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Convulsant doses of penicillin shorten the lifetime of GABA-induced channels in cultured central neurones

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1 The influence of sodium benzylpenicillin (PCN) on membrane channels activated by γ-aminobutyric acid (GABA) was studied in cultured spinal neurones of the mouse by the extracellular patch clamp technique.

2 In whole-cell, current clamp recordings, concentrations of PCN above 0.2 mM significantly reduced the amplitude of the GABA response.

3 Single channel currents activated by GABA were studied in outside-out patches of neuronal membrane. In both the absence and presence of PCN, cumulative open time distributions for GABA-activated channels were well fitted by the sum of two exponential terms, characterized by fast (τ_f) and slow time constants (τ_s).

4 PCN (2 mM) reduced the mean value of τ_s from 4.29 ± 0.56 ms (mean ± s.e.mean) to 1.12 ± 0.09 ms but had no significant effect on τ_f.

5 The mean open time of GABA-activated channels, calculated from the double exponential fits, decreased from 1.39 ± 0.35 ms to 0.53 ± 0.02 ms in the presence of 2 mM PCN.

6 The reduced mean open time of GABA-sensitive channels seen in the presence of PCN may contribute to the convulsant action of the drug *in vivo*.

Introduction

Penicillin (PCN) can cause adverse neurological reactions when administered in sufficient dose to man and laboratory animals. The neurological symptoms of penicillin poisoning include confusion, twitching, multifocal myoclonus and localized or generalized epileptiform seizures (Mandell & Sande, 1980). In mammals, application of millimolar concentrations of PCN to the cerebral cortex results in a pattern of seizure activity which is widely used as a model of epilepsy (Davenport *et al.*, 1978; Gjerstad *et al.*, 1981; Krnjević, 1983).

A number of mechanisms have been proposed to account for the convulsant action of PCN. The drug has been reported to increase the excitability of presynaptic nerve terminals (Prince, 1978), and to enhance the excitatory effect of the neurotransmitter candidate L-glutamate on central neurones (Macon & King, 1979). In addition, PCN has been shown to antagonize the inhibitory effects of the putative transmitter γ-aminobutyric acid (GABA) in the mammalian brain (Avoli, 1984) and spinal cord, studied both *in vivo* (Davidoff, 1972) and *in vitro* (MacDonald & Barker, 1977).

Quantitative studies indicate that PCN exerts a noncompetitive type of block on the GABA response in central neurones (Pickles & Simmonds, 1980). It has therefore been proposed that PCN may interact with the Cl^- permeable ion channel which mediates the response to GABA in these cells (McBurney & Barker, 1978; Pickles & Simmonds, 1980). This view is supported by the observation that convulsant doses of PCN alter the power spectral density of current fluctuations induced by GABA in spinal cord neurones of the mouse (Barker *et al.*, 1981). The form and amplitude of such spectra are held to reflect the average lifetime and conductance of GABA-induced chloride channels in the open state (McBurney & Barker, 1978).

In the present experiments, the extracellular patch clamp method was used to resolve currents flowing through single, GABA-sensitive Cl^- channels in the membrane of mouse spinal cord neurones in culture (Hamill *et al.*, 1983). This approach has shown directly

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that convulsant doses of PCN do indeed modify the gating of these channels.

Method

Primary dissociated cultures of spinal cord (SC) neurones were obtained from 13 day mouse (C57BL) embryos (Ransom *et al.*, 1977). Cultures were fed twice weekly with Minimum Essential Medium (Gibco) containing Earle's salts and 10% horse serum (Gibco). This medium did not contain penicillin or any other antibiotic. All cultures were incubated at 37°C for 3–4 weeks before use.

