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AN ANALYSIS OF THE INHIBITORY POST-SYNAPTIC CURRENT IN THE VOLTAGE-CLAMPED CRAYFISH MUSCLE

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SUMMARY

1. Inhibitory post-synaptic currents (i.p.s.c.s) were recorded from the feed-back current through a wire electrode inserted longitudinally into the opener muscle fibre of the claw in the crayfish (*Cambarus clarkii*).

2. I.p.s.c. rose to its peak in about 3–4 msec and decayed approximately exponentially. The decay time constant at -100 mV was 9·4 msec.

3. The decay time constant decreased as the membrane was hyperpolarized and increased during depolarization. The time constant (τ) depends on voltage (V) according to the relation $\tau = a \exp (AV)$, with $a = 18{\cdot}6$ msec and $A = 0{\cdot}0065\ \mathrm{mV}^{-1}$. Voltage dependence was opposite in direction to that seen at frog end-plates, but in the same direction as that of e.p.s.c. in crayfish muscle.

4. At lower temperatures, the rise and fall times of i.p.s.c.s were prolonged. Q_{10} for the decay time constant was 2·4 between 22·6 and 12·5 °C.

5. When pH was decreased from 7·2 to 5·5, the decay time constant increased by about 50%, with little change in the voltage dependence of the time course.

6. When chloride in the solution was changed to iodide, the decay time constant was increased by a factor of 3, while voltage dependence of the time course was not changed. In bromide solution the decay time constant increased by about 50%.

7. Peak amplitudes of i.p.s.c.s were approximately linear as the membrane was depolarized, but they levelled off as the membrane was hyperpolarized beyond the reversal potential. The non-linear I–V relation did not result from inadequate voltage clamping, nor from a change in the inside concentration of chloride. After equilibration with iodide solution the I–V relation was approximately linear.

8. The decay time constant was increased after repetitive nerve stimulation. This prolongation became more pronounced at lower temperatures.

9. The kinetic process of the transmitter action is discussed. It is suggested that the rate limiting process for i.p.s.c. is binding and unbinding of the transmitter to the receptor.

INTRODUCTION

Kinetics of the transmitter action has been most extensively studied in excitatory synapses, particularly in vertebrate neuromuscular junctions (for review see Gage, 1976). At inhibitory synapses the same principles seem to be involved. However, relatively little is known about time course of the inhibitory synaptic current. This

is largely due to difficulties in measuring the inhibitory post-synaptic current (i.p.s.c.). (*a*) The reversal potential of i.p.s.c. is close to the resting potential and therefore the i.p.s.c. must be recorded at membrane potentials some distance away from the resting potential. (*b*) In muscles inhibitory synapses tend to be diffusely distributed over the cell surface, while in ganglion cells they are at some distance from the cell body where the voltage clamp is applied. Despite these difficulties i.p.s.c. has been recorded in several synapses, including *Onchidium* neurones (Hagiwara & Kusano, 1961), spinal motoneurones of the frog (Araki & Terzuolo, 1962) and buccal ganglia of *Aplysia* (Adams, Gage & Hamill, 1976). In the present experiments the opener muscle of the crayfish claw was cannulated with a longitudinal metal electrode and the membrane potential was voltage-clamped (Onodera & Takeuchi, 1975). This enables one to clamp the muscle fibre membrane uniformly over its entire length, so that the diffusely distributed inhibitory synapses are clamped at the same membrane potential.

The crustacean neuromuscular junction is suitable for the study of the inhibitory synapse, because the inhibitory mechanism has been well investigated (Fatt & Katz, 1953; Boistel & Fatt, 1958; Dudel & Kuffler, 1961*b*) and the inhibitory transmitter has been identified as γ-aminobutyric acid (GABA) (for reviews see Gerschenfeld, 1973; Kuffler & Nicholls, 1976). It has been observed that the time course of the inhibitory post-synaptic potential (i.p.s.p.) at the crustacean neuromuscular junction is several times longer than that of the excitatory post-synaptic potential (e.p.s.p.) (Fatt & Katz, 1953; Dudel & Kuffler, 1961*b*; Takeuchi & Takeuchi, 1965). When L-glutamate and GABA are applied ionophoretically to the crayfish muscle the rise and fall of the GABA potential is about five times slower than that of the glutamate potential (Takeuchi & Takeuchi, 1965). It seems therefore that the slow time course of i.p.s.p. is not due to prolonged transmitter release, but to different kinetic processes in the post-synaptic mechanism.

Preliminary accounts of some of the results described here have already appeared (Onodera & Takeuchi, 1976). While the manuscript of this paper was being prepared Dudel's work appeared (Dudel, 1977), which was done with different experimental procedures.

METHODS

The opener muscle of the dactylpodite of the claw in the crayfish (*Cambarus clarkii*) was cannulated longitudinally with a stainless-steel wire of 70 μm in diameter and the voltage clamp method was employed (Onodera & Takeuchi, 1975). The potential changes were recorded differentially between an intracellular micro-electrode and a KCl-filled micro-electrode placed in the bath. The intracellular micro-electrode was filled with 3 M-KCl or 0·6 M-K_2SO_4. The inhibitory axon was dissected at the meropodite and stimulated with a pair of silver electrodes. The stimulation frequency was usually 10 Hz and was decreased to 5 Hz when i.p.s.c.s. were recorded at lower temperatures or in iodide solution. When the membrane potential was voltage-clamped at the resting potential, the synaptic potential was reduced to less than 5% of its original amplitude. Membrane potentials and membrane currents were displayed on the oscilloscope for photography and were also stored on an FM tape recorder (with 5 kHz bandwidth) for later analysis. The time course of i.p.s.c.s. was measured by averaging four to eight single records from the tape and displaying them on the *X-Y* recorder. In order to change the membrane potential a command voltage of about 2 sec in duration was applied to the summing point of the feed-back amplifier. Each time before and after changing the membrane potential, control i.p.s.c.s. were measured at the holding potential.

The bath solution flowed continuously throughout the experiment. The standard solution used was (mM): NaCl 207·5; KCl, 5·4; $CaCl_2$, 18·8 and the pH was adjusted to 7·2 with Tris maleate buffer. When the pH of the solution was altered, it was increased to 9 by Tris maleate and lowered to 5·5 by MES (2(N-morpholino) ethanesulphonic acid). When the effect of foreign anions was studied, NaCl in the solution was replaced with equimolar Na salt of various anions. In some experiments dantrolene sodium (1-[5-(*p*-nitrophenyl) furfurylidineamino] hydantoin) (6 μg/ml.) was added to the solution to reduce the muscle contraction which occurred when the membrane potential was depolarized below about −50 mV (Odette & Atwood, 1974). The experiments were performed at room temperature (23 °C). When the bath temperature was lowered, chilled saline solution was perfused and the temperature was measured with a small thermistor placed in the bath.

