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Dual mechanisms of $GABA_A$ response inhibition by β-lactam antibiotics in the pyramidal neurones of the rat cerebral cortex

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1 The effects of β-lactam antibiotics on the γ-aminobutyric acid (GABA)-induced Cl^- current were investigated in pyramidal neurones freshly dissociated from the rat frontal cortex by the use of a nystatin-perforated patch recording mode under voltage-clamp conditions.

2 The GABA-induced inward current increased in a concentration-dependent manner with an EC_{50} of 6.7×10^{-6} M at a holding potential of −40 mV. The GABA response was accompanied by an increase in the membrane conductance and reversed at near the Cl^- equilibrium potential.

3 All β-lactams (penicillin, imipenem, aztreonam and cefotiam) inhibited the 10^{-5} M GABA-induced response in a concentration-dependent manner with an IC_{50} and Hill coefficient of 1.3×10^{-3} M and 0.64 for penicillin, 9.6×10^{-4} M and 0.83 for imipenem, 2.5×10^{-3} M and 9.99 for aztreonam, and 2.9×10^{-4} M and 1.03 for cefotiam.

4 Imipenem inhibited the GABA-response competitively while penicillin inhibited the same response in a noncompetitive fashion.

5 The inhibitory action of imipenem showed no voltage-dependency, whereas the effect of penicillin was voltage-dependent.

6 It is thus proposed that some classes of β-lactams, including imipenem, may have a mechanism that is different from penicillin and competitively affects the $GABA_A$ receptor.

Keywords: Rat cortical pyramidal neurones; GABA-induced Cl^- current; β-lactam antibiotics

Introduction

The β-lactam antibiotics are compounds which have a tetracarvon-ring (β-lactam ring) as a common structure (Figure 1) and are frequently used in the treatment of various infectious diseases. These antibiotics have been known to induce serious adverse effects clinically on the central nervous system (CNS) such as confusion, twitching and seizures (Mandell & Sande, 1980). Among these compounds, the effect of penicillin G (PCG) on neurones has been well investigated (Prince, 1968; Hochner *et al.*, 1976; Dunn & Somjen, 1977; Krnjevic *et al.*, 1977). The application of PCG to the mammalian cerebral cortex evokes seizure activity (Krnjevic, 1983) and this effect seems to be caused by the suppression of inhibitory postsynaptic responses (Davidoff, 1972; Meyer & Prince, 1973). This response is mainly mediated by γ-aminobutyric acid (GABA) in various brain areas, which thus suggests that GABA is involved in the neuroexcitatory effects of PCG. Indeed, PCG has been shown to inhibit the GABA response in the mammalian brain (Avioli, 1984), and the mechanism by which PCG reduces the GABA-induced current is an open channel block of the Cl^- channel incorporated with the $GABA_A$ receptor (Pickles & Simmonds, 1980; Twyman *et al.*, 1992).

In the clinical field, some of the newly developed β-lactams (Imipenem(IPM) or cefotiam(CTM) etc.) can also cause symptoms similar to PCG (Calandra *et al.*, 1985; Tse *et al.*, 1987; Schliamser *et al.*, 1988). Recently, a radioligand binding study showed that imipenem and cephazoline, new β-lactams, inhibited the receptor binding of GABA (Shimada *et al.*, 1992), thus suggesting that these compounds can also reduce the GABA-mediated inhibitory response, but by a different mechanism from that observed with PCG. However, the electrophysiological and pharmacological characteristics of these new antibiotics on the $GABA_A$ receptor-Cl^- channel complex have not been fully elucidated. In the present study, we examined the effects of the newly developed β-lactam antibiotics (IPM, CTM and aztreonam(AZT)) on the $GABA_A$-receptor-mediated response in the pyramidal neurones acutely dissociated from the rat cortex.

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Methods

Preparation

Single pyramidal neurones of the cortex were acutely dissociated from 2-week-old Wistar rats, according to procedures reported elsewhere (Ito *et al.*, 1991). Briefly, the rats were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}, s.c.) and decapitated. The brain was removed and the area between 2.5 and 5 mm from the anterior tip of the brain was cut into coronal slices (400 μm) with a microslicer (DTK-1000, D.S.K., Kyoto, Japan). These slices were pre-incubated in a solution well-saturated with 5% CO_2:95% O_2 gas for 40-50 min at room temperature (22-25°C). Thereafter, the slices were treated first in an oxygenated standard solution with 0.015% pronase and collagenase for 20 min and then with 0.015% thermolysin for 20 min at 31°C. The frontal cortex was then micropunched out and triturated with fire-polished glass pipettes in a small plastic culture dish (Falcon). The dissociated neurones adhered to the bottom of the dish within 30 min. The neurones demonstrating the original morphological features of pyramidal neurones, including a pyramidal shape and a prominent apical dendritic process, were used.

