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γ-AMINOBUTYRIC-ACID- AND PENTOBARBITONE-GATED CHLORIDE CURRENTS IN INTERNALLY PERFUSED FROG SENSORY NEURONES

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SUMMARY

1. γ-Aminobutyric-acid- (GABA) and pentobarbitone-induced Cl^- currents (I_{Cl}) were studied in isolated frog sensory neurones after suppression of Na^+, K^+ and Ca^{2+} currents using a suction-pipette technique combining internal perfusion with voltage clamp.

2. All GABA-sensitive neurones responded to pentobarbitone. Both GABA- and pentobarbitone-induced I_{Cl} reversed at the Cl^- equilibrium potential (E_{Cl}).

3. The dose–response curve for maxima of GABA-induced I_{Cl} was sigmoidal with a mean concentration producing a half-maximum response, K_a of 2×10^{-5} M at a Hill coefficient of 1·8. In the presence of pentobarbitone, the GABA dose–response curve shifted to the left without affecting the saturating maximum current.

4. At high concentrations, both GABA and pentobarbitone could also potentiate the pentobarbitone- and GABA-induced I_{Cl} respectively, while pre-treatment with one of the two markedly attenuated currents induced by the other, indicating a 'cross-desensitization'.

5. In the presence of pentobarbitone, the augmented response was voltage dependent and this augmentation was much greater in the inward-current direction than outward.

6. In producing I_{Cl}, pentobarbitone and its stereoisomers were potent in the order of (−) isomer > (±) racemic mixture > (+) isomer. A stereospecific facilitatory action of pentobarbitone on GABA responses was also found in the same order.

7. Responses to GABA, homotaurine, taurine, β-alanine, 5-aminovaleric acid, (+)- and (−)-γ-amino-β-hydroxybutyric acid and muscimol were equally enhanced by pentobarbitone, though its action on glycine-induced I_{Cl} was less effective.

8. Picrotoxin inhibited the GABA- and pentobarbitone-induced I_{Cl} from either side of membrane, while internal application of GABA and pentobarbitone did not exert any effect.

9. It was concluded that pentobarbitone binds to the 'barbiturate receptors' located close to the GABA receptor–Cl^- channel complex, and directly affects the GABA–GABA receptor interactions rather than the ionic channels.

INTRODUCTION

γ-Aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the C.N.S. which depresses the electrical excitation in individual neurones by increasing Cl^- conductance and by modifying the transmitter secretion at specific nerve terminals, resulting in presynaptic inhibition (Schmidt, 1963). Pentobarbitone, one of the general anaesthetics, enhances presynaptic inhibition in the spinal cord of the frog and cat (Eccles, Schmidt & Willis, 1963; Schmidt, 1963). According to Willow & Johnston (1980), pentobarbitone potentiated GABA binding by increasing the apparent affinity of GABA receptor sites to GABA, though some questions remain as to whether GABA binding sites used in the binding assays are really specifically related to Cl^- channels. Binding of [^{3}H]muscimol to GABA receptor sites in rat cerebral cortex was also enhanced by pentobarbitone (Supavilai, Mannonen & Karobath, 1982). On the other hand, electrophysiological studies directly showed that in the sensory neurones of frog (Nicoll, 1975), rat (Evans, 1979; Connors, 1981) and cat (Higashi & Nishi, 1982), and also in the hippocampal pyramidal cells of the mouse (Alger & Nicoll, 1982), the GABA-induced responses were enhanced and prolonged in the presence of pentobarbitone. Moreover, pentobarbitone itself can induce GABA-mimetic responses (Nicoll, 1975; Barker & Ransom, 1978; Nicoll & Wojtowicz, 1981; Connors, 1981). These responses were associated not only with an increase in Cl^- conductance but also with a decrease in K^+ conductance (Higashi & Nishi, 1982).

Uptake processes of exogenous GABA into cellular elements are known to exist in vertebrate ganglia (Iversen & Neal, 1968; Iversen & Johnston, 1971; Krogsgaard-Larsen & Johnston, 1975; Aickin & Deisz, 1981) and influence the access of GABA to GABA receptors. GABA uptake in neurones was depressed in the presence of the GABA uptake blocker, nipecotic acid, or in low-Na^+ solution (Brown & Galvan, 1977). In the post-synaptic membrane of rat brain, Na^+ has a regulatory action on the binding of GABA to the receptor (Kurioka, Kimura & Matsuda, 1981). These results suggest some participation of the GABA binding and uptake process and/or interactions between other ionic conductances and the pentobarbitone-potentiated GABA responses. These possibilities cannot be entirely excluded, as previous experiments were performed with an unknown intracellular ionic environment and in the presence of external Na^+, K^+ and Ca^{2+}. Such difficulties certainly complicate interpretation of these results.

The primary afferent neurone is considered as a model for GABA-induced inhibition of transmitter release from sensory nerve terminals (Feltz & Rasminsky, 1974; Gallagher, Higashi & Nishi, 1978; Barker & Mathers, 1981). Exogenous GABA appears to act on the soma membrane in a similar way to that generating depolarization in the intraspinal sensory nerve terminals (Levy, 1977). Therefore, the present experiments were designed to elucidate the ionic basis of GABA and pentobarbitone responses in the frog sensory neurones by using a modified suction-pipette technique, which permits internal perfusion so as to be able to separate ionic currents other than Cl^- currents (I_{Cl}) (Akaike, Nishi & Oyama, 1983; Hattori, Akaike, Oomura & Kuraoka, 1984; Ishizuka, Hattori & Akaike, 1984).

METHODS

Preparation. Dorsal root ganglia dissected from the decapitated bull-frog (*Rana catesbeiana*) were used throughout the experiments. The thick connective tissue surrounding the ganglion was carefully stripped off with microforceps and the capsules enveloping the ganglion masses were digested in 10 ml normal Ringer solution containing 0·3 % collagenase and 0·05 % trypsin at pH 7·4 for about 15 min at 37 °C. During the enzyme treatment, the preparation was shaken gently by bubbling 95 % O_2 + 5 % CO_2. The ganglia were then stored in normal Ringer solution at 4 °C. The neurones which remained viable for 2 days were used for the internal-perfusion experiments.

TABLE 1. Ionic composition of frog Ringer solution (mM)

Solution	NaCl	KCl	$CaCl_2$	TrisCl	CsCl	$MgCl_2$	TEACl	4-AP	K Asp	Cs Asp	Glucose	EGTA
NES	112	2	2	—	—	—	—	—	—	—	5	—
120 mM-Cl^- ES	—	—	—	83	2	5	25	3	—	—	5	—
NIS	—	30	—	—	—	—	—	—	100	—	—	0·5
15 mM-Cl^- IS	—	—	—	—	—	—	15	—	—	115	—	0·5
30 mM-Cl^- IS	—	—	—	—	5	—	25	—	—	100	—	0·5
60 mM-Cl^- IS	—	—	—	—	35	—	25	—	—	70	—	0·5
120 mM-Cl^- IS	—	—	—	—	95	—	25	—	—	10	—	0·5

NES, normal external solution; NIS, normal internal solution; K Asp, K aspartate; Cs Asp, Cs aspartate.

Solutions. The isolated nerve cell body was perfused externally and internally with standard solutions containing 120 and 30 mM-Cl^-, respectively. Cl^- currents were separated after Na^+, K^+ and Ca^{2+} currents were eliminated by substitution of Tris(hydroxymethyl)aminomethane ($Tris^+$) for Na^+, Mg^{2+} for Ca^{2+} and Cs^+ for K^+ in both external and internal solutions. The ionic compositions of test solutions are listed in Table 1. The pH of all solutions was adjusted to 7·4 with Tris or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES).

