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MEMBRANE DEPOLARIZATION AND PROLONGATION OF CALCIUM-DEPENDENT ACTION POTENTIALS OF MOUSE NEURONS IN CELL CULTURE BY TWO CONVULSANTS: BICUCULLINE AND PENICILLIN

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SUMMARY

The convulsant compounds bicuculline (BICUC) and penicillin (PCN) are antagonists of GABA-mediated synaptic inhibition. In addition, we have shown that BICUC and PCN produced membrane depolarization of mouse spinal cord neurons in primary dissociated cell culture by blocking a potassium conductance, a non-synaptic direct effect. Both compounds also prolonged calcium-dependent action potentials of mouse dorsal root ganglion and spinal cord neurons in cell culture. Thus, BICUC and PCN had both synaptic and non-synaptic actions. The possibility that both synaptic and non-synaptic actions of BICUC and PCN are involved in their convulsant mechanism of action is discussed.

INTRODUCTION

Bicuculline (BICUC) and penicillin (PCN) produce generalized seizures when administered systemically[24,41,51]. Topical application of convulsant compounds to cerebral cortex or hippocampus leads to development of epileptiform discharges or 'spikes' recorded with surface electroencephalographic (EEG) electrodes[15,18,32]. Such convulsant-induced spikes are similar in form to those recorded in the interictal EEG of patients with epilepsy. Intracellular recordings obtained from neurons within convulsant-induced seizure foci demonstrated large depolarizations evoking volleys of action potentials which occurred randomly and were correlated with the occurrence of

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surface EEG spikes[15,32,42]. These depolarizing events were termed paroxysmal depolarization shifts (PDS)[30–32]. Two basic hypotheses of convulsant mechanism of action have been proposed: one is based on synaptic action, the other on non-synaptic action. (1) *Synaptic action.* BICUC[8–10,24] and PCN[7,11–13,22,28] both antagonize GABA-mediated inhibition. BICUC antagonizes GABA-mediated inhibition by binding to[35] and displacing[9,17,56] GABA from its neuronal receptor binding sites. PCN does not displace specific GABA-binding and therefore may antagonize GABA responses by modifying chloride channel activation or by blocking GABA-coupled chloride channels. Antagonism of GABA-mediated inhibition could produce PDS by at least two different mechanisms. Reduction of GABA-mediated inhibition by PCN[16,53] allowed orthodromic synaptic stimulation to depolarize dendrites and activate an intrinsic dendrite voltage-dependent calcium conductance in hippocampal pyramidal neurons[53]. It has been suggested that the resultant *calcium-dependent action potentials* of dendritic origin were equivalent to PDS[43,53]. Alternatively, it has been suggested that reduction of synaptic inhibition increases the efficacy or recruitment of recurrent excitatory pathways which results in synchronous excitatory transmitter release. PDS would then represent *giant excitatory postsynaptic potentials* (EPSPs)[4,25]. However, antagonism of GABA-mediated inhibition may not be the only action of convulsants. (2) *Non-synaptic action.* PCN has been shown to increase in inward current probably carried by calcium ions in spinal cord neurons at concentrations that produced paroxysmal bursting but not antagonism of GABA-mediated inhibition[46]. PCN also produced membrane depolarization and decreased membrane conductance in crustacean muscle[22]. BICUC produced membrane depolarization with a decrease in membrane conductance in lobster giant axon[19] and muscle[47] and crayfish muscle[48]. It has been argued that alterations of membrane properties by convulsants could produce negative slope conductance regions in membrane current–voltage curves and thus produce bursting[46]. The bursting would occur due to a persistent inward calcium-current induced by the convulsants, and thus PDS would again represent *calcium-dependent action potentials.*

In mouse spinal cord neurons in primary dissociated cell culture, BICUC and PCN produce paroxysmal depolarizing events (PDEs)[28] that are similar to paroxysmal bursts induced in spinal cord anterior horn cells by convulsants and to PDS produced in cortical neurons. Analogous to their action in other preparations, BICUC and PCN had synaptic actions to antagonize GABA-mediated responses on spinal cord neurons[28]. In the present study, we have demonstrated that BICUC and PCN also have non-synaptic actions on mouse neurons in cell culture. Both convulsants reduced a membrane potassium conductance, produced membrane depolarization and prolonged calcium-dependent action potentials. We shall argue that both synaptic and non-synaptic properties of BICUC and PCN may be involved in their convulsant actions.

METHODS

Primary dissociated cell culture

Primary dissociated neuronal cell cultures were prepared from dissected spinal

cords and attached dorsal root ganglia from 12–14-day-old fetal mice as described previously[44]. Following trypsinization and mechanical dissociation by trituration, the cells were plated on collagen-coated 35 mm culture dishes. The cultures were maintained in a growth medium containing 80% minimal essential medium, 10% fetal calf serum and 10% heat-inactivated horse serum at a pH of 7.3–7.4 and osmolarity 340 mOsm. Between days 2 and 4 uridine and 5′-fluoro-2′-deoxyuridine were added to suppress growth of non-neuronal cells. The growth medium was changed twice a week. The cultures were incubated at 35 °C in an atmosphere enriched with 10% carbon dioxide for 4–6 weeks prior to electrophysiological recording.

Intracellular recording

Neurons were visualized on the stage of an inverted phase contrast microscope modified to maintain the culture plate at 35–37 °C. Intracellular recordings were made from the somata of dorsal root ganglion and large multipolar spinal cord neurons (> 20 μm diameter) using glass microelectrodes filled with either 4 M potassium acetate (KAc) or 3 M potassium chloride (KCl) (25–50 MΩ). A unity gain high impedence amplifier and conventional bridge cricuit (WPI M707) was used to permit simultaneous measurement of membrane potential and injection of current through single microelectrodes. 'Membrane conductance' (reciprocal of resistance) was determined by applying brief, low amplitude constant current pulses through the recording micropipette and measuring the resultant voltage responses. Data were recorded on a 6-channel polygraph and photographed from the screen of a storage oscilloscope. Measurements from the film were made using a film magnifier.

Action potential duration was measured at half maximal amplitude of the action potential. Percentage prolongation of action potential duration was the percentage increase of duration after application of convulsant compound compared to the control action potential duration.