Electrical measurements

The whole-cell membrane currents were recorded from the somata of the cortex neurones by the use of the nystatin per-

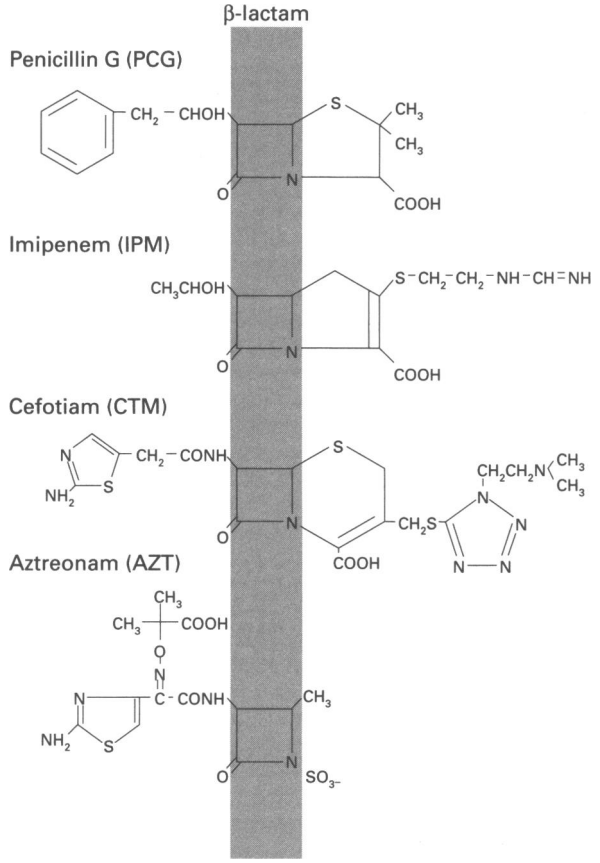

Figure 1 The structures of the 4 β-lactam antibiotics used in the present experiments. Penicillin G(PCG), imipenem(IPM) which is a carbapenem, cefotiam(CTM) which is a cephalosporin, and aztreonam(AZT) which is a monobactam. They all have a tetracarbon-ring which is called 'lactam' as a common structure (hatched part in figure).

forated patch recording mode (Horn & Marty, 1988) with some modifications (Akaike & Harata, 1994). Patch pipettes were prepared from glass capillaries (Narishige, 1.5 mm outer diameter) on a two-stage puller (Narishige, PB-7). The resistance between the patch-pipette filled with the internal solution and the reference electrode in the external solution was 4–8 MΩ. After the formation of a stable perforated patch, the series resistance ranged from 10 to 20 MΩ. The currents and voltage were measured with a patch-clamp amplifier (List Electronic, EPC-7) and were monitored simultaneously on an oscilloscope (Iwatsu, MS-5100A) and a pen-recorder (San-ei, RECTI-HORIT-8K). For the voltage-ramp experiment, a function generator (FG-121B, NF electronic instruments) was used. For an off-line analysis, the currents were stored on a video cassette recorder after the signal was digitized via a digital audio processor (Nihon Kohden, PCM-501ES). All experiments were performed at room temperature (22–25°C).

Solutions and their application

The standard external solution contained (in mM) NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. The pH was adjusted to 7.4 with tris (hydroxy-methyl) aminomethane base (Tris base). The incubation solution contained (in mM): NaCl 124, KCl 5, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, and glucose 10, aerated with 95% O_2: 5% CO_2 gas to a final pH of 7.4. The internal (patch-pipette) solution contained (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris base. A fresh stock solution of nystatin 10 mg ml^{-1} in methanol kept at −20°C was dissolved in the perforated patch-pipette solution at a final concentration of 200 μg ml^{-1} just before use. The final pipette solution was made fresh every 2 or 3 h. To examine the current-voltage (*I-V*) relationship, KCl in the internal solution was replaced by an equimolar CsCl.

Drugs were applied with a rapid application system termed the 'Y-tube' method, as described elsewhere (Murase *et al.*, 1989; 1990). This system completely exchanges the solution surrounding a neurone within 20 ms.

Drugs

The drugs used in the present study consisted of nystatin, penicillin G(PCG) and imipenem (IPM) [Banyu], cefotiam (CTM) [Takeda] and azactam (AZT) [Ezai]. All drugs were dissolved either in the pipette or the standard external solution just before use.

