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Kinetic and pharmacological properties of the GABA-induced chloride current in *Aplysia* neurones: a 'concentration clamp' study

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1 γ-Aminobutyric acid (GABA) was applied by the 'concentration clamp' technique to isolated neurones of *Aplysia*. GABA induced a chloride current (I_{Cl}) due to activation of a single class of chloride-channel.

2 The concentration-response curve for the peak I_{Cl} gave an apparent dissociation constant of 6.4×10^{-5} M and a Hill coefficient of 0.88. The current-voltage relationship was linear in the voltage range examined (−40 to +10 mV).

3 The activation phase of the I_{Cl} could be fitted to a single exponential function and desensitization followed the sum of two exponential functions. The time constants of activation and desensitization decreased with increasing concentrations of GABA but were voltage-independent. The recovery process from desensitization also followed the sum of two exponential functions.

4 As for the rate-limiting step of the channel activation, the hyperbolic relationship between the activation rate and GABA concentration showed that the rapid binding assumption holds, suggesting that the isomerization step is rate-limiting. The apparent channel closing rate constant was estimated to be $10\,s^{-1}$ from the ordinate intercept of the linear part of the above relationship at lower concentrations.

5 Muscimol and β-alanine induced a I_{Cl}, which cross-desensitized with that evoked by GABA. The GABA-I_{Cl} was not enhanced by diazepam (10^{-6} M) or α-chloralose (10^{-3} M), in fact depressant effects were evident.

6 Pentobarbitone decreased the GABA-I_{Cl} non-competitively without altering activation or desensitization kinetics. The concentration-inhibition curve gave a K_D value of 8.9×10^{-5} M and a Hill coefficient of 1.0.

7 These results suggest that GABA activates a single class of Cl channel in *Aplysia* neurones, which have one binding site for the agonist. The GABA receptor-Cl channel complex in *Aplysia* is pharmacologically and perhaps structurally different from that in vertebrates.

Introduction

γ-Aminobutyric acid (GABA) is an important neurotransmitter in central and peripheral tissues of vertebrates (Barker & Ransom, 1978a; Nicoll & Wojtowicz, 1980; Akaike *et al.*, 1985; Kaneko & Tachibana, 1986; Randle & Renaud, 1987) and invertebrates (Takeuchi & Takeuchi, 1966; Yarowsky & Carpenter, 1978). To elucidate the mode of interaction between the transmitter and the receptor-channel complex, kinetic studies with perturbation or fluctuation analysis are indispensable. Bath application and iontophoretic or pressure application have been used to characterize GABA responses in neuronal systems of various species. In bath application experiments, however, the peak amplitude of the current response was obscured by a rapidly developing desensitization (Feltz, 1971; Matsumoto *et al.*, 1986) and the iontophoretic or pressure application technique does not yield information on the exact agonist concentration at the postsynaptic receptor site (Peper *et al.*, 1976). Although the characteristics of invertebrate GABA receptors have been extensively examined (Takeuchi

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& Takeuchi, 1966; 1969; Feltz, 1971; Yarowsky & Carpenter, 1978; Kaneko & Tachibana, 1986), few kinetic data are available.

We have developed a new type of concentration jump technique, termed a 'concentration clamp' technique (Akaike *et al.*, 1986) which combines intracellular perfusion and the rapid exchange of the external solution under single-electrode voltage-clamp conditions. With this technique the external solution surrounding the single neurone can be replaced with fresh test solution containing a known concentration of the agonist with a time constant of only 2 to 3 ms; thus the kinetics of both activation and desensitization processes can be analyzed. The peak of the current induced by a given concentration of the agonist can be measured before desensitization takes place, and a precise concentration-response curve obtained.

Neurones of the sea mollusc *Aplysia* have membrane receptors and associated ionic channels that are gated by a variety of neurotransmitter substances. Individual neurones not only have receptors for multiple neurotransmitters, but may also have multiple receptors for a single neurotransmitter. Yarrowsky & Carpenter (1984) noted five different types of response to iontophoretic application of GABA in *Aplysia* neurones. We report here data on the kinetic and pharmacological properties of the GABA-induced chloride current in isolated neurones of *Aplysia*, obtained using the 'concentration clamp' technique.

Methods

Preparation

Aplysia kurodai were collected on the beach of Shikanoshima Island in Fukuoka City. Circum-oesophageal and abdominal ganglia were dissected from the animal and the connective tissue surrounding each ganglion was carefully stripped off with micro-scissors and forceps. The capsules enveloping the ganglia were digested in artificial sea water containing dispase (10,000 protease unit per 5 ml; Godo Shusei Co. Ltd, Japan) for 60 to 80 min at 37°C. During the enzyme treatment, the preparation was moved gently by bubbling air through the bathing medium. Neurones were then isolated mechanically with finely polished pins and these single neurones were left for a few hours at room temperature in a culture medium consisting of equal parts of Eagle's MEM (Nissui Co. Ltd., Japan) and artificial sea water (composition, mM): NaCl 450, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55, tris-hydroxymethylaminomethane (Tris) Cl 10 and with pH adjusted to 7.8 with Tris-base and N-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES). The majority of cells used in the experiments had diameters of about 50 μm.

Solutions

To isolate the chloride current from sodium and potassium currents, Na^+ and K^+ in both external and internal solutions were replaced with $Tris^+$ and Cs^+, respectively. The Na^+- and K^+-free external solution had the following composition (mM): Tris-Cl 340, Tris-base 100, CsCl 10, $CaCl_2$ 10, $MgCl_2$ 55 and HEPES 5 with pH adjusted to 7.8, while the Na^+-, K^+- and Ca^{2+}-free internal solution was composed of (mM): Tris-base 300, aspartic acid 275, CsCl 300 and EGTA 5, with pH adjusted to 7.2 with Tris-base and HEPES. The neurone was internally perfused at a constant flow rate of 0.5 to 1 ml min^{-1} for the first 20 min after aspiration to the tip of the electrode and the reversal potential of the GABA response (E_{GABA}) approached close to the equilibrium potential for Cl ions (E_{Cl}).