Solutions

All recordings were made in balanced salt solutions after removal of growth medium. Heavy paraffin oil was applied to the surface of the bathing solution to retard evaporation. In experiments on calcium-dependent action potentials, neurons were bathed in Tris-HCl buffered balanced salt solutions (TBS) with pH adjusted to 7.30–7.40 and osmolarity to 305–325 mOsm (the higher values were with solutions containing the potassium channel blocker 3-aminopyridine (3-AP)[27,40,55] at a concentration of 5 mM). TBS consisted of (in mM): NaCl 137.5; KCl 5.3; $CaCl_2$ 5.0; glucose 5.6; $MgCl_2$ 0.8; Tris-HCl 13. When direct membrane potential actions of BICUC were investigated, a phosphate-buffered saline (PBS) medium was used consisting of (in mM): NaCl 137.0; KCl 2.8; $CaCl_2$ 1.0; $MgCl_2$ 10.0; glucose 5.6; Na_2HPO_4 15.0; KH_2PO_4 1.5. When the potassium channel blocker tetraethylammonium chloride (TEA-Cl)[2,3] was added to the bathing medium, NaCl concentration was lowered to keep the solution osmolarity constant. Neurons deteriorated morphologically and electrophysiologically in TEA concentrations greater than 25 mM; therefore bathing solutions with 25 mM TEA-Cl and 5 mM 3-AP were sometimes used. The sodium

channel blocker tetrodotoxin (TTX)[36] was added from a 1 mM stock directly to the bathing solutions (final concentration was either 1 or 3 μM prior to addition to the culture plate).

Solutions of convulsant compounds were always prepared on the day of the experiment in the following manner. Dry BICUC was dissolved in 0.02 or 0.05 N HCl to form a 10 mM stock. Aliquots were removed and added to bathing medium to give concentrations between 10 and 200 μM. The pH was measured and adjusted to between 7.30–7.40 when necessary. BICUC is unstable at neutral pH[37,38] and therefore we used BICUC solutions for at most 1–2 h before replacing it with newly prepared BICUC. Stock concentrations of PCN (100 mM) were made by adding PCN to bathing medium from which 100 mM NaCl was deleted to maintain control osmolarity. The stock solutions were then diluted to 2.5–80 mM by adding control bathing medium. PCN solutions were used for two hours before replacement with newly prepared PCN solution. Osmolarity and pH were determined for all solutions.

Miniperfusion

Known concentrations of convulsants were applied to the somata of individual neurons during intracellular recording by the technique of miniperfusion. A microelectrode whose tip was manually broken to a diameter of 2–10 μm was filled with the test solution, and the open end of the miniperfusion pipettes were connected to a pressure regulator set between 0.5 and 2.0 pounds per square inch (psi) by tight fitting polyethylene tubing. Pressure pulse durations were regulated by a voltage-activated 3-way valve. Closure of the valve rapidly switched the miniperfusion pipette pressure from atmospheric pressure to that selected on the pressure regulator. Test solutions were applied to the recorded neuron by positioning the miniperfusion pipette 10–50 μm from the neuronal surface following generation of a control action potential and removing it 1–3 s before the next stimulated action potential. Small hyperpolarizing artifacts (< 5 mV) were occasionally produced by miniperfusion but these could be minimized by using small diameter miniperfusion pipettes, low miniperfusion pressure, more distant placement of the electrode and removal of the electrode prior to stimulation. No change in the membrane conductance was seen during the hyperpolarizing artifact. Miniperfusion of control solutions also produced a 10–20% reduction of calcium-dependent action potential duration which was minimized by regulating miniperfusion pipette tip size and pressure. The miniperfusion pipettes (usually 3) and recording microelectrode were held by Leitz micromanipulators. To decrease leakage of convulsant compound into the bathing medium, the tips of the miniperfusion pipettes were kept in the oil phase between drug application trials. They were lowered into the aqueous phase only during the interstimulus interval when drug application was desired.

Superfusion

Using the technique of superfusion the bathing solution in the culture dish was completely exchanged by other solutions at physiological pH, osmolarity and temperature. Solutions were delivered to and removed from the culture dish using a

peristaltic pump adjusted to a rate of 0.5–1 ml/min. Exchange was considered complete only after 3 times the original bathing solution volume was superfused through the culture dish. In many cases 10 or more complete exchanges were performed while recording intracellularly from a single neuron.

RESULTS

Sodium- and calcium-dependent action potentials

Action potentials recorded from spinal cord neurons in control bathing solution were brief in duration (0.6 ms) (Fig. 1A) and were abolished either by addition of 1 μM TTX to the bathing solution or removal of sodium from the bathing solution (not shown). When the potassium channel blocker TEA was added in addition to TTX, action potentials could be evoked by somatic stimulation, but action potential durations were prolonged (100 ms) (Fig. 1C). In dorsal root ganglion neurons, action potentials recorded in normal bathing solution were of longer duration (2.0 ms) with an inflection on the falling phase during repolarization (Fig. 1B). Addition of TTX did not appreciably alter the action potential. However, addition of TEA to the bathing medium considerably prolonged the action potential (100 ms) (Fig. 1D). We have previously shown that long duration action potentials evoked from spinal cord and dorsal root ganglion neurons bathed in TEA are calcium-dependent[21].

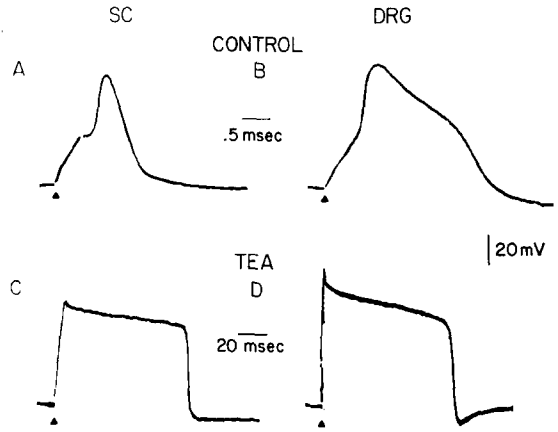

Fig. 1. Action potentials elicited from spinal cord (SC) and dorsal root ganglion (DRG) neurons in control bathing solution (CONTROL) and bathing solution containing tetraethylammonium (TEA). Action potentials were elicited from resting membrane potential (RMP) after brief depolarizing stimuli; stimulus onset in this and subsequent figures was denoted by filled triangles. The action potentials were recorded intracellularly in different bathing solutions in either SC (A and C) or DRG (B and D) neurons. A and B were recorded in control bathing solution (CONTROL); C and D in bathing solution containing 25 mM TEA and 1 μM TTX (TEA). In C and D, sodium chloride was reduced to 112.5 mM to maintain constant osmolarity. The composition of 'control' was as described in Methods. Pulse durations were 0.4 ms in A, 0.5 ms in B, and 2 ms in C and D. RMPs were: A, —62 mV; B, —46 mV; C, —55 mV; D, —42 mV. These and subsequent figures were retouched to remove grid markings, and to fill in the rising phase of the action potentials when reproduced faintly.