Statistical analysis

The data are presented as the mean standard error of the mean(s.e.) in the text, and the s.e. is indicated by a vertical bar in the figures.

The continuous theoretical curves for the concentration-response relationships were constructed according to a modified Michaelis-Menten equation (1) using a least-squares fitting routine:

$$I = I_{max} \frac{C^{n_H}}{C^{n_H} + EC_{50}^{n_H}} \quad (1)$$

where I is the drug-induced current amplitude and C is the corresponding drug concentration. EC_{50} and n_H denote the half-maximum concentration and Hill slope, respectively. The equation for the concentration-inhibition curve is the mirror image of the Michaelis-Menten equation:

$$I = 1 - \frac{C^{n_H}}{C^{n_H} + IC_{50}^{n_H}} \quad (2)$$

where I is the current amplitude normalized by that of the control and IC_{50} denotes the half-inhibition concentration of antagonists.

Results

GABA-induced responses

The dissociated cortical neurones were superfused with the standard external solutions at a holding potential (V_H) of −40 mV. The application of GABA evoked an inward Cl^- current (I_{GABA}) in a concentration-dependent manner (Figure 2a(i)). This current response was enhanced by benzodiazepine receptor agonists and was blocked by 10^{-4} M bicuculline (data not shown), which thus suggested that I_{GABA} was mediated by the $GABA_A$ receptor. At higher concentrations, the I_{GABA} developed desensitization. The relationship between the peak current amplitude and the GABA concentration is shown in Figure 2a(ii). In the figure, all responses are normalized to the peak current amplitude induced by 10^{-5} M GABA. The EC_{50} and Hill coefficient in the GABA concentration-response curve were 6.7×10^{-6} M and 0.93, respectively.

The current-voltage (*I-V*) relationships for I_{GABA} at various GABA concentrations (3×10^{-6}, 10^{-5} and 10^{-4} M) were investigated by the ramp-clamp method. The internal and external solutions contained 150 and 161 mM Cl^-, respectively. The voltage-dependent Ca^{2+} and Na^+ channels were suppressed by adding 10 μM $LaCl_3$ and 0.1 μM tetrodotoxin (TTX) to the external solution, respectively. Both $LaCl_3$ and TTX at the used concentrations had no effect on I_{GABA}. Under these conditions, a voltage ramp of 2 s duration from −60 to 40 mV was applied both before and immediately after the GABA application (Figure 2b, inset). Figure 2b shows the subtraction of the control *I-V* plot from that in the current