Electrical measurements

The suction-pipette technique was used for voltage clamp and internal perfusion of the neurones (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1985). The inner diameter of the fire-polished tip of the suction pipette was between 10 and 12 μm and the electrode resistance was 200 to 400 kΩ (Ikemoto *et al.*, 1987). The membrane potential was controlled with the single electrode-voltage clamp amplifier of the sample-and-hold type (Ishizuka *et al.*, 1984). GABA was applied by the 'concentration clamp' technique, as described by Akaike *et al.* (1986). Each application was separated by an interval longer than 3 min unless otherwise noted. All experiments were carried out at room temperature (about 20°C). Both voltage and current were monitored on an oscilloscope (Tektronix 5113, USA) and a chart recorder, and were digitized and stored on magnetic tapes for later analysis. Curve fittings were performed with a microcomputer (PC 9801XA, NEC, Japan).

Results

GABA-induced I_{Cl} and the concentration-response relationship

A sustained application of GABA induced a chloride current (I_{Cl}) which was rapidly activated and then desensitized completely (Figure 1a). The desensitization became more rapid as the concentrations of GABA were increased. In Figure 1b, the peak amplitude of the I_{Cl} is plotted against the GABA concen-

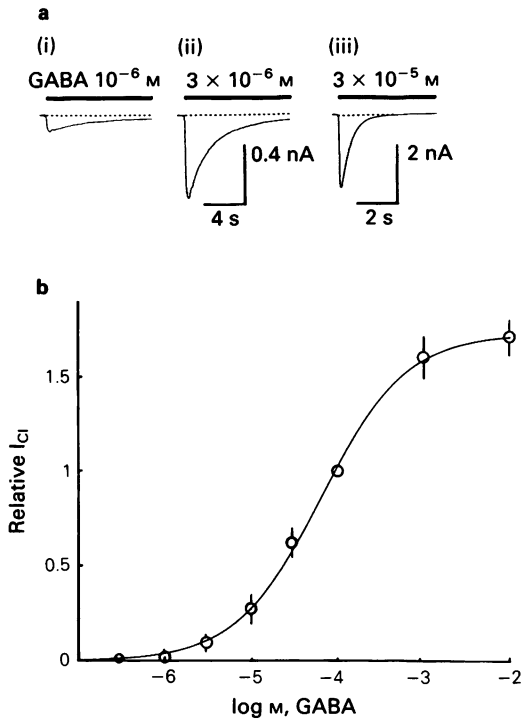

Figure 1 The GABA-induced I_{Cl} as a function of GABA concentration, and the concentration-response relationship. (a) An isolated neurone was voltage-clamped at a holding potential (V_h) of −20 mV and GABA was applied by the concentration clamp technique (shown by the solid lines above the responses). GABA elicited a rapidly activating inward current, which decayed almost to baseline during sustained application. It is clear that desensitization became more rapid with increasing concentrations of GABA. (b) The peak I_{Cl} is normalized to that evoked by 10^{-4} M GABA and plotted against the concentration. The points are fitted with a least squares best fit programme to give a K_D of 6.4 × 10^{-5} M and a Hill coefficient of 0.88. The continuous line is drawn using those values and I_{max} = 1.7. Each point is the mean of 7 to 8 experiments and bars indicate one s.e.mean when larger than the symbol.

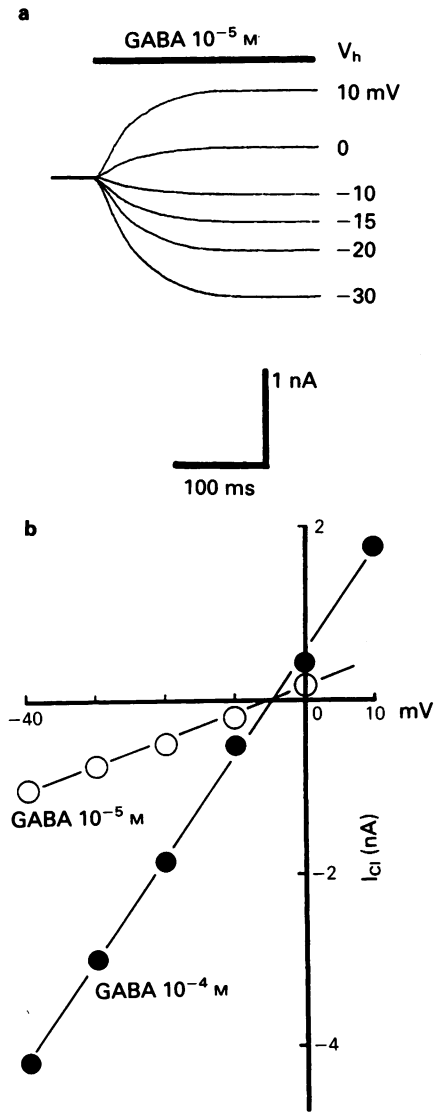

Figure 2 Current-voltage (I_{Cl}-V) relationship. (a) GABA (10^{-5} M) was applied to a cell voltage-clamped at various holding potentials (V_h). The I_{Cl} reversed its polarity between −10 and 0 mV. (b) I_{Cl}-V relationships in a neurone with 10^{-5} M (○) and 10^{-4} M (●) GABA. The relationship was linear between −40 and 10 mV, and both lines crossed the voltage axis at −5 mV.

tration: the GABA responses were normalized for the peak I_{Cl} elicited by 10^{-4} M GABA. The GABA concentration-response relationship was in accord with the conventional expression:

$$I = I_{max} \cdot C^n/(C^n + K_D^n) \quad (1)$$

where, I is the observed GABA-induced I_{Cl}, I_{max} the maximum value of the I_{Cl}, C the GABA concentration, K_D the dissociation constant, and n the Hill coefficient. The least square fit gave a K_D value of 6.4 × 10^{-5} M and a Hill coefficient of 0.88. The continuous line in Figure 1B was drawn according to equation (1) using I_{max} = 1.7, K_D = 6.4 × 10^{-5} M and Hill coefficient = 0.88.

