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SOME CHARACTERISTICS OF PRE- AND POST-SYNAPTIC INHIBITORY RECEPTORS AT THE HERMIT CRAB NEUROMUSCULAR JUNCTION

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1 The effects of γ-aminobutyric acid (GABA), β-guanidinopropionic acid (βGP) and picrotoxin on the pre- and post-synaptic receptors of the hermit crab neuromuscular junction were studied quantitatively using electrophysiological techniques. Reductions in excitatory junction potential (e.j.p.) amplitude and membrane resistance were measured simultaneously from the same cells.

2 The pre- and post-synaptic receptors were activated by the same order of concentration of GABA, whereas βGP stimulated the pre-synaptic receptors at a concentration ten times lower than was required to affect the post-synaptic membrane.

3 Picrotoxin appeared to antagonize the pre-synaptic action of βGP in a competitive manner. The affinity constants (± s.e. mean) for picrotoxin 5×10^{-6}M and 2×10^{-4}M were 6.80 (± 0.46) $\times 10^{5}$ M^{-1} and 6.42 (± 1.8) $\times 10^{5}$ M^{-1} respectively.

4 The effect of GABA on e.j.p. amplitude also appeared to be antagonized competitively by picrotoxin whereas the post-synaptic effect was antagonized in a non-competitive manner.

5 Possible differences in the nature of the pre- and post-synaptic receptors are discussed.

Introduction

In a previous publication we demonstrated that both pre- and post-synaptic mechanisms of inhibition exist at the hermit crab neuromuscular junction (Earl & Large, 1974). Furthermore, we considered that neural inhibition was achieved largely by the pre-synaptic mechanism, whereas the effect of exogenous γ-aminobutyric acid (GABA), the inhibitory transmitter at the crustacean neuromuscular junction, could be accounted for to a great extent by its ability to reduce the post-synaptic membrane resistance.

The pre- and post-synaptic inhibitory sites have certain features in common; they are both blocked by picrotoxin (Takeuchi & Takeuchi, 1969; Earl & Large, 1974) and in both cases the action of GABA brings about a selective increase in chloride conductance (Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1966). However, there appears to be at least one difference; at the crayfish neuromuscular junction, the GABA analogue β-guanidino-propionic acid (βGP) attenuates excitatory junction potentials (e.j.ps) without affecting the post-synaptic membrane resistance (Dudel, 1965) thus indicating a purely pre-synaptic inhibitory action. Furthermore, βGP antagonizes the action of GABA at the post-synaptic site (Feltz, 1971). Therefore, βGP appears to be a GABA-like agonist pre-synaptically but a competitive antagonist post-synaptically. Presumably there is a difference between either the pre- and post-synaptic receptors or the receptor-ionophore links.

In this study we carried out experiments to make a quantitative assessment of the sensitivity of pre- and post-junctional receptors to GABA-mimetics and picrotoxin.

Methods

Measurement of e.j.ps and membrane resistance

The abductor muscle of the large claw of the hermit crab (*Eupagurus bernhardus*) was used to study e.j.ps and membrane resistance. Details of the techniques are described elsewhere (Earl & Large, 1974). Briefly, e.j.ps were evoked by stimulation of the thin nerve bundle via a suction electrode and were recorded in the conventional manner with an intracellular microelectrode filled with 3M KCl. For measurement of membrane resistance, this electrode was used to record the

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voltage displacements produced by passing hyperpolarizing current pulses through a second intracellular KCl electrode. Input resistance was read as the slope of the current-voltage plot obtained when both electrodes were impaled in the middle of the fibre, less than 100 μm apart. Membrane resistance was calculated by the simplified method of Takeuchi & Takeuchi (1967). After successive applications of Ringer solution containing increasing concentrations of GABA or βGP, dose-response curves of percentage reduction of membrane resistance or e.j.p. amplitude against log concentration of GABA or βGP were plotted. One minute after the addition of each concentration of agonist, readings were taken of both current-voltage relationship and e.j.p. amplitude from the same fibre. The latter was estimated by recording several trains of e.j.ps, to allow time for complete facilitation, and then averaging the amplitude of the last three e.j.ps within a train. In those experiments in which the blocking action of picrotoxin was investigated, the dose-response curves were plotted after the preparation had been exposed to picrotoxin for 2 minutes. The composition of the Ringer solution was (mM): NaCl 445, KCl 12.2, $CaCl_2$ 29.6, $MgCl_2$ 5.75, $NaHCO_3$ 1.79.

