H) of -40 mV to various membrane potentials (Fig. 5A). In a neuron for which the result is shown in Fig. 5B, E_{GABA} was about -60 mV. The average value of E_{GABA} for 7 neurons was 57.2 ± 4.8 mV (mean $\pm$ S. E.). The E_{GABA} was not affected by either substituting Tris^+ for Na^+ or increasing external K^+ concentration from 9.6 to 19.2 mM.

4. Effect of changing external Cl^- concentration

Change of extracellular Cl^- concentration ($[\text{Cl}]_o$) directly affected the GABA-

Fig. 5. Determination of GABA equilibrium potential under voltage clamp condition. A: GABA-induced current changes measured at different membrane potentials. Downward deflections indicate conductance changes which were induced by 5 mV hyperpolarizing pulses of 500 msec at 4 sec intervals. B: current-voltage relationship for determination of GABA equilibrium potential (E_{GABA}). The application of GABA at 1 mM concentration was repeated on the same neuron at 30 min intervals, and in sequence numbered (1–8) beside each filled circle. The graph yields an E_{GABA} of -61 mV.

Fig. 6. Dependency of E_{GABA} on the external Cl⁻ concentration ($[Cl]_o$). Each point represents the average value of 7 neurons. Vertical bar for each point shows ± 1 S.D. of the mean. A straight line represents a slope of 58 mV predicted by the Nernst equation for Cl⁻ electrode. Arrow indicates the theoretical intracellular Cl⁻ concentration ($[Cl]_i$).

induced current and its equilibrium potential. When $[Cl]_o$ was decreased, E_{GABA} became more positive. A semi-logarithmic plot of the average E_{GABA} as a function of $[Cl]_o$ is shown in Fig. 6. A plot of E_{GABA} versus $[Cl]_o$ yielded a straight line having a slope of 58 mV for a tenfold change in $[Cl]_o$ as predicted by the Nernst equation for the Cl⁻ equilibrium potential (E_{Cl}). Therefore, the GABA-induced current in this neuron is due to a selective increase in Cl⁻ permeability across the cell membrane. Therefore, if it is assumed that E_{GABA} is equal to E_{Cl} in Ca²⁺-free high Mg²⁺ medium, the intracellular Cl⁻ concentration is estimated to be 57 mM for this neuron.

DISCUSSION

In peripheral and central mammalian neurons, LRS is a GABA-like neuronal depressant but is inactive at the postsynaptic, bicuculline- and picrotoxin-sensitive GABA receptor (CURTIS *et al.*, 1974; DAVIES and WATKINS, 1974; KROGSGAARD-LARSEN *et al.*, 1977; AULT and EVANS, 1978; BOWERY *et al.*, 1980; WADDINGTON and CROSS, 1980; Higashi, Inokuchi, and Nishi, personal communication). It has been suggested that, the receptor for LRS action is located presynaptically (BOWERY *et al.*, 1980). It is well known that synaptic transmitter release is blocked by bathing in low Ca²⁺ and high Mg²⁺ medium (KATZ and MILEDI, 1965; HUBBARD *et al.*, 1968; LLINAS and NICHOLSON, 1975; ERULKAR *et al.*, 1978). In our previous paper (OOMURA *et al.*, 1982), the effects of GABA and LRS on the identified *Onchidium* neuron were investigated in either normal artificial sea water or sea water containing half Ca²⁺ and 3 times Mg²⁺. Both solutions gave the same results, in that GABA induced two successive hyperpolarizations and LRS-induced a long-lasting hyperpolarization. Therefore, we concluded that these hyperpolarizations were elicited through direct action on the postsynaptic cell membrane.

In the present experiments, however, these long-lasting hyperpolarizations were not affected by the complete removal of Na^+ from normal external solution but blocked by Ca^{2+} -free high Mg^{2+} solution containing Co^{2+} . Therefore, it may be concluded that the GABA- and LRS-induced long-lasting hyperpolarizations are due to the presynaptic origin. However, we can not completely rule out the possibility that GABA and LRS can directly activate Ca^{2+} influx through the postsynaptic cell membrane, which would activate Ca^{2+} -dependent K^+ current and hyperpolarize the membrane (MEECH, 1978; AKAIKE *et al.*, 1983). Such questions require future study. On the other hand, it seems clear that the GABA-induced early hyperpolarization is due to the selective increase of Cl^- conductance in the postsynaptic membrane since the amplitude of the hyperpolarization is not different with or without Ca^{2+} in the external solution.

When GABA or LRS was added to a Na^+ - and Ca^{2+} -free medium bathing the internally perfused frog dorsal root ganglion cell, only GABA evoked a short-lasting current which was followed by desensitization. The GABA-induced current was not diminished by a successive substitution of Cs^+ for K^+ in either external or internal medium, but depended on the external or internal Cl^- concentrations (unpublished data). The results are in agreement with NISHI and his coworkers' reports by using microelectrode technique (1974). It is clear from these results that GABA has a direct action on the postsynaptic soma membrane and that GABA-induced current results from the increase of Cl^- permeability.

In the muscle fibers of crayfish where the desensitization of GABA-induced current is less pronounced, the Hill coefficients of 2 and 3 have been reported by TAKEUCHI and TAKEUCHI (1967) and FELTZ (1971), respectively. In the present study, GABA-induced I_{Cl} of the *Onchidium* neuron gave Hill coefficient, $n=1$, for the dose-response curve. Such a low Hill coefficient may be explained from the rapid desensitization in this identified neuron.

