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'CONCENTRATION-CLAMP' STUDY OF γ-AMINOBUTYRIC-ACID-INDUCED CHLORIDE CURRENT KINETICS IN FROG SENSORY NEURONES

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SUMMARY

1. Kinetics of the activation and desensitization phases of γ-aminobutyric acid (GABA)-induced Cl^- current (I_{Cl}) were studied in single frog sensory neurones using the 'concentration-clamp' technique which enables perfusion of drugs with the time constant of about 3 ms.

2. Both activation and desensitization phases of GABA response consisted of a single exponential at low concentrations and a double exponential at high concentrations. The time constant of the fast kinetic component in each phase was relatively stable, about 5 ms for activation and 3 s for desensitization over concentrations from 3×10^{-5} to 3×10^{-4} M, whereas those of the slow kinetic component decreased with increasing concentrations. The two kinetic components in both phases showed the same reversal potential.

3. The slow and fast activation components recovered sensitivity from desensitization with different time courses: the recovery rate of the fast activation component was slow and that of the slow one, rapid.

4. The peak I_{Cl} elicited at GABA concentrations below 10^{-5} M increased disproportionally at more negative membrane potentials, thereby suggesting that the activation kinetics is voltage dependent.

5. The steady-state I_{Cl}–voltage relationship obtained with less than 10^{-5} M-GABA showed a non-linearity, probably due to voltage dependence of activation rather than that of desensitization kinetics.

6. These results suggest the presence of at least two different GABA receptor–Cl^- ionophore complexes with a different affinity and kinetics.

INTRODUCTION

Kinetic studies of the action of neurotransmitter substances are indispensable for elucidating underlying molecular mechanisms. Two complementary approaches have been used: perturbation techniques and fluctuation analysis. The perturbation studies require the production of a change in the average number of membrane

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channels that are opened by transmitter action; the change has to occur instantaneously, relative to the time scale of the process under investigation. The equilibrium between open and closed states of channels depends on agonist concentration and membrane potential. Rapid changes in the agonist concentration are difficult to produce, and neither of the two methods presently available is satisfactory. The first approach involves nerve stimulation and relies on the rapid removal of transmitter from the cleft, by diffusion and inactivation processes (Magleby & Stevens, 1972; Onodera & Takeuchi, 1976; Dudel, 1977; Kuba & Nishi, 1979; Rang, 1981; Segal & Barker, 1984*b*). The second method uses a ligand, Bis-Q, that has two conformations (*cis* and *trans*) with different affinities to the receptor. Rapid changes to the active *trans* isomer can be made by brief light flashes of appropriate wave-lengths (Lester & Chang, 1977; Weinstock, 1983). These two methods, however, have common disadvantages; the time course of the concentration transient is uncertain, and the study is limited to the case of simpler systems, such as the neuromuscular junction and the single ligand *trans*-Bis-Q.

There are also problems in the application of fluctuation analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973). First, this method can be used for studying the kinetics only during a steady-state response. However, the agonist response usually comprises rapid rising and slow decaying phases, the decaying phenomenon known as desensitization (Katz & Thesleff, 1957). Therefore, the steady-state kinetics may not reflect the kinetics during the activation and desensitization phases. Secondly, if the flucutation is produced by two or more distinct receptor–ion channel complexes, the result is not straightforward for a valid interpretation (Yasui, Ishizuka & Akaike, 1985).

We developed a 'concentration-clamp' approach which combines internal perfusion (Hattori, Akaike, Oomura & Kuraoka, 1984; Ishizuka, Hattori & Akaike, 1984) and rapid solution change techniques (Krishtal, Marchenko & Pidoplichko, 1983). With the approach, extracellular solution could be changed with the time constant of about 3 ms thereby facilitating a reliable and efficient means to study the kinetics of agonist-induced ionic currents in the activation phase. To analyse the kinetics of γ-aminobutyric acid (GABA)-activated Cl^- current (I_{Cl}) we used the frog dorsal root ganglion cells. This current is thought to play a role in the inhibitory mechanism in the mammalian central nervous system (Krnjević, 1974). We now report that there are at least two components during the activation and desensitization phases of I_{Cl}, as induced by GABA concentrations over 6×10^{-5} M.

METHODS

Preparation. Dorsal root ganglia dissected from the decapitated bull-frog (*Rana catesbiana*) were used throughout the experiments. The thick connective tissue surrounding the ganglion was carefully stripped off with micro-forceps and the capsules enveloping the ganglion masses were digested in 10 ml normal Ringer solution containing 0·3 % (w/v) collagenase and 0·05 % (w/v) trypsin at pH 7·4 for about 18 min at 37 °C. During the enzyme treatment, the preparation was shaken gently by bubbling 99·9 % O_2. Thereafter, single cells were isolated from the ganglion mass, with finely polished pins, under binocular observation, and left overnight at room temperature (about 20 °C) in a culture medium consisting of equal parts of Ringer solution and Eagle MEM (Nissui, Japan).

Solution. To isolate the I_{Cl} from all other current components, Na^+, K^+, and Ca^{2+} were replaced

by tris(hydroxymethyl)aminomethane ($Tris^+$), Cs^+ and Mg^{2+} in both external and internal solutions. In previous experiments (Akaike, Hattori, Inomata & Oomura, 1985) where the intracellular and extracellular Cl^- concentrations were 15 and 120 mM, a shift in the reversal potential for GABA-induced Cl^- response (E_{GABA}) during GABA responses was found to strongly influence the time course of the desensitization onset. Therefore, both the intracellular and extracellular Cl^- concentrations were kept at 120 mM in the present study. The ionic compositions of the solutions were (in mM): internal, CsCl, 95; Cs aspartate, 10; TEA Cl, 25; EGTA, 2·5; external, Tris Cl, 83; CsCl, 2; $MgCl_2$, 5; TEA Cl, 25; 4-aminopyridine, 3; glucose, 5. The pH of all solutions was adjusted to 7·4 with Tris base or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES).