Internal perfusion by a suction-pipette technique and its adequacy. The enzymatically treated ganglion was transferred into a chamber filled with 0·15 ml solution, pinned to the Sylgard-covered floor of the chamber, and superfused with the external solution at a flow rate of 1·3 ml/min. Individual nerve cell bodies were clearly visible under binocular magnification of 80×. A suction-pipette technique (Akaike, Nishi & Oyama, 1981; Oyama, Nishi & Akaike, 1982*a*; Hattori *et al.* 1984; Ishizuka *et al.* 1984) was used for voltage clamp and internal perfusion. Pyrex glass having 3 mm outer diameter was pulled to a shank length of 2·5–3 mm. The tip of the pipette was cut at an outer diameter of about 40 μm and then fire-polished to give an inner diameter of about 10 μm. A part of the individual neurone (30–40 μm diameter) was aspirated by the suction pipette with negative pressure of about 30 cmHg. The aspirated membrane was electrically ruptured by applying 5–20 nA square-wave pulses of depolarizing current for 10–50 ms. Thereafter, the neurone was separated from the ganglion by retracting the suction electrode to break the axon. The isolated cell body was then internally perfused at a constant flow rate of 1 ml/min.

Adequacy of the internal perfusion with the present suction-pipette technique was evaluated from the following two experiments. (1) The reversal potential for GABA-induced Cl^- response (E_{GABA}) became equal to the Cl^- equilibrium potential (E_{Cl}) calculated from the Nernst equation based on external and internal ionic activities in test solutions of Cl^-. Here, the Cl^- activities were estimated by using F1012C1 Cl^- electrode connected to an ION85 Ion Analyzer (Radiometer A/S, Copenhagen). (2) The internal perfusion of F^-, which is known to block Ca^{2+} current (I_{Ca}) in snail neurones (Kostyuk, Krishtal & Pidoplichko, 1975; Akaike *et al.* 1983) as well as in unfertilized tunicate eggs (Takahashi & Yoshii, 1978), also completely depresses I_{Ca} in the frog sensory neurone, as shown by Ishizuka *et al.* (1984).

Electrical measurements. The membrane potential was measured through a Ag–AgCl wire in a Ringer–agar plug which was mounted on the suction-pipette holder. The reference electrode was also a Ag–AgCl wire connected to the bathing medium through a Ringer–agar bridge. The resistance

was 200–300 kΩ between the suction pipette filled with standard internal solution and the bath electrode in normal Ringer solution. Both electrodes were led to a voltage-clamp circuit, and the membrane potential was controlled by single-electrode voltage clamp switching at a frequency of 10 kHz and passing current for 36% of the cycle. Clamp currents were measured as the voltage drop across a 10 MΩ resistor in the feed-back loop of headstage amplifier. In this system, the suction electrode could carry time-averaged currents exceeding 100 nA at a switching frequency of 10 kHz without showing signs of polarization or other artifacts. A phase-compensation feed-back loop was used to stabilize the clamp circuit. The voltage clamp was tuned to give the fastest possible response as follows. The holding potential was set at the resting potential, the gain was gradually increased and the compensation circuit was adjusted. As the gain reached its maximum level, the circuit was delicately adjusted until the voltage trace was critically clamped. The series resistance (R_s) did not affect the performance. Both current and voltage were monitored on a storage oscilloscope (Tektronix, type 5113), and simultaneously recorded on an ink-writing recorder (Rikadenki, type R-22) and stored on a FM data recorder (Sony, type PFM-15). The current–voltage (I–V) relation was recorded at the peak of each drug-induced response by passing a depolarizing triangular voltage pulse (with slope of 250 mV/s), followed immediately by a mirror-image hyperpolarizing pulse so that the repolarizing arm of the depolarizing pulse was continuous with the hyperpolarizing pulse. The pulse was obtained by using a function generator (Kikusui, type 459AL). The resultant data were stored on the FM data recorder and also plotted directly on an X–Y recorder (National, type 6400A) to show the desired relation.

Drugs. Drugs employed in the present experiments were: GABA, (±)-pentobarbitone, tetraethylammonium and 4-aminopyridine (Tokyo Kasei), trypsin (Sanko) and collagenase type I (Sigma), taurine (Katayama), β-alanine and glycine (Ishizu), 5-aminovaleric acid (Aldrich) and muscimol (Sigma). Both (−)- and (+)-pentobarbitone were kindly supplied by the National Institute on Drug Abuse, U.S.A. Homotaurine, (−)- and (+)-γ-amino-β-hydroxybutyric acid (GABOB) were presented by Dr A. Mori. They were dissolved in the test solutions just before use. The pH of test solutions was adjusted to 7·4 by Tris and HEPES. All experiments were carried out at room temperature (18–22 °C).

RESULTS

Current-clamp experiments

GABA- and pentobarbitone-induced Cl^- responses. Frog sensory neurones were adequately perfused with Na^+-, K^+- and Ca^{2+}-free external and internal standard solutions containing 120 and 60 mM-Cl^- respectively, using a modified suction-pipette technique (Table 1). The membrane potential was held at −70 mV by direct current injection. Each bath application of GABA or racemic mixture of pentobarbitone isomers ((±)-pentobarbitone is shown as pentobarbitone in this text) at various concentrations was repeated at 5–10 min intervals. When GABA ranging between 10^{-7} and 10^{-3} M in concentration was superfused for a few minutes, the minimum GABA-induced depolarization appeared at 10^{-7} M. At 3×10^{-6} M and lower concentrations, neurones were slowly depolarized and the responses reached steady-state values with no detectable desensitization. The continuous perfusion of GABA at 10^{-5} M and higher concentrations produced a rapid depolarization which was immediately followed by desensitization, as shown in Fig. 1*A*. GABA-induced depolarization showed a sigmoidal increase with increasing concentration of GABA. Fig. 1*B* shows pentobarbitone-induced depolarizations with concentrations between 3×10^{-5} and 10^{-3} M. Pentobarbitone depolarized the neurones slowly compared with the GABA-activated responses.

The peak responses produced by GABA and pentobarbitone at high concentrations reached the Cl^- equilibrium potential (E_{Cl}) level and then declined gradually, indicating little desensitization during the peak period of drug-induced depolarizations.

GABA- and pentobarbitone-induced depolarizations were accompanied by a considerable decrease in the 'input resistance' before the membrane repolarized completely within 5–15 min of washing with drug-free solution.

Voltage-clamp experiments

GABA- and pentobarbitone-induced Cl^- currents. Fig. 2*A* shows GABA-induced Cl^- currents (I_{Cl}) in a sensory neurone perfused with Na^+-, K^+- and Ca^{2+}-free external and internal solutions containing 120 and 60 mM-Cl^-, respectively. The current–

