References and Notes

1. W. Wishart, *Alberta Lands Forests Parks Wildlife* **13**, 4 (1970).
2. E. H. Dustman, L. F. Stickel, J. B. Elder, *Proceedings of the International Conference on Environmental Mercury Contamination, Ann Arbor, Michigan, 1970*, in press.
3. K. Borg, H. Wanntorp, K. Erne, E. Hanko, *Viltrevy* **6**, 301 (1969).
4. N. A. Smart and M. K. Lloyd, *J. Sci. Food Agr.* **14**, 734 (1963).
5. S. Tejning, *Oikos* (Suppl. No. 8) (1967).
6. J. S. Palmer, *J. Amer. Vet. Med. Ass.* **142**, 1385 (1963).
7. D. L. Leedy and C. R. Cole, *J. Wildlife Manage.* **14**, 218 (1950).
8. Ceresan M, registered name, Dupont de Nemours and Co., consists of 7.7 percent ethyl mercury *p*-toluene sulfonanilide as the active ingredient or a total of 3.2 percent as metallic mercury.
9. G. W. Snedecor and W. G. Cochran, *Statistical Methods* (Iowa State Univ. Press, Ames, ed. 6, 1967).
10. Mercury analysis involved acid digestion of tissue and extraction of mercury from the liquid digest with dithizone [H. E. Monk, *Analyst* **86**, 608 (1961)]. Mercury determination of dithizone extract is by flame atomic absorption with the sampling boat technique [H. L. Kahn, G. E. Peterson, J. E. Schallis, *At. Absorpt. Newsl.* **7**, 35 (1968)]. The limit of detection is approximately 0.10 ppm.

11. We thank C. Vance for carrying out aviculture and candling and E. F. Hill for manuscript review.

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Gamma-Aminobutyric Acid Antagonism and Presynaptic Inhibition in the Frog Spinal Cord

Abstract. *The convulsant alkaloid bicuculline blocked presynaptic inhibition, dorsal root potentials, primary afferent depolarization, and depolarizing effects of gamma-aminobutyric acid on dorsal root terminals of the amphibian spinal cord, but did not block effects of other putative amino acid transmitters. These actions of bicuculline suggest that gamma-aminobutyric acid may be the transmitter involved in spinal presynaptic inhibition.*

There has been little agreement as to the identity of the transmitter compound that is released from synapses on primary afferent fiber terminals and is responsible for both the primary afferent depolarization recorded as the dorsal root potential and for the phenomenon of presynaptic inhibition. In 1963, Eccles and his colleagues (*1*) postulated that gamma-aminobutyric acid (GABA) might be this transmitter in view of the evidence that it depolarized dorsal root terminals in the cat. Subsequently, the finding that GABA depolarizes primary afferent terminals has been confirmed (*2, 3*) and has been denied (*4*), and the latter evidence used to deny the possible role of GABA in presynaptic inhibition.

Bicuculline, an alkaloid known to have similar convulsant properties in mammals and amphibians (*5*), has been shown specifically to block the action of GABA at a variety of synaptic sites in the cat central nervous system (*6, 7*), the cat autonomic ganglion (*8*), and the crayfish stretch receptor (*9*). Presynaptic inhibition is probably generated by similar mechanisms in the spinal cord of mammals and amphibians (*10*), because there is a great similarity in the dorsal root potentials and in their pharmacological properties (*2*). There are conflicting reports, however, about the effects of bicuculline on presynaptic inhibition. Levy and co-workers (*11*) showed that this alkaloid blocked primary afferent depolarization in the cat spinal cord, whereas Curtis and co-workers (*6*) were unable to demonstrate any effect on presynaptic inhibition of the spinal monosynaptic reflex in the same species. This study is concerned with the effects of bicuculline and GABA on presynaptic inhibition in the isolated frog spinal cord and adds direct evidence to the hypothesis that GABA is the transmitter involved in spinal presynaptic inhibition.

The isolated frog spinal cord was employed because potential changes at the dorsal root terminals produced by various drugs and transmitters can easily be measured. In addition, this preparation circumvents difficulties seen in intact vertebrates when drugs and transmitters are administered intravascularly.