Figure 2a illustrates the I_{Cl} evoked by 10^{-5} M GABA at six different holding potentials (V_h). It is evident from this figure that the reversal potential for

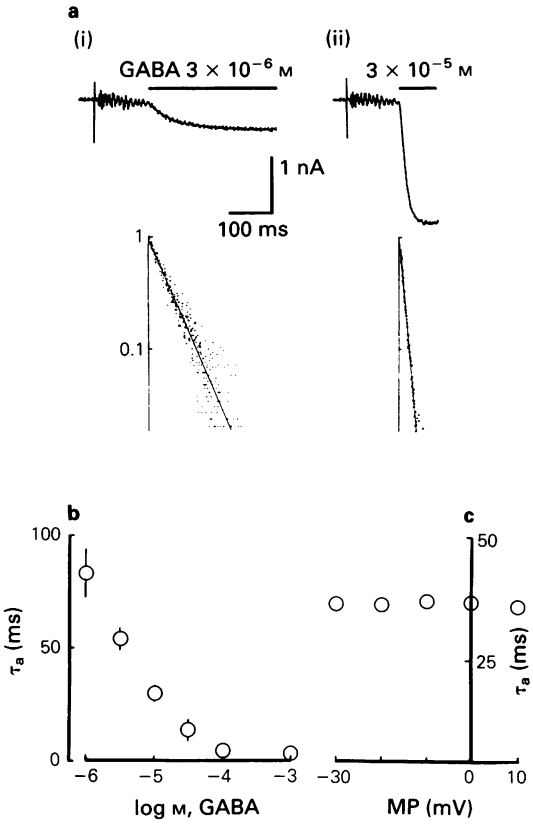

Figure 3 Activation kinetics of the I_{Cl}. (a) The I_{Cl} was evoked by 3×10^{-6} (i) and 3×10^{-5} M (ii) GABA at a V_h of -20 mV. The activation phase consisted of a single exponential even at the higher concentration, as shown by the semilogarithmic plot in the lower panel. The noises before the response are due to the opening of the magnetic valve and the beginning of the solution flow. It took about 110 ms for the GABA-containing solution to reach the neurone. (b) The activation time constant (τ_a) is plotted against the concentration of GABA. The time constant was reduced with increasing concentrations of the agonist. The points are the mean of 6 to 8 experiments and bars indicate one s.e.mean when larger than the symbol. (c) GABA (10^{-5} M) was applied to a neurone voltage-clamped at various membrane potentials (MP). The activation time constant was not altered by the membrane potential. This was also the case with other concentrations of GABA.

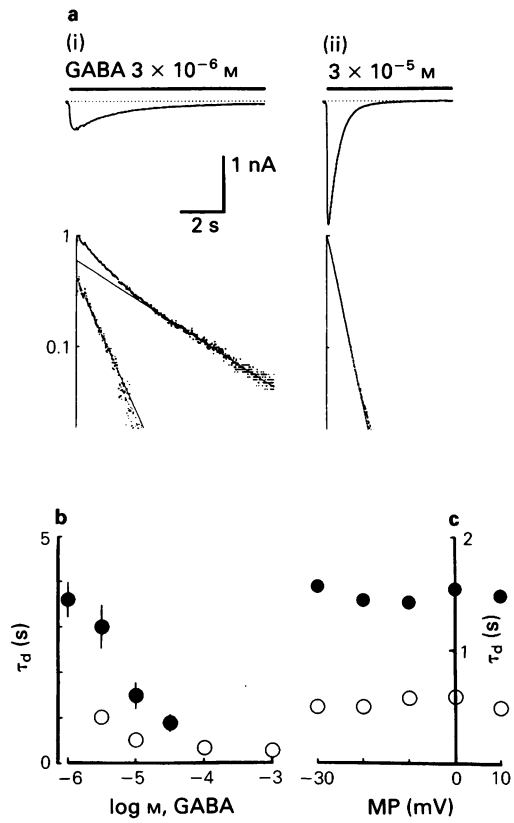

Figure 4 Desensitization kinetics of the I_{Cl}. (a) The I_{Cl} waned to null over time despite the sustained presence of 3×10^{-6} (i) and 3×10^{-5} (ii) M GABA at a V_h of -20 mV. The semilogarithmic plots in the lower panel indicate that the desensitization proceeded as the sum of two exponential functions. (b) The fast (○; τ_{df}) and the slow (●; τ_{ds}) time constants of desensitization are plotted in relation to the GABA concentration. Both time constants decreased with increasing concentrations of GABA. The concentration dependency of τ_{df} was less marked than that of τ_{ds}. Each point is the mean of 5 to 7 experiments and the bars indicate one s.e.mean when larger than the symbol. (c) Voltage-dependency of the desensitization time constants of the I_{Cl} elicited by 10^{-5} M GABA in a neurone. The two time constants were independent of the membrane potential.

the GABA-response (E_{GABA}) lies between -10 and 0 mV. The I_{Cl}-V relationship with 10^{-5} and 10^{-4} M GABA is depicted in Figure 2b, where the lines through the data points are linear in the voltage range between -40 and $+10$ mV and crossed the abscissa at -5 mV. The average E_{GABA} was -7.3 ± 1.6 mV (mean ± s.e.mean; $n = 11$), which was close to the E_{Cl} of -10.7 mV calculated from the Nernst equation, knowing the composition of the external and internal solutions and the interpolated activity coefficients for Cl^- ions (Radiometer, Denmark). This finding indicates the adequate internal perfusion and purity of I_{Cl}.

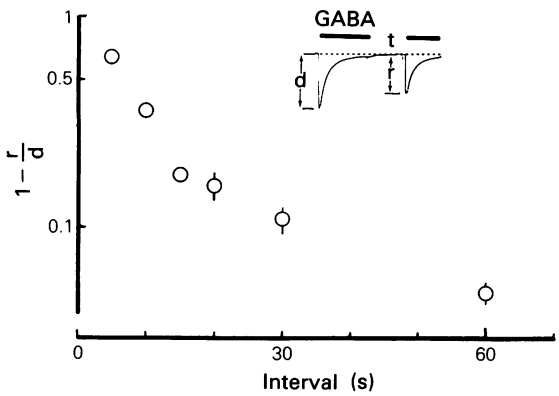

Figure 5 Recovery time course from the desensitization. GABA (10^{-5} M) was applied at −30 mV for 15 s, during which period the desensitization proceeded substantially (see inset), and GABA was washed out. During the interval (t) before the second application, the receptor-channel complex was allowed to recover from the desensitization. The I_{Cl} evoked by the second application became greater with prolongation of the interval. The unrecovered fraction (1 − r/d) is plotted semilogarithmically against the interval duration. The I_{Cl} recovered as the sum of two exponential functions with time constants of 8.5 and 50 s. Each point is the mean of 5 to 7 experiments and bars indicate one s.e.mean when larger than the symbol.

Concentration- and voltage-dependency of the I_{Cl} activation

Figure 3a(i),(ii) shows the activation phases of the I_{Cl} evoked by 3×10^{-6} and 3×10^{-5} M GABA at a V_h of −20 mV to fit a single exponential, except for the shoulder at the initial part of the response. The activation time constant (τ_a) markedly decreased with increasing concentrations of GABA (Figure 3b) but did not depend on the membrane potential (Figure 3c).