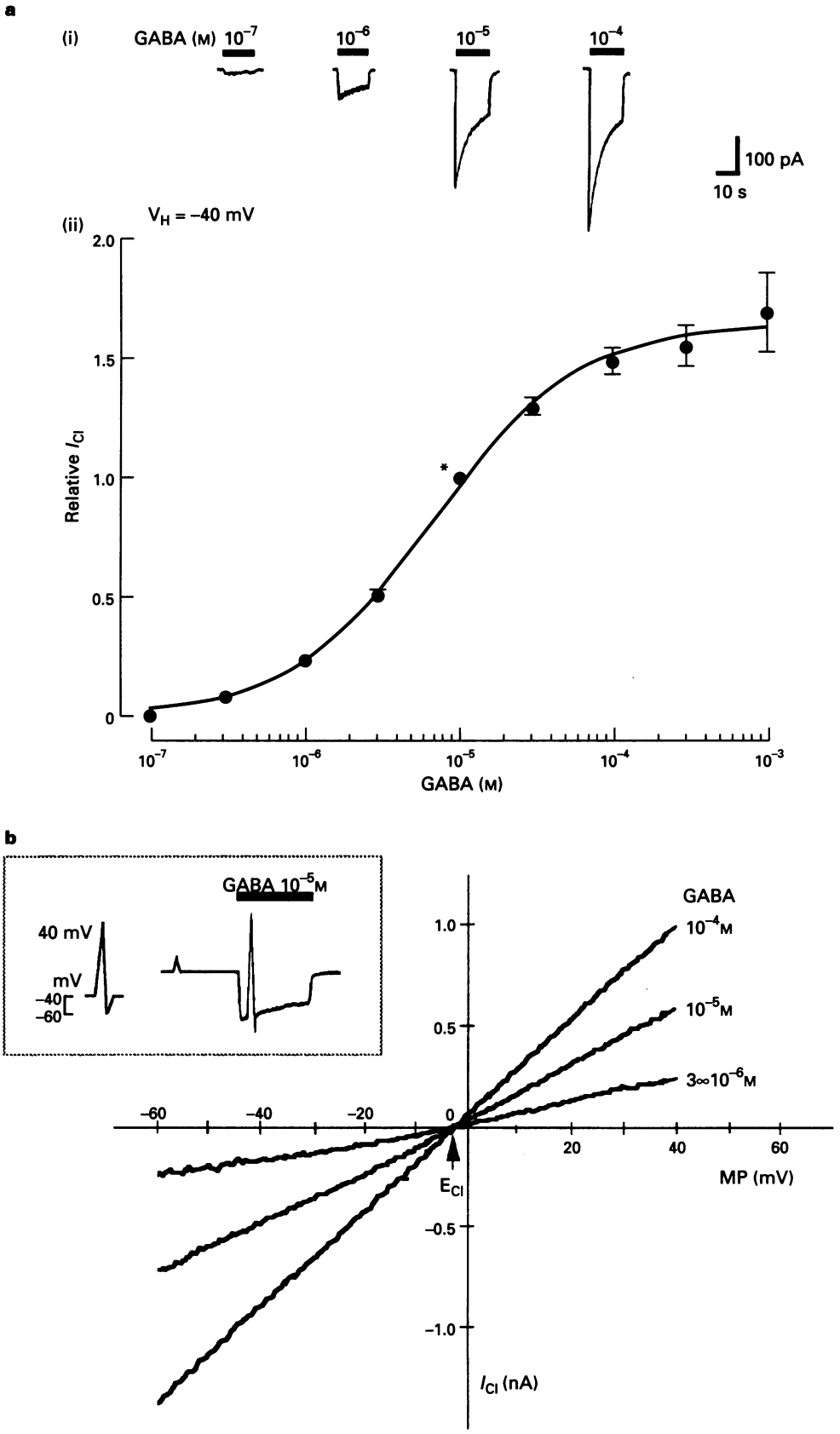

Figure 2 (a) GABA-induced Cl^- currents in the cortical neurones acutely dissociated from 2-week-old rats. The V_H was −40 mV. (i) Original chart paper traces of the GABA responses. Each horizontal bar indicates the period of a continuous GABA application. (ii) The concentration-response curve of GABA. Each point shows the average of 10 neurones. All responses induced by GABA at various concentrations were normalized to the current evoked by 10^{-5} M GABA alone (*). (b) *I-V* relationships for 3×10^{-6}, 10^{-5} and 10^{-6} M GABA-induced responses. V_H was −40 mV, and ramp-clamp commands between −60 and 40 mV with a duration of 3 s were added during application of GABA. The protocol of ramp command is shown in the inset. E_{Cl} is the Cl^- equilibrium potential.

response, which thus demonstrated the *I-V* relationships for I_{GABA}. The reversal potentials for I_{GABA} obtained by different GABA concentrations were -6.0 ± 3.0 mV ($n=4$, respectively), which was close to the Cl^- equilibrium potential (E_{Cl}) of -4.1 mV calculated from the Nernst equation, which indicated that I_{GABA} was carried by Cl^-.

Effect of β-lactam antibiotics on I_{GABA}

The effects of β-lactams (PCG, IPM, CTM and AZT) on I_{GABA} evoked by 10^{-5} M GABA at a V_H of -40 mV were investigated. As shown in Figure 3a, all β-lactams inhibited I_{GABA}. The concentrations of each β-lactam in Figure 3a were 10^{-3} M for PCG, 10^{-3} M for IPM, 3×10^{-3} M for AZT and 10^{-3} M for CTM. A small increase was observed in the currrent (hump) in the inhibitory responses by PCG and AZT when they were washed out (shown by arrows). In contrast, no hump was shown in the recording from the inhibition by IPM and CTM. AZT itself induced inward current. Figure 3b summarizes the concentration-inhibition curves of PCG(●), IPM(○), CTM(□) and AZT(■) on I_{GABA} elicited by 10^{-5} M GABA at a V_H of -40 mV. All