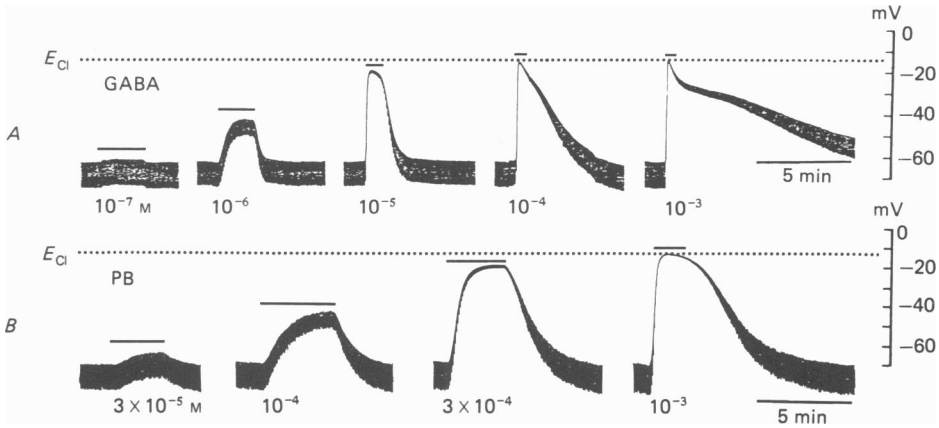

Fig. 1. *A*, GABA-induced Cl^- responses in Na^+-, K^+- and Ca^{2+}-free external and internal solutions containing 120 and 60 mM-Cl^- respectively, under current clamp. The neurone was briefly exposed to various concentrations of GABA for periods indicated by horizontal bar above each response. Downward brief deflexions in traces are electrotonic responses to 300 ms hyperpolarizing current pulses (0·5 nA) applied through the suction pipette every 2·5 s. The membrane potential was fixed at −70 mV by direct current injection. The desensitization was conspicuous at 10^{-5} M and higher GABA concentrations. *B*, Cl^- responses produced by the racemic mixture of pentobarbitone (PB). Dotted lines show E_{Cl} of −13·5 mV calculated from external and internal Cl^- activities using the Nernst equation. All recordings in *A* and *B* were obtained from the same neurone.

voltage (*I–V*) relations were recorded before adding the drugs and at the peak of 10^{-5} M-GABA- or 10^{-3} M-pentobarbitone-induced I_{Cl}, by passing a depolarizing triangular voltage step of 50 mV followed immediately by a mirror-image hyperpolarizing step. The *I–V* relations were plotted on a *X–Y* plotter to give the desired relation directly (Fig. 2*B*). When the reversal potentials of GABA- and pentobarbitone-induced responses (E_{GABA} and E_{PB}) were estimated from each intersect in the two *I–V* curves (with and without the drug), E_{GABA} was $-14{\cdot}5 \pm 0{\cdot}7$ mV (mean ± S.E. of mean, $n = 12$) and E_{PB} was $-14{\cdot}3 \pm 0{\cdot}6$ ($n = 6$), virtually the same as E_{GABA}. These E_{GABA} and E_{PB} values were quite close to the theoretical Cl^- equilibrium potential (E_{Cl}) of −13·5 mV calculated from the Nernst equation, knowing that the external solution contained 120 mM-Cl^- with an estimated activity coefficient of 0·73 and that the internal solution contained 60 mM-Cl^- with an estimated activity coefficient of 0·86. This indicates that both GABA- and pentobarbitone-induced currents are 'pure' I_{Cl} carried through Cl^- channels without

contamination by other ionic currents. Thus, the present series of quantitative studies about GABA and pentobarbitone were performed in Na^+-, K^+- and Ca^{2+}-free external and internal solutions using the suction-pipette technique.

Dose–response relations of GABA- and pentobarbitone-induced I_{Cl}. GABA-induced I_{Cl} increased as the dose increased from 3×10^{-7} to 3×10^{-4} M. At higher concentrations,

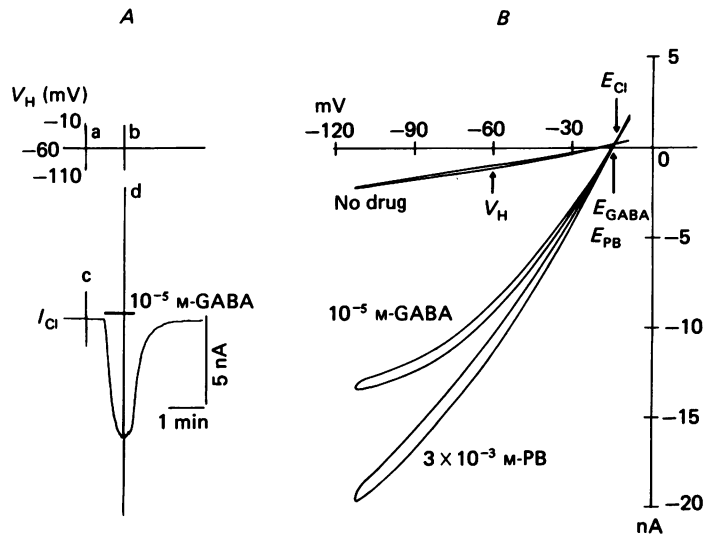

Fig. 2. *A*, measurement of *I–V* relations before GABA application and at the peak of GABA response by passing a depolarizing triangular voltage pulse followed by the mirror-image hyperpolarizing pulse. a and b show the changing holding potential as a result of the symmetrical triangular voltage pulse. c and d show I_{Cl} evoked by the triangular voltage pulses a and b, respectively. *B*, *I–V* relations. GABA- or pentobarbitone-induced I_{Cl} reverses direction near E_{Cl} of −13·5 mV. Holding potential (V_H) is −60 mV. E_{Cl}, E_{GABA} and E_{PB} show the Cl^-, GABA and pentobarbitone equilibrium potential, respectively.

beyond 6×10^{-6} M, I_{Cl} initially increased and subsequently decreased as a result of desensitization which conspicuously developed at concentrations higher than 10^{-5} M (Fig. 3). The maximum values (peak and steady-state values for higher and lower GABA concentrations, respectively) of the GABA-induced I_{Cl} were plotted as a function of GABA concentration (Fig. 4). The GABA dose–response relation accorded with the conventional expression:

$$I = I_{max} \frac{C^n}{C^n + K_a{}^n}. \quad (1)$$

Here, I is the observed GABA-induced I_{Cl}, I_{max} the maximum value of I_{Cl}, C the GABA concentration, K_a a constant, and n the Hill coefficient. In the Hill plot of the GABA responses shown in Fig. 7, the slope of a line determined by linear-regression analysis of all points from 6×10^{-6} M up to 10^{-4} M was 1·8. K_a, corresponding to the concentration of GABA producing a half-maximum response, was 2×10^{-5} M. Therefore, a continuous line in Fig. 4 was drawn according to eqn. (1) using $n = 1{\cdot}8$ and $K_a = 2 \times 10^{-5}$ M. The theoretical curve agrees well with the experimental data points measured as maximum I_{Cl} values unaffected by desensitization.

Pentobarbitone itself also induced I_{Cl} at 6×10^{-5} M and higher concentrations. When the drug concentration was increased to more than 6×10^{-4} M, desensitization appeared gradually (Fig. 3). The (−) isomer of pentobarbitone was more potent than the racemic mixture while the (+) isomer was less potent (Figs. 4 and 8*A*). The amplitude, time-to-peak and desensitization of I_{Cl} were all concentration dependent.

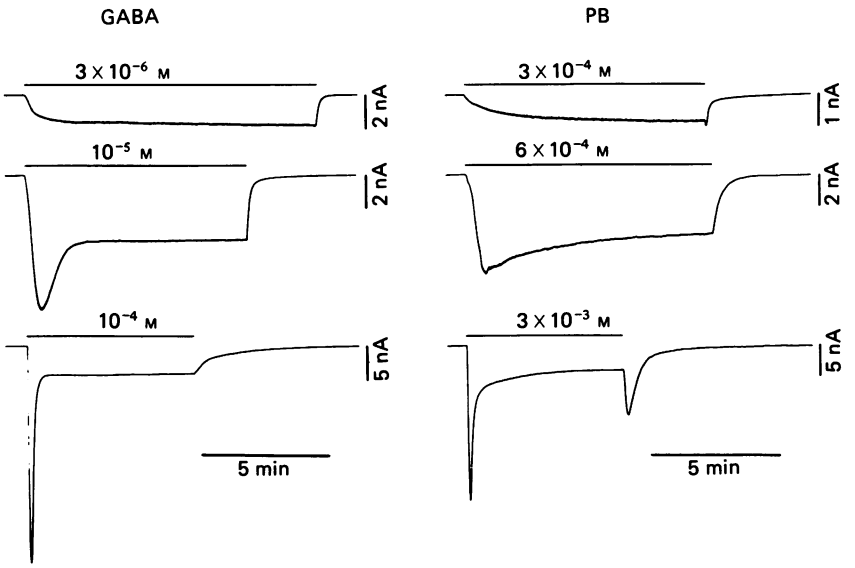

Fig. 3. GABA- or pentobarbitone-induced I_{Cl} under voltage clamp. All recordings were obtained from the same neurone perfused with 120 mM-external Cl^- and 60 mM-internal Cl^-. The holding potential was −60 mV. The neurone was superfused with GABA or pentobarbitone for a period indicated by horizontal bar above each recording. A marked desensitization occurred with GABA or pentobarbitone at high concentrations. Note the different current calibrations.