Patch clamp recordings took place at room temperature (22–24°C) using a List EPC-5 amplifier. Patch electrodes were made on a modified David Kopf 700 micropipette puller, coated to near the tip with 734 RTV sealant (Dow Corning) and fire polished immediately before use. The whole-cell, current clamp recording mode was used to study the effect of PCN on the amplitude of potential changes induced by GABA at the SC cell membrane (Hamill *et al.*, 1981). In these recordings, patch electrodes were filled with a solution containing (in mM): KCl 140, NaCl 3, $MgCl_2$ 1, EGTA 11, HEPES 10, pH 7.4. The external membrane face of the cells was exposed to a normal bathing solution of composition (mM): NaCl 140, KCl 4, $CaCl_2$ 1, $MgCl_2$ 10, HEPES 10, pH 7.4. Under these ionic conditions, the equilibrium potential for the chloride-mediated GABA response was markedly positive relative to the resting potential, enhancing the voltage response of the cells to small doses of GABA. GABA (Sigma, 10 μM) and sodium benzylpenicillin (Sigma, 50 μM–10 mM) were dissolved in normal bathing medium and applied to the somata of cells under study by pressure ejection from broken glass pipettes (external tip diameters, <5 μm, ejection pressure 30 p.s.i.). To produce a GABA response of about 15 mV, the GABA ejection pulse width was adjusted over the range 50–500 ms. After recording several control GABA responses evoked at a frequency of 0.1 Hz to minimize desensitization, GABA application was stopped. A 5 s pulse of PCN was then applied to the cell from an independent pressure pipette; 100 ms after the end of this pulse, the test applications of GABA were recommenced. This delay prevented flow interaction between the two pressure pipettes. PCN-induced inhibition of the GABA response was quantified using the relation $((C - P)/C) \times 100\%$, where C and P are the amplitudes of control and maximally depressed GABA responses respectively.

Single channel currents activated by GABA in isolated outside-out patches of neuronal membrane were measured with patch electrodes having resistances of 8–15 MΩ filled with a solution containing (in mM): Tris Cl 140; KCl 3; $MgCl_2$ 1; EGTA 11; HEPES 10; pH 7.4 (Hamill *et al.*, 1983). Outside-out membrane patches were characterized by an average membrane-electrode seal resistance of 155 ± 9 GΩ, mean ± s.e.mean., $n = 25$).

The outside face of these membrane patches was bathed in a solution of composition (in mM): Tris chloride 137, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, pH 7.4 (external Tris solution). GABA (1–1.25 μM) and sodium benzylpenicillin (0.2–2 mM) were dissolved in this solution and applied to the membrane patches by bath perfusion.

Patch currents were low-pass filtered at 2 kHz (8 pole Bessel) and stored on FM tape (Racal 4DS) for later analysis. Five second data segments were digitized at 4 kHz sampling rate and displayed on a Data Precision D-6000 wave form analyser. Digitized records were inspected visually and the amplitudes of 20–30 long duration (>3 ms) openings averaged to provide an initial estimate, i of the mean single channel current. Most records contained many small amplitude openings which were either too brief or too incomplete to reach the value of i. Similarly, many openings were punctuated by apparent channel closures too brief or incomplete to reach the zero current level. Some of these observations may be due to the existence of a substate conductance level for GABA-activated chloride channels in SC cells (Hamill *et al.*, 1983). In order to detect these small events, it was necessary to employ two threshold levels, T1 and T2, which were assigned the values $0.4i$ and $0.7i$ respectively. A negative going excursion of the patch clamp output signal across either T1 or T2 indicated the flow of inward membrane current and denoted a channel opening transition. Conversely, positive going excursions of the output signal across either threshold indicated a channel closure. This automatic event-finding routine was constantly monitored by displaying the calculated transitions as cursor brightened points in the original digitized data. Suspect transitions were edited out at this stage in the analysis.

The open times of several hundred single channel currents were measured and stored on magnetic disc. Frequency histograms of channel open times were constructed by use of a Hewlett-Packard HP-85 computer. Cumulative open time distributions were fitted by single or double exponential equations using the Osborne-Marquardt minimization method (Golub & Pereyra, 1972). This algorithm uses a separation of variables to accomplish least squares fitting to linear combinations of nonlinear functions. The relative tolerance for differences between two consecutive residuals was set at 0.02. The relative tolerance for the size of the correction was 0.001. Under these constraints, convergence usually occurred after 5–6 iterations. All fit curves were also assessed visually by plotting their equations over the original data points.