Suction pipette. Single cells sucked into a tapered capillary with a fire-polished tip, diameter about 50 μm, were transferred into a dish 3 cm in diameter. Single cells were clearly visible under binocular magnification of 80 ×. A suction-pipette technique was used for voltage clamp and internal perfusion (Hattori *et al.* 1984; Ishizuka *et al.* 1984; Akaike *et al.* 1985). A Pyrex glass with a 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. Part of an individual neurone (30–40 μm diameter) was aspirated by the suction pipette with negative pressure of about 30 cmHg. The aspirated membrane was ruptured spontaneously or was broken by applying 5–20 nA square-wave pulses of depolarizing current (10–50 ms). Thereafter, the neurone was internally perfused at a constant flow rate of 1 ml/min. Adequacy of the internal perfusion with the present suction-pipette technique was evaluated by determining how close the reversal potential for GABA-induced Cl^- response (E_{GABA}) was to the Cl^- equilibrium potential (E_{Cl}), about +4 mV, calculated from the Nernst equation based on the Cl^- activity in the external and internal solutions.

Electrical measurements. The membrane potential was measured through an Ag–AgCl wire in a Ringer–agar plug mounted on the suction pipette holder. The reference electrode was also an Ag–AgCl wire in a Ringer–agar plug as shown in Fig. 1. The resistance between the suction pipette filled with standard internal solution and the reference electrode was 200–300 kΩ. Both electrodes were led to a voltage-clamp circuit, and the membrane potential was controlled by a single-electrode voltage-clamp system switching at a frequency of 10 kHz and passing current for 36 % of the cycle (Ishizuka *et al.* 1984). Clamp currents were measured as the voltage drop across at 10 MΩ resistor in the feed-back path of a 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. Both current and voltage were monitored on a digital storage oscilloscope (National, type VP-5730A), and were simultaneously recorded on an ink-writing recorder (Rikadenki, type R-22) and stored on an FM data recorder. During the steady state of each drug-induced response, the current–voltage (*I–V*) relationship was recorded 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. These pulses were obtained by using a function generator (Kikusui, type 459AL). The resultant data were plotted directly on an *X–Y* recorder (National, type 6400A), which allowed an instant check of the ongoing experiment.

Application method. The 'concentration-clamp' method was developed to enable an extremely rapid application or switching of external solution containing agonist (Fig. 1). The cell-attached tip of the suction pipette was inserted into a plastic tube through a circular hole of approximately 1 mm diameter. The lower end of this tube could be exposed directly to external solutions by moving up and down the stage where drug-containing dishes were. The suction (−15 cmHg) applied to the upper end of the tube was controlled by an electromagnetic valve, which could be driven by 24 V d.c. The power supply was switched on for a desired duration by a stimulator (Nihon Kohden, type SEN-7103). The speed of solution exchange could be estimated from the time lag of current shift under the voltage-clamp condition after the change of external potassium concentration from 2 to 85 mM or vice versa using the 'concentration-clamp' technique. Fig. 1 shows one of the typical current shifts measured in sensory neurones having 30–40 μm diameter. The measured time constants varied between 2 and 4 ms, and any mechanical artifacts did not contaminate the current recordings in these conditions. This suggests that the time constants (more than about 5 ms) which were observed in this experiment faithfully reflect the true nature of interaction of GABA and its receptor.

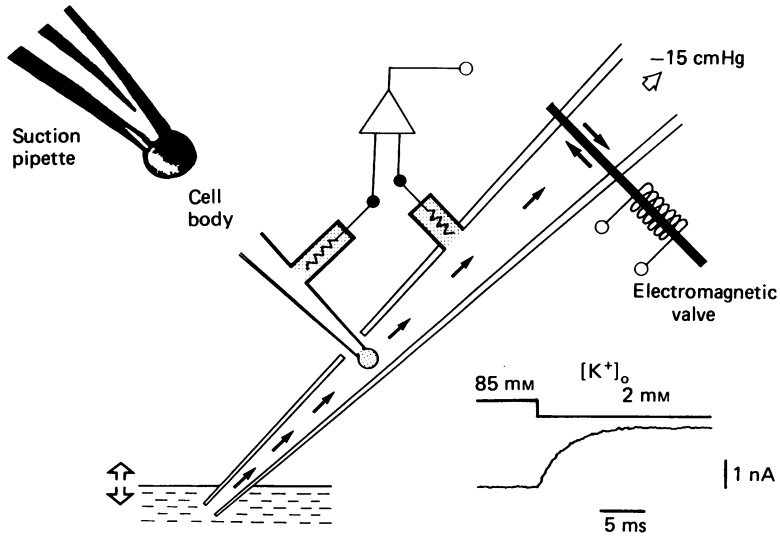

Fig. 1. The experimental arrangement. Part of the individual neurone was aspirated off by a suction pipette and the pipette was then inserted into the small tube through a hole. Electrical measurements were performed between the agar bridges (dotted part), using the single-electrode voltage-clamp technique. The solution in the tube was changed rapidly with suction (−15 cmHg), which was controlled by an electromagnetic valve. Inset shows that the holding current shifted to the new value with the time constant of about 3 ms when external K^+ concentration ($[K^+]_o$) decreased from 85 to 2 mM. The holding potential (V_H) was −70 mV.