However, no attempt was made to determine individual maximum values of I_{Cl} in isomers and the mixture solutions since the current diminished immediately due to rapid desensitization at concentrations higher than 10^{-3} M (Fig. 4).

Augmentatory interaction between GABA- and pentobarbitone-induced I_{Cl}. Pentobarbitone at low concentrations always enhanced GABA-induced I_{Cl} in neurones perfused with Na^+-, K^+- and Ca^{2+}-free external and internal solutions (external Cl^- 120 mM, internal Cl^- 60 mM, holding potential (V_H) −60 mV), and similarly GABA at low concentrations also enhanced pentobarbitone-induced I_{Cl} (Fig. 5*A* and *B*). Such potentiation effects were further increased by pre-treatment with pentobarbitone or GABA at low concentrations for a few minutes before exposure to a mixed solution of GABA plus pentobarbitone (Fig. 6*A*). These facilitatory actions were completely reversible. The reversal potentials in each *I–V* relation measured before and after adding pentobarbitone, GABA and GABA plus pentobarbitone (E_{PB}, E_{GABA} and $E_{GABA+PB}$) were the same (Fig. 5*C*) and close to E_{Cl}, indicating that all responses were mediated by an increase in the Cl^- conductance only. Furthermore, even at high drug concentrations which led to rapid desensitization, the mixtures of GABA and

pentobarbitone could still produce a transient conductance increase (Fig. 6*B*). However, when a large increase in Cl^- conductance was produced by either GABA or pentobarbitone at high concentrations, the further increase by subsequent addition of GABA or pentobarbitone alone was greatly attenuated (Fig. 6*B*), suggesting that there is a strong 'cross-desensitization' between the two drugs at their receptor level. These results also show that the degree of mutual enhancement

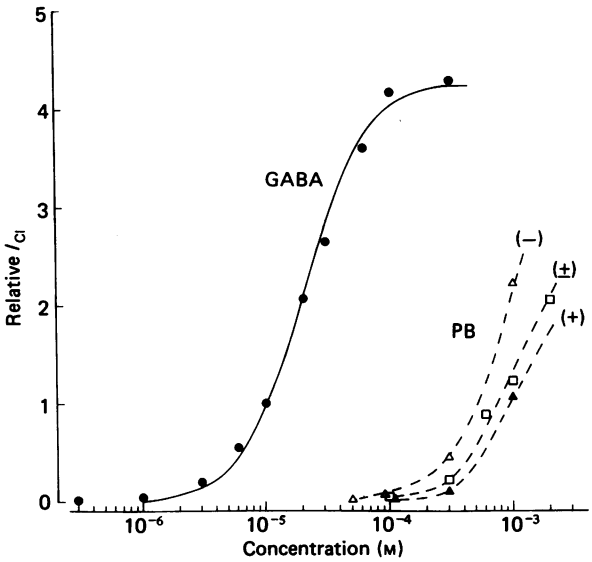

Fig. 4. Dose–response curves for GABA, pentobarbitone and its stereoisomers. Each point is the average of eight to fifteen neurones. The peak of I_{Cl} induced by each drug was normalized to the peak current produced by 10^{-5} M-GABA. External Cl^- 120 mM, internal Cl^- 60 mM, V_H −60 mV. A continuous theoretical curve for GABA responses was drawn according to the eqn. (1) using $n = 1{\cdot}8$ and $K_a = 2 \times 10^{-5}$ M (see Fig. 7*A*). Dotted lines for pentobarbitone and the isomers were fitted by eye.

between GABA and pentobarbitone actions depends not only on the concentration of each drug but also on the presence or absence of pre-treatment with one of the drugs. In our previous report, intracellular application of GABA could not evoke any I_{Cl} responses (Hattori *et al.* 1984). It is reasonable to also expect the site of enhancement of GABA responses by pentobarbitone to be external to the membrane, although the receptors are not necessarily the same for both drugs, and that internal perfusion of pentobarbitone should therefore have no effect upon GABA responses. Fig. 5*D* shows the currents recorded in a neurone externally perfused with 3×10^{-6} M-GABA or 3×10^{-6} M-GABA plus 3×10^{-4} M-pentobarbitone before and after a 20 min internal perfusion with 10^{-3} M-pentobarbitone. Internal application of pentobarbitone indeed had no effect on these currents. In contrast, internal perfusion with 10^{-5} M-picrotoxin caused a reversible reduction of these currents (Akaike, Hattori, Oomura & Carpenter, 1985).

The effects of adding 10^{-4} M-pentobarbitone (or 10^{-6} M-GABA) on the responses to GABA (or pentobarbitone) at various concentrations were studied quantitatively.

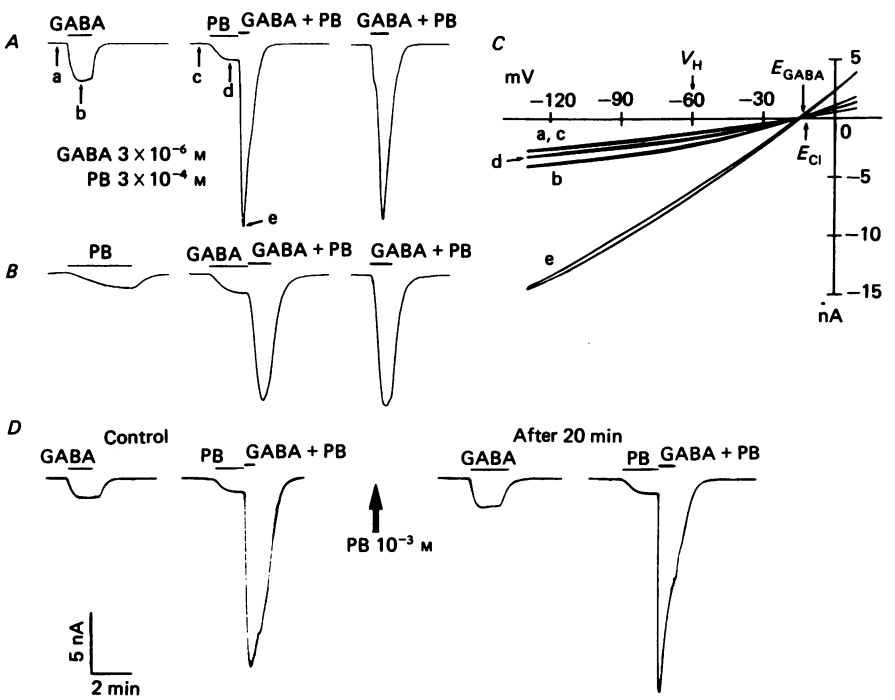

Fig. 5. *A*, facilitatory action of pentobarbitone upon GABA-induced I_{Cl}. External Cl^- 120 mM, internal Cl^- 60 mM, V_H −60 mV. Symmetrical triangular voltage pulses were applied at points a, b, c and d (see explanation of *C* in this Figure). *A* and *C* are recordings from the same neurone. *B*, enhancement of pentobarbitone-induced I_{Cl} by GABA pre-treatment. *C*, *I–V* relations measured in the absence of any drugs (a, c), and at the peak currents induced by 3×10^{-6} M-GABA alone (b), 3×10^{-4} M-pentobarbitone alone (d) and the mixture (e). *I–V* curves were obtained by passing a depolarizing triangular voltage pulse. All *I–V* curves before and after adding drugs intersect at the same reversal potential (−14 to −15 mV), which was very close to E_{Cl} (−13·5 mV) calculated from external and internal Cl^- activities. *D*, effect of internal application of pentobarbitone on 3×10^{-6} M-GABA- or 3×10^{-6} M-GABA plus 3×10^{-4} M-pentobarbitone-induced I_{Cl}. Control and 20 min after beginning internal perfusion of 10^{-3} M-pentobarbitone.