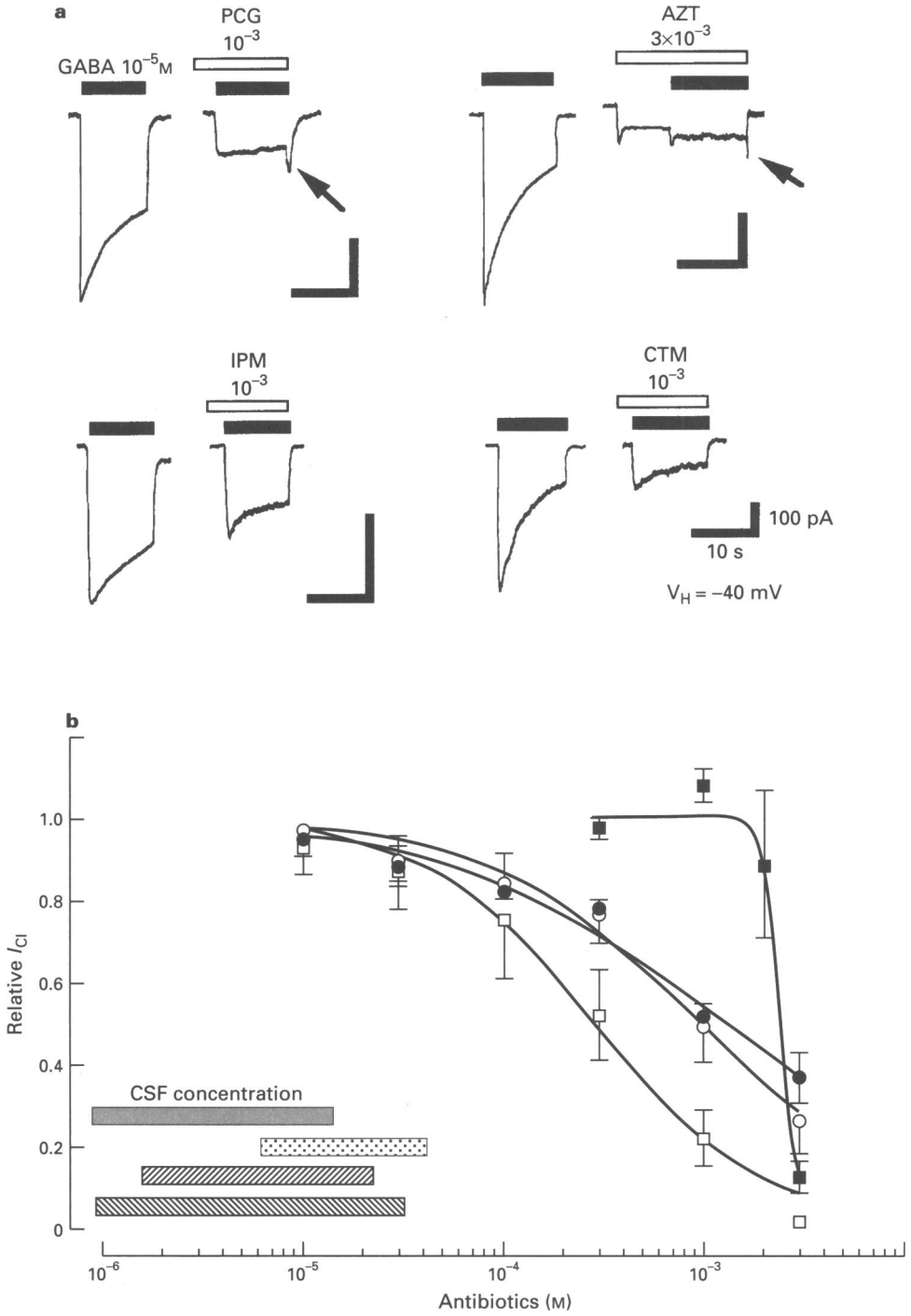

Figure 3 The effects of PCG, IPM, CTM and AZT on 10^{-5} M GABA-induced current. The V_H is -40 mV. (a) The original chart paper traces of the GABA-induced response in the absence and presence of 4 β-lactams. Each horizontal solid bar indicates the period of a continuous GABA application while the open bar indicates the period of each continuous β-lactam application. The hump was observed in the inhibitory response to PCG and AZT when GABA and either the PCG or AZT mixture were washed out (arrows). (b) The concentration-dependent inhibition curves of PCG(●), IPM(○), CTM(□) and AZT(■) on I_{GABA}. Each point is the average of 4–5 neurones. Each horizontal bar shows the CSF concentration range of 4 drugs used in clinical infectious cases (PCG ■, IPM ▦, CTM ▨, AZT ▧).