RESULTS

Fast and slow kinetic components and their dose–response relationship

When GABA was applied by the concentration-clamp technique, the time course of I_{Cl} showed either one or two exponentials, depending on the GABA concentration (Fig. 2*A*). The I_{Cl} produced by 6×10^{-6} M-GABA followed a single exponential (Fig. 2*B*), while the time course in the case of more than 10^{-5} M-GABA followed a double exponential, except that the initial part of the response had a shoulder of variable amplitude (Fig. 2*C*). The extrapolation toward zero time of the line responsible for the slow kinetics gives the value of slow component and the value of fast kinetic component was calculated by subtracting the slow component from the total, since the extrapolation of the fast kinetic line resulted in an over-estimate because of the variable shoulder at the beginning. The experimental points for the relationship between GABA dose and relative peak I_{Cl} fell close to the theoretical curve for the Hill number of 2·0 and an association constant (K_a) of 10^{-5} M (Fig. 3 upper). The dose–response relationships were obtained separately by kinetic analysis and the results are shown in Fig. 3 lower: the slow component (▲) was activated by less than 5×10^{-6} M-GABA and reached a maximum above 3×10^{-5} M while the fast component (●) appeared only at concentrations above 10^{-5} M-GABA.

Voltage dependence of I_{Cl} activation and I–V relationship

The action of acetylcholine (ACh) at the end-plate of frog muscle is voltage

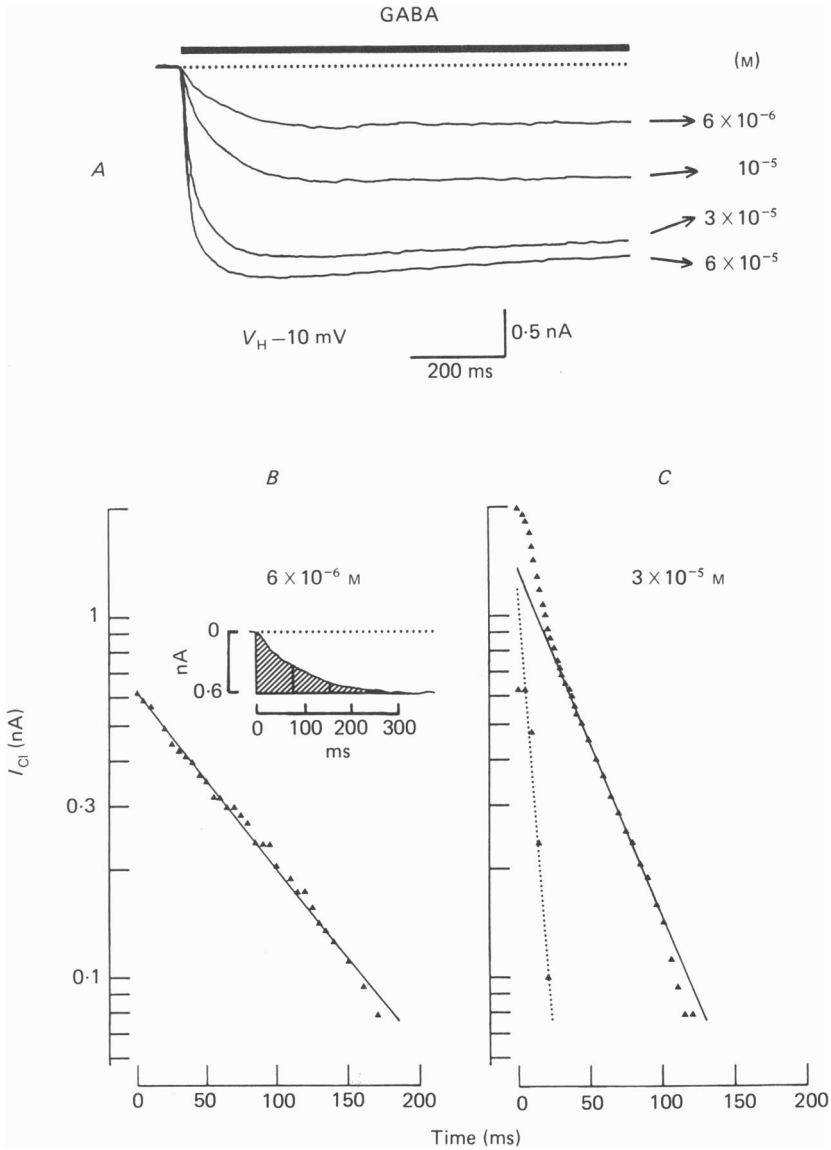

Fig. 2. The GABA-induced I_{Cl} recording as a function of GABA concentration (*A*), and two semilogarithmic plots of the rising phase of I_{Cl} produced by 6×10^{-6} M (B) and 3×10^{-5} M-GABA (*C*). In *A*, I_{Cl} flows into the cell so that Cl^- move outward. In *B* and *C*, $I_{Cl}(p) - I_{Cl}(t)$ are shown against time t (inset of *B*), where $I_{Cl}(p)$ denotes the maximum current at the peak and $I_{Cl}(t)$ was measured at time t after the beginning of the response. I_{Cl} can be fitted by a single exponential at 6×10^{-6} M-GABA and by a double exponential at 3×10^{-5} M-GABA.