Desensitization of the GABA-induced I_{Cl}

As shown in Figure 4a, the time course of desensitization could be fitted with the sum of two exponential functions at lower concentrations and a single at higher concentrations. The fast (τ_{df}) and the slow (τ_{ds}) time constants markedly decreased with increasing concentrations of GABA. Both τ_{df} and τ_{ds} of the I_{Cl} induced by 10^{-5} M GABA were voltage-independent (Figure 4c), as they were at all concentrations tested.

Figure 5 illustrates the recovery process from the desensitization. The first 15 s-application of 10^{-5} M GABA substantially desensitized the receptor-channel complex and the complex was allowed to recover during the interval before the second application. The protocol is depicted in the inset in Figure

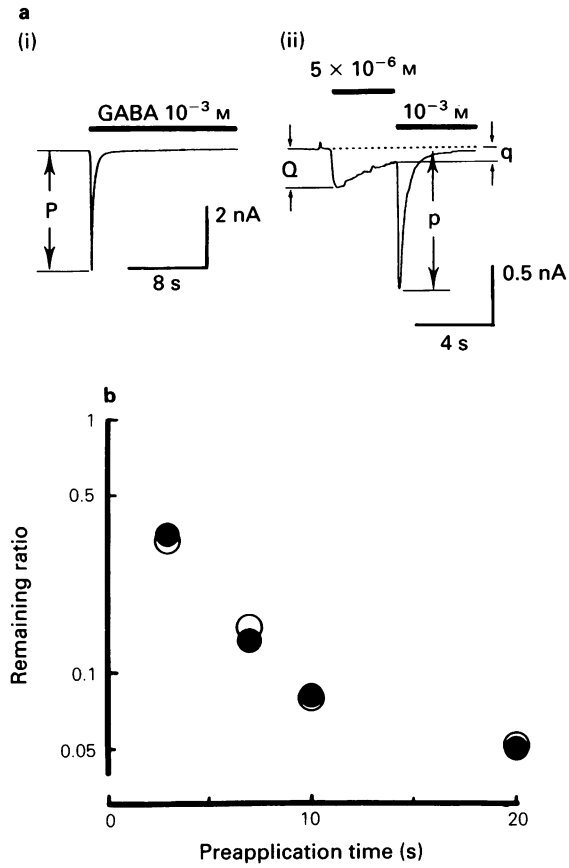

Figure 6 Activation of a single class of receptor-channel complexes by GABA. (a)(i) A high concentration of GABA (10^{-3} M) induced an almost fully activated I_{Cl} at −20 mV. P denotes the amplitude of the I_{Cl}. After various pre-application periods of a low concentration (5×10^{-6} M), 10^{-3} M GABA evoked a smaller I_{Cl} due to the progression of desensitization. The extent of desensitization was expressed by (q/Q), where Q and q are amplitudes of the I_{Cl} at the beginning and at the time when 10^{-3} M GABA was applied, respectively. The non-desensitized part which remained in the time course of desensitization was estimated by (p/P). (b) (q/Q) (○) and (p/P) (●) are plotted in relation to the pre-application period (t) of 5×10^{-6} M GABA. The time course of (q/Q) corresponds to the desensitization process itself of the I_{Cl} evoked by 5×10^{-6} M GABA. The time course of reduction in the remaining I_{Cl}, namely (p/P) − (t) relationship, overlaps that of desensitization, indicating that the I_{Cl} was due to activation of a single class of the channel (see Appendix).

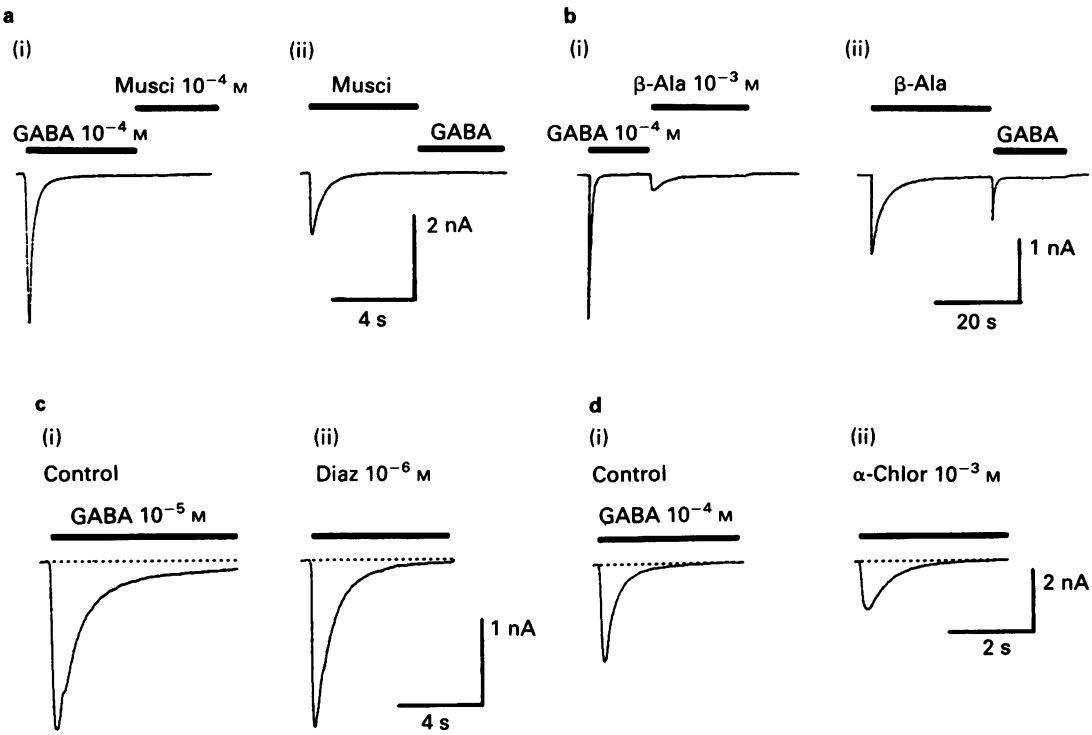

Figure 7 Pharmacological properties of the GABA-I_{Cl}. (a and b) Muscimol (Musci) (a) and β-alanine (β-Ala) (b) induced an I_{Cl} which cross-desensitized with the GABA-I_{Cl}. In one neurone out of four, the cross-desensitization of β-alanine-I_{Cl} with the GABA-I_{Cl} was incomplete as shown in (b). (c) Diazepam (Diaz, 10^{-6} M) did not augment the GABA-I_{Cl}. (d) α-Chloralose (α-Chlor) neither evoked an I_{Cl} nor enhanced the GABA-I_{Cl}, whereas, the agent did suppress the I_{Cl}.