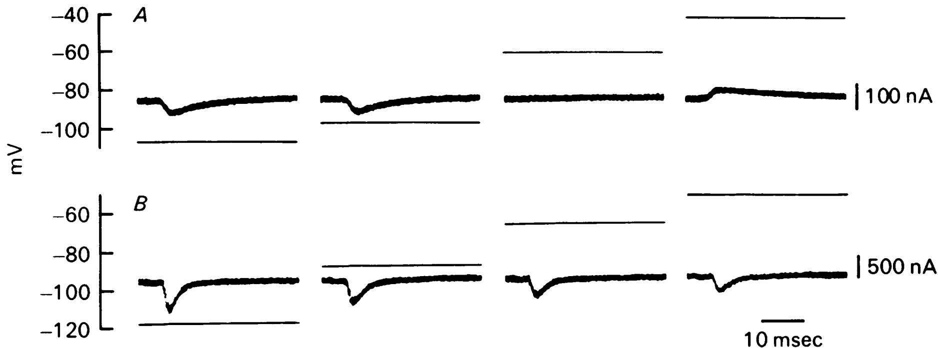

Fig. 1. I.p.s.c. and e.p.s.c. recorded at different membrane potentials. *A*, i.p.s.c.; *B*, e.p.s.c. Inward current is shown as a downward deflexion. Stimulus was applied at 10 Hz and about three traces were superimposed.

RESULTS

Inhibitory post-synaptic current

After the muscle fibre was cannulated with a longitudinal wire electrode, the membrane potential was clamped near the resting potential and the inhibitory nerve was stimulated. Direction and size of i.p.s.c. differed from fibre to fibre depending on the resting potential and the stimulation frequency. Fig. 1*A* illustrates the clamped membrane potentials and i.p.s.c.s. The inward current was indicated as a downward deflexion. The i.p.s.c. reversed its sign at −62 mV. Fig. 1*B* also shows e.p.s.c.s recorded from a different muscle fibre in the same muscle. The rise and fall times of i.p.s.c.s are longer than those of e.p.s.c.s. The i.p.s.c. rose to its peak in 3–4 msec and thereafter decayed approximately exponentially, total duration being 30–40 msec, while the peak time of e.p.s.c. was 1·5–2 msec and it lasted for less than about 10 msec. Another difference between e.p.s.c. and i.p.s.c. was their pattern of transmitter release. Amplitude of e.p.s.c. produced by each stimulus varied as expected from the quantal release of the transmitter (Dudel & Kuffler, 1971*a*), while the amplitude of i.p.s.c. was quite stable. The difference in the release pattern was more pronounced, when the i.p.s.c. and e.p.s.c. were recorded from a single synapse with an extracellular microelectrode (see also Takeuchi & Takeuchi, 1965).

Fig. 2*A* shows i.p.s.c.s recorded at five different membrane potentials. Four successive i.p.s.c.s produced at stimulation frequency of 10 Hz were averaged at each membrane potential. Upper traces indicate the clamped membrane potentials. The declining phases of i.p.s.c.s recorded at −40 mV (uppermost trace in Fig. 2*A*) and

at -119 mV (bottom trace) are plotted on the logarithmic scale in Fig. 2*B*. The declining phase was almost a single exponential function ($I = I_0 \exp(-t/\tau)$) and the time constant (τ) was 11·4 msec at -40 mV and 7·2 msec at -119 mV. The peak time of i.p.s.c. also changed as the membrane potential was altered (4 msec at

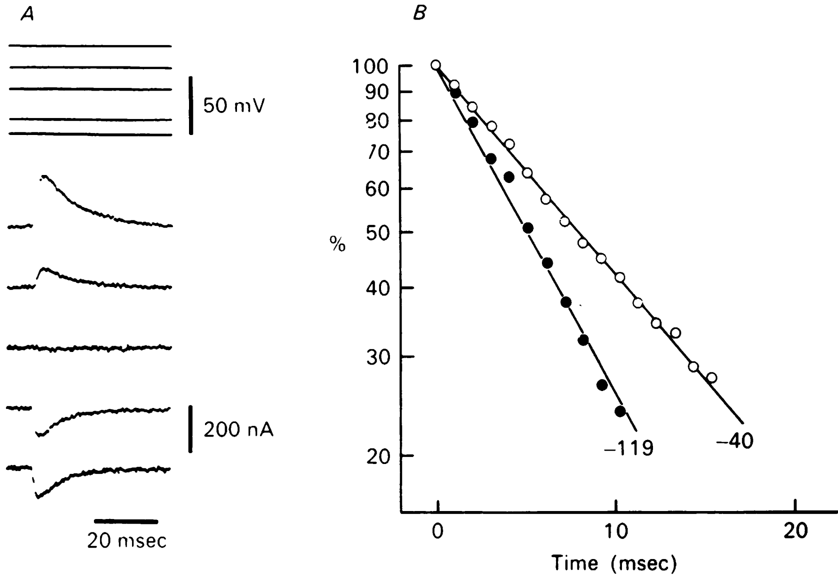

Fig. 2. Effect of membrane potential on the time course of i.p.s.c. *A*, i.p.s.c. and the clamped membrane potential. Membrane potential was changed from -40 mV (top trace) to -119 mV (bottom trace). Four single i.p.s.c.s were averaged. *B*, decay phase of i.p.s.c. was plotted on the semilogarithmic scale. Numbers attached indicate the clamped membrane potential. Origin of the time scale ocorresponds to 5 msec after the start of i.p.s.c.

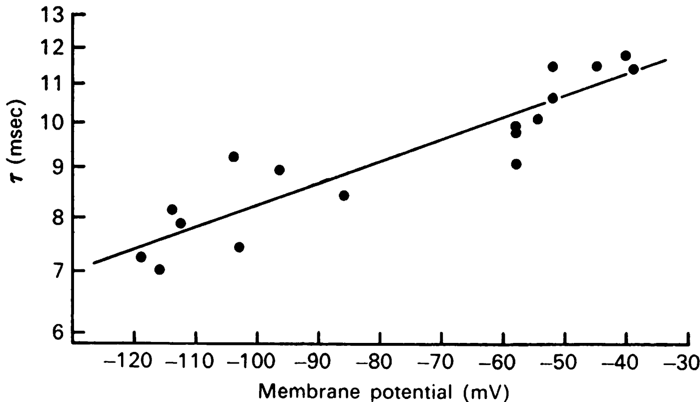

Fig. 3. Relationship between the time constant of decay phase and clamped membrane potential. Responses from experiment illustrated in part in Fig. 2. Plotted results are obtained from one muscle fibre.

-40 mV and 2·8 msec at -119 mV). Therefore, in Fig. 2*B* the amplitude at 5 msec, after the start of i.p.s.c. was taken as 100%.