dependent, as shown by the perturbation technique (Magleby & Stevens, 1972) and noise analysis (Anderson & Stevens, 1973). The GABA-operated Cl^- channel also has such a voltage dependence but its lifetime decreases with hyperpolarization, in contrast with that of ACh-activated cation channels (Onodera & Takeuchi, 1976; Dudel, 1977; Dudel, Finger & Stettmeier, 1980; Cull-Candy & Miledi, 1981; Segal

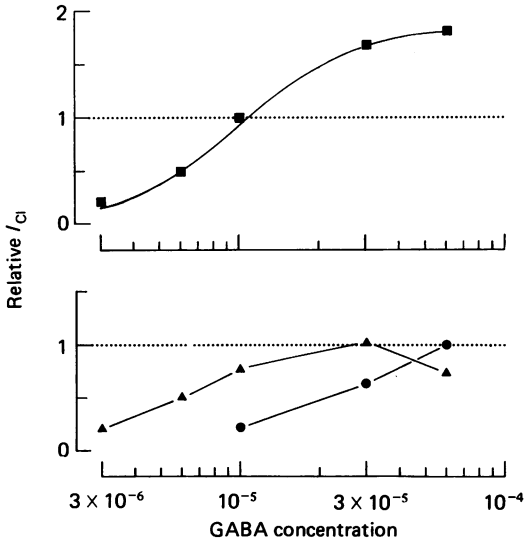

Fig. 3. Dose–response relationships: upper, dose–response plot of Fig. 2*A* and a theoretical fitting (Hill number, 2 and K_a, 10^{-5} M), where the response is the total current (■). Lower, dose–response relationships of the fast (●) and slow (▲) components which constitute the total response. The two components were determined from semilogarithmic plots of the activation phase (Fig. 2).

& Barker, 1984*a*,*b*). This nature of the GABA-operated Cl^- channel may account for the non-linear GABA-produced maximum *I–V* relationship under certain conditions (Fig. 4) (Cull-Candy & Miledi, 1981). The relationship in question obtained with 10^{-4} M-GABA was linear. As the GABA concentration was decreased, however, I_{Cl} at the response peak became progressively smaller than the estimate based on the assumption that the peak conductance change was constant (dotted line). This discrepancy means that the voltage dependence was enhanced as the membrane potential became more negative as well as when the GABA concentration was reduced.

The semilogarithmic plots of the activation I_{Cl} phase (Fig. 5*A*) produced by 3×10^{-5} M-GABA at five different membrane potentials reveal slow and fast components. The total, slow and fast currents are each seen to follow a straight line which consistently crosses the abscissa at E_{GABA} (Fig. 5*B*). The time constants of the fast and slow components denoted by $\tau_{m,f}$ and $\tau_{m,s}$ respectively showed very little voltage dependence in the present case of 3×10^{-5} M-GABA (Fig. 5*C*).

Activation time constants and GABA concentration

The time constant, τ_m, was determined at various concentrations of GABA. The fast components could be observed in all cases, at concentrations over 6×10^{-5} M-GABA and in some cases at lower levels. The plot of $\tau_{m,f}$ and $\tau_{m,s}$ at concentrations ranging from 6×10^{-6} to 3×10^{-4} M (Fig. 6) shows that $\tau_{m,s}$ had a definite concentration dependence whereas such was not as apparent with $\tau_{m,f}$.

Voltage dependence of desensitization onset

The amplitude of GABA-induced I_{Cl} decreased to a steady-state level when GABA was applied continuously. The time course of this decay phase could be fitted by either

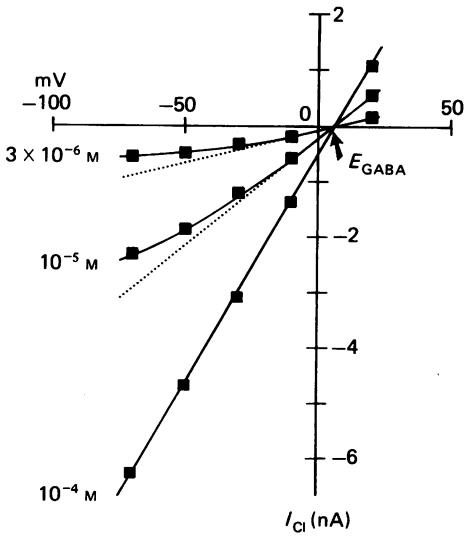

Fig. 4. Peak I_{Cl}–V relationships with 3×10^{-6}, 10^{-5} and 10^{-4} M-GABA. The three straight lines (two dotted, one continuous) are drawn through $E_{GABA} = +4$ mV. A marked voltage dependence developed as the concentration decreased and the membrane potential hyperpolarized.

