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The Cooperativity of γ-Aminobutyric Acid Action on the Membrane of Locust Muscle Fibers

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SUMMARY

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Dose-response curves of the action of γ-aminobutyric acid to increase the conductance of postsynaptic membrane were measured in fibers of the flexor tibialis muscle of the metathoracic leg of *Locusta migratoria*. The sigmoid shape of the curves is interpreted in terms of the cooperative binding of more than 1 γ-aminobutyric acid molecule to the receptor. The "function of state," which describes the particular case in which receptor activation is induced only after the binding of more than 1 ligand molecule, is compared with some other formulations of cooperative binding, and is shown to give a better fit to the experimental data. This function uniquely predicts that the limiting Hill slope at small concentrations equals n, the number of molecules required to activate a receptor. In the present experiments n appears to be 3.

INTRODUCTION

There is much evidence that γ-aminobutyric acid is a neurotransmitter mediating peripheral inhibition in the neuromuscular systems of arthropods (1-3). GABA[1] acts by selectively increasing the permeability of the postsynaptic membrane to chloride ions (4). The effect on permeability can be measured experimentally as an increase in membrane conductance. It was found that the dose-response curve for this action of GABA on crayfish muscle fibers is sigmoid in appearance (5), and is not described, therefore, by a Langmuir isotherm or Michaelis-Menten equation for 1:1 binding of GABA to receptor.

[1] The abbreviation used is: GABA, γ-aminobutyric acid.

In a brief report of our results with locust muscle fibers (6) we proposed that more than 2 molecules of GABA must interact with a receptor in order to activate it. Takeuchi and Takeuchi (7) proposed that the combination of 2 molecules of GABA is necessary to activate the receptor in crayfish, but they did not enter into the considerations bearing on this interpretation of their data.

In this paper we present in detail the results of our quantitative study of the action of GABA on the postsynaptic membrane of locust muscle fibers. An effort was made to measure dose-response curves over the widest possible response range. We examine some of the properties of the equation which describes the particular case in which

receptor activation occurs only after the binding of more than 1 ligand molecule. This equation is shown to be distinguishable from the sequential binding function (8, 9), appropriate to the case in which receptor activation occurs on binding of each ligand molecule, and is also shown to fit the conductance data more adequately.

The interpretation of these findings depends upon the relationship between receptor activation and conductance increase. If the assumption of a linear relationship (see Fig. 1) is justified, our results would indicate that the cooperative binding of at least 3 molecules of GABA is required to activate a receptor in the locust muscle preparation under study.

In the following paper (10) we describe the kinetics and temperature dependence of responses to GABA.

METHODS

The muscle studied was the flexor tibialis of the metathoracic (jumping) leg of *Locusta migratoria*. The femur of the jumping leg is largely filled with the bulky extensor tibialis muscle. The entire extensor, together with its surrounding cuticle, was cut away to expose the much smaller flexor tibialis muscle resting against one remaining face of the cuticle. The fibers of the flexor muscle are about 7 mm long and 20–50 μ in diameter. The preparation was mounted in a small Perspex chamber for superfusion. The stump of the tibia was clamped fully extended to fix the flexor muscle at its maximum physiological length.

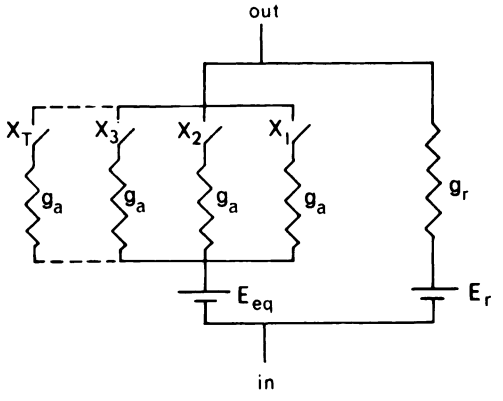

FIG. 1. *Hypothetical equivalent circuit of postsynaptic membrane*

E_r and g_r are the resting potential and resting conductance, respectively, and E_{eq} is the equilibrium potential of the chloride channels opened up by receptor activation. Closing each of the switches X_1, $X_2 \cdots X_T$ corresponds to activation of one receptor and adds unit conductance g_a to the over-all membrane conductance. Conductance increase is thus directly proportional to the number of receptors activated.