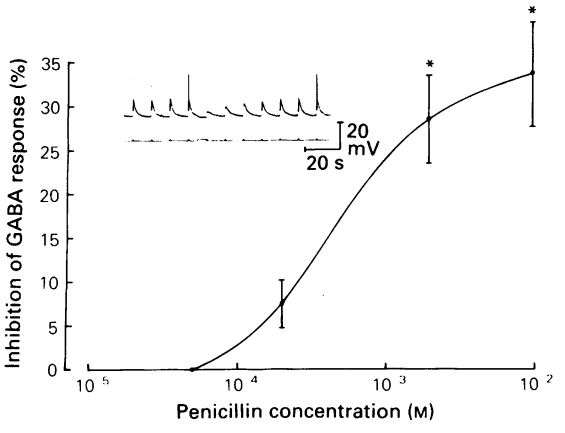

Figure 1 The effect of sodium benzylpenicillin (PCN) on membrane depolarizations evoked by γ-aminobutyric acid (GABA) at the somata of cultured SC neurones. These potentials were recorded by the whole-cell, current clamp variant of the extracellular patch clamp method. The upper trace of the insert shows a typical record of this kind (cell resting potential, −53 mV). The lower insert trace shows the duration and timing of pressure pulses (30 p.s.i.) used to eject 10 μM GABA (0.1 Hz, 0.5 s duration) and 2 mM PCN (single pulse, 5 s duration) from two independent pipettes. Larger GABA responses triggered action potentials that were greatly attenuated by the limited frequency response of the pen recorder (d.c. −100 Hz). The percentage inhibition of the GABA response by PCN was then plotted against the dose of PCN applied (logarithmic scale). Each data point represents the mean for 12 cells; s.e.mean shown by vertical lines. Asterisks indicate PCN concentrations which significantly depressed the GABA response with respect to control (Wilcoxon test, $P < 0.05$). The smooth curve was fitted to the data points by eye.

Results

Stable, whole-cell recordings were obtained from 45 SC neurones. The mean resting membrane potential of these cells was −54 ± 0.9 mV (mean ± s.e.mean). PCN 50 μM had no detectable influence on the amplitude of the GABA response as measured in 12 neurones. At higher concentrations, however, the drug reduced the amplitude of this response in a reversible, dose-dependent manner (Figure 1). When tested at concentrations up to 10 mM, no evidence was seen for a GABA-mimetic, agonist effect of PCN in any of the neurones examined.

Figure 2 shows the effect of PCN 2 mM on single channel currents activated by GABA in an outside-out patch of neuronal membrane. The figure indicates that the average open time of GABA-activated channels is apparently decreased in the presence of PCN. This observation was examined more closely by calculating frequency distributions for the open times of GABA-

