Cationic modulation of ρ_1 -type γ -aminobutyrate receptors expressed in *Xenopus* oocytes

(chloride channels/retina receptors/ Zn^{2+} inhibition/ La^{3+} potentiation)

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ABSTRACT A study was made of the effects of di- and trivalent cations on homomeric ρ_1 -type γ -aminobutyrate (GABA_{ρ_1}) receptors expressed in *Xenopus* oocytes after injection of mRNA coding for the GABA_{ρ_1} subunit. GABA elicited large currents with a $K_d \approx 1 \mu\text{M}$. The properties of these GABA_{ρ_1} receptors were similar to those of native bicuculline-resistant GABA receptors expressed by retinal mRNA. GABA_{ρ_1} currents showed very little desensitization, were blocked by picrotoxin but not by bicuculline, and were not modulated by barbiturates, benzodiazepines, or β -carbolines. Zn^{2+} reversibly decreased GABA_{ρ_1} responses ($\text{IC}_{50} = 22 \mu\text{M}$). Other divalent cations were also tested and their rank order of potency was: $\text{Zn}^{2+} \approx \text{Ni}^{2+} \approx \text{Cu}^{2+} \gg \text{Cd}^{2+}$, whereas Ba^{2+} , Co^{2+} , Sr^{2+} , Mn^{2+} , Mg^{2+} , and Ca^{2+} showed little or no effect. In contrast, La^{3+} reversibly potentiated the GABA currents mediated by homomeric GABA_{ρ_1} receptors, with an $\text{EC}_{50} = 135 \mu\text{M}$ and a maximal potentiation of about 100% (GABA, 1 μM ; La^{3+} , 1 mM). Other lanthanides showed similar effects ($\text{Lu}^{3+} > \text{Eu}^{3+} > \text{Tb}^{3+} > \text{Gd}^{3+} > \text{Er}^{3+} > \text{Nd}^{3+} > \text{La}^{3+} > \text{Ce}^{3+}$). Thus, GABA_{ρ_1} receptors contain sites for cationic recognition, and in particular, Zn^{2+} may play a role during synaptic transmission in the retina.

Inhibition in the vertebrate brain is mediated mainly by two well-known types of γ -aminobutyric acid (GABA) receptors: GABA_A and GABA_B (1, 2). GABA_A receptors are heteromeric complexes, of four or five subunits, that form ligand-gated Cl^- channels (3), whereas GABA_B receptors, whose structure is still unknown, regulate K^+ and Ca^{2+} channels through heterotrimeric guanine nucleotide-binding proteins (G proteins) (2).

A third type of GABA receptor was discovered by expressing retina mRNA in *Xenopus* oocytes (4) and its cDNA was isolated from a human retina library (5). Consequently, this receptor is sometimes called $\text{GABA}_{\text{retina}}$, GABA_{ρ} , or GABA_C . The physiological and pharmacological properties of the GABA_{ρ} receptors are very different from those of GABA_A or GABA_B receptors. For example, GABA_{ρ} receptors are not blocked by bicuculline, show little desensitization, and are not modulated by barbiturates, benzodiazepines, or other drugs that modulate GABA_A receptors (4–18).

It has been known for some time that polyvalent cations modulate transmitter action on some receptors. For example, Sr^{2+} increases the lifetime of the channels gated by acetylcholine at frog endplates (19, 20), and Ca^{2+} shortens the lifetimes of the channels gated by acetylcholine receptors in frog muscle and of the glutamate receptors in locust muscle (20, 21). More recently, it has been shown that Zn^{2+} , which is present in nerve terminals (22, 23) and is released following nerve stimulation (24, 25), modulates the action of GABA_A

receptors (25–29). Because of all this we thought it desirable to study the effects of Zn^{2+} and other polyvalent cations on the function of homomeric GABA_{ρ_1} receptors. This work, carried out in 1992, has been presented in preliminary form (30).

MATERIALS AND METHODS

Cloning, *in Vitro* Transcription, and Expression of Homomeric GABA_{ρ_1} Receptors. A 1-kb fragment of the ρ_1 gene (the gene encoding the GABA_{ρ_1} receptor) was amplified from a human retina cDNA library (kindly provided by J. Nathans, Johns Hopkins University School of Medicine) by PCR using primers derived from the published ρ_1 sequence (5). The fragment was cloned into pBluescript II KS (Stratagene), labeled by random priming (Boehringer Mannheim kit), and used to screen the library by hybridization to obtain the complete ρ_1 cDNA (A.E.V., M. Panicker, and R.M., unpublished work). ρ_1 cDNA was subcloned into the *EcoRI* site of pBluescript KS and transcribed *in vitro* to obtain sense ρ_1 mRNA. The RNA was microinjected into *Xenopus* oocytes, and this led to the expression of functional homomeric GABA_{ρ_1} receptors.