Calculation of the contribution of pre- and post-synaptic components to e.j.p. depression

Activation of post-synaptic inhibitory receptors by agonists will trigger a fall in the resistance of the post-synaptic membrane; if the contribution of this post-synaptic effect to the reduction of the e.j.p. amplitude can be estimated quantitatively, then pre-junctional inhibition can be detected from the excess depression of the e.j.p. The membrane current associated with an e.j.p. is partly a displacement current that charges the membrane capacity and partly an ionic current. In order to calculate the maximum possible contribution of the post-synaptic component of inhibition we have assumed that all the synaptic current passes through the membrane resistance. Using this analysis, a drug-induced reduction in e.j.p. amplitude which cannot be accounted for by a decrease in post-synaptic membrane resistance must be due to stimulation of the pre-synaptic inhibitory receptors. In fact the analysis will over-estimate the contribution of post-synaptic inhibition in reducing the amplitude of e.j.p. as will be discussed later.

If the membrane capacitance is ignored the equivalent electrical circuit of the post-synaptic membrane of the crab neuromuscular junction is given in Figure 1. The resting membrane potential of hermit crab fibres is usually around −70 mV

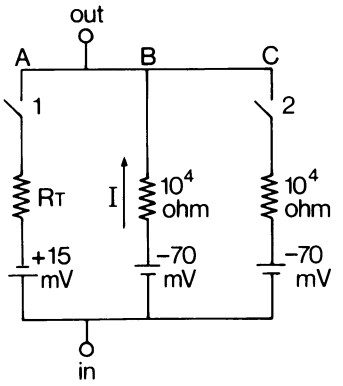

Figure 1 Equivalent electrical circuit of the post-synaptic membrane of the crustacean neuromuscular junction. Channel B represents the elements of the non-junctional membrane; A & C represent the pathways opened by the actions of the excitatory and inhibitory transmitters respectively.

and the membrane resistance $\triangleq 10^4\Omega$. The equilibrium potential for the excitatory transmitter, in the crayfish, is ~+ 15 mV (Takeuchi & Onodera, 1973) while that of the inhibitory transmitter is ~ −70 mV (Dudel & Kuffler, 1961). In channel A, R_T represents the synaptic resistance produced by the action of the excitatory transmitter. Closing switch 1 represents the action of the excitatory transmitter alone and sets up a current

$$I = \frac{15-(-70)}{R_T + 10^4}\ \text{mA}$$

this would lead to a depolarization of

$$\frac{85}{R_T + 10^4} \cdot 10^4\ \text{mV}$$

Since the amplitude of the unfacilitated e.j.p. is of the order of 1 mV, this means that R_T would have a value of 84.10^4 ohms, i.e. $R_T \gg R_m$, the resting membrane resistance. Let us now consider the effect of closing switches 1 and 2 together. By the use of simultaneous equations, the calculated depolarization (e.j.p.) produced by the excitatory transmitter is reduced from 1 mV to approximately 0.5 mV. The shunt resistance of 10^4 ohms, represented in the inhibitory channel C, (produced by the appropriate concentration of GABA) corresponds to an effective reduction of the membrane resistance by 50%, and therefore it can be concluded that the e.j.p. amplitude is directly proportional to the membrane resistance. When the % reduction in e.j.p. amplitude is greater than the concomitant depression of post-synaptic

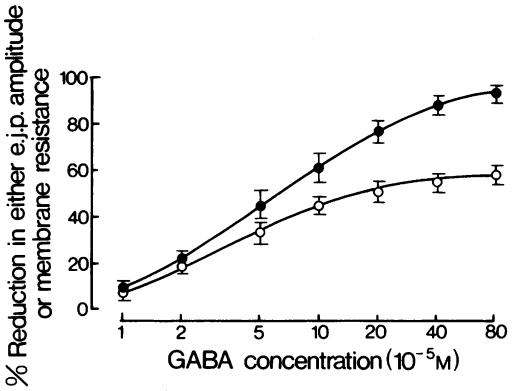

Figure 2 Effect of γ-aminobutyric acid (GABA) on e.j.p. amplitude and membrane resistance. The ordinate scale is the % reduction in either e.j.p. amplitude (•) or membrane resistance (○) and the abscissa scale is the GABA concentration plotted on a log scale. Each point represents the mean of 12 determinations. Vertical lines show s.e. mean.