The time constant of the declining phase was plotted against the membrane

potential in Fig. 3. There are large variations, but the time constant increased as the membrane was depolarized and decreased with hyperpolarization. The time constant (τ) depends on voltage (V) according to $\tau = a \exp(AV)$. The line in Fig. 3 was drawn by the method of the least squares with $a = 14 \cdot 0$ msec and $A = 0 \cdot 0053\ \mathrm{mV}^{-1}$ (correlation coefficient $r = 0 \cdot 92$, $P < 0 \cdot 01$, Student's t test). Mean value of a was $18 \cdot 6 \pm 0 \cdot 04$ msec and A was $0 \cdot 0065 \pm 0 \cdot 0002\ \mathrm{mV}^{-1}$ (S.E. of twenty-eight experiments at 23 °C). The time constant at -100 mV was on the average 9·4 msec.

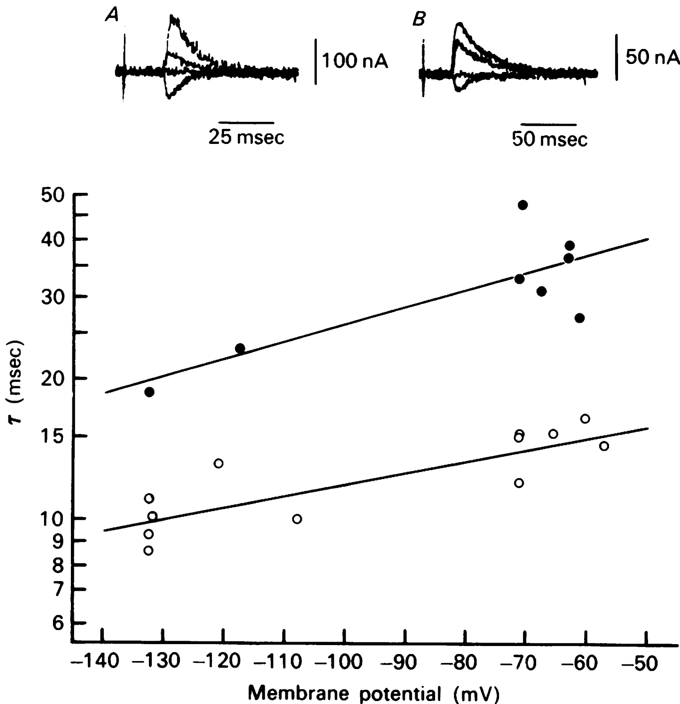

Fig. 4. Effect of temperature on the time course of i.p.s.c. *A*, sample records at 22·5 °C and *B*, at 13 °C recorded from the same muscle fibre. Eight single i.p.s.c.s were averaged. ○, decay time constant at 22·5 °C. ●, at 13 °C.

Effect of temperature on the time course of i.p.s.c.

The rise and fall of i.p.s.c. were prolonged as the bath temperature was decreased. Fig. 4 shows the decay time constant (τ) at two different temperatures. Open circles were recorded at 22·5 °C and filled circles at 13 °C and sample records (average of eight single records) are shown in Fig. 4*A* and *B*, respectively. The lines were fitted with $a = 21 \cdot 5$ msec, $A = 0 \cdot 0058\ \mathrm{mV}^{-1}$ for 22·5 °C ($r = 0 \cdot 70$, $P < 0 \cdot 01$) and $a = 61 \cdot 5$ msec, $A = 0 \cdot 0085\ \mathrm{mV}^{-1}$ for 13 °C ($r = 0 \cdot 70$, $P < 0 \cdot 05$). Mean value of Q_{10} for τ was $2 \cdot 4 \pm 0 \cdot 05$ (five experiments) between 22·6 and 12·5 °C at -100 mV. In Fig. 4 the voltage dependence was larger at the lower temperature; however, the average value of A at 13–15 °C was $0 \cdot 0067\ \mathrm{mV}^{-1}$ which was in the same range as at 22·5 °C. The Q_{10} for the decay phase of the i.p.s.c. was similar to those reported for frog and toad e.p.s.c.s. (Takeuchi & Takeuchi, 1959; Kordaš, 1972; Magleby & Stevens, 1972*b*; Gage & McBurney, 1975; Colquhoun, Large & Rang, 1977), crayfish

i.p.s.c. (Dudel, 1977) and crayfish e.p.s.c. (Onodera & Takeuchi, 1978). No appreciable change in the reversal potential was observed by changing the bath temperature.

Effect of pH on the time course of i.p.s.c.

The synaptic membrane conductance activated by GABA was increased by lowering pH and it decreased at higher pH (Takeuchi & Takeuchi, 1967). It is conceivable that the increase in the membrane conductance is due to an increased life time of the open channel. Fig. 5*A* and *B* show the i.p.s.c.s at pH 7·2 and 5·5 from the same muscle fibre (average of eight single records). Open circles in Fig. 5

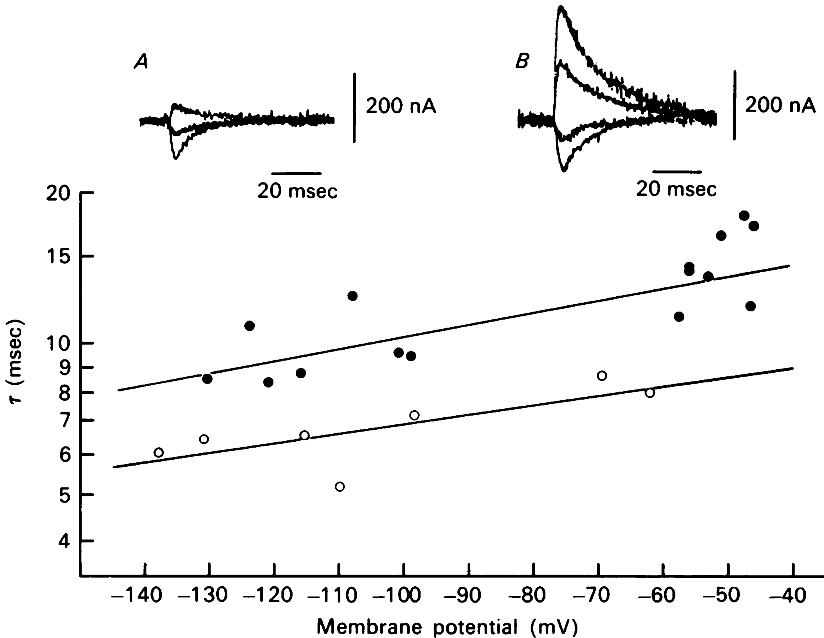

Fig. 5. Effect of pH on the time course of i.p.s.c. Sample responses recorded at pH 7·2 (*A*) and 5·5 (*B*) recorded from the same muscle fibre. Eight successive i.p.s.c.s were averaged. ○, decay time constant at pH 7·2. ●, at pH 5·5.

were obtained in pH 7·2 and filled circles in pH 5·5. Lines were fitted with $a = 10{\cdot}7$ msec, $A = 0{\cdot}0045\ \mathrm{mV}^{-1}$ ($r = 0{\cdot}79$, $P < 0{\cdot}05$) for pH 7·2 and $a = 18{\cdot}3$ msec, $A = 0{\cdot}0057\ \mathrm{mV}^{-1}$ ($r = 0{\cdot}69$, $P < 0{\cdot}01$) for pH 5·5. Voltage dependence of time constant was not very different between pH 5·5 and 7·2, but the time constant increased by about 50% in pH 5·5. The growth phase was also slightly prolonged in pH 5·5. When pH was increased to 9, a small decrease in the time course was sometimes observed, but no consistent change was detected. Within the range of pH tested, the reversal potential was not changed.