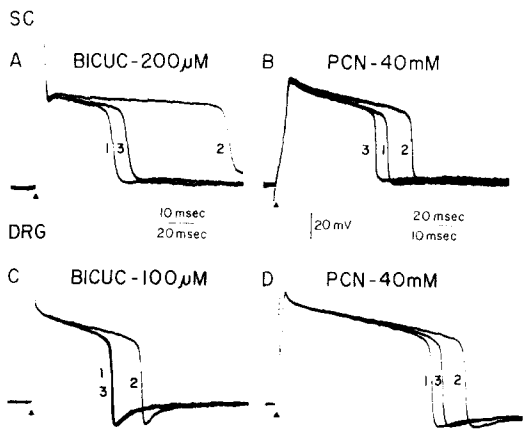

Fig. 2. Bicuculline (BICUC) and penicillin (PCN) prolonged calcium-dependent action potentials in SC and DRG neurons. Superimposed calcium-dependent action potentials prior to (1), 2 s after (2), and 22 s (3) after a 4–6 s pressure pulse (1 psi in A, B and D; or 2 psi in C) was applied to the end of a miniperfusion pipette filled with either BICUC or PCN at the designated concentration. The miniperfusion pipette was brought up to within 5 μm of the neuronal surface for application and then removed to the oil phase. 1, 2 and 3 are to the left of their respective action potentials. Stimulus pulse duration and RMP were: A, 4 ms, —64 mV; B, 6 ms, —62 mV (action potentials generated from —57 mV); C, 2 ms, —36 mV; D, 0.5 ms, —60 mV. Control bathing solution was used with 15 mM TEA-Cl, 3 μM TTX in A, B and D and 25 mM TEA-Cl, 1 μM TTX in C.

BICUC and PCN prolonged calcium-dependent action potentials

Calcium-dependent action potentials evoked in both dorsal root ganglion and spinal cord neurons (Fig. 2, labeled 1 in A–D) were prolonged when BICUC or PCN was applied to the soma by miniperfusion (Fig. 2, action potentials labeled 2 in A–D). A third action potential evoked 20–23 s after the miniperfusion pipette was removed returned to control duration (Fig. 2, action potential labeled 3 in A–D). Thus, action potential prolongation by both BICUC and PCN was reversible. The percentage prolongation of the action potential was dose-dependent for BICUC (Fig. 3) and PCN (Fig. 4) applied by superfusion or by miniperfusion. BICUC is unstable in solution at physiological pH[37,38]. Consistent with this, the potency of BICUC-induced prolongation deteriorated with time; within 2 h BICUC no longer prolonged the duration of the action potential. At 30–60 min after preparation BICUC produced a smaller percentage prolongation of calcium-dependent action potentials when applied by miniperfusion (Fig. 3A, filled squares) than BICUC made up within 5 min of superfusion onto spinal cord neurons (Fig. 3A, filled circles). PCN is stable in solution at physiological pH and, consistent with this, the potency of PCN-induced prolongation did not deteriorate for PCN solutions used 30–60 min after preparation (Fig. 4A, filled squares). However, our impression was that after about 2 h, the prolongation response decreased.

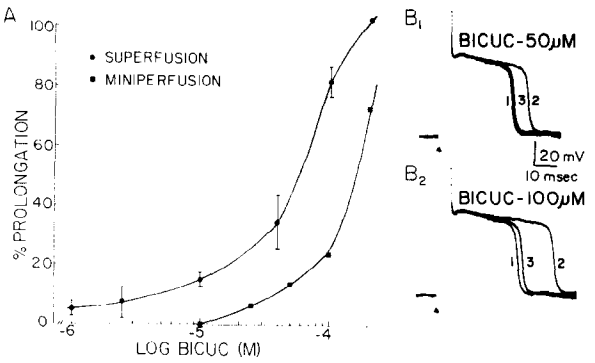

Fig. 3. BICUC prolongation of calcium-dependent action potentials was dose-dependent. BICUC was applied to spinal cord neurons by superfusion (A, filled circles) or by miniperfusion (A, filled squares; B_1, B_2). A: action potential duration was determined at half maximal amplitude. Percentage prolongation was the percentage increment in duration of the action potential after application of BICUC compared to the control duration. This percentage prolongation was plotted as a function of BICUC concentration. *Superfusion* (filled circles): each cell was superfused with test solution made just prior to application. Different doses of BICUC were applied onto the same neuron. Each *new* dose of BICUC was superfused only after the neuron had been returned to control bathing solution and the action potential duration returned to within 20% of its previous control value. Complete dose–response curves were generated for BICUC application to each neuron. The response at 200 μM BICUC was designated 100% prolongation, and the percentage prolongation at other concentrations were scaled appropriately. The filled circles were the averages of these scaled responses, and the bars above and below the filled circles their standard errors of the mean (S.E.M.). Each point is data from 4 or 5 neurons. *Miniperfusion* (filled squares and B_1, B_2): the filled squares denote the average (2–5 neurons) percentage prolongation to miniperfusion application of BICUC made 30–60 min beforehand. The pulse pressure and duration were 1 psi and 4 s. The stimulus was 4 ms in duration. Dose-dependent lengthening of calcium-dependent action potentials was shown in a spinal cord neuron at two concentrations of BICUC, 50 and 100 μM. 1, 2 and 3 designate the order of recording the consecutive, superimposed traces with 2 occurring 2 s, and 3, 22 s after removal of the test miniperfusion pipette. The bathing solution contained control solution adjusted for osmolarity and 15 mM TEA-Cl, 5 mM 3-AP and 3 μM TTX. RMP was —64 mV.