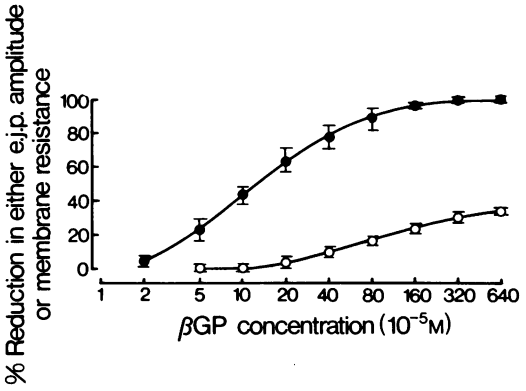

Figure 3 Effect of β-guanidinoproprionic acid (βGP) on e.j.p. amplitude and membrane resistance. The ordinate scale is the % reduction in either e.j.p. amplitude (•) or membrane resistance (○) and the abscissa scale is the βGP concentration plotted on a log scale. Each point represents the mean of 7 determinations. Vertical lines show s.e. mean.

membrane resistance, the difference must be due to a pre-synaptic effect.

Results

Effects of GABA and βGP on e.j.p. amplitude and post-synaptic membrane resistance

The threshold concentration of GABA for depression of both e.j.p. amplitude and membrane resistance was always 1-2 x 10^{-5}M (Figure 2). Whereas it was usually possible to attenuate the e.j.ps by 90%, the fall in membrane resistance was only 50-60%. These GABA-induced effects were not always totally reversed on washing, the maximal recovery taking 5-30 minutes. In those experiments where measurement of more than one dose-response curve was intended, e.j.p. depression was not allowed to proceed by more than 70%; in these instances, it was usually possible to attain the control e.j.p. amplitude and membrane resistance values on washing.

From the curves in Figure 2, it can be seen that for small doses of GABA (10^{-5} to 10^{-4}M), the reduction in post-synaptic membrane resistance can account for the major part of the e.j.p. attenuation, but for higher doses the pre-synaptic inhibitory component becomes increasingly more significant. However, the pre- and post-synaptic receptors appear to be activated by the same order of concentration of GABA, as in most experiments there was a small pre-synaptic inhibitory component (about 10% of the total inhibition) with small doses of GABA.

In contrast to the results with GABA, the threshold concentration of βGP (Figure 3) to reduce the post-synaptic membrane resistance was higher than that to attenuate the e.j.ps (in the order of 10-fold). Therefore, it appears that there is a difference between the pre- and post-synaptic inhibitory receptors in the hermit crab neuromuscular junction as in the crayfish. The inhibitory action of βGP is of an entirely pre-synaptic origin at concentrations below 20-40 x 10^{-5}M and even at higher concentrations, the post-synaptic effect accounts for only a small proportion of the inhibition.

Effect of picrotoxin on the inhibition mediated by GABA and βGP

The ability of picrotoxin to antagonize the pre- and post-synaptic inhibitory mechanisms was investigated. The two concentrations of picrotoxin used (5 x 10^{-6} and 2 x 10^{-4}M) were chosen because in earlier studies (Earl & Large, 1974) we had shown that picrotoxin 5 x 10^{-6}M produced a relatively weak antagonism of the post-synaptic action of GABA and it was predicted that 2 x 10^{-4}M would cause a marked block.

The first series of experiments was carried out with βGP since its action (at low concentrations) can be considered a purely pre-synaptic phenomenon. Figure 4 shows that picrotoxin produced a dose-dependent antagonism of βGP;

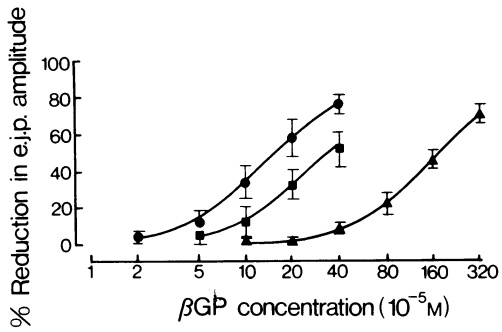

Figure 4 Effect of picrotoxin on the inhibitory action of β-guanidinoproprionic acid (βGP). The ordinate scale is the % reduction in e.j.p. amplitude and the abscissa scale is the βGP concentration plotted on a log scale. Each point represents the mean of 6 determinations. Vertical lines show s.e. mean. (●) Control; (■) 5×10^{-6}M picrotoxin; (▲) 2×10^{-4}M picrotoxin.