5. The fraction remaining unrecovered, (1 − r/d), is semilogarithmically plotted against the time interval (t). The recovery process followed the sum of two exponential functions with time constants of 8.5 and 50 s. The I_{Cl} evoked by 10^{-4} M GABA recovered with a similar time course, thereby indicating the concentration-independence of the recovery process.

Activation of a single class of chloride channels by GABA

In recordings of the whole cell current, the number of classes of receptor-channel complexes operating in the presence of an agonist is difficult to determine. Concerning the number of channel classes activated by GABA, we estimated the ratio of the I_{Cl} which remained non-desensitized during the time course of desensitization. Figure 6a(i) shows the I_{Cl} induced by 10^{-3} M GABA, which almost fully activated the channel. A pre-application of a lower concentration of GABA (5×10^{-6} M) evoked a smaller and desensitizing I_{Cl} (Figure 6a(ii)). After the pre-application, a subsequent application of 10^{-3} M GABA evoked a smaller I_{Cl}, compared with the control without pre-treatment (Figure 6a(i)). This corresponds to the non-desensitized part at the point of time. The remaining ratio during the progression of desensitization was calculated for the I_{Cl} evoked by 5×10^{-6} M and 10^{-3} M GABA as shown in Figure 6a, and plotted against the duration of pre-application period in Figure 6b. The ratio, q/Q, with 5×10^{-6} M GABA in the figure means the extent of desensitization. The ratio, p/P, with 10^{-3} M GABA decreased with the same time course as the progress of desensitization. This means that the non-desensitized part of I_{Cl} was always proportional to the I_{Cl} induced by 5×10^{-6} M GABA during the progression of desensitization. Since the desensitization rates are considerably smaller than the activation rate (compare τ_a with τ_d in Figures 3 and 4), it can be inferred that the I_{Cl} consists of a single population of receptor-channel complexes (Kijima & Kijima, 1982;

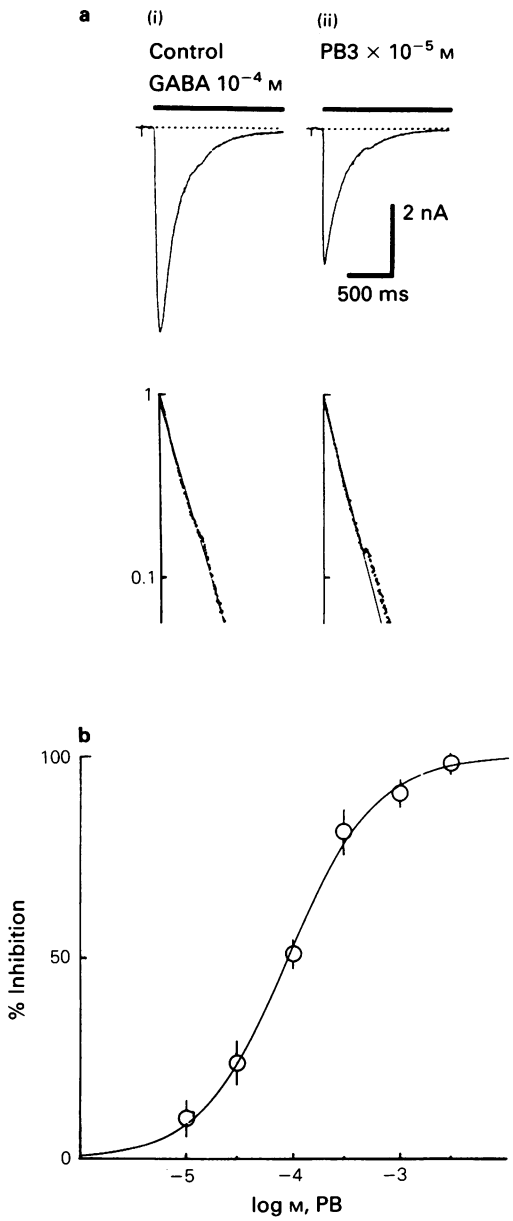

Figure 8 Pentobarbitone depression of the I_{Cl}. (a) Pentobarbitone (3×10^{-5} M) reduced the GABA-I_{Cl}. The semilogarithmic plot in the lower panel shows that the time course of desensitization phase was not affected. (b) A dose-inhibition curve was obtained for pentobarbitone with the I_{Cl} evoked by 10^{-5} M GABA. A least squares fit gave a K_D value of 8.9×10^{-5} M and a Hill coefficient of 1 for the inhibition. Each point is the mean of 5 to 6 experiments and bars indicate one s.e.mean.

1983). The rationale for this argument is given in the Appendix. The same results were obtained in three other experiments.

Pharmacological properties of the GABA-I_{Cl} in Aplysia *neurones*

Muscimol evoked a I_{Cl} which desensitized completely. Figure 7a depicts a cross-desensitization between GABA- and muscimol-induced I_{Cl}. β-Alanine also induced a similar I_{Cl} cross-desensitizing with the GABA-I_{Cl}. In one neurone out of four, the cross-desensitization was not complete (Figure 7b).

Diazepam enhances GABA response in vertebrates (Olsen, 1981). With *Aplysia* neurones, however, diazepam did not augment the GABA response at concentrations between 10^{-8} and 10^{-6} M (Figure 7c), rather the desensitization occurred more rapidly in the presence of diazepam.

α-Chloralose neither induced a I_{Cl} nor increased the GABA-I_{Cl} in *Aplysia* neurones but did depress the I_{Cl} (Figure 7d).

Depression of the I_{Cl} by pentobarbitone

Figure 8a shows that pentobarbitone suppressed the GABA response without altering activation or desensitization kinetics. The dose-inhibition curve is shown in Figure 8b. A least square fit gave a K_D value of 8.9×10^{-5} M and a Hill coefficient of 1 for the inhibition. Effects of pentobarbitone on the concentration-response curve are depicted in Figure 9a, and a double-reciprocal plot indicates that the mode of inhibition was non-competitive (Figure 9b).

Discussion

GABA-induced I_{Cl} and its activation kinetics

GABA induced a I_{Cl} which decayed with time in the presence of the agonist of constant concentration. Muscimol and β-alanine, GABA-mimetic agents, also evoked an I_{Cl} which cross-desensitized with the GABA-I_{Cl}. Therefore, these agents activated the same receptor-channel complex as GABA.