By the use of conventional methods (*12*), spinal cords and roots were removed from 9-cm-long frogs (*Rana pipiens*), were hemisected sagittally, and were perfused with oxygenated amphibian Ringer solution (*13*) in a temperature-controlled Lucite bath. A flow rate of 2.0 to 4.0 ml/min was used in different experiments. Dorsal and ventral roots 8 and 9 were pulled through slits in thin plastic partitions sealed with Vaseline. For a-c recordings and stimulation, the roots were placed on pairs of Ag-AgCl electrodes (interelectrode distance 12 to 15 mm) and covered with mineral oil to prevent desiccation. For d-c recording, nonpolarizing Ag-AgCl electrodes were used. Contact was made with the roots by means of a cotton wick (*14*). Stable recordings could be obtained from a preparation

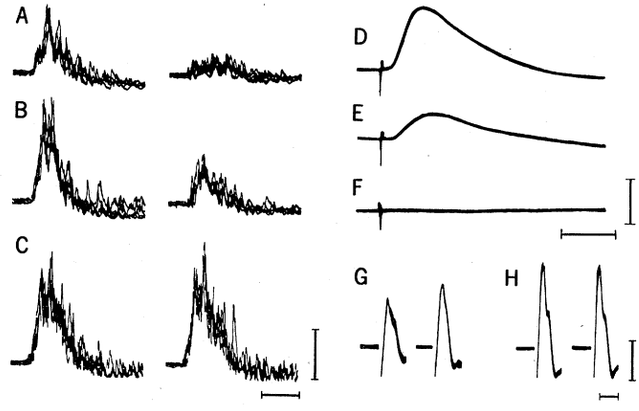

Fig. 1. (A–C) Effect of bicuculline on presynaptic inhibition. (Left) Polysynaptic ventral root reflexes produced by supramaximum stimuli applied to dorsal root. (Right) Inhibition of polysynaptic reflex when stimulation of adjacent ventral root preceded dorsal root stimulation by 50 msec. (A) Before, (B) 4 minutes, and (C) 11 minutes after addition of bicuculline (10 μg/ml) to Ringer solution. Records obtained by superimposing six traces. Bath temperature, 13°C. (D–F) Effects of bicuculline on dorsal root potential. (D) Dorsal root potential evoked by supramaximum stimulation of ventral root. (E) Ten minutes and (F) 20 minutes after bicuculline (10 μg/ml). Dorsal root potential completely abolished. Bath temperature, 15°C. (G–H) Effect of bicuculline on primary afferent depolarization. (G) (Left) Antidromic response recorded from dorsal root produced by constant-current, local stimulation in dorsal horn by a micropipette. (Right) Primary afferent depolarization as manifested by increased excitability of dorsal root terminals produced by stimulation of ventral root 50 msec before. (H) Sixteen minutes after bicuculline (10 μg/ml). Six superimposed traces in each figure. Bath temperature, 15°C. Vertical calibration: (A–H) 1 mv. Horizontal calibration: (A–C) 40 msec; (D–E) 100 msec; (G–H) 1 msec.

for 8 to 10 hours. Drugs were dissolved in standard Ringer solution and the *p*H was adjusted when necessary.

When bicuculline (5 to 20 μg/ml) was added to the Ringer solution bathing the spinal cord of the frog, it had distinct excitatory effects and produced a significant increase in the sizes of both the ventral root potential and the ventral root polysynaptic reflex evoked by stimulating the dorsal root of the same segment with a supramaximum stimulus (Fig. 1, A–C). This increased excitability can be attributed to at least two phenomena—depolarization of motoneurons (inferred from depolarizations of 0.5 to 1.0 mv recorded from the ventral root) and loss of presynaptic inhibition.

Stimulation of a ventral root can produce presynaptic inhibition of the polysynaptic reflex in the amphibian spinal cord (*10*) (Fig. 1A). This depressant effect on reflex transmission is presumably due to a synaptically generated depolarization of the primary afferent dorsal root fibers (*10, 15*), which produces a diminution in the size of the presynaptic terminal action potential and a consequent decrease in the amount of transmitter released from these terminals. The depolarization of primary afferent fibers may be recorded from the dorsal root (Fig. 1D) after electrotonic spread from the synaptically depolarized nerve terminals (dorsal root potential), and can be inferred from the increased excitability of dorsal root fiber terminals (Fig. 1G) (*16*). The time courses of all three associated phenomena—the presynaptic inhibition of reflex transmission, the duration of the dorsal root potential, and the duration of the increased afferent terminal excitability—are nearly identical (*10, 15, 17*).