Test solutions of 10^{-4} M-pentobarbitone plus GABA at various concentrations (or 10^{-6} M-GABA plus pentobarbitone at various concentrations) were superfused after pre-treatment with 10^{-4} M-pentobarbitone (or 10^{-6} M-GABA) for 2 min. The results are summarized in Fig. 7*A*. The presence of 10^{-4} M-pentobarbitone greatly enhanced the GABA-induced I_{Cl} over the full range of agonist concentrations tested. In the presence of 10^{-4} M-pentobarbitone, the GABA dose–response curve shifted to the left without changing the saturating maximum current, with the half-maximum dose (K_a) changed from 2×10^{-5} to 3×10^{-6} M. The maximum enhancement of GABA-induced I_{Cl} by pentobarbitone was observed at 3×10^{-6} M-GABA, and the facilitatory response was about 8 times greater than the simple algebraic sum of the individual GABA and pentobarbitone responses. Similarly, 10^{-6} M-GABA enhanced the pentobarbitone-induced I_{Cl} at all concentrations of pentobarbitone and also shifted

the threshold dose to the left. The maximum potentiation occurred at 10^{-4} M-pentobarbitone and the facilitation ratio was about 6 (Fig. 7*B*).

Enhancement of the GABA-induced I_{Cl} by pentobarbitone isomers. It is well known that in cultured mouse spinal neurones the (+) isomer of pentobarbitone evokes predominantly excitatory responses while the (−) isomer elicits inhibitory responses

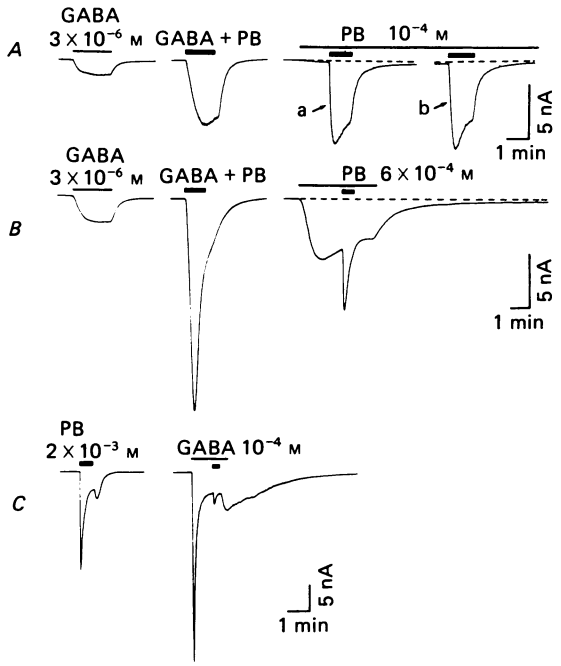

Fig. 6. Interactions between GABA- and pentobarbitone-induced I_{Cl} in neurones perfused with 120 mM-external Cl^- and 60 mM-internal Cl^- at V_H −60 mV. *A*, *B* and *C* were obtained from three different neurones. *A* and *B*, facilitatory action of pentobarbitone on GABA-induced I_{Cl}. The enhanced currents induced by GABA plus pentobarbitone increased with increasing pre-treatment time of pentobarbitone at a low concentration (*A*), but greatly attenuated at a high concentration (*B*). Time interval between records a and b is 10 min. *C*, the pre-treatment of GABA at a high concentration which caused a rapid desensitization in response to GABA plus pentobarbitone. Note a very small I_{Cl} induced by GABA plus pentobarbitone during the desensitization period of GABA-induced current.

(Barker, Huang, MacDonald & McBurney, 1980; Mathers & Barker, 1980). Therefore, we studied how GABA-induced Cl^- conductance is modified in the presence of the (+) or (−) isomer and the racemic mixture. Since the (−) isomer was consistently more potent than either the (+) isomer or the racemic mixture in the dose–response curves (Fig. 4), 2×10^{-4} M-(+)-, 10^{-4} M-(±)- and 5×10^{-5} M-(−)-pentobarbitone, concentrations which induced similar responses, were used. As seen in Fig. 8 the presence of purified pentobarbitone isomers ((+) and (−)) and the racemic mixture greatly enhanced the I_{Cl} induced by 3×10^{-6} M-GABA. The enhancing action was greater in the order of (−) > (±) > (+). There was a significant difference between the (+) and (−) stereoisomers at $P < 0.05$ (Fig. 8*C*).

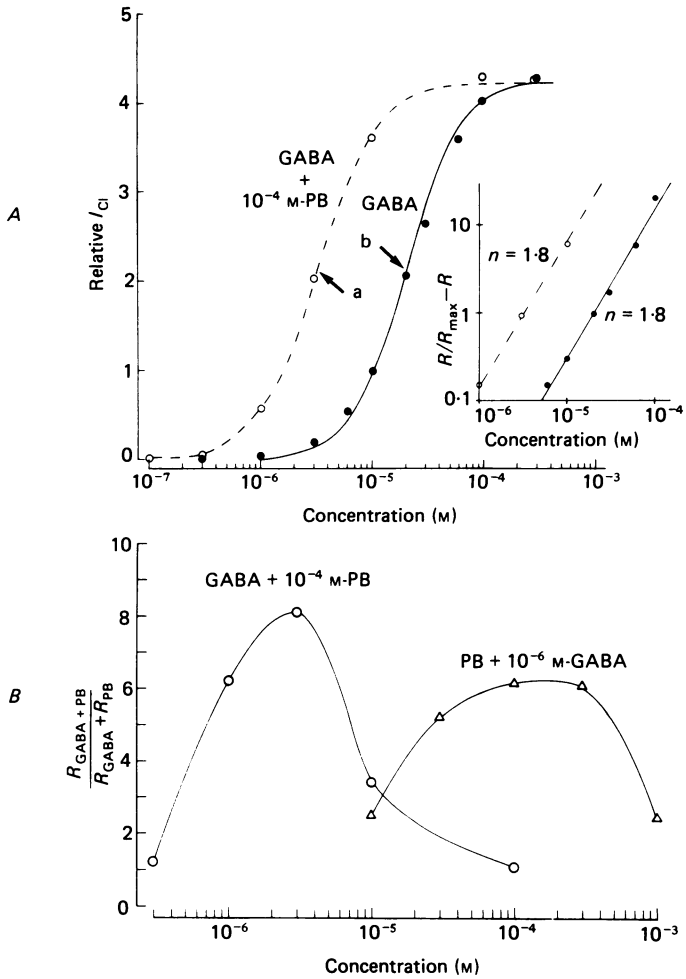

Fig. 7. *A*, dose–response curves of GABA-induced I_{Cl} in the absence (●) and presence (○) of pentobarbitone. The peak of each GABA-induced current is normalized to the peak current induced by 10^{-5} M-GABA alone. Theoretical curves were drawn using $n = 1{\cdot}8$ shown in the inset and $K_a = 3 \times 10^{-6}$ M for a and 2×10^{-5} M for b (see arrows). $R = I_{Cl}$ induced by various GABA concentrations, R_{max} = the maximal value of R. The dose–response curve of GABA alone is taken from Fig. 4. Each circle represents the average from eight to fifteen neurones. Note the shift of K_a to the left without affecting the maximum saturating current in the presence of pentobarbitone. *B*, facilitatory effect of 10^{-4} M-pentobarbitone or 10^{-6} M-GABA upon currents induced by GABA or pentobarbitone at various concentrations, respectively. Ordinate shows the enhancement ratio of GABA-, pentobarbitone- and GABA-plus-pentobarbitone-induced I_{Cl}, as R_{GABA}, R_{PB} and $R_{GABA+PB}$, respectively. Abscissa shows agonist concentration. Each point is the mean of at least eight measurements.