The concentration-response relationship of the GABA-induced I_{Cl} obtained using the 'concentration clamp' technique gave a K_D value of 6.4×10^{-5} M and a Hill coefficient of near unity (0.88), thereby suggesting that the GABA receptor of *Aplysia* neurones has one binding site for the agonist. The Hill coefficient for the GABA response in a variety of vertebrate preparations has been reported to be near 2 (Akaike *et al.*, 1985; 1986; Bormann & Clapham, 1985; Kaneko & Tachibana, 1986; Parker *et al.*,

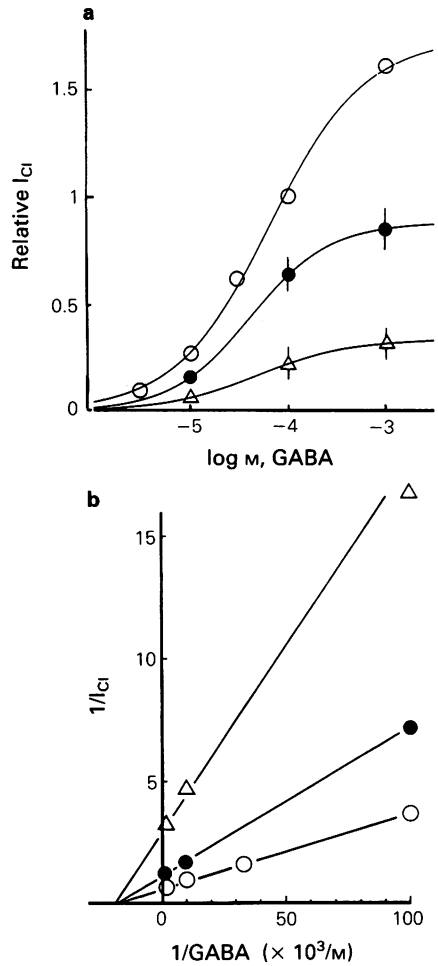

Figure 9 Non-competitive inhibition by pentobarbitone. (a) Open circles indicate the control concentration-response curve (○) obtained in Figure 1. Pentobarbitone, 10^{-4} (●) and 3×10^{-4} (△) M, suppressed the I_{Cl}. Each point is the mean of four experiments and bars indicate one s.e.mean. (b) Double reciprocal plots of the concentration-response curve indicate that the inhibition was non-competitive.

1986; Randle & Renaud, 1987). In invertebrates, the Hill coefficient varied from 1 to 4 (Takeuchi & Takeuchi, 1969; Feltz, 1971; Brookes & Werman, 1973; Constanti, 1977; Matsumoto, 1982; Shimizu *et al.*, 1983). Studies on molluscan neurones gave the coefficient of unity (Matsumoto, 1982; Shimizu *et al.*, 1983) in agreement with the present study. The discrepancy may be due to differences in species and technique of application of the agonist.

In frog sensory neurones, the activation phase followed the sum of two exponential functions, suggesting two major populations of the GABA receptor-Cl channel complex contributing to the whole current (Akaike *et al.*, 1986). Noise analysis and single channel recording with the same preparation revealed three components having different conductances (Yasui *et al.*, 1985). In *Aplysia* neurones, the main contributing population may be single, since the activation phase was single exponential and activation of a single class of the receptor-channel complex was suggested in Figure 6 in the present experiments (see Appendix).

The rapid exchange of external solution facilitated by our technique enables the instantaneous application of the agonist, relative to the time scale of the activation. A simple sequential model may help explain the agonist-induced current:

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O \underset{f_1}{\overset{d_1}{\rightleftharpoons}} D_1 \underset{f_1}{\overset{d_2}{\rightleftharpoons}} D_2 \qquad (2)$$

where C, O, D_1 and D_2 denote the activatable, open and two desensitized states, respectively; α and β are apparent closing and opening rate constants (see the next section); d_1 and d_2 are desensitization rate constants and f_1 and f_2 are rate constants of recovery from desensitization. In the sequential model, α was reported to depend on the membrane potential in the acetylcholine-activated channel at the muscle endplate (Magleby & Stevens, 1972) and at the *Aplysia* excitatory synapse (Ascher et al., 1978). In the present experiments with GABA, the I_{Cl}-V relationship was linear and the activation time constant, which can be expressed as $1/(\alpha + \beta)$, did not depend on the voltage. These findings suggest that α does not depend on the membrane potential in the voltage range examined. This result is consistent with that of GABA-I_{Cl} in frog sensory neurones (Akaike *et al.*, 1986). The difference may be due to the different natures of the receptor-channel complex.

The rate-limiting step of activation

The activation phase in the sequential model can be written as follows in more detail (del Castillo & Katz, 1957):

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AR^* \qquad (3)$$

where A and R are the agonist and the receptor-channel complex, respectively. AR denotes the bound state of R with A; AR isomerizes to form AR*, which represents the open state of the channel; k_1 and k_{-1} are the forward and backward rate constants of agonist binding and k_2 and k_{-2} are the forward and backward rate constants of isomerization, respectively. In the present GABA-I_{Cl} system

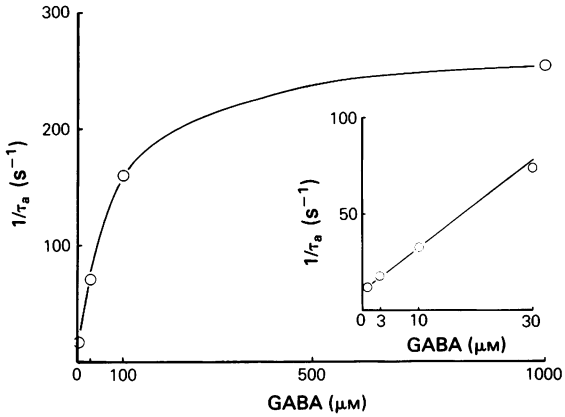

Figure 10 Relationship between the activation rate ($1/\tau_a$) and GABA concentration. Figure 3b is replotted as $1/\tau_a$ versus GABA concentration on normal scale. The relationship was hyperbolic and saturating, suggesting that the isomerization step is rate-limiting. The inset illustrates the relationship at lower concentrations of GABA, where points fall on a straight line giving the apparent closing rate constant of $10\,s^{-1}$ at the ordinate intercept.