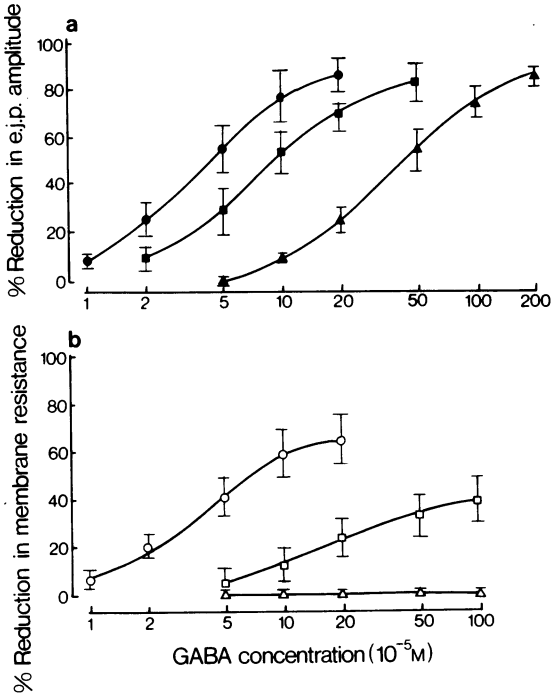

Figure 5 Effect of picrotoxin on the inhibitory action of γ-aminobutyric acid (GABA). The ordinate scales represent the % reduction in either e.j.p. amplitude (a) or membrane resistance (b) and the abscissa scale is the GABA concentration plotted on a log scale. Each point represents the mean of 6 determinations. Vertical lines show s.e. mean. (●) and (○) Control; (■) and (□) 5×10^{-6}M picrotoxin; (▲) and (△) 2×10^{-4}M picrotoxin.

there was a parallel shift to the right of the curves with increasing concentrations. It was possible to achieve maximal reduction in e.j.p. amplitude, even in the presence of 2×10^{-4}M picrotoxin, on the occasions when larger doses of βGP were added, thus suggesting that the antagonism is of a competitive nature. From the results of the individual experiments obtained with βGP it was possible to estimate the affinity constant, K_A, of picrotoxin for the pre-synaptic receptors. The values of K_A were calculated using the equation $r_A^n - 1 = X_B^m . K_A$ (Rang, 1971) where r_A is the dose-ratio, n is the number of molecules of agonist that combine with the receptor; X_B is the molar concentration of antagonist and m is the number of molecules of antagonist that combine with the receptor. Takeuchi & Takeuchi (1969) suggested that two molecules of GABA or one molecule of picrotoxin combine with the post-synaptic receptor, therefore we let $n = 2$ and $m = 1$ for our calculations. The calculated affinity constants for picrotoxin 5×10^{-6}M and 2×10^{-4}M with βGP as agonist were 6.80 (± 0.46) $\times 10^5 M^{-1}$ and 6.42 (± 1.8) $\times 10^5 M^{-1}$ respectively (mean ± s.e. of 6 determinations). These values were not significantly different at the 95% probability level when compared by Student's t-test. If other values of n, e.g. 1, 3 or 4 were used, there was less agreement between the K_A values of the high and low picrotoxin concentrations, which would be consistent with values for n and m used earlier.

When the effect of GABA on e.j.ps was challenged with the same concentrations of picrotoxin (Figure 5a), there was also a parallel shift in the curves. Also, as with βGP, it was possible to achieve the maximal effect on e.j.p. attenuation in the presence of picrotoxin. Further, if the affinity constants for picrotoxin 5×10^{-6}M and 2×10^{-4}M against GABA depression of e.j.ps are calculated from the curves in Figure 5a, one obtains the values $6.8 \times 10^5 M^{-1}$ and $5.0 \times 10^5 M^{-1}$ respectively. These are very similar to the values calculated for βGP which acts purely on the pre-synaptic receptors.

This is in direct contrast to the effect of GABA on the post-synaptic membrane resistance (Figure 5b). In these experiments (with picrotoxin 5×10^{-6}M), there was the characteristic non-parallel shift with a reduced maximum previously observed with picrotoxin (Takeuchi & Takeuchi, 1969; Earl & Large, 1974). In fact, in the presence of picrotoxin 2×10^{-4}M, there was total block of the post-synaptic GABA effect even though the e.j.ps had been reduced by 80-90%. It is of interest

that picrotoxin 5×10^{-6}M causes a larger shift of the curve for the post-synaptic GABA effect (Figure 5b) than it does with βGP a pre-synaptic agonist (Figure 4). As this antagonism of GABA by picrotoxin at the post-junctional receptor appears to be non-competitive, the equation used earlier for calculation of the affinity constant does not hold.

A further point is that there was a direct effect of the higher concentration of picrotoxin on e.j.p. amplitude; the amplitude was increased by a mean value of 35% (range 10-60% in 12 experiments), with little accompanying change in the post-synaptic membrane resistance. The most probable explanation for this effect is that there is a large tonic discharge of GABA from the pre-synaptic inhibitory terminals and little onto the post-synaptic receptors.