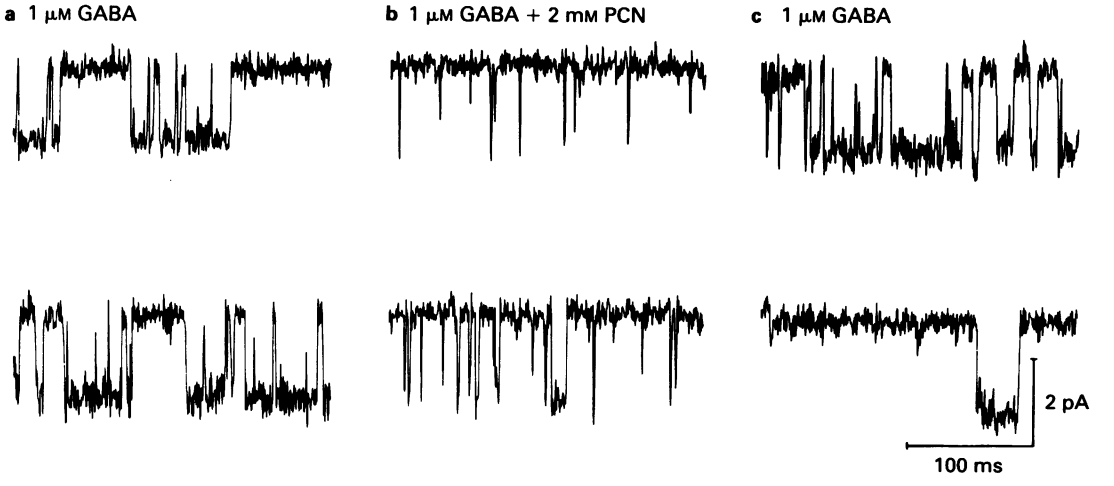

Figure 2 The effect of 2 mM sodium benzylpenicillin (PCN) on single channel currents evoked by application of 1 μM γ-aminobutyric acid (GABA) to an outside-out patch of neuronal membrane. The membrane patch was excised from a mouse spinal cord cell grown in culture for three weeks before use and was voltage-clamped to −80 mV. In all traces, inward membrane currents appear as downward deflections from baseline. Single channel currents were recorded in the presence of: (a) 1 μM GABA alone, (b) 1 μM GABA and 2 mM PCN, (c) 1 μM GABA following wash off of PCN. Temperature 22°C, bandwidth of recording d.c. −1 kHz.

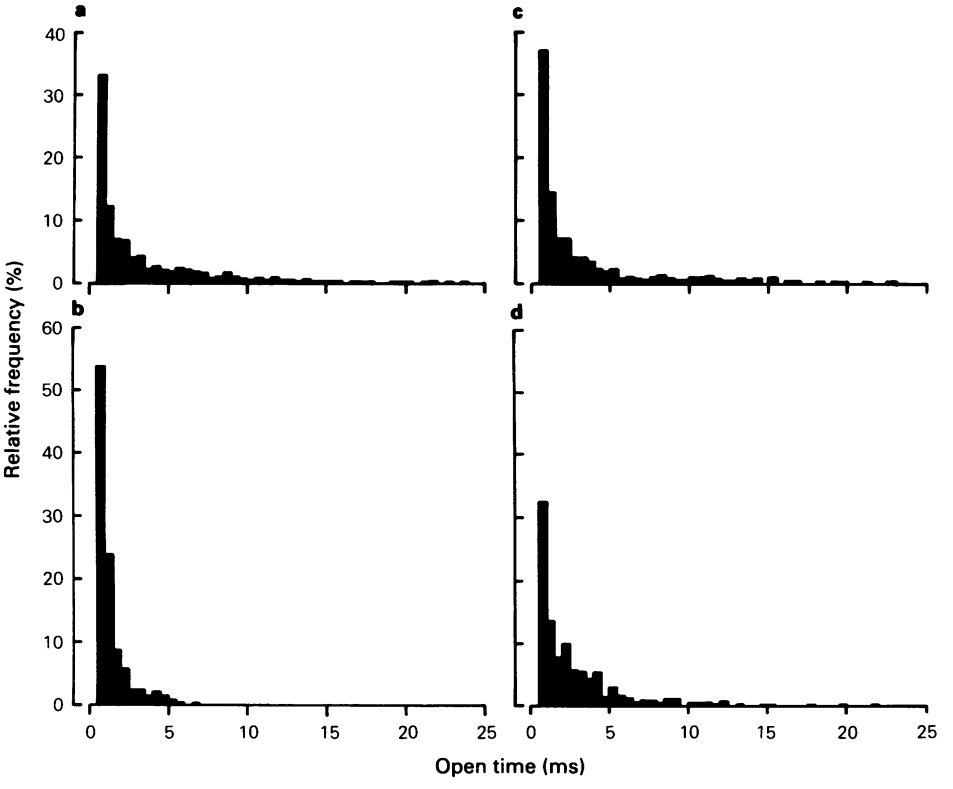

Figure 3 The effect of sodium benzylpenicillin (PCN) on the distribution of open times for γ-aminobutyric acid (GABA)-activated single channel currents recorded in an outside-out patch of neuronal membrane. To obtain these plots, non-cumulative frequency histograms of channel open time were first prepared. Then, the number of single channel currents briefer than 0.5 ms was subtracted from the total number of observed events, yielding the parameter n. Each distribution was then normalized by expressing the number of observations in each time bin as a percentage of n (relative frequency, ordinate scale). Finally a Chi square test was performed on the values of relative frequency obtained. The control data provided the expected frequency for each time bin, while the PCN-treated data contributed the observed frequencies. (a,b) The effect of 2 mM PCN on the open time distribution. Data were obtained from a single outside-out patch voltage-clamped to −60 mV. GABA 1 μM was applied in the absence, (a) or presence, (b) of 2 mM PCN. For (a), $n = 570$, in (b), $n = 538$. These distributions are significantly different ($P < 0.001$). (c,d) The lack of effect of 0.2 mM PCN on the open time distribution. Data were obtained from a single outside-out patch, different from that in (a), and voltage-clamped to −60 mV. GABA 1 μM was applied in the absence, (c) or presence (d) of 0.2 mM PCN. For (c), $n = 557$, in (d), $n = 345$. These distributions are not significantly different ($P < 0.45$).