The membrane conductance of superficial muscle fibers was measured using conventional equipment and techniques. A fiber was impaled with two glass micropipettes approximately one cell diameter apart. A linear ramp of inward current was passed across the membrane through one micropipette, and the resultant potential change was recorded by the second micropipette. The current ramps were slow enough for the potential always to remain in equilibrium with the instantaneous current across the membrane. Current and potential were applied across the horizontal and vertical plates of an oscilloscope in the *xy* mode, and conductance was obtained from the slope of the line displayed on the screen. There was usually no deviation from linearity in the current-voltage relationship of the membrane over the range studied (0–10-mV hyperpolarization). No evidence of rectification was seen in the range of polarization studied.

Micropipettes with tip diameters of less than 1 μ and resistances in the 20–100 megohm range were used. Those for passing current were filled with 1 M potassium citrate neutralized to pH 7 with citric acid, and those for potential measurement were filled with 0.6 M potassium sulphate. The recorded resting potentials of the fibers were generally in the range 40–60 mV, but these values are uncorrected for the tip potential of the micropipettes. All of the fibers impaled were on the exposed inner surface of the flexor tibialis muscle. These fibers always responded to applications of GABA with an increase in membrane conductance, and this increase could be detected uniformly over the fiber surface

At a typical resting input conductance (input conductance is hereafter referred to as conductance) of 2.7×10^{-6} mho the

length constant was found to be 4.5 mm, assuming infinite cable properties (11). It can be shown that negligible error is introduced by this assumption if the fiber length is more than about 1.5 times greater than the length constant. Thus, with the tips of the micropipettes placed not more than 50 μ apart, the overestimate of the true membrane conductance was less than 2% even when conductance was increased by a factor of 2 or 3 in the presence of higher concentrations of GABA.

The muscle was superfused continuously with bathing solution flowing at 20 ml/min through a 5-ml bath. The bathing solution was an unbuffered saline ("chloride saline") containing NaCl (140 mM), KCl (10 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (2 mM) at room temperature (20–25°). These conditions were suitable only for the measurement of responses to small concentrations of GABA. The maximum response was an apparent 5-fold or greater increase in conductance, and full recovery to the control value of resting conductance was rarely seen after such responses. Replacement of a large part of the NaCl content of the saline by sodium propionate brought about an "attenuation" of the responses to GABA and an extension of the range over which they were reversible. The composition of the "propionate saline" was C_2H_5COONa (112 mM), NaCl (28 mM), KCl (10 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (2 mM). Because of the alkalinity introduced by the propionate, the solution was adjusted to pH 6.5 by addition of propionic acid.

THEORY

We deal with the case in which n molecules of ligand, A, must bind to the receptor, X, in order to produce the conformational change mediating the specific effect on membrane permeability. The conditions may be summarized as follows: (a) none of the possible conformations of X or of the complexes $AX \cdots A_{n-1}X$ is effective; (b) the formation of A_nX is associated with the specific change in conformation.

If the dissociation constants K_1, $K_2 \cdots K_n$, for each step in the interaction, are defined conventionally, and y, the fraction of total receptor activated to the effective conformation, is put equal to $[A_nX]$, it follows that at equilibrium in the presence of concentration $[A]$ of ligand

$$y = \frac{[A]^n}{([A]^n + [A]^{n-1}k_1 + \cdots + [A]k_{n-1} + k_n)} \quad (1)$$

where $k_1 = K_n$, $k_2 = K_nK_{n-1}$, $k_3 = K_nK_{n-1}K_{n-2}$, etc.