of *Aplysia* neurones, one molecule of the agonist binds to the receptor to activate the channel, which consists of a single population. Since the activation time constant followed a single exponential function at all concentrations tested, three conditions can be postulated with the basic kinetic constants k_1, k_{-1}, k_2 and k_{-2} (Sakmann & Adams, 1979; Kijima & Kijima, 1982; 1983):

(a) steady-state assumption: $(k_{-1} + k_2) \gg (k_1[A] + k_{-2})$, where the activation rate ($1/\tau_a$) is expressed as the sum of apparent rate constants, $\alpha + \beta$, and [A] denotes the agonist concentration,

$$\alpha = \frac{k_{-1} \cdot k_{-2}}{k_2 + k_{-1}}, \quad \beta = \frac{k_1[A] \cdot k_2}{k_2 + k_{-1}}.$$

(b) rapid binding assumption: $(k_1[A] + k_{-1}) \gg (k_2 + k_{-2})$, where

$$\alpha = k_{-2}, \quad \beta = \frac{k_1[A] \cdot k_2}{k_1[A] + k_{-1}}.$$

(c) rapid isomerization assumption:

$$(k_{-1} + k_{-2}) \gg (k_1[A] + k_{-1}),$$

where

$$\alpha = \frac{k_{-1} \cdot k_{-2}}{k_2 + k_{-2}}, \quad \beta = k_1[A].$$

The τ_a-concentration relationship in Figure 3b is replotted as $1/\tau_a$ versus GABA concentration in Figure 10. The points fall on a hyperbolic saturating line up to 10^{-3} M, suggesting that the rapid binding assumption is correct. At lower concentrations of the agonist the relationship was linear, and the apparent closing rate constant, α, was estimated from the ordinate intercept to be $10\,s^{-1}$ (see inset of Figure 10). These arguments lead us to an inference that the isomerization step may be rate-limiting in the GABA-I_{Cl} system in *Aplysia* neurones, which is consistent with the inference of Sakmann & Adams (1979) that the rapid binding assumption held for the ACh-induced current at frog endplate.

Kinetics of desensitization

Desensitization of GABA response was noted in invertebrates (Feltz, 1971; Matsumoto, 1982) and in vertebrates (Numann & Wong, 1984; Akaike *et al.*, 1986; Kaneko & Tachibana, 1986; Cash & Subbarao, 1987). The 'concentration clamp' technique revealed that the desensitization of the GABA-I_{Cl} in *Aplysia* neurones proceeds very rapidly and follows the sum of two exponential components. The two-component desensitization has been noted with various ACh receptors, including those at the endplate (Chesnut, 1983) and in molluscan neurones (Andreev *et al.*, 1984). Numann & Wong (1984) reported a single exponential time course of desensitization in isolated hippocampal neurones of guinea-pig. Since they used a pressure application of GABA (10^{-4} M), the precise concentration and spatial homogeneity of the agonist concentration were unclear (Peper *et al.*, 1976). Using *Aplysia* neurones, Matsumoto *et al.* (1986) noted a single exponential desensitization. Bath application of GABA in their experiments, however, may have obscured the rapid phase of desensitization.

The desensitization time constants decreased with increasing concentrations of GABA, a finding consistent with data on various transmitter activated systems (Chesnut, 1983; Andreev *et al.*, 1984; Slater *et al.*, 1984; Akaike *et al.*, 1986) and which indicates that the rate constants of desensitization in the sequential model become larger with increasing concentrations of the agonist. The time constants were not affected by the membrane potential. These findings agree with the results of Andreev *et al.* (1984) who studied desensitization kinetics of the ACh-induced I_{Cl} in isolated *Lymnea stagnalis* neurones. Matsumoto *et al.* (1986) noted voltage-independency of the desensitization process of GABA-I_{Cl} in *Aplysia* neurones. On the other hand, the voltage dependency of the desensitization process was noted in the case of the frog endplate (Chesnut, 1983). Andreev *et al.* (1984) ascribed the discrepancy to the ion species flowing through the channel, namely, cations rather than anions. A fundamental difference, however, may exist between receptor-channel complexes in molluscan neurones and the vertebrate endplate, since

Slater *et al.* (1984) found a lack of voltage dependency of desensitization with the ACh-activated cation channel in *Aplysia* neurones. The recovery from desensitization proceeded as the sum of two exponential components and the time constants were not concentration-dependent. This finding, together with the two-component time course of progression, suggests the existence of two states of desensitization. It was not clear, however, whether the two desensitized states are in parallel or in a series.

Differences in pharmacological properties

Diazepam reportedly increases GABA-responses in vertebrate neurones (MacDonald & Barker, 1978; Study & Barker, 1981). In frog sensory neurones, diazepam markedly increased the GABA-I_{Cl} at a concentration as low as 3×10^{-8} M (Hattori *et al.*, 1986), while, in the present experiments with *Aplysia* neurones, diazepam did not augment the GABA-response even at 10^{-6} M. α-Chloralose evoked a Cl response and enhanced the GABA-induced Cl response in frog motoneurones (Nicoll & Wojtowicz, 1980) and in frog sensory neurones (Akaike *et al.*, unpublished observations). In *Aplysia* neurones, the agent neither induced an I_{Cl} nor enhanced the GABA-I_{Cl}, rather, suppression of the I_{Cl} was evident.

Pentobarbitone induces a Cl response (Nicoll & Wojtowicz, 1980; Akaike *et al.*, 1985) and augments the postsynaptic response to GABA in a variety of vertebrate preparations (Ransom & Barker, 1976; Barker & Ransom, 1978b; Willow & Johnston, 1983; Wong *et al.*, 1984; Akaike *et al.*, 1985; Parker *et al.*, 1986). The potentiation of the GABA response was mainly attributed to increase in the apparent binding affinity of the receptor to GABA in the presence of pentobarbitone (Ransom & Barker, 1976; Evans, 1979; Conners, 1981; Higashi & Nishi, 1982; Akaike *et al.*, 1985; Parker *et al.*, 1986). On the other hand, our results with *Aplysia* neurones show that pentobarbitone reduced the GABA response non-competitively with a K_D value of 8.9×10^{-5} M (Figures 8 and 9). These findings agree with the observations of Cote & Wilson (1980) that pentobarbitone depressed GABA-I_{Cl} of *Aplysia* neurones with an ED_{50} of 6.27×10^{-5} M. The present results further indicate that binding of one molecule of pentobarbitone was involved in the suppression of the GABA-I_{Cl}, as was the case for augmentation in vertebrates (Parker *et al.*, 1986). With another invertebrate system, the crayfish stretch receptor, Aikin & Deicz (1981) observed no significant effects of the agent on the postsynaptic GABA response.