Increase in the membrane conductance produced by GABA at lower pH may be partly attributed to the increase in the channel life time. However, the conductance change produced by GABA was almost doubled by lowering pH from 7·2 to 5·5 and it decreased to about one half by increasing pH from 7·2 to 9 (Takeuchi & Takeuchi, 1967). Therefore, the pH may also influence the channel conductance, e.g. by changing the charge density of the ion channel.

Effect of anions on the time course of i.p.s.c.

When GABA was ionophoretically applied to the crayfish muscle, the time course of the GABA potential was prolonged when the chloride in the bath solution was replaced with iodide (Fig. 7, Takeuchi & Takeuchi, 1971*b*). This result suggests that the channel life time might be prolonged in the I^- solution. Fig. 6*B* indicates sample records of i.p.s.c. recorded about 3 min after changing the solution to that containing I^-. The rise and fall of i.p.s.c. was prolonged in the I^- solution compared to that recorded from the same muscle fibre in the Cl^- solution (Fig. 6*A*). The declining phase of i.p.s.c. remained a single exponential function in I^- solution. At the holding

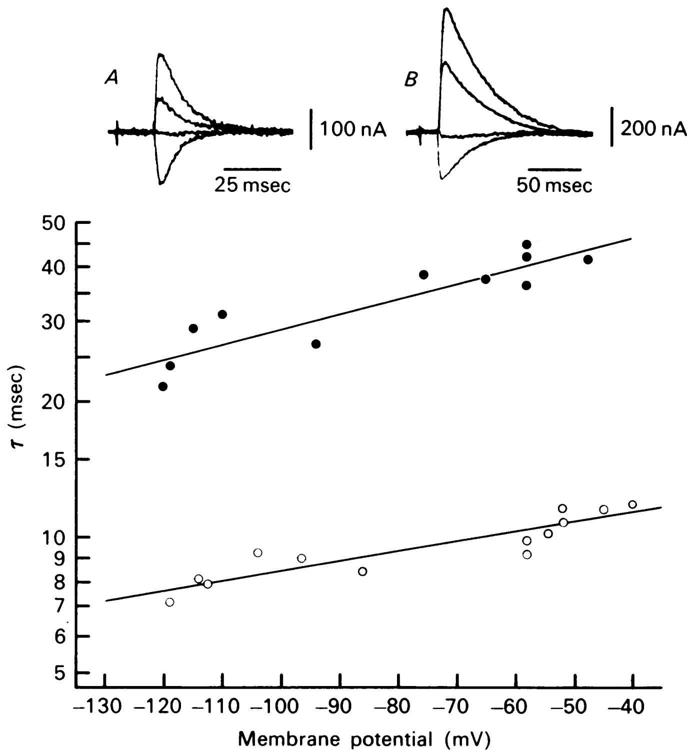

Fig. 6. Effect of I^- on the time course of i.p.s.c. *A*, control i.p.s.c. in normal solution. *B*, prolonged time course after 3 min in a solution containing iodide (with 43 mM-Cl). Eight single i.p.s.c.s were averaged. ○, recorded in Cl^- solution. ●, in I^- solution. Reversal potential was −71 mV in Cl^- and −87 mV in I^- solution.

potential of −58 mV the growth time (the time from 20 to 80% of the peak amplitude) in Cl^- solution was 1·2 msec, increasing to 1·8 msec in I^- solution. The time constant of the decay phase was more strikingly affected; it rose from about 9·5 msec at −58 mV in Cl^- solution to 31·9 msec within 3 min in I^- solution. Bathing in the I^- solution for an additional 15 min did not change the time constant. In the I^- solution the decay time constant increased by a factor of 3, ranging from 2·9 to 3·2. Fig. 6 illustrates the relation between the time constant and membrane potential. The lines were drawn with a = 13·6 msec, A = 0·0049 mV^{-1} (r = 0·90, P < 0·01)

for Cl^- solution (open circles) and $a = 63{\cdot}6$ msec, $A = 0{\cdot}0080\ mV^{-1}$ ($r = 0{\cdot}90$, $P < 0{\cdot}01$) for I^- solution (filled circles). The average value of A was $0{\cdot}0061\ mV^{-1}$ in I^- solution, which was approximately the same as that in the Cl^- solution.

It may be considered that permeant anions such as I^- enter the ion channel and change its properties, resulting in a prolongation of the channel life time. When Cl^-

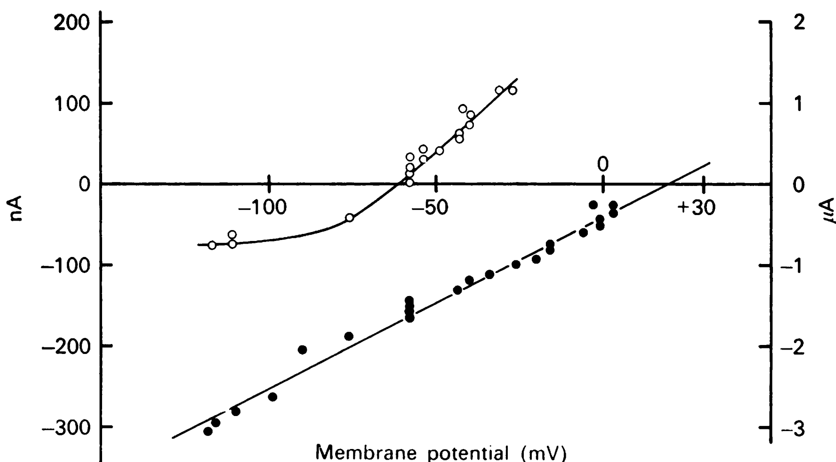

Fig. 7. Relationship between membrane potential and peak amplitude of i.p.s.c. and e.p.s.c. ○, amplitude of i.p.s.c. produced at 10 Hz. ●, amplitude of e.p.s.c. recorded from the same muscle fibre. Left ordinate is for i.p.s.c. and right one for e.p.s.c.

in the bath solution was replaced with foreign anions, it took about 10 min for the inside Cl^- to be exchanged (Takeuchi & Takeuchi, 1971*a*). In the case of Fig. 6 the i.p.s.c. was recorded within 3 min after replacing Cl^- with I^-, therefore, the inside anion may have been still largely Cl^-. (This assumption is supported by the observation that the reversal potential was at a hyperpolarized level in I^- solution: -71 mV in Cl^- solution and -87 mV in I^- solution (Takeuchi & Takeuchi, 1971*a*).) Accordingly the inwardly directed i.p.s.c. was predominantly carried by outward flux of Cl^-, while the outward i.p.s.c. was carried by inward flux of I^-. These results suggest that the presence of I^- in the bath solution is more important for determining the time course of the e.p.s.c., rather than I^- ions which carry synaptic current.