Voltage-Clamp Recording of Membrane Currents. Frogs were from *Xenopus* I (Ann Arbor, MI) or Nasco (Modesto, CA), and *Xenopus* oocytes were handled as described (31). Briefly, oocytes were injected with 5–50 ng of mRNA (in 50 nl), defolliculated with collagenase, and stored for 3–9 days in Barth's medium with gentamicin at 0.1 mg/ml. Voltage-clamp recordings (32) were made from oocytes in a chamber continuously superfused (5–10 ml/min) with frog Ringer solution (115 mM NaCl/2 mM KCl/1.8 mM CaCl_2 /5 mM Hepes, pH 7.00). Results are presented as the mean $\pm$ SEM. All the experiments were carried out at room temperature, 20–23°C. Solutions were made up fresh each day as concentrated (0.001–1 M) stocks in water. Chloride or sulfate salts of the various cations were dissolved in Ringer solution or Ringer solution containing GABA. The pH of each test solution was always checked and adjusted (7.00). All the salts were purchased from Sigma.

RESULTS

Pharmacological Profile of Cloned Homomeric GABA_{ρ_1} Receptors. GABAergic agonists and antagonists were assayed on the homomeric GABA_{ρ_1} receptors expressed in oocytes. The results essentially confirmed those previously reported (5, 17) but included additional tests of related compounds. The GABA_{ρ_1} responses were resistant to bicuculline and insensitive to GABA_A allosteric modulators such as pento-barbital, diazepam, chlordiazepoxide, flunitrazepam, RO 15-

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Abbreviations: GABA, γ -aminobutyric acid; TACA, *trans*-aminocrotonic acid; CACA, *cis*-aminocrotonic acid.

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1788, or methyl, ethyl, or butyl β -carboline up to 100 μ M. Furthermore, the rank order for conformational analogs [*trans*-aminocrotonic acid (TACA) > GABA > muscimol > *cis*-aminocrotonic acid (CACA)] was as found previously for receptors expressed by native or cloned mRNAs. Current-voltage relationships were fairly linear (e.g., Fig. 1D) and the reversal potentials (4, 9, 17) were around -20 mV, as expected for currents carried mainly by Cl^- ions (33).

It is known that cations can modulate GABA-mediated Cl^- currents in different types of native or cloned GABA_A receptors (34–36). We examined the modulation of GABA_A receptors by various polyvalent cations.

Inhibition of GABA_A Currents by Zn^{2+} . It is known that Zn^{2+} suppresses GABA responses in a variety of preparations (34–38) and that recombinant GABA_A receptors are blocked by Zn^{2+} (29, 36) with different efficacy. The present experiments were made to determine whether homomeric GABA_A receptors were also modulated by Zn^{2+} ions.

Zn^{2+} inhibited the GABA_A currents in a dose-dependent manner (Fig. 1). At 100 μ M, Zn^{2+} blocked almost completely the currents elicited by 1 μ M GABA, while 30 μ M Zn^{2+} reduced them to 33% ($\text{IC}_{50} = 21.9 \pm 1.0$ μ M). The current amplitude recovered fully after short periods (1–3 min) of wash with Zn^{2+} -free Ringer solution. Thus, the blocking effect was dose-dependent (Fig. 1B), and both the onset and decay of Zn^{2+} action were rapid. Dose-response curves in the absence or presence of 30 μ M Zn^{2+} (Fig. 1C) show that Zn^{2+} decreased the affinity (K_d) without affecting the maximum response to GABA (in the control, $K_d = 1.08 \pm 0.36$ μ M, Hill coefficient (h) = 2.02 ± 0.57 ; in the presence of Zn^{2+} , $K_d = 1.82 \pm 0.25$ μ M, $h = 1.77 \pm 0.43$). Furthermore, the inhibition of GABA-induced currents by Zn^{2+} was strongly dependent on the concentration of GABA, with the

blockage being less potent at high concentrations of agonist. The blockage was independent of the membrane potential (Fig. 1D).