activated membrane channels. The effect of PCN on these distributions was initially studied by the approach shown in Figure 3. The data were normalised by expressing the number of observations in each time bin as a percentage of the total number of observed events. Single channel currents briefer than 0.5 ms were not included in the analysis, since their detection rate was reduced by system bandwidth limitations. In the experiment shown Figure 3a,b, a Chi square test indicated that 2 mM PCN significantly altered the form of the open time distribution ($P < 0.001$). In addition, during PCN action, the arithmetic mean, m of all observed open times decreased from 3.6 ms (600 events) to 1.1 ms (608 events). Similar results were obtained with 2 mM PCN in four other patches from four cells. For all 5 patches, PCN significantly altered the form of the open time distribution, P being always less than 0.005. In the presence of PCN, the grand mean value of m (averaged from 5 patches) decreased from 2.7 ± 0.54 ms to 0.92 ± 0.09 ms, this difference being significant ($P < 0.05$, Wilcoxon test).

The effects of 0.2 mM PCN were tested in a similar fashion on two patches. As shown in Figure 3c,d, this concentration of PCN had no significant action on the open time distribution for GABA-sensitive channels ($P < 0.45$).

In both the presence and absence of 2 mM PCN, cumulative open time distributions were found to deviate from a single exponential form. The probability that the observed distributions deviated from a best one exponential fit solely by chance was always less than 0.0001 for both control and PCN-treated patches (5 patches, Chi square test). This result indicated that, in both the absence and presence of PCN, the closing of GABA-activated channels is not controlled by a simple first order process (Colquhoun & Hawkes, 1982).

Attempts were made to fit the observed distributions with the sum of two exponential terms, $y = N_f.e^{-t/\tau_f} + N_s.e^{-t/\tau_s}$ (Figure 4).This equation afforded a better fit to both control and PCN data, as judged by the uniformly high probability that deviations from the calculated curves were due solely to chance (Chi square test, P always greater than 0.3). Table 1 shows values of the fit parameters τ_f and τ_s calculated for data obtained from 5 outside-out patches. It can be seen that 2 mM PCN reduced the value of τ_s, the slow time constant of these fits from 4.29 ± 0.56 ms to 1.12 ± 0.09 ms, this difference being significant ($P < 0.05$, Wilcoxon test). However, no significant effect of PCN was seen in the case of τ_f, the fast time constant of these distributions (control, 0.49 ± 0.06 ms; PCN, 0.42 ± 0.02 ms, $P < 0.2$ Wilcoxon test). The mean open time, τ_m of GABA induced channels in these patches was calculated from the relation $\tau_m = N_f/(N_f + N_s).\ \tau_f + N_s/(N_f + N_s).\ \tau_s$, where N_f and N_s are the number of events in the fast and slow fit components respectively. The mean value of τ_m decreased from 1.39 ± 0.35 ms in control recordings to 0.53 ± 0.02 ms in the presence of 2 mM PCN, this difference being significant ($P < 0.05$, Wilcoxon test).