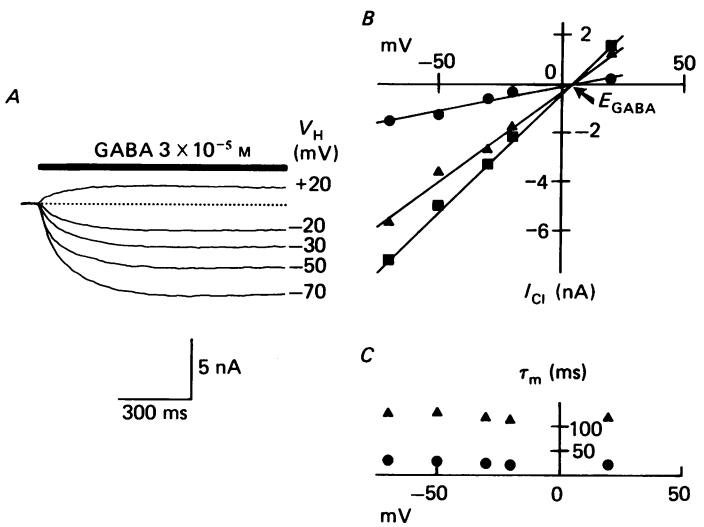

Fig. 5. Voltage dependence of activation time constants. *A*, I_{Cl} recordings at various membrane potentials. *B*, I_{Cl}–V relationships of the total peak current (■) and the slow (▲) and fast (●) components. Each plot is seen to cross the abscissa at the E_{GABA} (+4 mV). *C*, activation time constants as a function of membrane potential. $\tau_{m,f}$ and $\tau_{m,s}$ represent the fast and slow components, respectively; and they remained relatively constant over the voltage range examined.

a single or double exponential, depending on the concentration of GABA. The semilogarithmic plot of this time course at 3×10^{-5} M-GABA (inset in Fig. 7*A*) indicates a double exponential. The *I–V* relationships for the total, slow and fast currents all crossed the abscissa at the estimated E_{Cl} of 4 mV (Fig. 7*B*), as was the

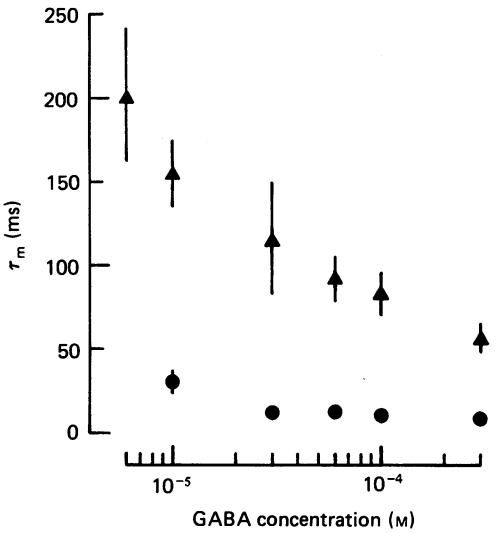

Fig. 6. Activation time constants as a function of GABA concentration. Mean (data points) and S.E. (bars) of mean. $\tau_{m,s}$ of the slow component (▲) showed a marked concentration dependence. The fast component (●) was observed in all neurones with over 6×10^{-5} M-GABA, in four of nine neurones (44 %) with 10^{-5} M-GABA and in two of six neurones (33 %) with 6×10^{-6} M-GABA.

case with activation. The time constants for the fast and slow desensitizing components, $\tau_{h,f}$ and $\tau_{h,s}$, seemed to be independent of membrane potential over the range from -80 to -10 mV (Fig. 7*C*), even though a hyperpolarizing shift of E_{GABA} observed during the steady-state GABA response became larger as the membrane potential became more negative (Fig. 7*D*).

The voltage dependence of the onset of desensitization was studied in detail for the response to 10^{-5} M-GABA, since the voltage dependence of the activation kinetics was revealed only by a low concentration of GABA (Fig. 4). The peak I–V relationship showed a marked voltage dependence at membrane potentials more negative than -20 mV (Fig. 8*B*). At -60 mV, the peak current was reduced to 82 % of the value which would be obtained if there were not voltage dependence (straight line) prediction in Fig. 8*B*. The similar reduction occurred with respect to the steady-state I_{Cl} (Fig. 8*B*); after correcting for the E_{GABA} shift (Fig. 8*D*), the steady-state current at -60 mV was about 75 % of the linear prediction. It can be said, therefore, that the voltage dependence of desensitization onset is either very slight or nil. The slow and fast desensitization time constants, $\tau_{h,f}$ and $\tau_{h,s}$, both remained relatively constant against changes in membrane potential (Fig. 8*C*).

Concentration dependence of desensitization onset

The onset of desensitization was accelerated as the GABA concentration was increased. Records of I_{Cl} elicited by GABA at various concentrations were collected while holding the membrane potential at -10 mV to prevent the shift of E_{GABA}. We found that the peak current increased in a sigmoidal fashion (Fig. 9*A*) over the range of 6×10^{-6}–10^{-4} M, whereas the steady-state Cl^- reached a saturating level at around 10^{-5} M. With regard to the desensitization time constants, $\tau_{h,s}$ of the slow component