Potentiation of GABA_A Currents by La^{3+} . Like native or cloned GABA_A receptors (39–44), homomeric GABA_A receptors are also modulated by La^{3+} ions. La^{3+} produced a rapid and reversible increase of the currents elicited by GABA (Fig. 2). This potentiation increased as the La^{3+} concentration was increased (Fig. 2B) ($\text{EC}_{50} = 135.5 \pm 28.8$ μ M), and the GABA dose-response curve in the presence of La^{3+} had a shift indicative of an increase in apparent affinity (Fig. 2C) (control, $K_d = 1.14 \pm 0.28$ μ M, $h = 1.88 \pm 0.12$ μ M; with La^{3+} , $K_d = 0.55 \pm 0.07$ μ M, $h = 2.4 \pm 0.58$). Moreover, as was the case for the inhibition caused by Zn^{2+} , the potentiation of GABA_A currents by La^{3+} was voltage-independent (Fig. 2D) and was less marked when higher GABA concentrations were used (Fig. 2C).

Effects of Other Cations. To see whether other ions besides Zn^{2+} and La^{3+} were capable of modulating GABA_A currents, we tested other transition metals and lanthanides. In general, the transition metals inhibited, whereas the lanthanides potentiated GABA_A currents. The most potent inhibitors were the period 4 elements Zn^{2+} , Ni^{2+} , and Cu^{2+} , whereas Cd^{2+} (period 5) was much less potent (Fig. 3A).

Divalent-cation effects were washed out rapidly in Ringer solution with or without GABA. Furthermore, Zn^{2+} did not affect greatly the rising and falling phases of the GABA-induced currents, in contrast to what has been found for locust muscle GABA receptors (26). However, long washing (1 hr) was required after blockage by Cu^{2+} in order to fully recover GABA_A responses. This suggests a different type of interaction between Cu^{2+} and GABA_A receptors.

FIG. 1. Inhibition of GABA_A currents by Zn^{2+} . (A) Effect of ZnCl_2 on currents elicited by 1 μ M GABA. For this and subsequent figures *Xenopus* oocytes were voltage-clamped at -60 mV and inward currents are denoted as downward deflections of the trace. Pulses were used to monitor conductance. (B) Inhibition of the current elicited by 1 μ M GABA by various concentrations of Zn^{2+} . (C) Dose-response curve for GABA alone or in the presence of 30 μ M Zn^{2+} . (D) Current-voltage relationship of currents elicited by 1 μ M GABA alone or in the presence of 30 μ M Zn^{2+} .

FIG. 2. Potentiation of GABA $_A$ currents by La^{3+} . (A) Effect of $100 \mu\text{M}$ La^{3+} on currents elicited by $1 \mu\text{M}$ GABA. (B) Curve of GABA $_A$ receptor sensitivity to La^{3+} . (C) Dose-response curve for GABA alone or in the presence of $100 \mu\text{M}$ La^{3+} . (D) Current-voltage relationships for $1 \mu\text{M}$ GABA with or without $100 \mu\text{M}$ La^{3+} .

In contrast to divalent ions such as Zn^{2+} , Ni^{2+} , and Cu^{2+} , all the trivalent lanthanides tested potentiated the effect of GABA, with slight differences among them, and their rough order of potency is shown in Fig. 3B. Nevertheless, it appears that the type of modulatory effect is not simply determined by ionic charge, since some divalent cations had little or no inhibitory effect, whereas Al^{3+} inhibited GABA $_A$ currents instead of potentiating them (Fig. 3A). Moreover, the modulation by cations did not depend upon the class of agonist used, since the conformationally restricted analogs of GABA (TACA and CACA) and β -alanine were also sensitive to the action of lanthanides (Fig. 4). In addition, the potentiation of the GABA $_A$ currents by Lu^{3+} differed from that of other lanthanides. The effect of Lu^{3+} was studied both by applying GABA after 1 min of preincubation with 1 mM Lu^{3+} and by applying Lu^{3+} once the GABA current had reached a plateau. The rate of rise of the GABA current was greatly increased by Lu^{3+} but not by La^{3+} or other lanthanides. Furthermore, Lu^{3+} caused the GABA currents to desensitize (Fig. 4).

As previously shown for GABA $_A$ receptors (44), Lu^{3+} also acts as a partial agonist on GABA $_A$ receptors. Similarly, small picrotoxin-sensitive GABA $_A$ currents (10 – 20 nA) were elicited by 1 mM Lu^{3+} alone. Other lanthanides gave similar but smaller responses (2 – 5 nA). On the other hand, tetraeth-

ylammonium ($100 \mu\text{M}$) or replacing Ca^{2+} by Mg^{2+} (2 mM) in the perfusing solution did not alter the responses to GABA.