Discussion

With low concentrations of GABA, although the major part of the e.j.p. reduction can be accounted for by a post-synaptic action, there was still a small pre-synaptic component suggesting that both populations of receptors were similar in their sensitivity to GABA. At the crayfish neuromuscular junction, βGP competitively antagonizes the action of GABA suggesting that the two compounds combine with the same receptor (Dudel, 1965; Feltz, 1971). Therefore, our finding that βGP stimulates the pre-synaptic receptor in lower concentrations than are required to activate the post-synaptic sites suggests that there is a difference between the two populations of receptors. It should be noted that, in the hermit crab, βGP produces a post-synaptic membrane conductance increase and therefore differs from the crayfish; presumably this is due to a species difference.

Furthermore, there appears to be a difference in the pre- and post-synaptic receptors with respect to their antagonism by picrotoxin. The results with βGP suggest that picrotoxin antagonizes the pre-synaptic inhibitory actions in a competitive way, while the post-synaptic effect of GABA is blocked in a non-competitive manner. There are two possible explanations for this. Firstly, as suggested above, the pre- and post-synaptic receptors are in some way chemically different, and/or, secondly, there are many 'spare receptors' pre-synaptically and few or none post-synaptically.

In the presence of picrotoxin the shift of the GABA post-synaptic membrane resistance curve was greater than that of either the GABA or βGP e.j.p. curves, suggesting that picrotoxin has a greater affinity for the post-synaptic than the pre-synaptic receptors. It is interesting that the qualitative and quantitative characteristics of the antagonism of GABA-induced e.j.p. attenuation by picrotoxin are similar to those of βGP (purely pre-synaptic). This would suggest that the inhibitory action of GABA is also largely pre-junctional, despite the accompanying large fall in post-synaptic membrane resistance. This can be accounted for if the rising phase of the e.j.p. is due largely to a capacitative current, with little current passing through the membrane resistance; in fact an equation can be derived where the e.j.p. amplitude is independent of the membrane resistance (see equation, (5a) in Ginsborg, 1973).

The observation that one can achieve total attenuation of e.j.p. amplitude in the presence of high concentrations of picrotoxin indicates the potency of the pre-synaptic inhibitory mechanism. Moreover, this offers a better explanation than limited access of picrotoxin, for the difficulty in blocking pre-synaptic rather than post-synaptic inhibition previously reported (Earl & Large, 1974).

References

BOISTEL, J. & FATT, P. (1958). Membrane permeability change during inhibitory transmitter action in crustacean muscle. *J. Physiol., Lond.*, **144**, 176-191.

DUDEL, J. (1965). Pre-synaptic effects of inhibitory drugs on crayfish neuromuscular junction. *Pflügers Arch. ges. Physiol.*, **283**, 104-118.

DUDEL, J. & KUFFLER, S.W. (1961). Pre-synaptic inhibition on the crayfish neuromuscular junction. *J. Physiol., Lond.*, **155**, 543-562.

EARL, JANET & LARGE, W.A. (1974). Electrophysiological investigation of GABA-mediated inhibition at the hermit crab neuromuscular junction. *J. Physiol., Lond.*, **236**, 113-127.

FELTZ, ANNE (1971). Competitive interaction of β-guanidinopropionic acid and γ-aminobutyric acid on the muscle fibre of the crayfish. *J. Physiol., Lond.*, **216**, 391-401.

GINSBORG, B.L. (1973). Electrical changes in the membrane in junctional transmission. *Biochimica et Biophysica Acta*, **300**, 289-317.

RANG, H.P. (1971). Drug receptors and their function. *Nature, Lond.*, **231**, 91-96.

TAKEUCHI, A. & ONODERA, K. (1973). Reversal potentials of the excitatory transmitter and L-glutamate at the crayfish neuromuscular junction. *Nature, New Biol.*, **242**, 124-126.

TAKEUCHI, A. & TAKEUCHI, NORIKO (1966). On the permeability of the pre-synaptic terminal of the crayfish neuromuscular junction during synaptic inhibition and the action of γ-aminobutyric acid. *J. Physiol., Lond.*, **183**, 433-449.

TAKEUCHI, A. & TAKEUCHI, NORIKO (1967). Anion permeability of the inhibitory post-synaptic membrane of the crayfish neuromuscular junction. *J. Physiol., Lond.*, **191**, 575-590.

TAKEUCHI, A. & TAKEUCHI, NORIKO (1969). A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. *J. Physiol., Lond.*, **205**, 377-391.

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