When Cl^- in the bath solution was replaced with Br^-, the i.p.s.c. was prolonged by about 50%. The average time constant at -100 mV was 13·5 msec (ranging from 15·2 to 12·0 msec) in the Br^- solution and 10·6 msec in Cl^- solution recorded from the same muscle fibres.

I.p.s.c.–membrane potential relationship

Peak amplitude of i.p.s.c. was plotted against the clamped membrane potential, outwardly directed i.p.s.c. being indicated as positive (Fig. 7, open circles). The relationship was approximately linear as the membrane potential was depolarized, but deviated from linearity with hyperpolarization beyond the reversal potential, and almost saturated at about -100 mV or beyond.

There are several possible errors which might cause an apparent non-linear relationship. First, if the voltage clamp was not adequate, the I–V relation may

level off upon hyperpolarization, because of a large conductance increase of the membrane during hyperpolarization (Ozeki, Freeman & Grundfest, 1966). This possibility seems excluded because if e.p.s.c.s were recorded from the same muscle fibre, under the same voltage clamp condition, the *I–V* relation of e.p.s.c. remained linear between −120 and 0 mV (Fig. 7, filled circles).

Secondly, the hyperpolarizing current through the crayfish muscle is carried largely by chloride (Ozeki *et al.* 1966) and the inside concentration of chloride may be decreased during hyperpolarization, resulting in a decreased synaptic current

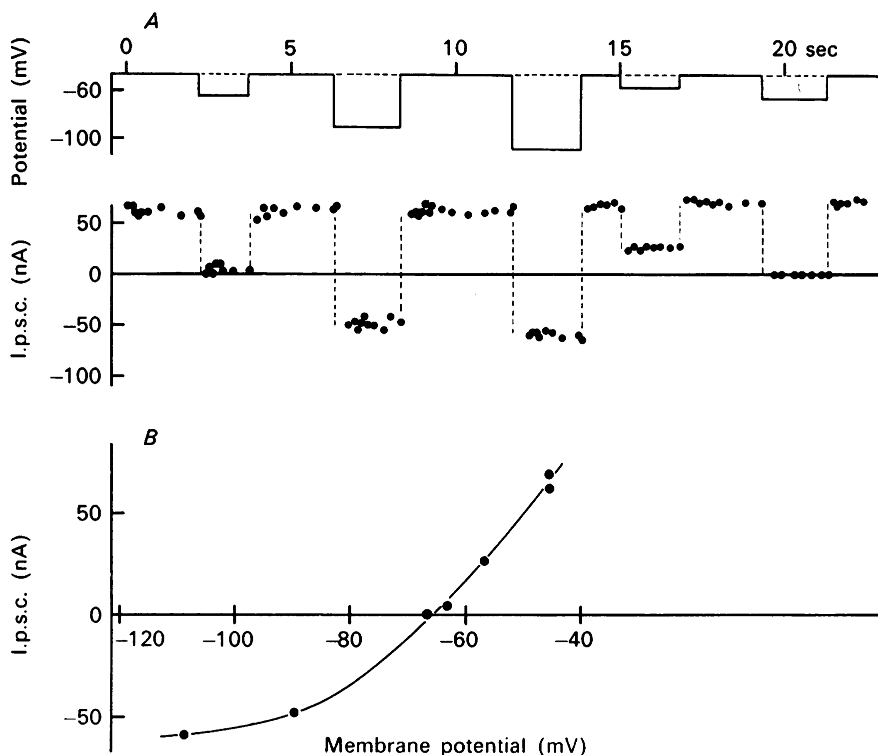

Fig. 8. Procedure for measurement of amplitude of i.p.s.c. *A*, upper trace: clamped membrane potential. Lower trace: peak amplitude of single i.p.s.c.s. *B*, relation between clamped membrane potential and mean amplitude of single i.p.s.c.s.

(Dudel, 1977). Fig. 8*A* illustrates the procedure for measurement of single i.p.s.c.s (circles). When the membrane potential was shifted to a new level, the amplitude of i.p.s.c. was changed and remained at the same value for about 2 sec. When mean amplitudes of single i.p.s.c.s were plotted against the clamped membrane potential (Fig. 8*B*) the *I–V* relationship was again non-linear. This result indicates that under the present experimental condition, the change in the inside concentration of chloride may be small and the non-linear *I–V* relation is not a secondary effect due to the changes in Cl^- concentration.

A large change in the internal chloride concentration observed by Dudel (1977) may be attributed to the different experimental procedures, where the i.p.s.c. was measured by averaging 128–4096 single records produced at 10 Hz. With this procedure it took 12 sec to several minutes

to measure an i.p.s.c. at a specific membrane potential. Such a prolonged potential change presumably changed the reversal potential, because in our experiments even a hyperpolarization for 10 sec caused a shift of several millivolts.

When the membrane potential was hyperpolarized to -120 mV, the holding current was of the order of 10 μA. A large part of this current is carried by Cl^- and the main pathway for this ion may be the tubular membrane (Girardier, Reuben, Brandt & Grundfest, 1963; Orentlicher & Reuben, 1971). In the present experiment the membrane potential was changed usually with a pulse of 2 sec in duration. During this period the inside Cl^- concentration may be decreased by 0·8 mM, assuming the fibre diameter of 250 μm and fibre length of 5 mm. This value would correspond to about 5% of the inside Cl^- concentration.

The i.p.s.c.–membrane potential relationship was also measured under various experimental conditions. When the pH or temperature was varied, $I-V$ relation was not markedly different from that in the control solution. When Cl^- in the bath solution was replaced with I^-, the $I-V$ curve was still non-linear immediately after replacing Cl^- with I^-. However, after soaking the muscle in the I^- solution for more than 10 min when the inside Cl^- may have also been replaced by I^-, the $I-V$ curve became almost linear.