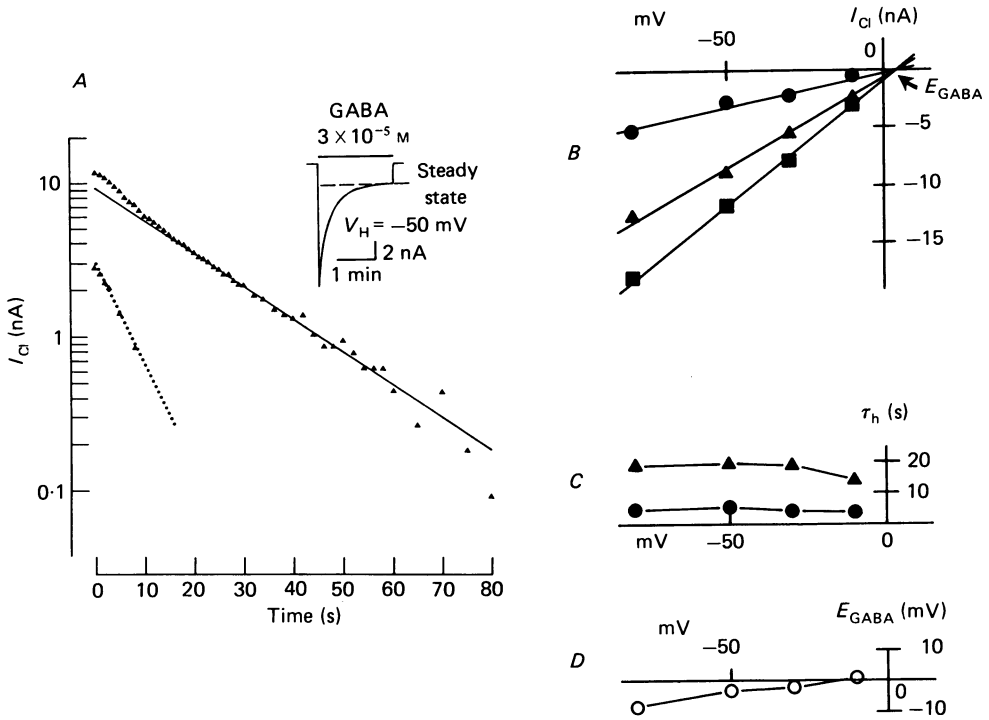

Fig. 7. Quantitative analysis of the desensitization. *A*, semilogarithmic plot of the decay to the steady state, indicating a double-exponential manner of the decay. Inset, an I_{Cl} trace recorded at −50 mV elicited by 3×10^{-5} M-GABA. *B*, linear I_{Cl}–V relationships of the total current (■) and the slow (▲, $\tau_{h,s}$), and fast (●, $\tau_{h,f}$) components. Each case is seen to have an E_{GABA} of 4 mV. *C*, desensitization time constants, $\tau_{h,f}$ (●) and $\tau_{h,s}$ (▲), at various membrane potentials; they were not dependent on the membrane potential. *D*, shift of E_{GABA} at the steady-state GABA response. E_{GABA} was estimated from the intercept between the two I_{Cl}–V curves; one obtained using a triangular voltage pulse during the control before GABA application and the other during the steady-state GABA response.

was markedly concentration dependent while $\tau_{h,f}$ (fast component) was not (Fig. 9*B*). Desensitization produced by GABA levels less than 3×10^{-6} M was too slow and small for an accurate analysis. The fast component of desensitization was present in all records with concentrations over 6×10^{-5} M but was found in only three of twenty-three tested cases with 10^{-5} M-GABA. Thus, the relevant threshold concentration is thought to be between 10^{-5} M and 6×10^{-5} M.

Recovery process from desensitization

Ionophoretic application is used to study the recovery from desensitization as bath application is not suited for examining the early phase of recovery that probably occurs in a few tens of seconds (Katz & Thesleff, 1957; Adams, 1975; Feltz & Trautmann, 1982). The experiments cited above indicate that the recovery process depends neither on the amount of agonist nor on the degree of desensitization; rather, the recovery is influenced by the time duration of agonist application. Therefore, we examined the recovery process by keeping a constant value for the duration of GABA

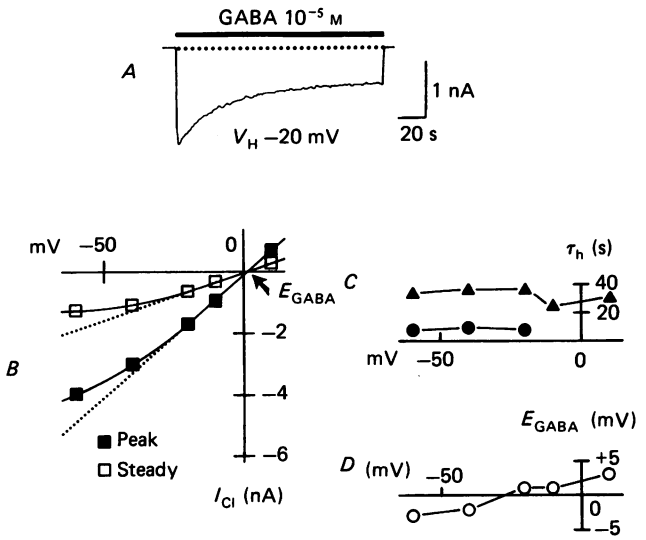

Fig. 8. Voltage dependence of the desensitization. *A*, I_{Cl} elicited by 10^{-5} M-GABA at −20 mV. *B*, peak and steady-state I_{Cl}–V relationships. The dotted straight lines are drawn through the E_{GABA} (+2 mV) point. *C*, desensitization time constants, $\tau_{h,f}$ (●) and $\tau_{h,s}$ (▲), of the fast and slow components, respectively. They are seen to be nearly constant over the voltage range tested. *D*, shift of E_{GABA} at the steady-state GABA response.