The authors thank Dr. H. Higashi and Dr. D. Carpenter for their helpful discussions in the preparation of this manuscript. The present study was partially supported by a Grant-in-Aid for Scientific Research (No. 577075) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- ADAMS, P. R. (1975) An analysis of the dose-response curve at voltage-clamped frog-endplate. *Pflügers Arch.*, **360**: 145-153.
- AKAIKE, N., BROWN, A. M., DAHL, G., HIGASHI, H., ISENBERG, G., TSUDA, Y., and YATANI, A. (1983) Voltage-dependent activation of K current in *Helix* neurons by endogenous cellular calcium. *J. Physiol. (Lond.)*, **334**: 309-324.
- AULT, B. and EVANS, R. H. (1978) Central depressant action of baclofen. *J. Physiol. (Lond.)*, **284**: 131P.
- BOWERY, N. G., DOBLE, A., HILL, D. R., HUDSON, A. L., SHAW, J., and TURNBULL, M. J. (1980) β -Chlorophenyl GABA (Baclofen) is a selective ligand for a novel GABA receptor on nerve terminals. *Brain Res. Bull. (Suppl. 2)*, **5**: 497-502.

- CURTIS, D. R., GAME, C. J. A., HOHNSTON, G. A. R., and McCULLOCH, R. M. (1974) Central effects of β -(*p*-chlorophenyl)- γ -aminobutyric acid. *Brain Res.*, **70**: 493-499.
- DAVIDOFF, R. A. (1978) Pharmacology of spasticity. *Neurology*, **28**: 46-51.
- DAVIES, J. and WATKINS, J. C. (1974) The action of β -phenyl-GABA derivatives on neurones of the cat cerebral cortex. *Brain Res.*, **70**: 501-505.
- ERULKAR, S. D., RAHAMIMOFF, R., and ROTSHENKER, S. (1978) Quelling of spontaneous transmitter release by nerve impulses in low extracellular calcium solutions. *J. Physiol. (Lond.)*, **278**: 491-500.
- FELTZ, A. (1971) Competitive interaction of β -guanidino-propionic acid and γ -aminobutyric acid on the muscle fibre of the crayfish. *J. Physiol. (Lond.)*, **216**: 391-401.
- FOX, S., KRNEVIC, K., MORRIS, M. E., PUIL, E., and WERMAN, R. (1978) Action of baclofen on mammalian synaptic transmission. *Neuroscience*, **3**: 495-515.
- HIGASHI, H. and NISHI, S. (1982) 5-Hydroxytryptamine receptors of visceral primary afferent neurones on rabbit nodose ganglia. *J. Physiol. (Lond.)*, **323**: 543-567.
- HUBBARD, J. I., JONES, S. F., and LANDAU, E. M. (1968) On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *J. Physiol. (Lond.)*, **196**: 75-86.
- KATZ, B. and MILEDI, R. (1965) The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. Lond. (Biol.)*, **161**: 496-503.
- KROGSGAARD-LARSEN, P., JOHNSTON, G. A. R., LODGE, D., and CURTIS, D. R. (1977) A new class of GABA agonist. *Nature*, **268**: 53-55.
- LLINAS, R. and NICHOLSON, C. (1975) Calcium in depolarization-secretion coupling: An aequorin study in squid giant synapse. *Proc. Natl. Acad. Sci. U.S.A.*, **72**: 187-190.
- MEECH, R. W. (1978) Calcium-dependent potassium activation in nervous tissues. *Annu. Rev. Biophys. Bioeng.*, **7**: 1-18.
- NAIK, S. R., GUIDOTTI, A., and COSTA, E. (1976) Central GABA receptor agonist: Comparison of muscimol and baclofen. *Neuropharmacology*, **15**: 479-484.
- NISHI, S., MINOTA, S., and KARCZMAR, A. G. (1974) Primary afferent neurones: The ionic mechanism of GABA-mediated depolarization. *Neuropharmacology*, **13**: 215-219.
- OOMURA, Y., MARUHASHI, J., SHIMIZU, N., and KATO, M. (1979) GABA receptors and ionic channels in *Onchidium* neuron. *Brain Res. Bull.*, **4**: 178-180.
- OOMURA, Y., OOYAMA, H., and SAWADA, M. (1974) Analysis of hyperpolarizations induced by glutamate and acetylcholine on *Onchidium* neurones. *J. Physiol. (Lond.)*, **243**: 321-341.
- OOMURA, Y., SHIMIZU, N., and MARUHASHI, J. (1982) Chemical structure of the GABA receptive membrane in *Onchidium* neurons studied by electrophysiological analysis. In: *Problems in GABA Research from Brain to Bacteria*, ed. by OKADA, Y. and ROBERTS, E. Excerpta Medica, Amsterdam, Oxford, and Princeton, pp. 69-79.
- POTASHNER, S. J. (1979) Baclofen: Effects on amino acid release and metabolism in slices of guinea pig cerebral cortex. *J. Neurochem.*, **32**: 103-109.
- TAKEUCHI, A. and TAKEUCHI, N. (1967) Anion permeability of the inhibitory postsynaptic membrane of the crayfish neuromuscular junction. *J. Physiol. (Lond.)*, **191**: 575-590.
- WADDINGTON, J. L. and CROSS, A. J. (1979) Baclofen and muscimol: Behavioural and neurochemical sequelae of unilateral intranigral administration and effects on ³H-GABA receptor binding. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **306**: 275-280.
- WADDINGTON, J. L. and CROSS, A. J. (1980) GABA-ergic properties of baclofen *in vivo* and *in vitro*. *Brain Res. Bull. (Suppl. 2)*, **5**: 503-505.