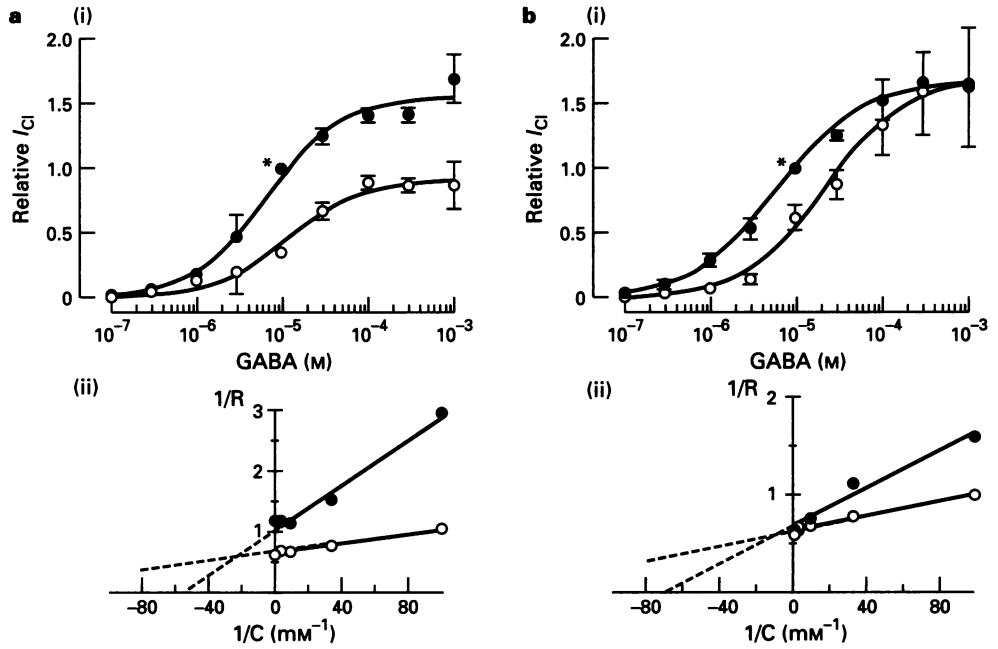

Figure 4 The inhibitory effect of 10^{-3} M PCG (a(i)) and 10^{-3} M IPM (b(i)) on the concentration-response curve of GABA. Each response was normalized to the peak current induced by 10^{-5} M GABA(*). The continuous lines of the sigmoidal curve were drawn by the Michaelis-Menten equation. (a(ii)), (b(ii)), Lineweaver-Burk plots. Each point represents the mean value of 4–5 neurones. (●) control; (○) in the presence of either 10^{-3} M PCG (a) or 10^{-3} M IPM (b).

drugs reversibly blocked the I_{GABA} in a concentration-dependent manner. The values of the half-inhibition doses (IC_{50}) and Hill coefficient were 1.3×10^{-3} M and 0.64 for PCG, 9.6×10^{-4} M and 0.83 for IPM, 2.5×10^{-3} M and 9.99 for AZT, 2.9×10^{-4} M and 1.03 for CTM, respectively. The solid bars show the reported normal CSF concentrations of each β-lactam used in clinical cases (Ory *et al.*, 1945; Bailey, 1961; Fujii, 1985; Schliamser *et al.*, 1988). In clinical use the IPM is associated with cilastatin, an inhibitor of dehydropeptidase, which is found in the kidney. In this study, cilastatin did not affect I_{GABA} (data not shown).

Inhibitory effects of PCG and IPM on I_{GABA}

Figure 4a and b shows the concentration-response curves of the GABA response with 10^{-3} M PCG or 10^{-3} M IPM at a V_H of −40 mV. All the GABA responses are normalized to the peak current amplitude induced by 10^{-5} M GABA alone. PCG suppressed the maximum response and the EC_{50} slightly shifted to a higher concentration (from 6.67×10^{-6} to 1.11×10^{-5} M). The Lineweaver-Burk plot (Figure 4a(ii)) shows that the inhibition by PCG has both elements of noncompetitive and competitive suppression. In contrast, IPM caused a parallel shift in the concentration-response curve toward the right without affecting the maximum response. The EC_{50} changed from 6.71×10^{-6} to 2.44×10^{-5} M. The Lineweaver-Burk plot (Figure 4b(ii)) thus indicates that IPM inhibits the GABA responses competitively.

Figure 5 shows the current-voltage (*I-V*) relationship to ramp voltage between −60 and 60 mV from a V_H of −40 mV, control (GABA 10^{-5} M) and during the application of 10^{-5} M GABA plus 10^{-3} M PCG (Figure 5a) or 10^{-3} M IPM (Figure 5b). PCG blocked the GABA response in a voltage-dependent manner, the depression being particularly pronounced at positive potentials. IPM suppressed the GABA response in a voltage-independent manner. Figure 5a(ii) and b(ii) show the relative I_{Cl} in the presence of 10^{-3} M PCG and 10^{-3} M IPM, respectively. All responses are normalized to the current amplitude evoked at −40 mV.