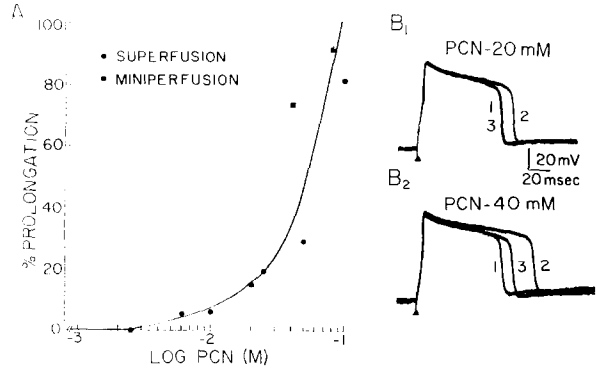

Fig. 4. PCN prolongation of calcium-dependent action potentials was dose-dependent. PCN was applied to spinal cord neurons by *superfusion* (A, filled circles) or by *miniperfusion* (A, filled squares; B_1, B_2). A: action potential duration was determined at half maximal amplitude and percentage prolongation, as described in Fig. 3. *Superfusion* (filled circles): each neuron was superfused with test solution made just prior to application. Different doses of PCN were applied to the same neuron. Only after each neuron was returned to the control bathing solution and returned to within 20% of control duration was a new dose of PCN superfused onto the neuron. Complete dose–response curves were generated for PCN application to most neurons. The response at 100 mM (filled circle) was the average percentage prolongation for 4 neurons. The remaining responses (filled circles) represent only one or two responses. The bathing solutions contained control bathing solution adjusted for osmolarity and 25 mM TEA-Cl, 5 mM 3-AP and 4 μM TTX. *Miniperfusion* (filled squares, and B_1, B_2): the filled squares denote the average (3 neurons) percentage prolongation to miniperfusion of PCN. The pulse pressure and duration were 2.5 psi and 4 s. The stimulus was 6 ms in duration. Dose-dependent lengthening of calcium-dependent action potentials is shown in a spinal cord neuron at two concentrations of PCN, 20 and 40 mM. 1, 2 and 3 designate the order of recording the consecutive, superimposed traces with 2 occurring 2 s and 3, 22 s after removal of the test miniperfusion pipette. Control bathing solution was adjusted for osmolarity and modified to contain 15 mM TEA-Cl, 5 mM 3-AP and 1 μM TTX. RMP was —62 mV.

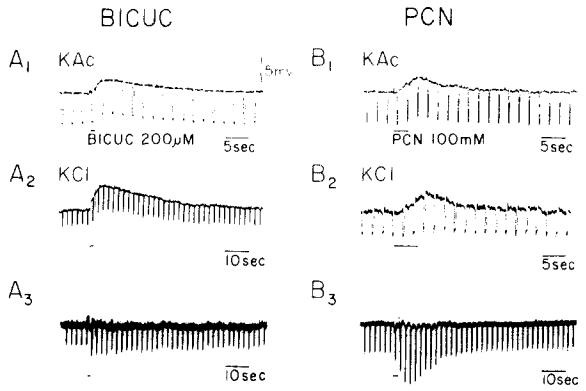

Fig. 5. BICUC and PCN had direct reversible actions on resting membrane properties. BICUC and PCN were applied onto spinal neurons by miniperfusion. Responses were recorded intracellularly at RMP with micropipettes filled with either 4 M KAc (A_1, A_3, B_1 and B_3) or 3 M KCl (A_2 and B_2). Superimposed on these responses were potentials that resulted from constant current pulses (300 ms duration). BICUC: freshly prepared 200 μM BICUC was miniperfused onto spinal cord neurons using KAc (A_1 and A_3) or KCl (A_2) microelectrodes for recording. A_3: steady hyperpolarizing current was passed through the recording electrode and adjusted manually to maintain the membrane potential at the initial RMP. RMPs were —71, —71 and —78 mV in A_1, A_2 and A_3 with neurons bathed in PBS with 10 mM $MgCl_2$ added to reduce synaptic activity. PCN: 100 mM PCN was miniperfused onto spinal cord neurons using KAc (B_1 and B_3) and KCl (B_2) microelectrodes for recording. B_3: steady hyperpolarizing current was passed through the recording electrode and adjusted manually to maintain the membrane potential at the initial RMP. RMPs were —54, —62 and —46 mV in B_1, B_2 and B_3 with neurons bathed in control bathing solution with 10 mM $MgCl_2$ added to reduce synaptic activity.

BICUC and PCN increased membrane resistance and produced membrane depolarization

Freshly prepared BICUC (Fig. 5A_1) or PCN (Fig. 5B_1) applied to spinal cord neurons by miniperfusion produced membrane depolarization. This direct depolarizing action of BICUC diminished with time, and application of BICUC 120–150 min after preparation produced no depolarization (not illustrated). The depolarization produced by both BICUC (Fig. 5A_1, A_2) and PCN (Fig. 5B_1, B_2) was associated with a decrease in membrane conductance (increase in resistance). At the peak of the membrane depolarization voltage responses to constant current pulses increased indicating decreased membrane conductance. This decreased membrane conductance was illustrated in another manner. Since the constant current pulses occurred every 4 s and the membrane response was slow, membrane potential could be 'clamped' manually by applying current through the recording electrode during the response. Under these conditions, voltage responses produced by superimposed constant current pulses still increased with application of BICUC (Fig. 5A_3) and PCN (Fig. 5B_3). Thus the increase in resistance was not due to a depolarization induced membrane rectification. Current–voltage curves obtained prior to and during superfusion with 40 μM BICUC (Fig. 6A) and 100 mM PCN (Fig. 6B) also demonstrated an increase in membrane resistance.