Bicuculline affected all three measures of presynaptic inhibition in a similar way. Concentrations of 5 to 20 μg/ml completely abolished the ventral root–dorsal root potential (Fig. 1F) and the increased excitability of dorsal root terminals produced by ventral root stimulation (Fig. 1H). The reflex inhibition produced by ventral root stimulation was also abolished (Fig. 1C). These effects occurred rapidly (8 to 12 minutes) but were irreversible despite prolonged (more than 2 hours) washing with normal Ringer solution.

The addition of GABA (Fig. $2A_2$) to the Ringer solution in concentrations of greater than $10^{-5}M$ produced a depolarization of 1 to 5 mv when

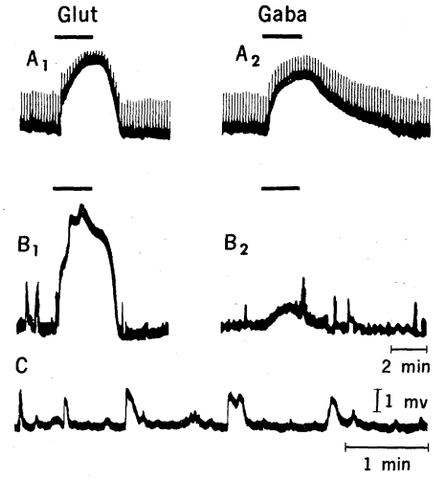

Fig. 2. Effect of bicuculline on depolarizations produced by glutamic acid (*Glut*) and GABA. (A) Glutamic acid ($10^{-3}M$) and GABA ($10^{-3}M$) added to Ringer solution and each applied for 2 minutes (horizontal bars) produced approximately equal depolarizations of dorsal root terminals. The ventral root was stimulated every 10 seconds and the dorsal root potential can be seen as an upward "spike." In d-c records in this figure plotted with an ink-writer, negativity is upward. (B) Twenty minutes after exposure of preparation to bicuculline (7.5 μg/ml). The alkaloid produced a 1-mv depolarization and loss of the dorsal root potential. Glutamic acid produced a larger depolarization, but bicuculline blocked most of the depolarizing effect of GABA. Spontaneous dorsal root depolarizations produced by bicuculline are seen in (B) and in (C), which was taken 5 minutes after the last application of GABA (B_2).

recorded between the bath and the peripheral end of the dorsal root. The depolarization, as expected, was associated with an increased excitability of dorsal root terminals (*17*) and was proportional to the concentration of GABA.

Other physiologically active amino acids, glutamic acid (Fig. $2A_1$), glycine (*18*), and aspartic acid produced similar depolarizations of dorsal root terminals. The actions of amino acids on intraspinal nerve terminals (*19*) had previously been attributed not only to a direct action of the amino acid on the terminal, but to effects on interneurons in the pathway to the presynaptic synapses (*2, 4*). However, the four above amino acids produced identical depolarizations when reflex transmission in the spinal cord was suppressed by the removal of Ca^{2+} and the addition of Mg^{2+} (10 to 20 mM) to the Ringer solution. This procedure precludes the possibility that an alteration in the activity of interneurons caused the depolarizations and demonstrates that the entire depolarizing activity of amino acids can be explained by their direct effects on afferent terminals.

Examination of the interactions between amino acids and bicuculline on dorsal root terminals revealed that addition of the alkaloid to the perfusing fluid effectively reduced the depolarizing action of GABA (Fig. $2B_2$) on all preparations tested. It usually potentiated the effects of glutamic acid and did not modify the depolarizations produced by glycine or aspartic acid. The GABA-blocking activity of bicuculline was irreversible.

Thus, GABA and several other amino acids can mimic the actions of the natural presynaptic inhibitory transmitter in depolarizing the membrane of dorsal root terminals. Antagonism by bicuculline, however, discriminates between the effects of GABA and the effects of the other amino acids tested, as has been shown in other neural sites (*5–7*). It is not possible, however, to state what the interactions between bicuculline and GABA are. The alkaloid would appear to have direct effects on dorsal roots, because it produced a slowly developing (5- to 20-minute), long-lasting depolarization (0.5 to 1.0 mv) of dorsal root terminals. In addition, the alkaloid frequently induced rapid, superimposed depolarizations (Fig. 2C) (*20*). High doses of bicuculline (> 30 μg/ml) sometimes produced a rapid, irreversible depolarization recorded at both ventral roots and dorsal roots. These latter effects make it difficult to determine whether the alkaloid blocked the action of GABA at the presynaptic receptor site or interfered with some other later part of the synaptic process. However, the simultaneous reduction by bicuculline of presynaptic inhibition and of the actions of GABA on dorsal root terminals suggests that GABA could be the spinal presynaptic inhibitory transmitter in amphibians. Such a role for this amino acid has already been postulated in the spinal cord of the cat (*11*).