Since Eq. 1 expresses the variation with concentration of the complex A_nX, and since the formation of A_nX is associated with a specific conformational state, we may consider Eq. 1 a "function of state." The corresponding "binding function" is that formulated by Adair (8) and by Koshland *et al.* (9), which may be expressed as follows:

$$y' = \frac{n[A]^n + (n-1)[A]^{n-1}k_1 + \cdots + [A]k_{n-1}}{n([A]^n + [A]^{n-1}k_1 + \cdots + [A]k_{n-1} + k_n)} \quad (2)$$

where y' is the fractional saturation of binding sites. The function of state (Eq. 1) describes the saturation curve of formation of the activated receptor conformation, while the binding function (Eq. 2) describes the saturation curve of ligand binding to the receptor.

The most useful property of Eq. 1 (12) that distinguishes it from Eq. 2 is that

$$\lim_{A \to 0} \frac{d \log y}{d \log [A]} = n \quad (3)$$

It follows that the Hill slope,

$$\{d \log [y/(1-y)]\}/(d \log [A])$$

also approaches a limiting value equal to n as concentration decreases. For Eq. 2, the functions developed by Monod *et al.* (13), and formulations based upon them (14, 15), the slopes of the Hill plots ($\log [y/(1-y)]$ vs. $\log [A]$) all approach 1 or 0 as ligand concentration approaches 0, irrespective of the value of n. Thus the applicability of Eq. 1 can be tested if we obtain accurate experimental data at the bottom of the curve relating receptor activation to ligand concentration. All the functions mentioned,

including Eq. 1, give a limiting Hill slope of 1 at high saturation.

The theoretically limiting form of Eq. 1 at infinite cooperativity, when the intermediate species $AX \cdots A_{n-1}X$ are negligible, is the same as for Eq. 2. Both reduce to the mass action expression for an nth-order reaction

$$y' = y = \frac{[A]^n}{[A]^n + K} \qquad (4)$$

When the free energy of interaction between binding sites is zero, each site is independent and has the same intrinsic dissociation constant, K'. The macroscopic dissociation constants are now related to the intrinsic constants by simple statistical factors (16). Whereas the binding function (Eq. 2) now reduces to the Michaelis- Menten expression (16), the function of state (Eq. 1) reduces to

$$y = \left\{\frac{[A]}{[A] + K'}\right\}^n \qquad (5)$$

Equation 5 is the form used by Dodge and Rahamimoff (17) to describe the interaction of Ca^{++} with a receptor at the presynaptic terminal in frog muscle (18).

From the above limiting forms it may be anticipated that the binding and state functions will be difficult to distinguish for highly cooperative interactions and most obviously distinguishable when cooperativity is low.

Figure 2 shows how the slope of the Hill plot varies over the experimentally accessible range in four specific instances with $n = 4$. The slope given by the binding function (*c*) is seen to approach 1 as responses get smaller, while the slope given by the function of state (*b*) approaches 4. The dissociation constants substituted in both functions are the same ($K_1 = K_2 = K_3 = 100 \times K_4$) and were chosen to introduce a high degree of cooperativity.

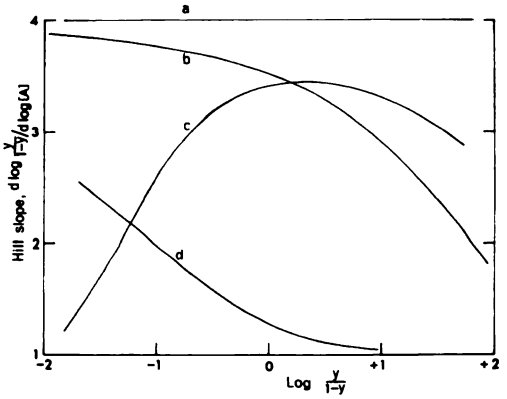

FIG. 2. *Characteristic Hill slope distributions given by different state and binding functions when* $n = 4$

Curve *a*, Eq. 4, the *n*th-order reaction; *b*, Eq. 1, the function of state ($K_1 = K_2 = K_3 = 100 \times K_4$); *c*, Eq. 2, the Adair-Koshland binding function ($K_1 = K_2 = K_3 = 100 \times K_4$); *d*, Eq. 5, zero cooperativity.