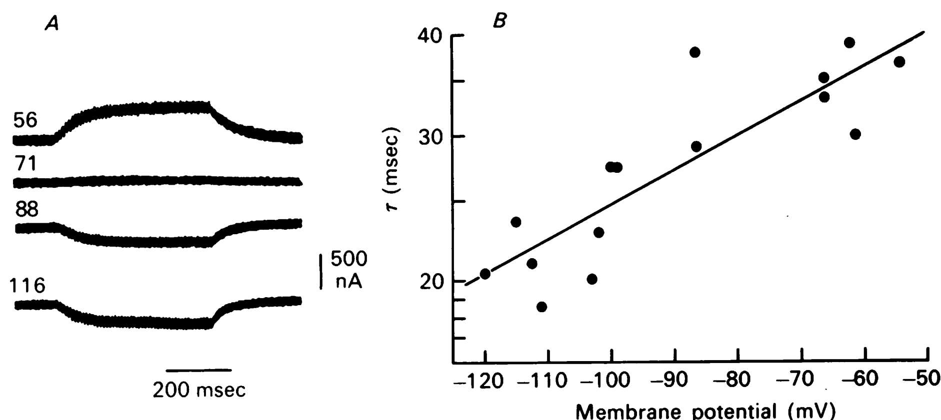

Fig. 9. Effect of repetitive stimulation on i.p.s.c. *A*, i.p.s.c.s produced by 100 Hz. Numbers indicate the clamped membrane potential. *B*, relation between membrane potential and time constant of decay phase.

Peak amplitude of i.p.s.c. was variable from fibre to fibre. When the synaptic membrane conductance (G) was expressed by the relation $G = I\ (V - V_r)^{-1}$, where V_r is the reversal potential and I, the peak synaptic current, the peak synaptic conductance decreased with hyperpolarization. At the reversal potential G was $5{\cdot}4 \pm 0{\cdot}04 \times 10^{-6}$ S, when produced at 10 Hz and the reversal potential was $-72{\cdot}3 \pm 0{\cdot}2$ mV (mean ± S.E. of fifteen experiments).

Effect of repetitive stimulation on the time course of i.p.s.c.

Fig. 9*A* illustrates the i.p.s.c. produced at a stimulation frequency of 100 Hz and recorded at various clamped membrane potentials, as indicated on the left side. I.p.s.c.s gradually built up to a plateau and after cessation of stimulation they decayed approximately exponentially. The declining phase was prolonged by depolarization and became shorter by hyperpolarization. The relation between decay time constant and clamped membrane potential was fitted with $a = 65{\cdot}0$ msec and

$A = 0{\cdot}0097\ \mathrm{mV}^{-1}$ ($r = 0{\cdot}86$, $P < 0{\cdot}01$) (Fig. 9). The voltage dependence of decay phase was similar to that of single i.p.s.c. It appears that the relatively long decay phase after repetitive stimulation depends on the number of stimuli. Fig. 10*A* illustrates i.p.s.c.s produced by 22 stimuli at a stimulation frequency of 90 Hz, while

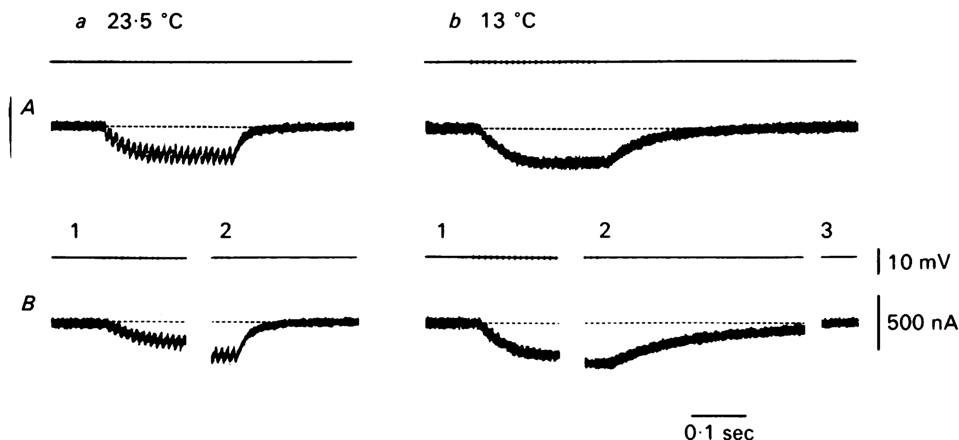

Fig. 10. Effect of number of stimuli on the time course of i.p.s.c. *a*, at 23·5 °C. *A*, application of 22 stimuli at 90 Hz. *B*, beginning and end of 240 stimuli at 90 Hz. *b*, at 13 °C. *A*, application of 22 stimuli at 90 Hz. *B*, beginning and end of 204 stimuli at 90 Hz recorded from the same muscle. Upper traces indicate the clamped membrane potential (−74 mV) and small spikes are stimulus artifact.

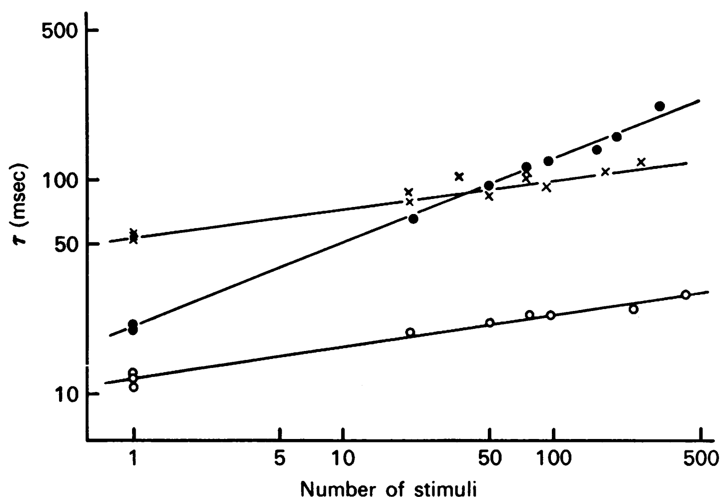

Fig. 11. Relation between the decay time constant and the number of stimuli, plotted on logarithmic scales. ○, decay time constant in Cl^- solution at 23·5 °C. ●, in Cl^- solution at 13 °C, recorded from the same muscle fibre. ×, recorded from a different muscle fibre in I^- solution at 23·5 °C. Stimulus frequency was 90 Hz.

Fig. 10*B* shows i.p.s.c.s at the beginning and end of a train of 240 stimuli at the same frequency. Both records were made at the same membrane potential of −74 mV. The decay time constant was 19·4 msec after 22 stimuli and increased to 24·3 msec

after 240 stimuli. At lower temperatures, such as at 13 °C, the declining phase was strikingly prolonged (Fig. 10*b*) recorded from the same muscle fibre. In addition the plateau level was much smoother if compared with Fig. 10*a* and the decay time constant was 64·9 msec after application of 22 stimuli and 157·3 msec after 204 stimuli.