Note added in proof: Curtis and his colleagues in recent investigations [*Nature* **231**, 187 (1971); *Brain Res.* **32**, 69 (1971)] have shown that bicuculline does block long duration inhibition of monosynaptic reflexes and the dorsal root potential in the cat.

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References and Notes

1. J. C. Eccles, R. Schmidt, W. D. Willis, *J. Physiol. London* **168**, 500 (1963).
2. R. F. Schmidt, *Arch. Ges. Physiol. Menschen Tiere* **277**, 325 (1963).
3. A. K. Tebĕcis and J. W. Phillis, *Comp. Biochem. Physiol.* **23**, 553 (1967); *ibid.* **28**, 1303 (1969); N. Davidson and C. A. P. Southwick, *J. Physiol. London* **210**, 172p (1970).
4. D. R. Curtis and R. W. Ryall, *Exp. Brain Res.* **1**, 195 (1966).
5. A. D. Welch and V. E. Henderson, *J. Pharmacol. Exp. Therap.* **51**, 482 (1934).
6. D. R. Curtis, A. W. Duggan, D. Felix, G. A. R. Johnston, *Nature* **226**, 1222 (1970).
7. D. R. Curtis, A. W. Duggan, D. Felix, *Brain Res.* **23**, 117 (1970); D. R. Curtis, D. Felix, H. McLennan, *Brit. J. Pharmacol.* **40**, 881 (1970); A. W. Duggan and H. McLennan, *Brain Res.* **25**, 188 (1971); D. Felix and H. McLennan, *ibid.*, p. 661.
8. W. C. De Groat, P. M. Lalley, M. Block, *ibid.*, p. 665.
9. H. McLennan, *Nature* **228**, 674 (1970).
10. A. D. Grinnell, *J. Physiol. London* **182**, 612 (1966).
11. R. A. Levy, A. Repkin, E. G. Anderson, *Fed. Proc.* **30**, 317 (1971).
12. A. K. Tebĕcis and J. W. Phillis, *Exp. Physiol. Biochem.* **2**, 361 (1969).
13. The composition of the Ringer solution was: NaCl, 100 m*M*; KCl, 2.5 m*M*; Na_2HPO_4, 2.5 m*M*; NaH_2PO_4, 0.45 m*M*; $CaCl_2$, 1.9 m*M*; $NaHCO_3$, 12.0 m*M*; glucose, 2.8 m*M*. This was equilibrated with 5 percent CO_2 and 95 percent O_2. The *p*H remained constant at 7.4 ± 0.1 unit. The temperature varied in different experiments from 13° to 15°C.
14. Differential d-c recordings were either made between the most proximal and the most distal ends of the roots with a Ag-AgCl ground in the bath or between the bath and the most distal end of the root. In the latter case the bath was left ungrounded. No qualitative differences were seen between the two methods of recording. Slow potentials were displayed on a Brush paper writer.
15. J. Bergmans, J. Colle, Y. Lafere, *J. Physiol. Paris* **56**, 219 (1964).
16. P. D. Wall's technique [*J. Physiol. London* **142**, 1 (1958)] was employed to measure excitability of intraspinal afferent terminals. This technique is based on the concept that when primary afferent depolarization of dorsal root terminals is produced, dorsal root fibers are more easily discharged by intraspinal stimulation. This leads to an increase in the amplitude of the compound antidromic spike potential recorded from the dorsal root. A low-resistance, sodium chloride-filled glass capillary microelectrode was used for stimulation after insertion into the dorsal horn close to the terminals of the primary afferent fibers. Recording of the extracellular field potential was used to correct the location of the electrode tip in the spinal cord. Submaximum stimuli were used.
17. R. A. Davidoff, in preparation.
18. On rare occasions glycine hyperpolarized the dorsal root terminals. Bicuculline did not affect this phenomenon.
19. Amino acids have little or no effect on spinal afferent fibers proximal to the terminals (*4*).
20. Small, spontaneous dorsal root depolarizations have been described on exposure of the spinal cord to convulsants (*2*) and to excitatory agents (*21*).
21. K. Koketsu, A. G. Karczmar, K. Kitamura, *Int. J. Neuropharmacol.* **8**, 329, (1969).