RESULTS

Nature of response to GABA. When a solution containing GABA was introduced into the chamber, the membrane conductance of the impaled fiber increased slowly over a period of minutes to reach an equilibrium value (10). This response showed no sign of desensitization in the continued presence of GABA. The membrane potential generally responded with transient hyperpolarization, followed by depolarization to below the initial resting level. The hyperpolarization was more marked in propionate saline and was frequently absent in chloride saline. The development of the conductance increase appears to proceed independently of the associated small changes in membrane polarization (10).

The detailed innervation of the flexor tibialis muscle has not to our knowledge been studied since the initial work of Hoyle (19). The following experimental evidence suggests that the measured responses were the result of a direct inhibitory neurotransmitter-like action on the postsynaptic membrane. (a) Sensitivity to the action of GABA is comparable to that found in other arthropod muscles for which there is good evidence of postsynaptic inhibition by a GABA-like transmitter. (b) The increase in conductance is specific to chloride ions. Replacement of chloride by a large anionic species such as propionate largely eliminated the conductance increase. (c) The resting conductance is not affected by addition of 0.1 mM picrotoxin [a specific antagonist of GABA and of peripheral inhibition in the

locust (2, 20)]. This indicates that there is no tonic release of an endogenous GABA-like inhibitory transmitter and, therefore, that the measured responses are not modified by a presynaptic action to modulate such release.

Dose-response curves. The log-log plots of averaged responses vs. GABA concentration were found to be linear in each experiment for concentrations below 0.1 mM. Their mean slope (10 experiments) was 2.77 ± 0.15 (standard error).

The concentration of GABA was changed in steps with no intermediate washout. No attempt was made to randomize the concentrations, since the useful life of an impaled fiber (2–3 hr) was not very long in relation to the period of equilibration (7–8 min). It was commonly observed that the equilibrium conductance increases, g', were larger on descent through the concentrations than on ascent. The data were therefore divided into "ascending" and "descending" curves and replotted as shown in Fig. 3.

The regression line through the mean ascending curve (nine experiments) shown in Fig. 3 has a slope of 3.15 ± 0.06 (standard error of estimate). The slope of the mean descending curve (10 experiments) increases somewhat as concentration decreases (the slope of the regression line through the points is 2.45 ± 0.11). The indicated maximum response [1.40 (±0.21) × 10^{-5} mho] in Fig. 3 was estimated by averaging both experimentally observed maxima and extrapolated maxima (the method of extrapolation is described below).

Figure 3 shows that for responses down to about 1% of the maximum there is no sign that the log-log plot turns towards a limiting slope of 1, as required by the binding function (Eq. 2). The plot behaves, rather, in the manner required by the function of state (Eq. 1). Given the assumption of a linear relationship between receptor activation and conductance increase (see DISCUSSION), we would infer from the limiting slope that 3 or more molecules of GABA are necessary to activate the receptor.

Chloride saline (see METHODS) was used in the above experiments, and apart from efforts to determine maxima, the largest changes measured corresponded to an approximate doubling of the resting conductance [mean g_r = 2.67 (±0.17) × 10^{-6} mho]. Larger responses often were not fully reversible. The response to GABA can be made smaller by replacing chloride with a large anion such as propionate (5, 10). Propionate saline (see METHODS) was used in the remaining experiments in order to measure responses at higher GABA concentrations. In this saline the resting conductance [mean g_r = 3.57 (±0.30) × 10^{-6} mho] was increased only by a factor of 2 or 3 at full saturation with GABA. Reversibility appears to depend more upon the size of the response than upon the concentration of GABA.

