Br. J. Pharmacol. (1988), **95**, 805–810

Amino acid pharmacology in neocortical slices: evidence for bimolecular actions from an extension of the Hill and Gaddum-Schild equations

[1]Thelma L. Williams, D.A.S. Smith, N.R. Burton & T.W. Stone

Dept. of Physiology and Neurosciences Research Group, St. George's Hospital Medical School, University of London, London SW17 0RE

1 The equivalent circuit of the cortical slice preparation has been analysed to show that the measured d.c. voltages can be used to estimate the Hill coefficient for ligands acting on the pyramidal cell bodies.

2. The Gaddum-Schild equation for agonist-antagonist interactions has been modified for applications in which the Hill coefficient is not equal to 1.

3 The equations obtained have been applied to recent data of Burton *et al.* (1988). The results provide evidence for a bimolecular action of the agonists N-methyl-D-aspartate (NMDA) and quinolinic acid and the antagonists kynurenic acid and 2-amino-5-phosphono-pentanoic acid at NMDA receptors on mouse neocortical pyramidal cells.

Introduction

The quantitative analysis of drug-receptor interactions developed by Gaddum (1937) and Arunlakshana & Schild (1959) is widely used for comparisons of agonist or antagonist action. It does not yield any information on the stoichiometry of the agonist-receptor interaction. For this a Hill plot is required (Hill, 1910) the slope of which provides a measure of the cooperativity of the reaction. In the Hill model, based on simple mass action (Clark, 1926), the slope is an integer and represents the number of agonist molecules required for receptor activation. Alternative models are available which are consistent with a slope which is not integral (see Colquhoun, 1973).

The construction of a Hill plot is based on the assumption that the response measured is directly proportional to the number of receptors activated, but this assumption cannot be made for most experimental systems. Indeed the only measurements to which the Hill analysis can reasonably be applied are those of membrane conductance changes during the application of known concentrations of agonist. Voltage changes occurring secondary to the conductance change would normally be unsatisfactory for the determination of Hill coefficients.

Harrison & Simmonds (1985) have described a technique for measuring the d.c. potential between a fragment of cerebral cortex and the associated underlying white matter containing the efferent axons of pyramidal cells. This method is ideally suited to quantitative analysis of the actions of substances acting on the pyramidal cell bodies.

In the present paper we analyse the equivalent circuit of the recording system used and show that this method is suitable for constructing Hill plots and assessing agonist molecule cooperativity. We then show how the classical Gaddum-Schild plot can be modified for situations in which the agonist:receptor stoichiometry is not 1:1 as traditionally assumed. Finally we illustrate the application of our model to data recently obtained by Burton *et al.* (1988) on the actions of excitatory amino acids in the neocortical slice preparation.

The Gaddum-Schild derivation

The classical model for the interaction between receptors and their agonists (Hill, 1910; Clark, 1926) is based on the law of mass action. Thus if the reaction between agonist and receptor is described by

$$nC + R \rightleftharpoons C_nR$$

where C signifies the agonist molecule, R the unbound receptor and C_nR the agonist-receptor

[1] Author for correspondence.

complex, then at equilibrium

$$[C]^n[R] = K_C[C_nR] \quad (1)$$

where K_C is the dissociation constant for the reaction and [C], [R], and $[C_nR]$ are the concentrations of the respective species. Furthermore, the response mediated by the receptor complex is assumed to be proportional to the concentration of occupied receptors, so that

$$y = \frac{[C_nR]}{[R] + [C_nR]} \quad (2)$$

where y gives the response as the fraction of the maximal response obtained.

Combining equations (1) and (2) gives

$$y = \frac{[C]^n}{[C]^n + K_C} \quad (3)$$

from which it follows that

$$\frac{y}{1-y} = \frac{[C]^n}{K_C} \quad (4)$$

which leads to

$$\log \frac{y}{1-y} = -\log K_C + n \log [C] \quad (5)$$

A plot of $\log \{y/(1-y)\}$ against log [C] should yield a straight line with slope n and intercept pK_C, the negative logarithm of the dissociation constant.

In the presence of a competitive antagonist the following reactions occur simultaneously:

$$nC + R \rightleftharpoons C_nR \quad (6)$$

$$mA + R \rightleftharpoons A_mR \quad (7)$$

where m signifies the number of antagonist molecules, A, acting at the receptor.