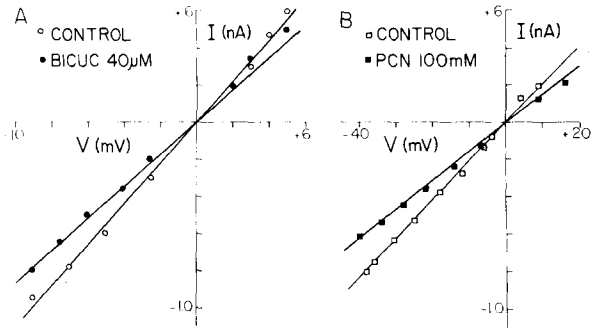

Fig. 6. BICUC and PCN increased input resistance (R_{in}) in spinal cord neurons A: R_{in} was measured in control (9 MΩ) (A, empty circles) and after superfusion of 40 μM BICUC (12 MΩ) (A, filled circles). The current–voltage curve was linear to 60 ms hyperpolarizing pulses of current. '0' mV was at RMP (—68 mV). Superfusing with CONTROL solution restored R_{in} toward control. B: R_{in} was measured in control (46 MΩ) (B, empty squares) and after superfusion of 100 mM PCN (64 MΩ) (B, filled squares). The current–voltage curve was linear to 70 ms hyperpolarizing pulses of current. '0' mV was at RMP (—58 mV). Superfusing with control bathing solution restored R_{in} toward control. Control bathing solution was adjusted for osmolarity and modified to contain 25 mM TEA-Cl, 5 mM 3-AP, and 1 μM TTX.

In general, membrane depolarization could be produced either by an *increase* in an ionic conductance whose equilibrium potential is depolarized relative to resting membrane potential such as for sodium or calcium, or by a *decrease* in an ionic conductance whose equilibrium potential was relatively hyperpolarized such as for chloride or potassium. Since BICUC and PCN decreased membrane conductance, it was likely that BICUC and PCN reduced either chloride or potassium conductance. If BICUC or PCN reduced chloride conductance, reduction of the chloride equilibrium potential from —65 to —20 mV by injecting chloride ions intracellularly from a 3 M KCl-containing micropipette would change the response from depolarizing to hyperpolarizing. Application of either BICUC (Fig. $5A_2$) or PCN (Fig. $5B_2$) while using KCl-containing recording micropipettes failed to invert the depolarizations suggesting that neither BICUC nor PCN altered chloride conductance; therefore, they probably blocked potassium conductance. Application of control solution without convulsant produced no depolarization.

To further investigate the ionic mechanism of depolarization evoked by BICUC and PCN, we determined the change in amplitude of the depolarizing responses as a function of membrane potential. It was difficult to depolarize or hyperpolarize the membrane more than 20 mV using a single recording micropipette and the bridge technique; therefore, in many cases we were unable to reverse the responses. Both KAc- and KCl-containing micropipettes were used. When the membrane was depolarized, response amplitude for BICUC (Fig. 7) and PCN (Fig. 8) increased, and when the membrane was hyperpolarized, response amplitude decreased. Extrapolated reversal potentials for the BICUC and PCN responses were 8–15 mV larger (more hyperpolarized) than resting membrane potential.

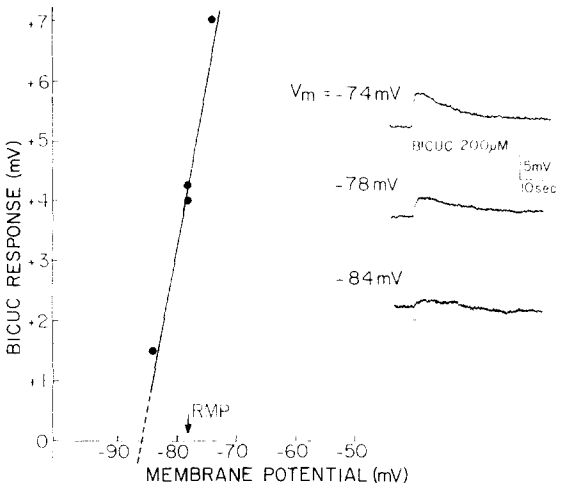

Fig. 7. BICUC direct responses had extrapolated reversal potentials more negative than RMP. Application of 200 μM BICUC onto a neuron in PBS (see Methods) at membrane potentials more negative (—84 mV) and less negative (—74 mV) than RMP (—78 mV) altered the amplitude of the depolarizing responses (specimen records to right of plot). The extrapolated reversal potential was between —80 and —90 mV. Data shown was from a recording with a KAc-filled micropipette. Recordings made with KCl-filled recording micropipettes had similar extrapolated reversal potentials.

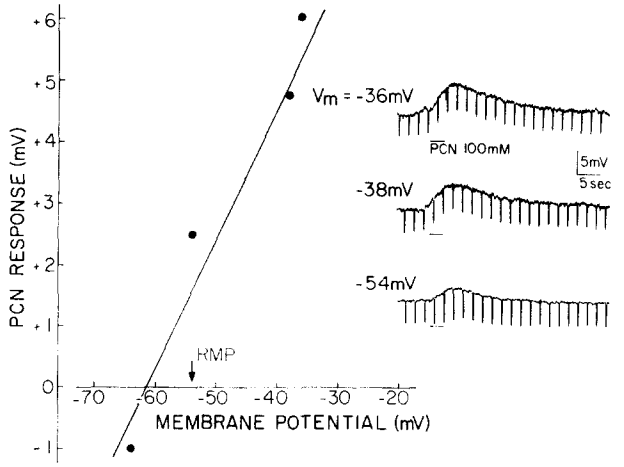

Fig. 8. PCN direct responses had extrapolated reversal potentials more negative than RMP. Application of 100 mM PCN onto a neuron in control bathing solution with 10 mM $MgCl_2$ at membrane potentials more negative (—65 mV) and less negative (—36, —38 mV) than RMP (—54 mV) altered the amplitude of the depolarizing responses (specimen records to right of plot). The extrapolated reversal potential was between —60 and —70 mV. Data shown was from a recording with a KAc-filled micropipette. Recordings made with KCl-filled recording micropipettes had similar extrapolated reversal potentials.

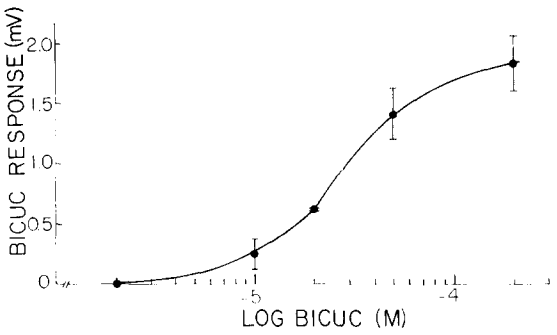

Fig. 9. BICUC produced dose-dependent membrane depolarization. Intracellular recordings were made from spinal cord neurons bathed in PBS with KAc-filled micropiettes. All responses were elicited from RMP and represent the average response ± S.E.M. of 3–7 neurons (average 4.2) to freshly prepared BICUC (less than 2 h old).