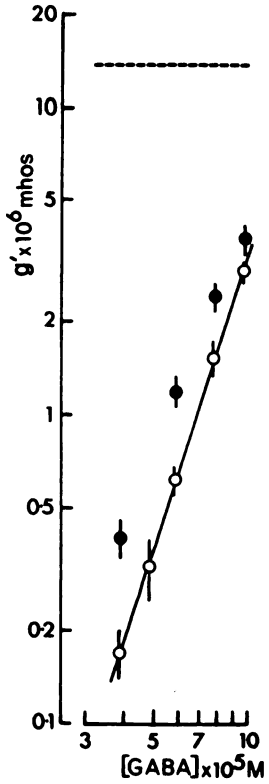

FIG. 3. *Experimental limiting slope of log-log plot of conductance increase, g', against GABA concentration (chloride saline)*

○, mean points (± standard error, nine experiments) measured on cumulative ascent of the concentrations; ●, mean points (± standard error, 10 experiments) measured on descent through the concentrations. The slope of the regression line is 3.15 ± 0.06 (standard error of estimate). - - -, estimated mean maximum response.

Theoretically, if substitution of propionate merely reduces the unit conductance change, g_a (Fig. 1), it should be possible to use the measured maxima in propionate saline to estimate a maximum for chloride saline. Small responses in the two salines differ by an approximate factor of 5.5. When the mean experimental maximum in propionate saline [5.76 ($\pm$0.40) $\times$ 10^{-6} mho] is multiplied by this factor, the result (3.2 $\times$ 10^{-5} mho) is more than double the value determined in chloride saline (1.4 $\times$ 10^{-5} mho; see above). It appears that the role of the anion may not be entirely passive.

Hill plot. Figure 4 shows the difference between log dose-response curves measured on ascent and descent of the concentrations. It should be noted that in this particular experiment, descending concentrations were used first, but that the effect was the same when the order was reversed. Although there is a shift along the concentration axis, the curves are approximately parallel, and this is confirmed by the slopes of the Hill plots (log $[y/(1 - y)]$ vs. log $[A]$), which are not consistently different. Thus to construct the mean Hill plot (nine experiments) shown in Fig. 5, ascending and descending curves were pooled.

The maximum slope of the Hill plot was taken as the slope of the regression line through the lower five points. This slope is 2.78 $\pm$ 0.05 (standard error of estimate). The function of state requires that the slope of the Hill plot shall nowhere be steeper than the limiting slope at small concentrations, and this condition is therefore fulfilled. On the other hand, the binding function will in general give a maximum Hill slope in the region of half-saturation (21).

Curve fitting. There is experimental justification (Fig. 3) for extending the linear portion of the Hill plot (Fig. 5) down to $y/(1 - y) \cong 0.01$. This Hill plot can be approximately fitted by Eq. 1 when $n = 3$ and $K_1 = 5 \times K_2 = 25 \times K_3$. When $n = 4$,

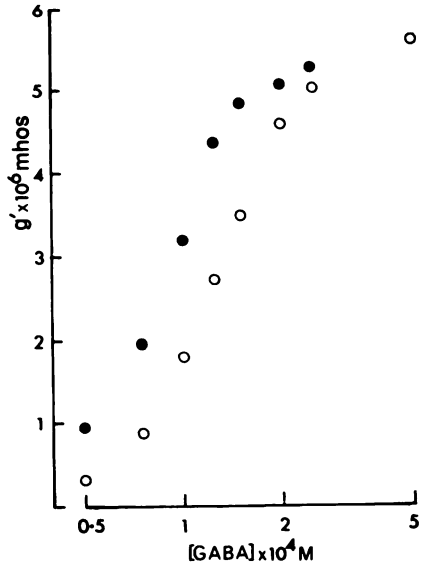

FIG. 4. *"Ascending" and "descending" log dose-response curves of GABA action (propionate saline)*

An example of the shift observed between points measured on cumulative ascent through the concentrations (○) and on descent (●) in the same experiment.