Pentobarbitone action on I_{Cl} produced by GABA-related compounds. In order to know whether the enhancing effect of pentobarbitone upon GABA-induced I_{Cl} is specific or not, the pentobarbitone actions on I_{Cl} produced by muscimol and other amino acids, such as homotaurine, taurine, β-alanine, (+)-GABOB, (−)-GABOB, glycine and 5-aminovaleric acid were examined. GABA-induced I_{Cl} was markedly enhanced

by 10^{-4} M-pentobarbitone. Similarly, the responses to homotaurine, taurine, β-alanine, (+)-GABOB, (−)-GABOB, 5-aminovaleric acid and muscimol were equally potentiated in the presence of 10^{-4} M-pentobarbitone, whereas the response to glycine was less enhanced (Figs. 9, 10). However, there was no facilitatory interaction among these amino acids themselves including GABA and muscimol.

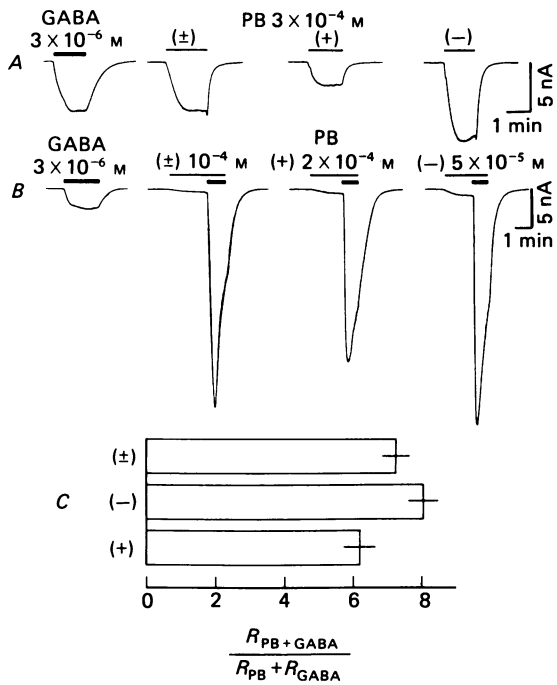

Fig. 8. *A*, direct actions of pentobarbitone and stereoisomers on the neurone perfused with external and internal solutions containing 120 and 60 mM-Cl^- respectively at V_H −60 mV. Recordings were obtained from the same neurone. *B*, potentiation of GABA-induced I_{Cl} by stereoisomers of pentobarbitone and the racemic mixture. 3×10^{-6} M-GABA was added after the pre-treatment of this neurone with pentobarbitone for 2 min. Note the marked potentiation of GABA-induced I_{Cl}. Data were obtained from the same neurone. *C*, facilitatory actions of pentobarbitone isomers and the racemic mixture on the response to GABA. Each column represents mean ± S.E. of mean of six neurones. Abscissa shows the facilitation ratio in which R_{PB}, R_{GABA} and $R_{PB+GABA}$ indicate the peak response induced by pentobarbitone ((−), (±) or (+)) alone, GABA alone and pentobarbitone plus GABA, respectively.

Voltage dependency of GABA-, pentobarbitone- and GABA-plus-pentobarbitone-induced I_{Cl}. The effects of 10^{-6} M-GABA, 10^{-4} M-pentobarbitone and their mixture on the neurones perfused with Na^+-, K^+- and Ca^{2+}-free external and internal solutions containing 120 and 60 mM-Cl^- respectively were examined at different holding potentials (V_H). The individual outward currents with each of these three solutions at V_H +40 mV were greater than the corresponding inward currents at V_H −75 mV in spite of the nearly symmetrical driving forces (from E_{Cl} −13·5 mV), although the potentiation ratio of GABA-induced I_{Cl} by pentobarbitone was much greater for the

inward-current direction (Fig. 11). Therefore, the facilitatory effect of 10^{-4} M-pentobarbitone on the response to 10^{-6} M-GABA was investigated more closely by varying V_H between -100 and $+50$ mV. In these experiments, membrane potentials were held at each V_H value for 15–20 min, during which time GABA, pentobarbitone and GABA plus pentobarbitone were superfused. Maximum currents evoked by each

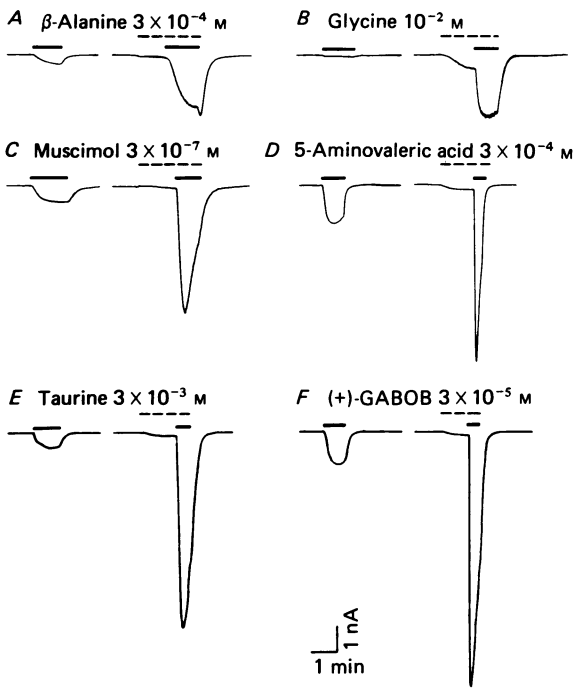

Fig. 9. Facilitatory actions of pentobarbitone on I_{Cl} produced by muscimol and various amino acids. All data were obtained from different neurones. These neurones were perfused with external and internal solutions with 120 and 60 mM-Cl^-, respectively and V_H -60 mV. The GABA analogues at various concentrations and 10^{-4} M-pentobarbitone were applied externally for periods indicated by horizontal bar and dotted bar above each response, respectively.

test solution at V_H of -80, -60, -40, 0, $+20$ and $+40$ mV were plotted as a function of the membrane potential (Fig. 12*A*). The measured currents were greater in the outward direction than the inward direction even at symmetrical driving forces from E_{Cl}, while the GABA-potentiating action of pentobarbitone became much greater as the potential became more negative with respect to E_{Cl}, and smaller at more positive potentials. There was a linear relation between the augmentation of GABA-induced I_{Cl} by pentobarbitone and the membrane potential (Fig. 12*Ba*). The dose–response relation for GABA alone appears to be independent of V_H (unpublished observation).