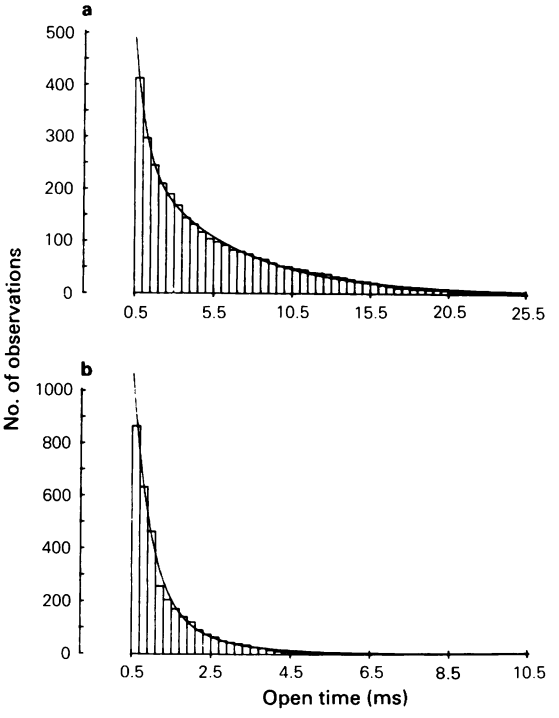

Figure 4 The effect of 2 mM sodium benzylpenicillin (PCN) on the cumulative open time distribution for γ-aminobutyric acid (GABA)-activated single channel currents recorded in an outside-out patch of neuronal membrane. The patch was voltage-clamped to −80 mV throughout at a temperature of 21°C. Cumulative open time distributions for single channel currents induced by 1.25 μM GABA were obtained in the absence of PCN (a) and in the presence of 2 mM PCN (b). Note the different time and amplitude scales in (a) and (b). Scales were chosen to show fits clearly. In both cases, the distributions were well fit by the sum of two exponential terms, $y = N_f.e^{-t/\tau_f} + N_s.e^{-t/\tau_s}$ (smooth curves). In (a), the best fit parameters were $y = 482.e^{-t/0.72\,ms} + 271.e^{-t/5.92\,ms}$. In (b) the fit parameters were $y = 2600.e^{-t/0.44\,ms} + 339.e^{-t/1.38\,ms}$. In both distributions, events shorter than 0.5 ms were excluded from the analysis.

Table 1 The influence of 2 mM sodium benzylpenicillin (PCN) on the time constants obtained by fitting double exponential curves to cumulative frequency distributions for open times of γ-aminobutyric acid (GABA)-activated channels

Membrane patch	*Drug*	τ_s(ms)	τ_f(ms)	τ_m(ms)
1	GABA	4.88	0.47	1.61
	GABA + PCN	1.02	0.45	0.59
2	GABA	3.28	0.37	0.60
	GABA + PCN	0.84	0.42	0.52
3	GABA	4.58	0.46	1.41
	GABA + PCN	1.17	0.35	0.50
4	GABA	2.81	0.43	0.76
	GABA + PCN	1.20	0.44	0.49
5	GABA	5.92	0.72	2.59
	GABA + PCN	1.38	0.44	0.54

Data were obtained from 5 outside-out patches from 5 different cultured neurones. Patch potential, −80 mV, temperature 22–24°C. See text for definitions of τ_s, τ_f and τ_m.

Discussion

The present experiments show that PCN antagonizes the chloride-dependent response of cultured SC neurones to GABA, as measured by the whole-cell, current clamp method. This result confirms earlier findings obtained in the same preparation with conventional intracellular recording techniques (MacDonald & Barker, 1977). In addition, the present data indicate that concentrations of PCN in excess of 0.2 mM are required to produce significant antagonism of the GABA response in cultured SC cells. Similar PCN concentrations (0.1 mM and above) are needed to antagonize GABA-evoked responses recorded from rat olfactory bulb and cuneate nucleus cells *in vitro* (Pickles & Simmonds, 1980).

PCN could reduce GABA responses by blocking chloride channels previously opened by GABA. A mechanism of this kind may account for the depressant effects of local anaesthetics at the vertebrate endplate (Neher & Steinbach, 1978; Neher, 1983). If PCN does indeed act as a channel blocker, then the drug is expected to decrease the mean open time of the GABA-sensitive channel. This is in fact seen in the present data. A channel block model of PCN action is also consistent with the noncompetitive antagonism indicated by analysis of GABA dose-response curves (Pickles & Simmonds, 1980).

The benzylpenicillin moiety exists predominantly as an anion at physiological pH (pK = 2.76). In the present experiments, the negative field established across the patch would be expected to repel a blocking anion entering the channel from the external membrane face. However, it should be noted that a blocking anion would experience little of this field if its receptor site were located near the outside face of the membrane, for example in a channel atrium (Neher & Steinbach, 1978). It seems likely that PCN will prove a useful probe to study the GABA-activated chloride channel, particularly in view of the many available analogues of the drug with well defined physicochemical properties.

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