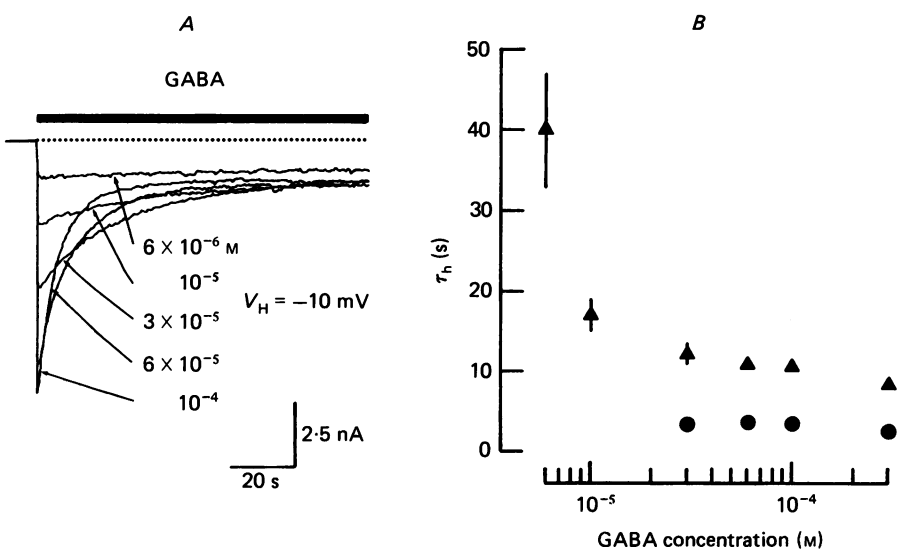

Fig. 9. Concentration dependence of the desensitization. *A*, I_{Cl} elicited by various GABA concentrations at −10 mV. The peak I_{Cl} increased sigmoidally with increasing GABA concentration, whereas the steady-state I_{Cl} reached a maximum already around 10^{-5} M-GABA. *B*, desensitization time constants ($\tau_{h,f}$ (●) and $\tau_{h,s}$ (▲)) *versus* GABA concentration; mean over ten neurones and S.E. of mean. $\tau_{h,s}$ of the slow desensitization component showed a marked concentration dependence.

application as well as for the GABA concentration. Fig. 10*A* shows I_{Cl} responses recorded after various GABA-free intervals from virtually complete desensitization resulting from a continuous GABA application for 3 min. The semilogarithmic plot of the activation phase revealed that the slow and fast activation components recovered their sensitivity, with different time courses (Fig. 10*B*). The fast component was detectable only after more than 15 s from the complete desensitization. The

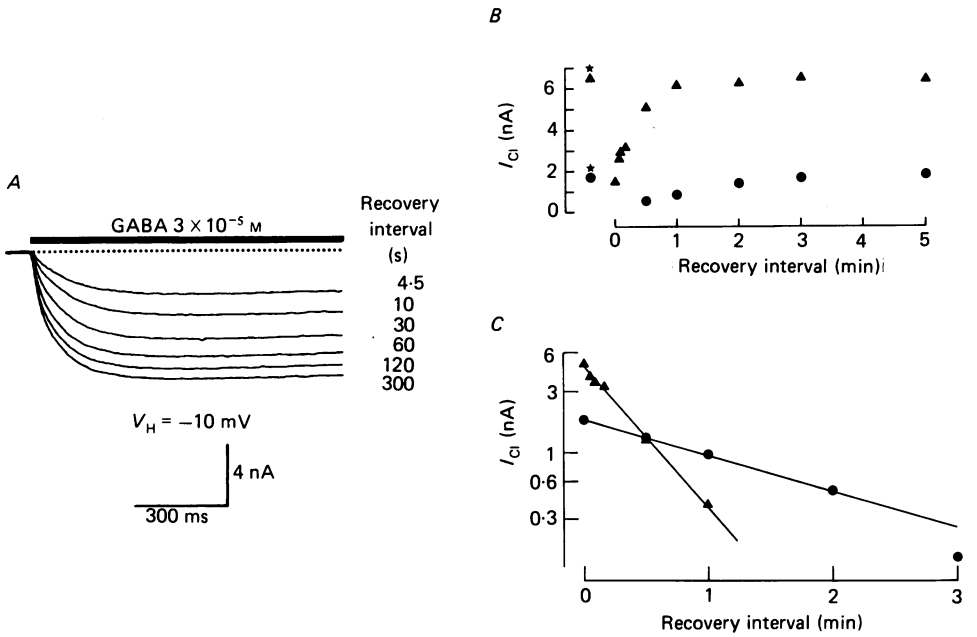

Fig. 10. Recovery from the desensitization. *A*, I_{Cl} elicited by 3×10^{-5} M-GABA after various GABA-free intervals following a complete desensitization, which was accomplished by 3 min application of GABA. *B*, recovery process of the slow (▲) and fast (●) activation components, as expressed in their sensitivity, determined from the semilogarithmic plot (Fig. 2) of the activation phase. The fast activation component was not detectable before the recovery interval of about 10 s. Stars mark the control value for the fast and slow activation components. *C*, semilogarithmic plot of $I_{Cl}(max) - I_{Cl}(t)$ against the time t where $I_{Cl}(max)$ is the control current without desensitization and $I_{Cl}(t)$ the current recorded after GABA-free interval t from desensitization, and the steady-state I_{Cl} is assumed to reflect the slow component only.

control responses are marked by stars for the slow and fast components. The semilogarithmic plot of Fig. 10*C* was obtained from *B* by assuming that the steady-state I_{Cl} reflected only the slow component. It follows from this plot that the fast and slow components of activation, respectively, take about 92 and 23 s (in time constant) to recover from desensitization. These values in turn are consistent with the assumption of the steady state representing the slow component, since the ratio (0·003) of $\tau_{h,f}$ (3 s) of the fast kinetic component to the recovery time constant (92 s) approximates the proportion of I_{Cl} at the steady state to the peak value.