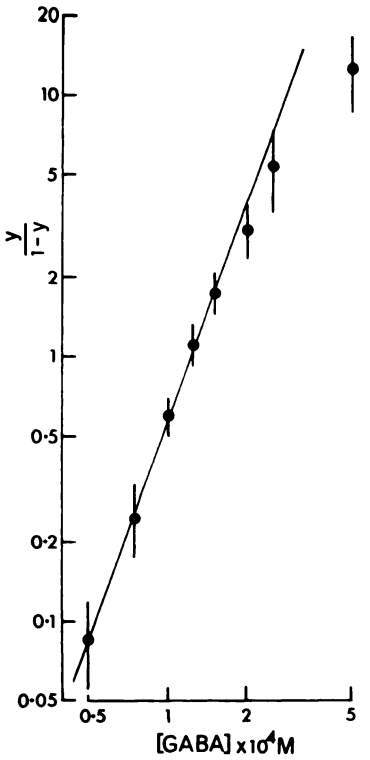

FIG. 5. *Experimental Hill plot for GABA (propionate saline)*

The regression line was computed from the lower five points (slope = 2.78 $\pm$ 0.05). These are the mean results of nine experiments ($\pm$ standard error of the mean).

it appears to be necessary that the affinity for the first GABA molecule be high in relation to the affinity for the second (negative cooperativity) in order to fit Eq. 1 to the curve.

The three most useful quantities for characterizing the dose-response curve are (a) the maximum response, g_{max} ; (b) the concentration at half-maximum response, $[A]_{0.5}$; and (c) the Hill slope. It would be advantageous to estimate these without the necessity of measuring maximum responses since, as already mentioned, such responses quite often are not fully reversible. We therefore examined the errors involved in assuming that the dose-response curve is described by Eq. 4 (nth-order reaction), which may be written in the following form:

$$\frac{1}{g'} = \frac{1}{g_{max}}\left(1 + \frac{K}{[A]^n}\right) \qquad (6)$$

The value of n was computed which minimizes the sum of squares of deviations from linearity in the double-reciprocal plot of $1/g'$ vs. $1/[A]^n$. The data treated were those used to construct Fig. 5, but excluding points above 0.24 mM GABA. Values of g_{max} and $[A]_{0.5}$ were obtained from the intercepts on the axes of the linearized double-reciprocal plot. The statistical weight of the points is not equal in the double-reciprocal plot, and this is a possible source of error. An internal check on the method was afforded by using the estimated g_{max} to construct a Hill plot and establishing both that the Hill slope did not deviate from the computed value of n and that the standard error of estimate of the Hill slope was small. In Table 1 are shown the mean values of Hill slope, $[A]_{0.5}$, and g_{max} obtained from eight experiments treated in this way. A further three experiments yielded absurd values (i.e., g_{max} either less than submaximal responses or, in one case, more than 10 times greater than the usual maxima). The mean computed value of n was 2.90 ± 0.23, which is sufficiently close to the mean Hill slope (2.80 ± 0.24), and the mean standard error of estimate of the Hill slopes was only 3.9%.

By comparison with the experimental values also shown in Table 1, the above procedure is seen to give reasonably good estimates of the Hill slope and of $[A]_{0.5}$, but to underestimate somewhat the maximum response. Use is made of this procedure in the following paper (10), in which it is shown that there is a sharp dependence of $[A]_{0.5}$ on temperature.

DISCUSSION

If, as has often been suggested, receptors are multi-subunit proteins (15, 22, 23), how much can one infer about the mechanism of receptor activation from the equilibrium relationship between activation and ligand concentration alone? We show that one can hope to decide whether receptor activation is induced only after binding to more than one site. This is possible because the appropriate equation for this case (Eq. 1) uniquely predicts that the slope of the plot of log y vs. log $[A]$ (and of the Hill plot) will increase monotonically towards a limiting integral value greater than 1 as $[A]$ decreases (12).

Figure 6 allows a comparison of the Hill plots given by Eq. 1, which we have called a function of state, and the Adair-Koshland binding function (Eq. 2), when $n = 3$ and

TABLE 1

Comparison of experimental and extrapolated values of maximum response, g_{max} , concentration at half-maximum response, $[A]_{0.5}$, and Hill slope (propionate saline)

Value	g_{max}	$[A]_{0.5}$	Hill slope
	mhos × 10^6	*M* × 10^4	
Experimental	5.76 ± 0.40[a]	1.18[b]	2.78[b]
Extrapolated[c]	4.99 ± 0.43[d]	1.08 ± 0.10[d]	2.80 ± 0.24[d]

[a] Standard error of six experiments.