The direct depolarizing responses were dose-dependent over the range 2–200 μM for BICUC (Fig. 9), and over the range 5–100 mM for PCN (Fig. 10) at resting membrane potential.

Dose-dependency of BICUC and PCN actions

BICUC and PCN had two different effects on spinal cord and dorsal root ganglion neurons including prolongation of calcium-dependent action potentials, and reduction of resting membrane conductance resulting in membrane depolarization. While both of these actions were dose-dependent, there was a great difference in the relevant concentration range for the action of BICUC and PCN. Prolongation of calcium-dependent action potentials (Fig. 11, filled circles) and membrane depolarization (Fig. 11, filled squares) occurred over the same concentration ranges. However, larger concentrations of PCN were needed to produce these effects compared to the concentrations of BICUC required (Fig. 11).

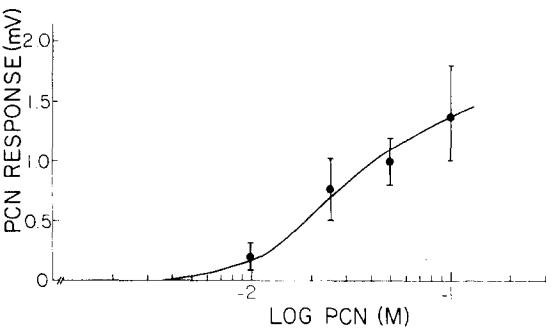

Fig. 10. PCN produced dose-dependent membrane depolarization. Intracellular recordings were made from spinal cord neurons bathed in control bathing solution with 10 mM $MgCl_2$ with and without 1 μM TTX with KAc-filled micropipettes. All responses were elicited from RMP and represent the average response ± S.E.M. of 4–8 neurons (average 5).

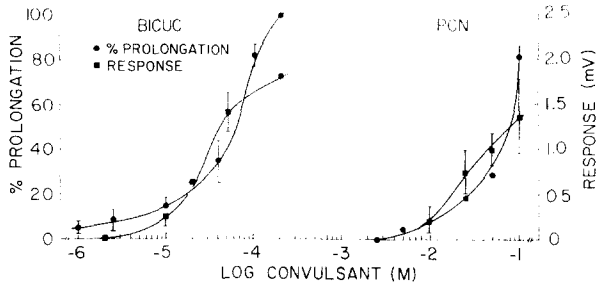

Fig. 11. Dose-dependent BICUC and PCN actions to increase calcium-dependent action potential duration and produce membrane depolarization. BICUC prolonged calcium-dependent action potential duration above 1 μM, and produced direct membrane depolarization above 2 μM. PCN prolonged calcium-dependent action potential duration above 2.5 mM, and produced direct membrane depolarization above 10 mM. Application of BICUC and PCN was by miniperfusion for direct response, and by superfusion for percentage prolongation of the calcium-dependent action potential. The bars above and below data points are $\pm$ values of S.E.M. Only 1–4 spinal cord neurons were studied for prolongation of calcium-dependent action potentials with superfusion of PCN. Bathing solution and details of intracellular recordings are found in Figs. 3, 4, 9 and 10 for percentage prolongation and response in BICUC and PCN.

DISCUSSION

We have demonstrated that the convulsant compounds BICUC and PCN produced membrane depolarization of mouse spinal cord neurons in primary dissociated cell culture. The depolarization was most likely due to blockade of a potassium conductance since the depolarization: (1) was produced by an increase in membrane resistance; (2) was not inverted following intracellular injection of chloride ions, and (3) had an extrapolated reversal potential more negative than resting membrane potential. At least 3 potassium conductances have been described in vertebrate neurons: (1) a voltage-dependent potassium conductance (delayed rectification)[23]; (2) a calcium-dependent potassium conductance[5,33,34], and (3) a voltage-dependent muscarine-sensitive potassium conductance[6]. In addition, resting voltage-independent 'leak' conductance may have a potassium conductance component. We could not determine from our data which potassium conductance was reduced by the convulsants.

In addition, we have demonstrated that BICUC and PCN prolonged calcium-dependent action potentials in both spinal cord and dorsal root ganglion neurons. Calcium-dependent action potentials have been described in several neuronal cell types[14,20,26,29,45,54] and repolarization of these action potentials is probably due to an increase in voltage- and/or calcium-dependent potassium conductance. Calcium dependent action potential prolongation could be produced either by *increasing* calcium conductance and/or by *reducing* a repolarizing conductance such as potassium or chloride conductance. Since BICUC and PCN blocked potassium conductance over the concentration range producing calcium-dependent action potential prolongation, it was likely that reduction of potassium conductance resulted in the action potential prolongation. While we cannot exclude the possibility that BICUC and PCN increased

calcium conductance, the finding that they decreased potassium conductance is probably sufficient to explain their effect on calcium-dependent action potential duration.

In addition to these direct or non-synaptic actions, BICUC and PCN also have been shown to antagonize GABA-mediated inhibition in spinal cord neurons in cell culture (a synaptic action)[28]. Are both synaptic and non-synaptic actions involved in production of PDE and PDS? As discussed in the introduction, PDS may represent calcium-dependent action potentials of dendritic origin which under abnormal circumstances invade neuronal somata[43]. Orthodromically activated excitatory postsynaptic potentials (EPSPs), produced calcium-dependent action potentials only if inhibitory postsynaptic potentials (IPSPs) (presumably GABA-mediated) were reduced by PCN[53]. In computer models of hippocampal and cortical pyramidal neurons which included dendritic calcium-dependent action potentials, PDS could be generated not only by reducing inhibitory input but also by prolonging calcium-dependent action potentials[49,50], although until now no physical examples have been available. Therefore, antagonism of GABA-mediated inhibition and prolongation of calcium-dependent action potentials could be involved in producing PDS and PDE.