22. I thank V. Grayson and A. Warren for technical assistance.

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Control of Aggregation in Dictyostelium discoideum by an External Periodic Pulse of Cyclic Adenosine Monophosphate

Abstract. *We have induced and controlled normal aggregation of* Dictyostelium discoideum *amoebas by electrophoretic release of pulses of cyclic adenosine monophosphate from a microelectrode. This has yielded information about the sequence of development of aggregation competences during interphase. We believe that modifications of the technique will have wide application in investigations of other developing systems.*

The cellular slime mold *Dictyostelium discoideum* was discovered by Raper (*1*). His classic paper (*2*) is a valuable source for details of its development (*3*). Amoebas of *D. discoideum* live in damp environments eating bacteria which they find by chemotaxis (*4, 5*). When the food supply is exhausted the amoebas go through a period of differentiation, called interphase, lasting for 6 to 8 hours (*6*). At the end of interphase some amoebas, distributed at random, begin to emit pulses (*7*) of an attractant (*8*), almost certainly cyclic adenosine monophosphate (AMP) (*9*). Neighboring amoebas respond to these periodically repeated pulses by emitting their own pulse approximately 15 seconds after being signaled (*10*), and then moving toward the original signal source. The movement lasts for about 100 seconds (*11*), while the period between pulses is approximately 300 seconds (*10, 12*). During the movement each amoeba is apparently refractory to further stimulation. These features of the signal and response mechanisms guarantee the outward propagation of a wave of inward movement (*7, 10–13*). As each amoeba acts as a local signal source, there is a tendency for amoebas to move first toward their nearest neighbors, forming streams (*14*), which then continue to move toward the original signal source, finally leading to aggregation. There are many further complexities in the signaling and response mechanisms which lead to aggregation of *D. discoideum*; a detailed discussion is not appropriate here, but may be found elsewhere (*2, 3, 10–12, 14*).

We have made a model of wave propagation during the aggregation of *D. discoideum*, and from this model have been able to extract quantities for the duration and amplitude of the cyclic AMP signal (*10*). In order to test our model and calculations we decided to attempt to initiate and control aggregation with an external signal source.

While the only properly investigated periodic signaling mechanism for the control of development is that of *D. discoideum*, there are many developmental processes involving movement having a component with a period in the order of minutes (*15*). It has been suggested that this periodicity might represent the activity of an underlying control signal of the sort considered in Goodwin and Cohen's theoretical model for the control of development (*13, 15*). This gives the experimental control of *D. discoideum* aggregation added point.

Amoebas of *D. discoideum* strain NC4-H were cultured on high growth agar (*3*) with *Escherichia coli* B/r and centrifuged free of the food bacteria (*16*). Amoebas were then plated out in a plastic chamber on 2 percent agar at a density of approximately 600 per square millimeter. The thickness of the aqueous film on the agar surface was adjusted to ~1 μm. The tip of a glass micropipet, with an internal diameter of approximately 5 μm, was introduced into the surface film. The micropipet contained 1 m*M* cyclic AMP, and 1 m*M* fluorescein in a buffered salt solution, *p*H = 6.7 (*17*), the same as that used to make up the agar. Its impedance was approximately 1 to 3 megohms. The fluorescein was used as a visible marker in initially setting up the system, as it dissociates to produce a negatively charged ion with approximately the same ionic weight as cyclic AMP. The electrolyte in the micropipet was biased, in order to maintain the column of cyclic AMP stationary in the pipet, through a chlorided wire via a 10-megohm resistor to approximately ½ volt above ground, to which the agar in the experimental chamber was referred, also via a silver chlorided wire. Approximately every 4 minutes and 25 seconds the bias was reversed for 2 seconds to approximately 10 to 30 volts negative with respect to ground, depending on electrode impedance, driving out the negatively charged cyclic AMP and fluorescein ions. Estimates of the partial conductance from the cyclic AMP ions indicated that a pulse of cyclic AMP