DISCUSSION

The bath perfusion method of drug application will not always yield a valid dose–response relationship or activation kinetics, since a significant amount of desensitization may occur before the drug equilibrates with the receptors. Another conventional method, ionophoretic application, also has a problem in that the concentration may very well be a complicated function of space and time. This makes it difficult to quantify the effective amount of a drug delivered from the pipettes. In the present work, we used the concentration-clamp technique which we developed and which reduces problems caused by other approaches regarding concentration and speed of solution exchange. The method allows drugs to equilibrate with receptors with time constants of about 3 ms. This means that aspects of activation kinetics with a time scale greater than about 5 ms should be detected.

Two distinct GABA receptor–ionophore complexes

In general, two components were observed in the activation and decay phases of 3×10^{-5} M-GABA-produced responses. This suggests the possibility that the activation and desensitization phases were not mutually independent, in terms of their respective components. In fact, this view was supported by the experiment on the recovery from desensitization; the fast kinetic component observed in the activation phase desensitizes at a fast rate while the slow kinetic one decays at a slow rate. There appear to be at least three hypotheses for these kinetics. (1) The kinetics could be an artifact resulting from inadequate control of the membrane potential. (2) The two components might represent distinct types of GABA-operated ionic channels. (3) There might be a single receptor–ionophore complex with a complex pattern of opening and closing. Hypothesis (1), that a recording artifact was responsible, is unlikely for several reasons. (a) The reversal potential was the same for both components, suggesting that the membrane potential was well held at the same level. (b) The activation and decaying phases were accurately fitted by two exponential components and did not consist of a fast phase followed by an indeterminate tail. In addition, the desensitization onset proceeded in the sequential order of the fast and slow phases, as in the rising phase. (c) The time constant of each component was fairly constant over the range of membrane potential examined. We conclude, therefore, that the recorded current faithfully represented the transmembrane current at the cell soma. This places us in a position to consider whether the complex characteristics resulted from activation of the two types of receptor–ionophore complexes or from the property of a single type. In attempting to distinguish between hypothesis (2) (two distinct types of GABA receptor–ionophore complexes) and hypothesis (3) (a single type of complex controlled by a multi-step reaction schema), a recapitulation of our study on the recovery is warranted. As shown in Fig. 10, the fast kinetic component took more time for the recovery than did the slow one. This point seems to strongly support hypothesis (2). Furthermore, data from patch-clamp experiments using the same preparation agree with hypothesis (2) in that the distribution of GABA-activated single Cl^- conductances was composed of at least two groups (Yasui *et al.* 1985).

Activation kinetics

The GABA-induced Cl^- conductance (i.e. the slope of the I–V curve) decreased progressively with membrane hyperpolarization (Figs. 4 and 8). Furthermore, this voltage dependence became more evident when the GABA concentration was low. At concentrations higher than 3×10^{-5} M, however, the peak current increased linearly with the membrane potential range examined (Figs. 5*B* and 7*B*). This concentration-dependent voltage dependence can be explained in the following manner. Consider simple kinetics relating the open and closed states;

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O, \tag{1}$$

where C and O represent the closed and open states, respectively, α and β are the closing and opening rate constants, respectively. It follows from eqn. (1) that

$$P = \frac{\beta}{\alpha+\beta} \tag{2}$$

at the equilibrium condition. Here, P denotes the probability for a channel in the open state (the whole-cell current is proportional to P). As is the case of the ACh-activated channel at the end plate (Magleby & Stevens, 1972; Anderson & Stevens, 1973), we assume that β increases with GABA concentration, and that α depends on membrane potential but not on concentration. In a variety of preparations, the decay of GABA-mediated synpatic current (whose time constant is inversely proportional to α) is facilitated at hyperpolarized membrane potentials, implying the corresponding increase in the closing rate constant (Onodera & Takeuchi, 1976; Dudel, 1977; Segal & Barker, 1984*b*). If such is the case in our present set-up, then membrane hyperpolarization should increase α and thus decrease P, which in turn should decrease the membrane conductance. At higher GABA concentrations, however, we have

$$\alpha \ll \beta \left(\text{or } \frac{\beta}{\alpha+\beta} \approx 1 \right),$$

which means from eqn. (2) that the open-state probability is close to unity regardless of the α value. This explains the linear I–V relationships observed at more than 3×10^{-5} M-GABA.

Desensitization kinetics

The desensitization process produced by ACh in the frog end-plate is affected by the membrane potential and the extracellular Ca^{2+} concentration (Magazanik & Vyskocil, 1970; Scubon-Mulieri & Parsons, 1977, 1978; Chesnut, 1983). On the other hand, Bregestovski, Bukharaeva & Iljin (1979) studied the ACh-induced I_{Cl} of *Aplysia* neurones and observed that the decay rate of the ACh response was related neither to the membrane potential, nor to the extracellular Ca^{2+} concentration. They, therefore, concluded that the voltage- and the Ca^{2+}-dependent nature of the desensitization process is due not to the ACh receptor, but to the cation channel activated by ACh. Our experimental data favours their conclusion in that the

non-linear *I–V* relationship of our steady-state 10^{-5} M-GABA response may be ascribed to the voltage dependence of activation kinetics, but not to that of desensitization kinetics.

The agonist concentration is also one of the factors influencing the time course of the desensitization process. Similar to our result, recent experiments have shown that ACh-induced desensitization in the end-plate is comprised of two phases; i.e. the fast and slow kinetic ones (Anwyl & Narahashi, 1980; Feltz & Trautmann, 1980; Chesnut, 1983). The effect of agonist concentration on these two components is not clear. Anwyl & Narahashi (1980) noted a marked facilitation of the slow component with increasing ACh concentrations, while Chesnut (1983) showed that the decay of the fast component was facilitated at a higher ACh concentration.

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