The relationship between decay time constant and number of stimuli at frequency of 90 Hz is shown in Fig. 11 at 13 °C (filled circles) and at 23·5 °C (open circles). The decay time constant of single i.p.s.c. was 12 msec and it increased with the number of

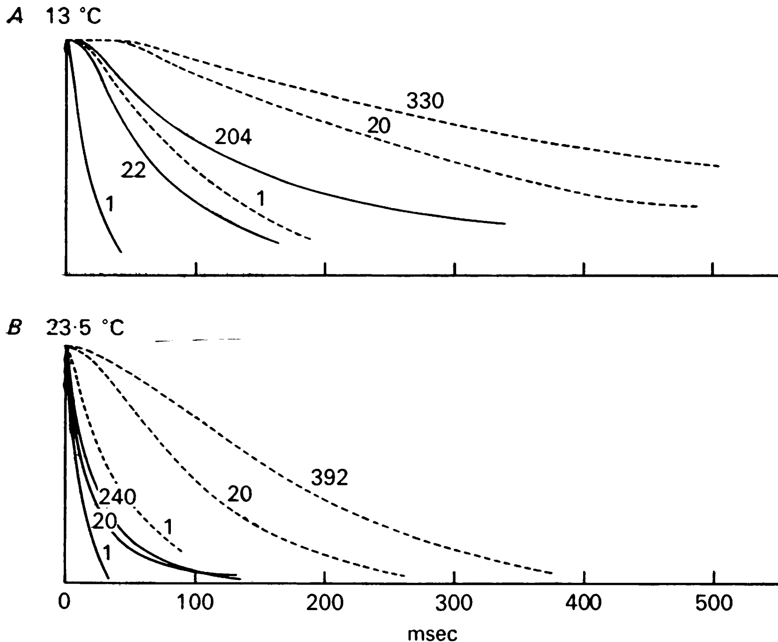

Fig. 12. Tracing of the decay phase after application of repetitive stimuli. *A*, at 13 °C. *B*, 23·5 °C. Number of stimuli at 90 Hz are indicated above the curves. Continuous curves are from records in Cl^- solution and broken curves from I^- solution.

stimuli. The time constant reached 24 msec after 240 stimuli. At 13 °C the prolongation of the decay time constant was more pronounced as the number of stimuli was increased. It rose from 21 msec for a single i.p.s.c. to 200 msec after 300 stimuli.

Effect of iodide on the repetitive i.p.s.c.

As already mentioned, the time course of an i.p.s.c. in I^- solution was about three times longer than in Cl^- solution. When repetitive stimulation was applied in the I^- solution, the decay phase was further increased (Fig. 11, crosses). The decay time constant of single i.p.s.c. recorded at −58 mV was 52 msec and it increased to about 90 msec after application of 50 stimuli at 90 Hz. The slope of the curve in Fig. 11 was approximately the same as that obtained in chloride solution at 23·5 °C.

A comparison of the declining phase of i.p.s.c.s in Cl^- (continuous lines) and in I^- solution (interrupted lines) is shown in Fig. 12 at 13 and 23·5 °C. The numbers above the traces indicate the number of stimuli. In the normal solution the i.p.s.c. started

to decline immediately after the ending of the stimulation. However, in the I^- solution the i.p.s.c. stayed at the same level for about 10 msec after the end of 20 stimuli and then decayed approximately exponentially with a larger time constant than in the Cl^- solution. At lower temperature the stationary level was prolonged and lasted for about 40 msec after the end of 20 stimuli and then declined with a long half-decay time of about 300 msec. At 13 °C even in Cl^- solution the declining phase of i.p.s.c. was convex upwards after repetitive stimulation (Fig. 12*A*). These observations are similar to those seen after large ionophoretic application of GABA to the synapse in the iodide solution. The GABA potential showed a saturation and then decayed with much slower time course than that produced by small amounts of GABA (Takeuchi & Takeuchi, 1971*b*).

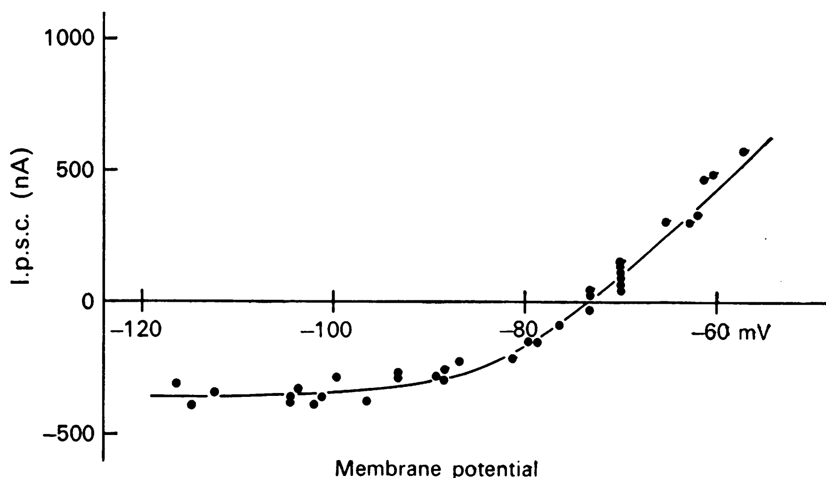

Fig. 13. Relationship between membrane potential and the plateau level of repetitive i.p.s.c. Stimulus frequency was 100 Hz for 450 msec recorded at 23·5 °C.

Current–voltage relationship of repetitive i.p.s.c.

Plateau levels of i.p.s.c. produced by simulation at 100 Hz for 450 msec are plotted against the clamped membrane potential (Fig. 13). The $I-V$ relation was essentially the same as that obtained by plotting the peak amplitude of single i.p.s.c.s (Figs. 7 and 8). The membrane conductance of the plateau level depended on stimulus frequency, but it was larger than that of single i.p.s.c. The membrane conductance (G) at the reversal potential was $28{\cdot}7 \times 10^{-6}$ S in Fig. 13.

The $I-V$ relation became more linear after the muscle was equilibrated with I^- solution. Essentially the same $I-V$ relation was also obtained when GABA was applied ionophoretically to the clamped muscle.

DISCUSSION

A large part of the present results is descriptive and they must be compared with previous works in which excitatory and inhibitory synaptic currents have been analysed in other tissues.

Time course of i.p.s.c. The time course of the i.p.s.c. was about four times longer than that of the e.p.s.c. and is similar to that reported by Dudel (1977) for crayfish

muscle and i.p.s.c.s in *Onchidium* (Hagiwara & Kusano, 1961) and *Aplysia* ganglia (Adams *et al.* 1976). On the other hand the time course of i.p.s.c. in spinal motoneurones is a fast process comparable to that of e.p.s.c. (Araki & Terzuolo, 1962).