Spinal cord motor neurons develop paroxysmal bursting after application of PCN[46]. Calcium-dependent action potentials have not been recorded from motoneurons unless potassium channel blockers have been applied[1,5]. How do convulsants produce bursting in these neurons? The PCN-induced paroxysmal activity was due to a slow persistent inward current which was probably carried by calcium ions[46]. When the inward current was present there was a negative slope conductance region in the membrane current–voltage curve and such current–voltage relationships are known to produce bursting. The negative slope conductance region could be produced by a reduction of outward potassium current by at least two mechanisms: (1) a decrease in the potassium equilibrium potential, or (2) a reduction in potassium conductance. In spinal cord glycine, not GABA, is probably the major inhibitory neurotransmitter[52] and glycine-mediated inhibition is not blocked by PCN[28]. Thus the synaptic action of PCN may be insufficient to produce bursting. Our results therefore suggest that PCN and BICUC may produce bursting, at least in part, by blocking a membrane potassium conductance as well as GABA-mediated inhibition and inducing a negative slope conductance region in the membrane current–voltage curves. Thus in neurons which do not have well developed calcium conductances, blockade of both potassium conductance and GABA-mediated increases in chloride conductance may be required to induce bursting.

If enhanced excitatory transmitter release underlies PDS generation (giant EPSP hypothesis), both synaptic and non-synaptic factors could be of importance. Antagonism of GABA-mediated inhibition could enhance the efficacy or recruitment of excitatory pathways. Reduction of potassium conductance at presynaptic terminals might increase calcium entry and thus enhance transmitter release at individual synapses.

In summary, we have shown that two convulsant compounds, BICUC and PCN, have both synaptic and non-synaptic actions. We have argued that in certain regions

of the nervous system such as hippocampus, which contain neurons with well developed dendritic calcium conductance, the synaptic convulsant action to antagonize GABA-mediated inhibition may be sufficient to produce PDS. However in other regions of the central nervous system such as spinal cord or cortex, where calcium conductance may not be as well developed, the non-synaptic action of the convulsants to block the repolarizing potassium conductance may be required in addition to the synaptic action to produce PDS.

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REFERENCES

1 Alvarez-Leefmans, F. J. and Miledi, R., Voltage sensitive calcium entry in frog motoneurones, *J. Physiol. (Lond.)*, 308 (1980) 241–257.
2 Armstrong, C. M., Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons, *J. gen. Physiol.*, 58 (1971) 413–437.
3 Armstrong, C. M. and Binstock, L., Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride, *J. gen. Physiol.*, 48 (1965) 859–987.
4 Ayala, G. F., Dichter, M., Gumnit, R. J., Matsumoto, H. and Spencer, W. A., Genesis of epileptic interictal spikes: new knowledge of cortical feedback systems suggests a neurophysiological explanation of brief paroxysms, *Brain Research*, 52 (1973) 1–17.
5 Barrett, E. F. and Barrett, J. N., Separation of two voltage-sensitive potassium currents and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurones, *J. Physiol. (Lond.)*, 225 (1976) 737–774.
6 Brown, D. A. and Adams, P. R., Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone, *Nature (Lond.)*, 283 (1980) 673–676.
7 Clarke, G. and Hill, R. G., Effects of a focal penicillin lesion on responses of rabbit cortical neurons to putative neurotransmitters, *Brit. J. Pharmacol.*, 44 (1972) 435–441.
8 Curtis, D. R., Duggan, A. W., Felix, D. and Johnston, G. A. R., Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat, *Brain Research*, 32 (1971) 69–96.
9 Curtis, D. R., Duggan, A. W. Felix, D., Johnston, G. A. R. and McLennan, H., Antagonism between bicuculline and GABA in the cat brain, *Brain Research*, 33 (1971) 57–73.
10 Curtis, D. A. and Felix, D., The effect of bicuculline upon synaptic inhibition in the cerebral and cerebellar cortices of the cat, *Brain Research*, 34 (1971) 301–321.
11 Curtis, D. R., Game, C. J. A., Johnston, G. A. R., McCulloch, R. M. and MacLachlan, R. M., Convulsive action of penicillin, *Brain Research*, 43 (1972) 242–245.
12 Davidoff, R. A., Penicillin and presynaptic inhibition in the amphibian spinal cord, *Brain Research*, 36 (1972) 218–222.
13 Davidoff, R. A., Penicillin and inhibition in the cat spinal cord, *Brain Research*, 45 (1972) 638–642.
14 Dichter, M. A. and Fischbach, G. D., The action potential of chick dorsal root ganglion neurones maintained in cell culture, *J. Physiol. (Lond.)*, 267 (1977) 281–298.
15 Dichter, M., Spencer, W. A., Penicillin-induced interictal discharges from the cat hippocampus. I. Characteristics and topographical features, *J. Neurophysiol.*, 32 (1969) 649–662.