DISCUSSION

Our experiments show that homomeric GABA $_A$ receptors, like other GABA receptors, contain sites for cationic recognition and that the cations produce changes in the currents gated by the receptors. Negative, as well as positive, modulation of the Cl^- currents by specific cations was observed. Thus, Zn^{2+} , Ni^{2+} , Cu^{2+} , and Cd^{2+} inhibited GABA $_A$ currents whereas the lanthanides potentiated them.

It has been postulated that Zn^{2+} and La^{3+} act on at least two different sites on the GABA $_A$ receptor complex—one for positive regulation by lanthanides and the other for negative regulation by divalent cations—and both sites appear to be different from the modulatory sites for benzodiazepines and barbiturates (41–44). However, the way cations affect the different GABA receptors is not identical (28, 29, 35, 36). It is important to note here that GABA $_A$ receptors, which show very little desensitization, also possess sensitivity to Zn^{2+} . Thus, the inhibitory action of Zn^{2+} is probably not linked to fast desensitization processes as has been suggested for some GABA $_A$ receptors (35, 37). Furthermore, the fast onset and washing out of both the inhibition and the potentiation of

FIG. 3. Effect of cations on GABA_{p1} currents. The concentration of GABA was 1 μM for both A and B and the bars show mean ± SEM current amplitudes in 4–18 oocytes. (A) Effects of Li⁺, Al³⁺, and divalent cations. C, control. (B) Effects of lanthanides.

GABA_{p1} currents by the cations support an extracellular site of action.

The sensitivity of GABA_{p1} receptors to Zn²⁺ was similar to that of GABA_A receptors from newborn rat dorsal root ganglion neurons (GABA_{p1}, 22 μM; GABA_A, 19 μM) (38). Changes in the Zn²⁺ sensitivity during development have been reported for GABA_A receptors and are probably due to developmental differences in the subunit composition of the receptors (38). For example, for recombinant GABA_A receptors, it was shown that the potency of the inhibitory action of Zn²⁺ depended strongly on the presence of the γ₂ subunit (29, 36).

Our results show clearly that lanthanides potentiate the responses of homomeric GABA_{p1} receptors, which are not modulated by benzodiazepines. This suggests that the sites for La³⁺ and benzodiazepine modulation are different, as has been suggested also for GABA_A receptors (43, 44).

FIG. 4. Potentiation by lanthanides (300 μM La³⁺ or Lu³⁺) of GABA_{p1} currents elicited by the GABA receptor agonists. TACA (1 μM), CACA (10 μM), and β-alanine (1 mM).

In conclusion, GABA_{p1} receptors are modulated by Zn²⁺ and lanthanides in a voltage-independent manner. The modulation by Zn²⁺ is particularly interesting, because it is known that Zn²⁺ is present in retinal cells (45), from which it may be released in sufficient amounts to exert a strong effect on synaptic transmission mediated by GABA_{p1} receptors.

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- Olsen, R. W. & Tobin, A. J. (1990) *FASEB J.* 4, 1469–1480.
- Bowery, N. G. (1993) *Annu. Rev. Pharmacol. Toxicol.* 33, 109–147.
- Seeburg, P. H., Wisden, W., Verdoorn, T. A., Pritchett, D. B., Werner, P., Herb, A., Luddens, H., Sprengel, R. & Sakmann, B. (1990) *Cold Spring Harbor Symp. Quant. Biol.* 55, 29–40.
- Polenzani, L., Woodward, R. M. & Miledi, R. (1991) *Proc. Natl. Acad. Sci. USA* 88, 4318–4322.
- Cutting, G. R., Lu, L., O'Hara, B. F., Kasch, L. M., Montrose-Rafizadeh, C., Donovan, D. M., Shimada, S., Antonarakis, S. E., Guggino, W. B. & Uhl, G. R. (1991) *Proc. Natl. Acad. Sci. USA* 88, 2673–2677.
- Woodward, R. M., Polenzani, L. & Miledi, R. (1992) *Mol. Pharmacol.* 41, 89–103.
- Woodward, R. M., Polenzani, L. & Miledi, R. (1992) *Mol. Pharmacol.* 41, 1107–1115.
- Woodward, R. M., Polenzani, L. & Miledi, R. (1992) *Mol. Pharmacol.* 42, 165–173.
- Woodward, R. M., Polenzani, L. & Miledi, R. (1993) *Mol. Pharmacol.* 43, 609–625.
- Woodward, R. M., Polenzani, L. & Miledi, R. (1994) *J. Pharmacol. Exp. Ther.* 268, 806–817.
- Feigenspan, A., Wassle, H. & Bormann, J. (1993) *Nature (London)* 361, 159–162.
- Qian, H. & Dowling, J. E. (1993) *Nature (London)* 361, 162–164.
- Matthews, G., Ayoub, G. S. & Heidelberger, R. (1994) *J. Neurosci.* 14, 1079–1090.
- Lukasiewicz, P. D., Maple, B. R. & Werblin, F. S. (1994) *J. Neurosci.* 14, 1202–1212.
- Lukasiewicz, P. D. & Werblin, F. S. (1994) *J. Neurosci.* 14, 1213–1223.