Discussion

In the present study, we investigated the effects of newly developed β-lactam antibiotics (β-lactams) on the $GABA_A$-induced current in neurones of the rat frontal cortex. In the cortex, a high density of the $GABA_A$ receptor is present and the pyramidal neurones receive a high density of GABAergic fibres. These neurones are considered to be highly involved in the adverse effect of β-lactams.

The β-lactams could be divided into two groups based on the shape of the current when the drugs were removed (Figure 3a). PCG and AZT elicited a transient inward hump current on washing out the GABA and drug mixture (shown by arrows). This phenomenon has also been observed in other preparations in the case of PCG and has been considered to result from the removal of the channel blockade (Adams, 1975). In contrast, IPM and CTM did not induce any hump, which suggested that this latter group might inhibit I_{GABA} by a mechanism which is different from that of the former group.

The inhibition of I_{GABA} by PCG has been reported to be competitive at low concentrations and non-competitive at higher concentrations (Hochner *et al.*, 1976; Yakushiji *et al.*, 1987). In the current study, the effects of 10^{-3} M PCG were non-competitive. The non-competitive blockade is considered to result from the Cl^- channel blockade. The voltage-dependence and transient hump observed in the PCG action are also consistent with this mechanism. In addition, PCG slightly shifted the EC_{50} to the right. Thus, PCG may act not only on the Cl^- channel but also on the $GABA_A$ receptor. Binding studies show that PCG inhibits the receptor binding of GABA. In contrast to PCG, IPM suppressed the I_{GABA} in a competitive manner. The *I-V* relationship for the effects of IPM seemed to have no remarkable voltage-dependency. At more negative potentials than −50 mV, the inhibition of I_{GABA} by IPM was apparently strong. This phenomenon was not due to the voltage-dependency of IPM, but might correspond to the *I-V* property at low GABA concentrations, as shown in Figure 2b, which may result from the decreased open probability of the Cl^- channel at negative potentials (Kaneda *et al.*, 1989; Akaike