[b] These values were taken from the regression line shown in Fig. 5.

[c] Method of extrapolation is described in the text.

[d] Standard error of eight experiments.

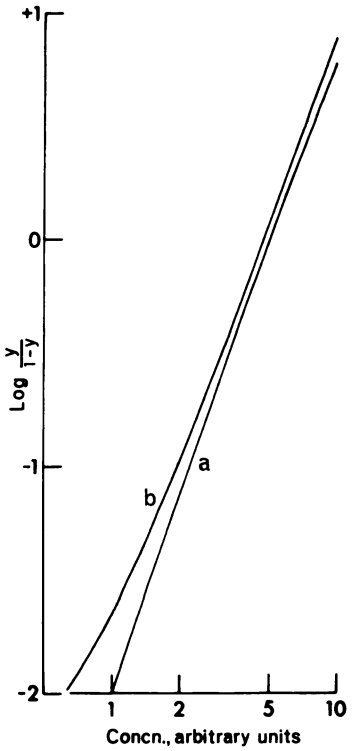

FIG. 6. *Limiting Hill slopes of state and binding functions*

The theoretical curves were obtained by putting $n = 3$ and $K_1 = 3 \times K_2 = 100 \times K_3$ in Eqs. 1 and 2. Even at this high degree of cooperativity the binding function (*b*) curves towards unity slope within the experimentally accessible range.

the dissociation constants introduce a high degree of cooperativity ($K_1 = 3 \times K_2 = 100 \times K_3$). It can be seen that the Hill slope given by the binding function drops visibly towards unity for small responses within the experimental range (curve *b*). Figure 6 also shows that if the function of state is in fact applicable, then the concentration $[A]_{0.5}$, at half-maximal response will be greater than the concentration at half-saturation of binding sites. The concentration at half-saturation is a widely used measure of over-all ligand affinity, and it will be overestimated if use is made of $[A]_{0.5}$ in its stead. The two values converge when cooperativity is high and the concentration of intermediate complexes falls.

Our experimental results with GABA can be taken to indicate that 3 molecules of GABA are required to activate the receptor. However, this interpretation raises the question of the extent to which increases in conductance are a direct measure of receptor activation and, thus, whether the theoretical equations considered in this paper are applicable to the experimental dose-response curves.

A simple view of GABA receptors is that they are identical membrane-bound protein molecules which, on interaction with GABA, independently undergo a characteristic change in conformation to produce anion-specific channels in the membrane. According to this view the equivalent circuit illustrated in Fig. 1 is appropriate to the changes in membrane conductance on the conditions that (a) net movements of ions do not alter the unit conductance (g_a) and (b) small changes in membrane polarization do not affect permeability. Evidence that these conditions are fulfilled in the present experiments is discussed in the following paper (10). Even though there is a possibility that channel permeability is in some way dependent on GABA concentration (24), it is more likely (12, 25) that, under favorable conditions, conductance increase may be related linearly to receptor activation.

In the locust muscle studied we observed none of the desensitization phenomena found in crustacean muscle (26, 27). To the contrary, some apparent metastability in the responses to GABA was observed (Fig. 4). Further possible evidence of metastability is noted in the following paper (10).

The study of GABA receptors is at an early stage, but the approach used here may have some general bearing on current attempts at the isolation and characterization of receptor protein (28, 29). Our results suggest that binding data obtained with isolated receptor should not necessarily match the concentration dependence of receptor activation *in situ*. An important test of the isolation of functional receptor will involve the incorporation of the purified material into artificial bileaflet membranes (12, 30). Measurements of membrane conductance changes as a function of ligand concentration can then be made for comparison with corresponding data obtained from intact cells.

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