16. Shimada, S., Cutting, G. & Uhl, G. R. (1992) *Mol. Pharmacol.* **41**, 683–687.
17. Kusama, T., Spivak, C. E., Whiting, P., Dawson, V. L., Schaeffer, J. C. & Uhl, G. R. (1993) *Br. J. Pharmacol.* **109**, 200–206.
18. Kusama, T., Wang, T. L., Guggino, W. B., Cutting, G. R. & Uhl, G. R. (1993) *Eur. J. Pharmacol.* **245**, 83–84.
19. Dodge, F. A., Miledi, R. & Rahamimoff, R. (1969) *J. Physiol. (London)* **200**, 267–283.
20. Miledi, R. & Parker, I. (1980) *J. Physiol. (London)* **306**, 567–577.
21. Cull-Candy, S. G. & Miledi, R. (1980) *J. Physiol. (London)* **303**, 74P.
22. Haug, F. M. (1967) *Histochemistry* **8**, 355–368.
23. Holm, I. G., Andreasen, A., Danscher, G., Perez-Clausell, J. & Nielsen, H. (1988) *Histochemistry* **89**, 289–293.
24. Assaf, S. Y. & Chung, S. H. (1984) *Nature (London)* **308**, 734–736.
25. Howell, G. A., Welch, M. G. & Frederickson, C. J. (1984) *Nature (London)* **308**, 736–738.
26. Smart, T. G. & Constanti, A. (1982) *Proc. R. Soc. London B* **215**, 327–341.
27. Westbrook, G. L. & Mayer, M. L. (1987) *Nature (London)* **328**, 640–643.
28. Mayer, M. L. & Vyklicky, L., Jr. (1989) *J. Physiol. (London)* **415**, 351–365.
29. Draguhn, A., Verdoorn, T. A., Ewert, M., Seeburg, P. H. & Sakmann, B. (1990) *Neuron* **5**, 781–788.
30. Calvo, D. J., Vazquez, A. E. & Miledi, R. (1994) *FASEB J.* **8**, 659 (abstr. 3824).
31. Miledi, R. & Woodward, R. M. (1989) *J. Physiol. (London)* **416**, 601–621.
32. Miledi, R. (1982) *Proc. R. Soc. London B* **215**, 491–497.
33. Kusano, K., Miledi, R. & Stinnakre, J. (1982) *J. Physiol. (London)* **328**, 143–170.
34. Smart, T. G. & Constanti, A. (1990) *Br. J. Pharmacol.* **99**, 643–654.
35. Legendre, P. & Westbrook, G. L. (1991) *Mol. Pharmacol.* **39**, 267–274.
36. Smart, T. G., Moss, S. J., Xie, X. M. & Haganir, R. L. (1991) *Br. J. Pharmacol.* **103**, 1837–1839.
37. Celentano, J. J., Gyenes, M., Gibbs, T. T. & Farb, D. H. (1991) *Mol. Pharmacol.* **40**, 766–773.
38. Smart, T. G. (1992) *J. Physiol. (London)* **447**, 587–625.
39. Xie, X. M. & Smart, T. G. (1993) *J. Physiol. (London)* **460**, 503–523.
40. Reichling, D. B. & MacDermott, A. B. (1991) *J. Physiol. (London)* **441**, 199–218.
41. Im, M. S., Hamilton, B. J., Carter, D. B. & Im, W. B. (1992) *Neurosci. Lett.* **144**, 165–168.
42. Ma, J. Y. & Narahashi, T. (1993) *Brain Res.* **607**, 222–232.
43. Im, W. B. & Pregenzer, J. F. (1993) *Eur. J. Pharmacol.* **245**, 111–117.
44. Ma, J. Y. & Narahashi, T. (1993) *J. Neurosci.* **13**, 4872–4879.
45. Wu, S. M., Qiao, X., Noebels, J. L. & Yang, X. L. (1993) *Vision Res.* **33**, 2611–2616.