There are two extreme models for the time course of transmitter action (see e.g. Colquhoun & Hawkes, 1977). Binding and unbinding to the receptor are rapid and the rate limiting process is the conformational change (Magleby & Stevens, 1972*a*, *b*). Alternatively, the conformational change is rapid and binding is the rate limiting process (Kordaš, 1969; Katz & Miledi, 1973; Adams, 1977). At present we have no evidence to decide among these alternatives. However, if the slow process after repetitive stimulation is determined by the slow diffusional loss of the transmitter from the synaptic region, the latter possibility seems more attractive.

Noise analysis of GABA action on the crayfish muscle shows fast and slow processes (Dudel, Finger & Stettmeier, 1977) and it was suggested that the fast component is of synaptic origin while the slow component is due to the extrasynaptic receptor. The time constant of the decay phase in the present experiments was larger than that of the fast process in noise analysis. It is not known whether this difference is attributed to the different preparations or to other causes, e.g. the transmitter remaining at the synapse for a relatively long period of time (see also Colquhoun & Hawkes, 1977).

Voltage dependence of the time course. Voltage dependence of the decay time constant of i.p.s.c. was the same in value but opposite in direction to the e.p.s.c. in frog and toad neuromuscular junctions (Kordaš, 1969; Magleby & Stevens, 1972*a*, *b*; Gage & McBurney, 1975) and i.p.s.c. in *Aplysia* ganglia (Adams *et al.* 1976), while e.p.s.c. in crayfish muscle has the same voltage dependence as i.p.s.c. (Dudel, 1974; Onodera & Takeuchi, 1978). These results indicate that the voltage dependence is not related to the charge of ions which carry the synaptic current. It seems interesting that i.p.s.c. and e.p.s.c. induced by the action of ACh have a negative voltage dependence (end-plate current: Kordaš, 1969; Magleby & Stevens, 1972*a*, *b*; Gage & McBurney, 1975; i.p.s.c. in *Aplysia* ganglia: Adams *et al.* 1976; i.p.s.c. in parasympathetic neurones: Hartzell, Kuffler, Stickgold & Yoshikami, 1977); on the other hand e.p.s.c. and i.p.s.c. produced by amino acids have a positive value (Anderson, Cull-Candy & Miledi, 1976; Dudel, 1974, 1977; Onodera & Takeuchi, 1976, 1978). Further experiments are needed to decide whether the voltage-dependent time course is due to the characteristics of the receptor. Alternatively, if the voltage dependence is attributed to the conformational change of the transmitter-receptor complex, the charge distribution in the macromolecule would be opposite to that of the vertebrate end-plate and *Aplysia* ganglia. Whatever the mechanism of the voltage dependence, it is worth noting that the voltage dependence of the i.p.s.c. decay was not altered very much under conditions where the time course was prolonged up to threefold (Figs. 4–6). This result suggests that the mechanism for the rate limiting process and for the voltage dependence might be different.

Voltage dependence of the i.p.s.c. amplitude. In the frog end-plate the e.p.s.c. displays an over-all non-linear relation over a wide range of the membrane potential and this is explained by the slightly voltage-dependent rate constants for opening and closing of the channel (Magleby & Stevens, 1972*b*; Dionne & Stevens, 1975). In the crayfish

the voltage dependence of GABA effectiveness was approximately the same when GABA was released by single nerve impulse and when it was applied by repetitive stimuli. The $I-V$ relation was more or less linear at voltage more positive than the reversal potential but the non-linearity was much larger than that of frog end-plate upon hyperpolarization. If the $I-V$ relation of i.p.s.c. is explained in terms of voltage-dependent rate constants, the closing rate constant increases as the membrane is hyperpolarized, whereas the opening rate constant decreases with hyperpolarization and depends about three times more strongly upon voltage than the rate for closing.

Recent observations suggest that the voltage sensitivity of the opening rate constant is negligible in the frog and toad end-plates and in electroplaques (Gage & McBurney, 1975; Neher & Sakmann, 1975; Trautmann & Zilber-Gachelin, 1976; Sheridan & Lester, 1977). If this is also the case for i.p.s.c., the non-linear $I-V$ curve may be attributed to the rectification of the channel conductance. There are several instances in which the $I-V$ relation shows a marked non-linearity as observed in the present experiments (e.p.s.c. and ACh current in the electroplaque (Lassignal & Martin, 1976, 1977; Sheridan & Lester, 1977); potassium channel of *Aplysia* ganglia activated by carbachol (Ginsborg & Kado, 1975); light-induced Na conductance in the barnacle photoreceptor (Brown, Hagiwara, Koike & Meech, 1970); see however Dionne & Ruff, 1977 for end-plate).

Effect of repetitive stimulation. The declining phase after repetitive stimulation was remarkably long and had a large termperature dependence. The Q_{10} for the decay phase of single i.p.s.c. was 2·4, while after application of about 100 stimuli, Q_{10} was almost 6. Very slow decay after repetitive stimulation is similar to the observations in the frog and snake end-plates after acetylcholinesterase (AChE) has been inhibited. It is thought that when AChE is inhibited, the transmitter is removed by diffusion which is retarded by the binding of the transmitter to the receptor (Katz & Miledi, 1973; Hartzell, Kuffler & Yoshikami, 1975). By analogy the present experiments suggest that in the crayfish inhibitory synapse there is a mechanism which removes the transmitter from the synaptic region and at lower temperatures this mechanism is inhibited. Since no evidence has been obtained about the enzymic breakdown of GABA, the removal may be made through an uptake system (Iversen & Kravitz, 1968). As suggested in the end-plate, the diffusional loss of the transmitter from the synaptic region may be a relatively slow process which is retarded by the binding of the transmitter to the synaptic receptor (Katz & Miledi, 1973; Crank, 1956).

This assumption qualitatively explains the slow decay of i.p.s.c., but quantitative explanation seems required. First of all it is necessary to study the effect of a competitive inhibitor on the declining phase (Katz & Miledi, 1973). Unfortunately, however, inhibitors which are comparable to D-tubocurarine have not been found for the inhibitory synapse. At lower temperatures the decay time constant increased up to ten times that of single i.p.s.c. It must be confirmed that this large increase can be explained by the diffusional loss only. In any way, in the course of further experiments other possibilities need to be considered; for example, the transmitter release may continue from the nerve terminals for a relatively long time after the end of stimulation and that the transmitter may diffuse to the extrasynaptic receptor.

Effect of foreign anions. One of the most remarkable properties of i.p.s.c. is a large change in the time course by the action of foreign anions. The degree of prolongation was in the order of $I^- > Br^- > Cl^-$. These anions are permeable through the synaptic channel and it is conceivable that they change the channel properties, thereby prolonging the time course. Alternatively, the foreign anions may affect the receptor macromolecule and prolong the binding and unbinding processes. The marked prolongation of the decay phase after repetitive stimulation might be explained by the latter possibility. It was tentatively assumed that these permeable anions change the binding process as well as the permeability properties of the synaptic membrane. A similar change in the channel life time has also been observed for permeant cations at the end-plate of the toad (Van Helden, Hamill & Gage, 1977).

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