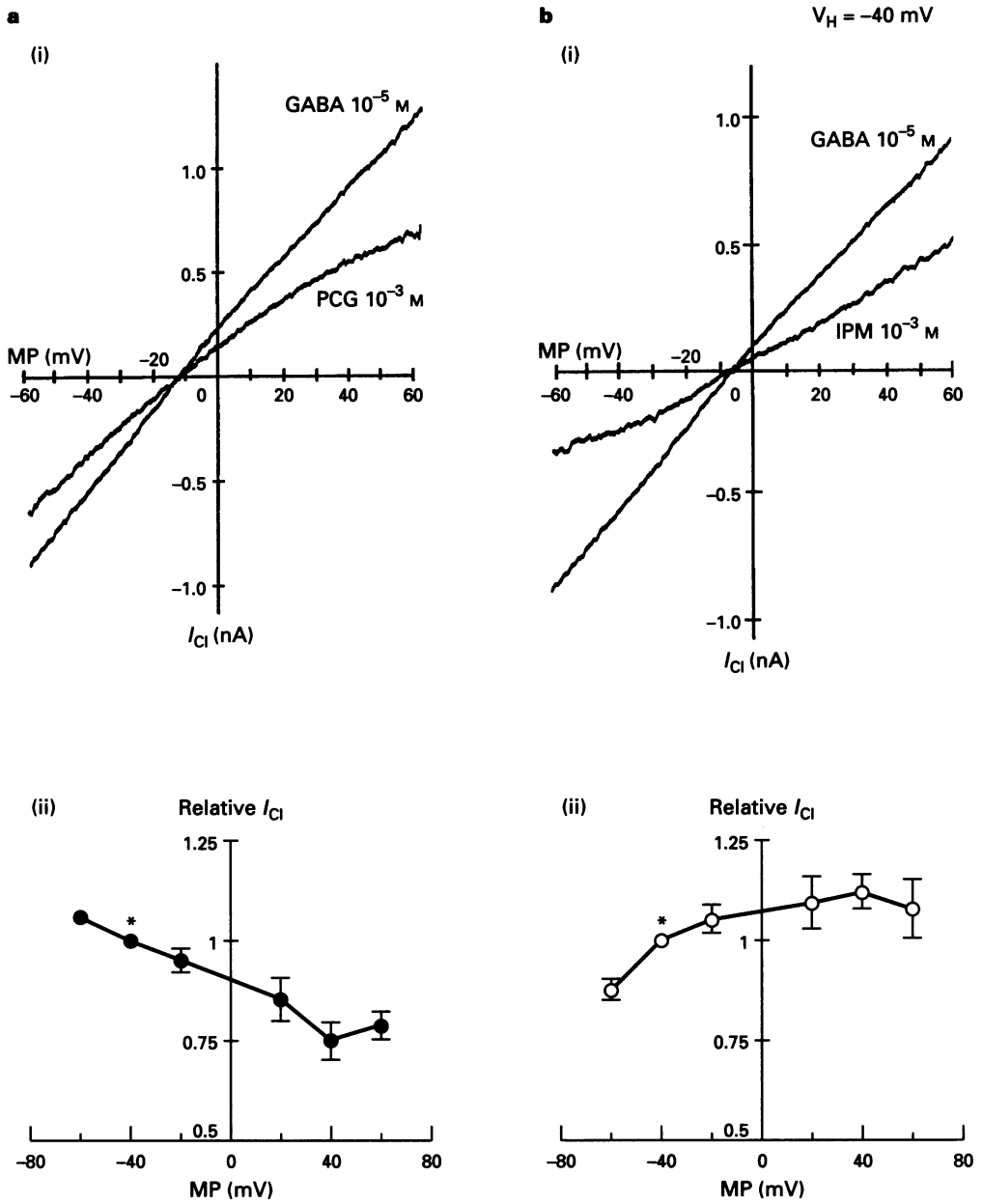

Figure 5 The inhibitory effect of 10^{-3} M PCG (a) and 10^{-3} M IPM (b) on the ramp *I-V* curve obtained by ramp-commands between −60 and 60 mV in 3 s in the presence of 10^{-5} M GABA only (control) or 10^{-5} M GABA plus either PCG or IPM. V_H is −40 mV. The PCG-mediated inhibition of the GABA response was voltage-dependent whereas the IPM-mediated inhibition was voltage-independent. (a(ii)), (b(ii)): the relative I_{Cl} in the presence of 10^{-3} M PCG(●) and 10^{-3} M IPM(○), respectively. Each point shows the average of 3–4 neurones. All responses were normalized to the current amplitude evoked at −40 mV(*).

et al., 1990). These facts suggested that IPM interacted either with the $GABA_A$ receptor or with an allosteric site linked to the receptor and did not act on the Cl^- channel.

The structural-convulsive activity relationship is of much interest in the clinical aspect. So far, several authors have reported this relationship for β-lactam antibiotics. As for penicillins, it has been found that the ability to produce seizures was abolished after incubation with penicillinase, an enzyme which splits the β-lactam ring of penicillins (Gerald *et al.*, 1973; Hartesvelt *et al.*, 1975), which thus suggests that the β-lactam ring is an indispensible structure for epileptogenic activity of penicillin. In addition, the modification of the side chains at the 6 position of β-lactam ring of benzylpenicillin was associated with epileptic potency (Gutnic *et al.*, 1976). Regarding cephalosporins, the presence of heterocyclic rings at position 3 and 7 of 7-aminocephalosporanic acid has been supposed to induce the epileptogenic activity of compounds (Kamei *et al.*, 1983; Sarro *et al.*, 1989). However, these authors discussed all drugs together without considering their mechanisms of action, such as interaction with a $GABA_A$ receptor site which we showed in this study, or channel blockade. If the convulsion induced by PCG results predominantly from the blockade of the Cl^- channel, then the structural features as mentioned above may be related to the channel blocking effect.

Recently, a molecular modelling study of some $GABA_A$ antagonists revealed that they all share a cationic and an anionic site of interaction, distant by about 5Å, that delimits the specific interaction core with the $GABA_A$ receptor. In addition, the nature and orientation of additional binding sites determine the selectivity and affinity for the $GABA_A$ receptor. The location in the elongation of the cationic site confers a higher selectivity for the $GABA_A$ receptor (Rognan *et al.*, 1992). Both IPM and CTM have carboxyl as a 'cationic site' (position 2 of carbapenem and position 4 of 7-aminocephalosporanic acid nucleus, respectively). Moreover, they also have formimidoylamine and dimethylaminoethyl

(position 2 of tetrazol) as an 'anionic site', respectively. Their structures may thus be candidates to interact with the $GABA_A$ receptor site. In contrast, PCG and AZT do not have such structures. Further investigations are called for to clarify this supposition.

In short, β-lactam antibiotics suppress the I_{GABA} not only by a channel-blockade but also by interaction with the $GABA_A$ receptor site. Thus even if drugs belong to the same group, 'β-lactams', and cause similar adverse effects, they have different structures and do not necessarily demonstrate the same mechanism of action. Therefore, when new drugs are designed it is important to consider carefully their structures and mechanisms.

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