Effects of change in internal Cl^- concentration on the GABA-potentiating action of pentobarbitone. The effects of change in the internal Cl^- concentration on the enhancement of GABA-induced I_{Cl} by pentobarbitone were examined with a

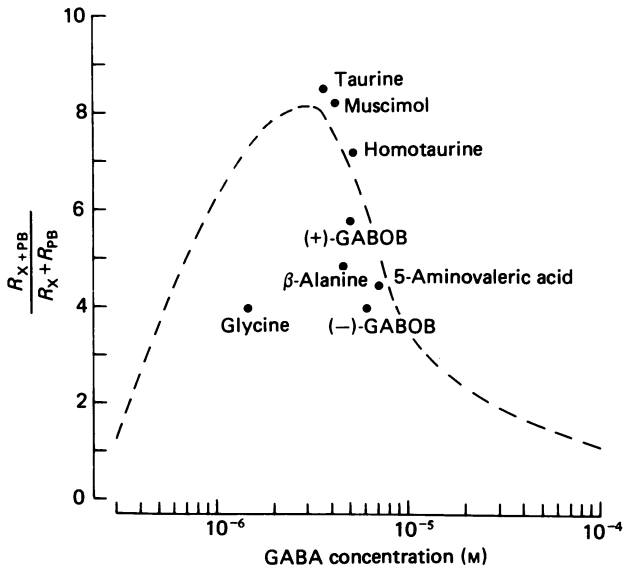

Fig. 10. Facilitatory effects of pentobarbitone upon the responses produced by muscimol and various amino acids. Ordinate shows the enhancement ratio of test-drug-, pentobarbitone and test-drug-plus-pentobarbitone-induced I_{Cl} as R_X, R_{PB} and R_{X+PB}, respectively. Abscissa shows GABA concentrations. Test-drug applications induced I_{Cl} and these points are plotted as the GABA concentration which produced the equivalent I_{Cl} amplitude. Each point is the average value of four experiments. The dotted curve was taken from Fig. 7*B*. The following concentrations were used in the experiments: taurine, 3×10^{-3} M; homotaurine, 10^{-5} M; β-alanine, 3×10^{-4} M; 5-aminovaleric acid, 3×10^{-4} M; (+)-GABOB, 3×10^{-5} M; (−)-GABOB, 10^{-5} M; glycine, 10^{-2} M; and muscimol, 3×10^{-7} M.

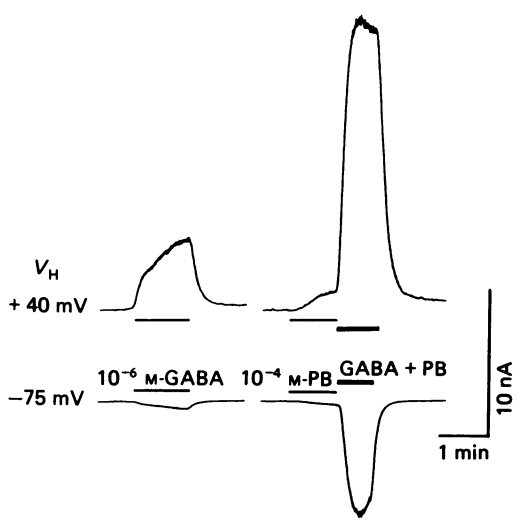

Fig. 11. Voltage dependence of GABA and pentobarbitone responses in the internally perfused neurone. External Cl^- 120 mM, internal Cl^- 60 mM, E_{Cl} −13·5 mV. All data were obtained from the same neurone.

constant external Cl^- concentration of 120 mM. When the internal Cl^- concentration was increased from 30 to 120 mM while keeping external Cl^- at 120 mM during 20 min internal perfusion, the E_{GABA} shifted from $-29{\cdot}8 \pm 2{\cdot}3$ mV (mean $\pm$ S.E. of mean, $n = 4$) to around +4 mV (Fig. 13*A*). Thus, E_{GABA} closely followed E_{Cl}. Fig. 13*B* shows the currents induced by 3×10^{-6} M-GABA, 10^{-4} M-pentobarbitone and the

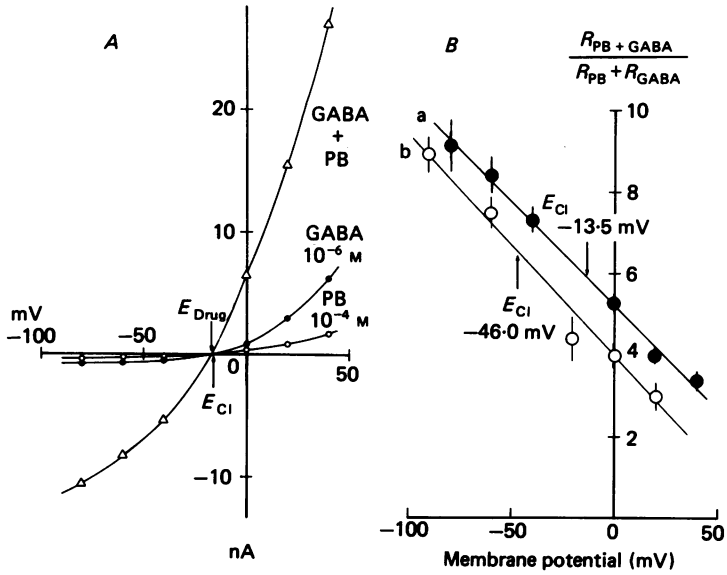

Fig. 12. I_{Cl} induced by GABA, pentobarbitone and GABA plus pentobarbitone at various holding potentials in neurones perfused with 120 mM-external Cl^- and 60 mM-internal Cl^-. *A*, voltage dependence of I_{Cl} induced by 10^{-6} M-GABA, 10^{-4} M-pentobarbitone and their mixture. The mixture was always applied after pre-treatment with 10^{-4} M-pentobarbitone for 2 min. Each data point represents the average of seven separate experiments. *B*a, relation between membrane potential and potentiation ratio of GABA-induced I_{Cl} by pentobarbitone in neurones perfused with 120 mM-external Cl^- and 60 mM-internal Cl^-. *B*b, neurones were perfused with 120 mM-external Cl^- and 15 mM-internal Cl^-. Each point and vertical bar gives mean and S.E. of mean of seven experiments. Note a marked increase in the GABA-potentiating action of pentobarbitone with hyperpolarizing potential.

mixture before and after the increase in internal Cl^- concentration at V_H −20 mV. As clearly shown in this Figure, the GABA-potentiating action of pentobarbitone appeared in both outward and inward directions, but the facilitation was greater with the inward currents. A similar result was obtained when the internal Cl^- concentration was reduced from 60 to 15 mM. Fig. 12*B*b also shows the voltage dependency of the pentobarbitone-induced GABA potentiation observed in neurones having E_{GABA} of $-46{\cdot}0 \pm 2{\cdot}6$ mV (mean $\pm$ S.E. of mean, $n = 5$), in which E_{Cl} predicted from the Nernst equation was $-45{\cdot}9$ mV at 120 mM-external Cl^- and 15 mM-internal Cl^- (after correcting for the activity coefficient: see the Methods section). The *I–V* relation shifted in parallel to negative potential as E_{GABA} changed from $-14{\cdot}5$ to $-46{\cdot}0$ mV.

DISCUSSION

In the internally perfused frog sensory neurone, the slope of the dose–response relation for the GABA-induced I_{Cl} yielded $n = 1{\cdot}8$ for the Hill coefficient, suggesting that two GABA molecules bind to one receptor site in order to open one Cl^- channel. Similar values have been reported in crayfish muscle, $n = 2$ (Takeuchi & Takeuchi, 1969) or $n = 3$ (Felts, 1971); cat sensory neurone, $n = 2$–3 (Gallagher *et al.* 1978); lamprey spinal interneurone, $n = 2$–3 (Homma & Rovainen, 1978); and in locust muscle, $n = 3$ (Werman, 1973).