16 Dingledine, R. and Gjerstad, L., Reduced inhibition during epileptiform activity in the in vitro hippocampal slice, *J. Physiol. (Lond.)*, 305 (1980) 297–313.
17 Enna, S. J. and Snyder, S. H., Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions, *Brain Research*, 100 (1975) 81–97.
18 Enomoto, T. F. and Ajmone-Marsan, C., Epileptic activation of single cortical neurons and their relationship with electroencephalographic discharges, *Electroenceph., clin. Neurophysiol.*, 11 (1959) 199–218.
19 Freeman, A. R., Electrophysiological analysis of the actions of strychnine, bicuculline and picrotoxin on the axonal membrane, *J. Neurobiol.*, 4 (1973) 567–582.
20 Gelduldig, D. and Junge, D., Sodium and calcium components of action potentials the *Aplysia* giant neurone, *J. Physiol. (Lond.)*, 199 (1968) 342–365.
21 Heyer, E. J., Macdonald, R. L., Bergey, G. K. and Nelson, P. G., Calcium-dependent action potentials in mouse spinal cord neurons in cell culture, *Brain Research*, 220 (1981) 408–415.
22 Hochner, B., Spira, M. E. and Werman, R., Penicillin decreases chloride conductance in crustacean muscle: a model for the epileptic neuron, *Brain Research*, 107 (1976) 85–103.
23 Hodgkin, A. L. and Huxley, A. F., A quantitative description of membrane current and its application to conduction and excitation in nerve, *J. Physiol. (Lond.)*, 117 (1952) 500–544.
24 Johnston, G. A. R., Beart, P. M., Curtis, D. R., Game, C. J. A., McCulloch, R. M. and Maclachlan, R. M., Bicuculline methochloride as a GABA antagonist, *Nature New Biol.*, 240 (1972) 219–220.
25 Johnston, D. and Brown, T. H., Giant synaptic potential hypothesis for epileptiform activity, *Science*, 211 (1981) 294–297.
26 Llinas, R. and Hess, R., Tetrodotoxin-resistant dendritic spikes in avian Purkinje cells, *Proc. nat. Acad. Sci. U.S.A.*, 73 (1976) 2520–2523.
27 Llinas, R., Walton, K. and Bohr, V., Synaptic transmission in squid giant synapse after potassium conductance blockage with external 3- and 4-aminopyridine, *Biophys. J.*, 16 (1976) 83–86.
28 Macdonald, R. L. and Barker, J. L., Specific antagonism of GABA-mediated postsynaptic inhibition in cultured spinal cord neurons: a common mode of convulsant action, *Neurology*, 28 (1978) 325–330.
29 Matsuda, Y., Yoshida, S. and Yonezawa, T., A Ca-dependent regenerative response in rodent dorsal root ganglion cells cultured in vitro, *Brain Research*, 115 (1976) 334–338.
30 Matsumoto, H., Intracellular events during the activation of cortical epileptiform discharges, *Electroenceph. clin. Neurophysiol.*, 17 (1964) 294–307.
31 Matsumoto, H., Ayala, G. F. and Gumnit, R. J., Neuronal behavior and triggering mechanism in cortical epileptic focus, *J. Neurophysiol.*, 32 (1969) 688–703.
32 Matsumoto, H. and Marsan, C. A., Cortical cellular phenomena in experimental epilepsy: interictal manifestations, *Exp. Neurol.*, 9 (1964) 286–304.
33 Meech, R. W., The sensitivity of *Helix aspersa* neurones to injected calcium ions, *J. Physiol. (Lond.)*, 237 (1974) 259–277.
34 Meech, R. W. and Standen, N. B,., Potassium activation in *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx, *J. Physiol. (Lond.,)*, 249 (1975) 211–239.
35 Mohler, H. and Okada, T., GABA receptor binding with ^{3}H(+)-bicuculline-methiodide in rat CNS, *Nature (Lond.)*, 267 (1977) 65–67.
36 Narahashi, T., Chemical tools in the study of excitable membranes, *Physiol. Rev.*, 54 (1974) 813–889.
37 Olsen, R. W., Ban, M. and Miller R., Studies on the neuropharmacological activity of bicuculline and related compounds, *Brain Research*, 102 (1976) 283–299.
38 Olsen, R. W., Ban, M., Miller, T. and Johnston, G. A. R., Chemical instability of the GABA antagonist bicuculline under physiological conditions, *Brain Research*, 98 (1975) 383–387.
39 Olsen, R. W., Ticku, M. K., VanNess, P. L. and Greenlee, D., Effects of drugs on γ-aminobutyric acid receptors, uptake, release and synthesis in vitro, *Brain Research*, 139 (1978) 277–294.
40 Pelhate, M. and Pichon, Y., Selective inhibition of potassium current in the giant axon of the cockroach, *J. Physiol. (Lond.)*, 242 (1974) 90–91.
41 Pong, S. F. and Graham, L. T., N-methyl bicuculline, a convulsant more potent than bicuculline, *Brain Research*, 42 (1972) 486–490.
42 Prince, D. A., The depolarization shift in 'epileptic' neurons, *Exp. Neurol.*, 21 (1968) 467–485.
43 Prince, D. A., Neurophysiology of epilepsy, *Ann. Rev. Neurosci.*, 1 (1978) 395–415.
44 Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N. and Nelson, P. G., Mouse spinal cord in

cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties, *J. Neurophysiol.*, 40 (1977) 1132–1150.
45 Schwartzkroin, P. A. and Slawsky, M., Probable calcium spikes in hippocampal neurons, *Brain Research*, 135 (1977) 157–161.
46 Schwindt, P. and Crill, W., Role of a persistent inward current in motoneuron bursting during spinal seizures, *J. Neurophysiol.*, 43 (1980) 1296–1318.
47 Shank, R. P., Pong, S. F., Freeman, A. R. and Graham, L. T., Bicuculline and picrotoxin as antagonists of γ-aminobutyrate and neuromuscular inhibition in the lobster, *Brain Research*, 72 (1974) 71–78.
48 Takeuchi, A. and Onodero, K., Effect of bicuculline on the GABA receptor of the crayfish neuromuscular junction, *Nature New Biol.*, 236 (1972) 55–56.
49 Traub, R. D., Neocortical pyramidal cells: a model with dendritic calcium conductance reproduces repetitive firing and epileptic behavior, *Brain Research*, 173 (1979) 243–257.
50 Traub, R. D. and Llinas, R., Hippocampal pyramidal cells: significance of dendritic ionic conductances for neuronal function and epileptogenesis, *J. Neurophysiol.*, 42 (1979) 476–496.
51 Welch, A. D. and Henderson, V. E., A comparative study of hydrastine, bicuculline and adlumine, *J. Pharmacol exp. Ther.*, 51 (1934) 482–491.
52 Werman, R., Davidoff, R. A. and Aprison, M. H., Inhibitory action of glycine on spinal neurons in the cat, *J. Neurophysiol.*, 31 (1968) 81–95.
53 Wong, R. K. S. and Prince, D. A., Dendritic mechanisms underlying penicillin-induced epileptiform activity, *Science*, 204 (1979) 1228–1231.
54 Wong, R. K. S., Prince, D. A. and Basbaum, A. I., Intradendritic recordings from hippocampal neurons, *Proc. nat. Acad. Sci. U.S.A.*, 76 (1979) 986–990.
55 Yeh, J. E., Oxford, G. S. and Narahashi, T., Interactions of aminopyridines with potassium channels of squid axon membranes, *Biophys. J.*, 16 (1976) 77–81.
56 Zukin, S. R., Young, S. R. and Snyder, S. H., Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system, *Proc. nat. Acad. Sci. U.S.A.*, 71 (1974) 4802–4807.