These arguments suggest that GABA receptors of vertebrates and invertebrates are pharmacologically and perhaps structurally different. The Hill coefficient for inducing the I_{Cl} has been reported to be 2 in vertebrates and the GABA receptor-Cl channel complex is positively coupled with pentobarbitone and/or diazepam receptor (Olsen, 1981). In *Aplysia* neurones, one molecule of GABA binds to the receptor to activate the channel. The diazepam receptor is either lacking or uncoupled and the pentobarbitone receptor is negatively coupled with the channel. Figure 11 presents a schematic illustration showing interactions among these receptors in vertebrates and molluscs.

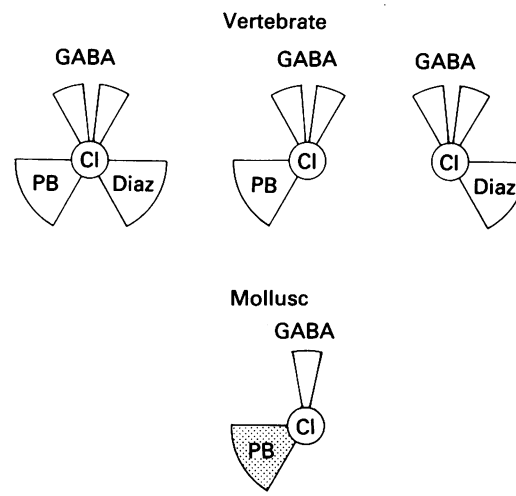

Figure 11 Schematic illustration showing differences in GABA receptor-Cl channel complexes of vertebrates and molluscs. In our recent patch clamp experiments using 'inside-out' preparations of frog dorsal root ganglion cells, the majority of recordings showed prolongation of the mean open time of the GABA-activated Cl channel both by pentobarbitone (PB) and diazepam (Diaz), but some showed prolongation only by pentobarbotone or diazepam. The GABA-Cl channels of vertebrates may be activated by two molecules of GABA and positively coupled with pentobarbitone and/or diazepam receptors (upper panel) (Olsen, 1981). In molluscan neurones, one molecule of GABA may bind to activate the Cl channels which have no diazepam receptor and are negatively coupled with the pentobarbitone receptor (lower panel).

Appendix

Let us assume first that only one class of GABA gated Cl channel exists. The transition states between the non-desensitized states (activatable closed state(s) and open state(s)) are reversible in general, and are rapid enough compared with the desensitization processes (cf. Figures 3 and 4). Then, the channel in the non-desensitized states are in quasi-equilibrium with each other and the propor-

tion of the channel in each non-desensitized state is nearly the same as those in true equilibrium (Colquhoun & Hawkes, 1977; Kijima & Kijima, 1982; 1983).

When a definite concentration, C, of GABA is applied, the proportion of the channels in the open state to those in the non-desensitized states is constant at any time, t, after the peak response (at t = 0), because the channels in the non-desensitized states are in quasi-equilibrium. Thus, the following relations hold:

$$\frac{N_o(t, C)}{N_n(t)} = \frac{N_o(0, C)}{N_n(0)} = K(C) \qquad (A\text{-}1)$$

or

$$\frac{N_n(t)}{N_n(0)} = \frac{N_o(t, C)}{N_o(0, C)} = L(t) \qquad (A\text{-}1')$$

where, $N_n(t)$ and $N_o(t, C)$ are the number of channels in the non-desensitized state(s) and open state(s) at time, t, after the peak, respectively. $K(C)$ is a constant dependent on C and $L(t)$ is a constant independent of C, but dependent on t.

In the experiment in Figure 6,

$$q/Q = \frac{N_o(t, 5 \times 10^{-6})}{N_o(0, 5 \times 10^{-6})} = \frac{N_n(t)}{N_n(0)} = L(t) \qquad (A\text{-}2)$$

The current value P is proportional to the total number of non-desensitized channels, $N_n(0)$, and p is proportional to that at time, t, $N_n(t)$. Thus,

$$p/P = \frac{N_n(t)}{N_n(0)} = L(t) = q/Q \qquad (A\text{-}3)$$

Figure 6b shows that this relation (A-3) held throughout the time course of desensitization. This is consistent with the above assumption of a single class of channels.

Next, we assume that there are two classes of GABA-gated channels with different dose-response relations and desensitization time courses. Then, we can show that the relation (A-3) does not hold as follows.

Since both the dose-response relation and the desensitization time course are different,

$$\frac{N_o^{(1)}(t, C)}{N_n^{(1)}(t)} = K^{(1)}(C) \neq \frac{N_o^{(2)}(t, C)}{N_n^{(2)}(t)} = K^{(2)}(C) \qquad (A\text{-}4)$$

and

$$\frac{N_n^{(2)}(t)}{N_n^{(1)}(t)} = f(t) \qquad (A\text{-}5)$$

where numbers in parentheses designate the class of channels and f(t) is a function of time (not constant). Then,

$$q/Q = \frac{N_o^{(1)}(t, C_1) + N_o^{(2)}(t, C_1)}{N_o^{(1)}(0, C_1) + N_o^{(2)}(0, C_1)} = \frac{K^{(1)}(C_1)N_n^{(1)}(t) + K^{(2)}(C_1)N_n^{(2)}(t)}{K^{(1)}(C_1)N_n^{(1)}(0) + K^{(2)}(C_1)N_n^{(2)}(0)} \qquad (A\text{-}6)$$

and

$$p/P = \frac{K^{(1)}(C_2)N_n^{(1)}(t) + K^{(2)}(C_2)N_n^{(2)}(t)}{K^{(1)}(C_2)N_n^{(1)}(0) + K^{(2)}(C_2)N_n^{(2)}(0)} \qquad (A\text{-}7)$$

where $C_1 = 5 \times 10^{-6}$ M and $C_2 = 10^{-3}$ M. Under the conditions of (A-4) and (A-5),

$$p/P \neq q/Q \qquad (A\text{-}8)$$

Thus, the relation shown in Figure 6b does not hold, except in cases where the relative dose-response relations or the desensitization time courses of the two classes of channels happen to be the same (i.e. $K^{(1)}(C) = K^{(2)}(C)$ or $N_n^{(2)}(t)/N_h^{(1)}(t) =$ constant).

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