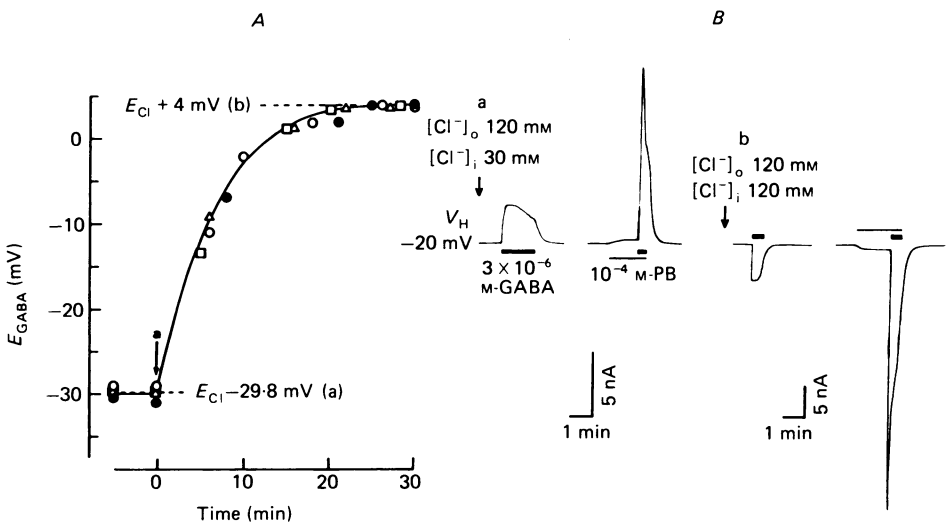

Fig. 13. *A*, changes in E_{GABA} after change in internally perfused Cl^-. Internal Cl^- concentration was switched from 30 to 120 mM (arrow a). E_{GABA} was estimated from the intersection in the two *I–V* relations which were obtained before and after adding 10^{-5} M-GABA by passing a symmetrical triangular voltage pulse (see explanation of Fig. 2*B*). Four different symbols correspond to four different neurones. There was no difference between E_{GABA} and E_{Cl} which was calculated from Cl^- activities in the external and internal solutions. *B*, effect of internal Cl^- concentration change on the potentiation of GABA-induced I_{Cl} by pentobarbitone at V_H −20 mV. Recordings were obtained from the same neurone. Measurement of inward I_{Cl} was made after the internal perfusion with 120 mM-Cl^- for 20 min. Note the different current calibrations.

GABA induced dual actions in C.N.S. neurones such as cultured spinal neurones (Nicoll, Padjen & Barker, 1976; Barker & Ransom, 1978; Obata, Oide & Tanaka, 1978) and hippocampal pyramidal cells (Alger & Nicoll, 1979, 1982; Andersen, Dingledine, Gjerstad, Langmoen & Mosfeldt Laursen, 1980; Thalmann, Peck & Ayala, 1981). In fact, two types of GABA receptor were reported in the spinal cord of the new-born rat (Allan, Evans & Johnston, 1980). However, GABA *in vivo* produced only depolarizing responses in dorsal root ganglion cells (Gallagher *et al.* 1978; Hattori *et al.* 1984), sympathetic ganglion cells (Adams & Brown, 1975), olfactory cortical neurones (Brown & Scholfield, 1979) and dentate gyrus granule neurones (Assaf, Crunelli & Kelly, 1981). In the present experiments, pentobarbitone

enhanced GABA-induced I_{Cl} over a wide range of voltage-clamped potentials. The enhancement was greater in inward current than outward current, and thus the effect is asymmetric. In addition, the voltage dependence of GABA potentiation by pentobarbitone shifted to the left along the membrane-potential axis as internal Cl^- was reduced from 60 to 15 mM (Fig. 12). These results indicate that potentiation of GABA currents by pentobarbitone depends not only upon the membrane potential but also on the direction of I_{Cl} across the cell membrane.

Our results suggest the existence of a 'barbiturate receptor' located close to a GABA receptor and directly modulating interactions between GABA and its receptor complex. This idea conforms to the biochemical findings that barbiturates enhance the binding of GABA to its receptor and decrease the dissociation rate (Willow & Johnston, 1980, 1981*a*, *b*; Olsen & Snowman, 1982).

Pentobarbitone at 10^{-4}–10^{-3} M can reduce the voltage-dependent inward Ca^{2+} current in the internally perfused snail neurone from either side of the plasma membrane (Oyama *et al.* 1982*a*; Nishi & Oyama, 1983). Over a similar concentration range, pentobarbitone could facilitate GABA- and pentobarbitone-induced I_{Cl} of the frog sensory neurone only from the external membrane surface (Fig. 5*D*). On the other hand, picrotoxin, a specific Cl^- channel blocker (Takeuchi & Takeuchi, 1969; Nicoll & Wojtowicz, 1981; Akaike & Oomura, 1984; Akaike *et al.* 1985), reduced the GABA- and pentobarbitone-induced I_{Cl} from both sides of the membrane (Akaike *et al.* 1985).

In the tissue-cultured mouse spinal neurone, pentobarbitone selectively facilitated the GABA response but did not measurably affect the sensitivity to β-alanine or glycine (Barker & Ransom, 1978). According to Choi, Farb & Fischbach (1981), chlordiazepoxide, one of the benzodiazepine derivatives, also selectively potentiated the GABA response in the cultured spinal neurone of chick, while the responses to glycine, β-alanine and taurine were not affected by chlordiazepoxide. However, pentobarbitone equally enhanced I_{Cl} produced by GABA, homotaurine, taurine, β-alanine, (+)-GABOB, (−)-GABOB, 5-aminovaleric acid and muscimol in all internally perfused sensory neurones tested in the present experiments. Diazepam also augmented the responses in the sensory neurone produced not only by GABA but also by these amino acids or muscimol (N. Akaike, K. Hattori & Y. Oomura, unpublished observations). However, the effects of both pentobarbitone and diazepam on glycine-induced I_{Cl} were less potent. In addition, in the sensory neurone, GABA-induced I_{Cl} was not enhanced by any other amino acid or muscimol. These results suggest that in the sensory neurone, muscimol and neutral amino acids except glycine may share a GABA receptor since the potentiation of amino-acid- and muscimol-induced I_{Cl} by either pentobarbitone or diazepam was not selective, unlike in C.N.S. neurones.

Both excitatory and inhibitory actions of barbiturates have been reported in many preparations (Sato, Austin & Yai, 1967; Nicoll, 1975; Kleinhaus & Prichard, 1977; Bowery & Dray, 1978; Cote, Zbicz & Wilson, 1978; Nicoll & Iwamoto, 1978; Schlosser & Franco, 1978). Stimulant and depressant actions of stereoisomers of pentobarbitone were also reported in mice *in vivo* studies (Büch, Grund, Buzello & Rummel, 1969; Waddell & Baggett, 1973). According to Barker *et al.* (1980), in cultured mouse spinal neurones, the (+) isomer also produced predominantly excitation through a direct activation of both excitatory conductance and transmitter release, and then elicited

sedation by activating inhibitory conductance at higher concentrations. In contrast, the (−) isomer evoked predominantly inhibitory responses by increasing Cl^- conductance and/or inhibiting transmitter release and also by potentiating the GABA-induced current. In the present experiments, stereoisomers and the racemic mixture of pentobarbitone increased the Cl^- conductance dose dependently in the order of (−) > (±) > (+). These three types of pentobarbitone at near-threshold doses could also enhance markedly the GABA-induced I_{Cl} in the same order. This result implies some stereospecific requirement for the pentobarbitone actions. However, the difference between (+) and (−) isomers in the activation of Cl^- conductance as well as in the augmentation of GABA-induced I_{Cl} was much less than expected from *in vivo* or cultured preparations which retain synaptic connexions. Therefore, the difference reported in previous studies performed under uncontrolled external and internal ionic environments might have been due to different actions of these isomers on other ionic conductances and/or transmitter-release mechanisms rather than on Cl^- conductance alone.

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