At equilibrium, equation (1) applies and also

$$[A]^m[R] = K_A[A_mR] \quad (8)$$

where K_A is the dissociation constant of the antagonist-receptor reaction. In the presence of antagonist, equation (2) must be modified so that the response (as a fraction of the maximal) is given by

$$y = \frac{[C_nR]}{[R] + [C_nR] + [A_mR]}. \quad (9)$$

Equations (1), (8) and (9) can be combined to give

$$y = \frac{[C]^n}{K_C + [C]^n + [A]^m K_C/K_A}. \quad (10)$$

If reactions (6) and (7) are first-order (n and m both equal to 1) then from equations (3) and (10), for concentrations [C] and [c] of agonist producing the same response in the presence and absence, respectively, of antagonist

$$\frac{[C]}{[c]} = 1 + [A]/K_A.$$

This is the Gaddum-Schild equation, which can also be expressed as

$$\log (DR - 1) = pK_A + \log [A]. \quad (11)$$

where DR is the dose-ratio.

If time is allowed for equilibrium, a plot of log (DR − 1) against log [A] should give a straight line with unit slope and intercept of pK_A, the negative logarithm of the dissociation constant of the antagonist-receptor complex. Since K_C has been eliminated in this analysis the result should be independent of the agonist used, as long as it acts at the given receptor. This analysis is valid only if both n and m of equations (3) and (10) can be assumed to be equal to 1. We will obtain an equation similar to equation (11) but for any combination of integers n and m. Putting [c] in equation (3) for the concentration of agonist required for a given response in the absence of antagonist, equating the response to that given by equation (10) and rearranging (Rang, 1971),

$$([C]/[c])^n = 1 + [A]^m/K_A. \quad (12)$$

In a later section we will show that if it is possible to estimate n, the number of agonist molecules needed at the receptor site, equation (12) can be put into a form which can be used to estimate m, the number of antagonist molecules which bind at the receptor site.

Analysis

Conductance and voltage in the cortical slice preparation

The neocortical slice preparation, originally described using rat tissue by Harrison & Simmonds (1985) and subsequently for mouse brain by Burton *et al.* (1988) consists of slices of cerebral tissue containing cerebral cortex and corpus callosum, mounted in a two-chambered bath. The ventral margin of the cortex passes through a greased slot such that the corpus callosum is in one chamber and almost all of the cortical tissue in the other. In Figure 1 we have drawn an equivalent circuit of this preparation. The diagram is drawn as if for a single pyramidal cell but the same equivalent circuit pertains to many cells in parallel, if their properties are similar. Furthermore, the cable properties of the

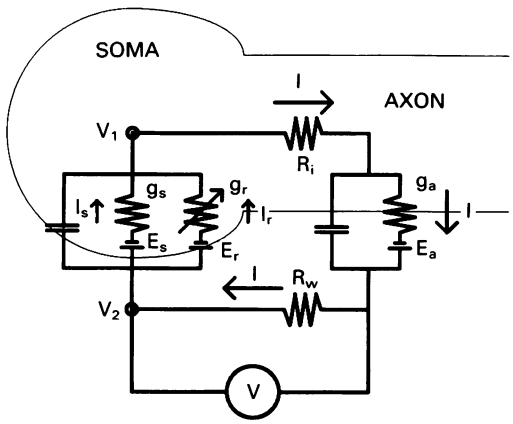

Figure 1 Equivalent circuit of the cortical slice preparation. The somata of the pyramidal cells are in one chamber while the greater part of their axons is in the other. A grease seal (represented by R_w) separates the extracellular contents of the two chambers. See text for symbol definitions.

axons do not affect the form of the equations but only the values of the lumped parameters R_i and g_a.

There are two types of current pathway connecting the two chambers, the first through the axons of the pyramidal cells which enter the corpus callosum (R_i) and the second an extracellular pathway limited by the grease seal (R_w). All the voltage measurements are made in the steady state, so that the membrane capacitance does not enter the circuit equations. We intend to show how the measured d.c. voltage difference between the two compartments (V) depends upon the conductance of the ionic pathway opened by receptor activation. The following symbols will be used:

E_a = resting potential of the axonal membrane.
E_r = reversal potential of the receptor-activated channel.
E_s = resting potential of the somatic membrane.
g_a = conductance of the axonal membrane.
g_r = conductance of the receptor-activated channel.
G_r = maximal value of this conductance.
g_s = resting conductance of the somatic membrane.
I = the current flow around the loop, taken as positive in the directions shown by the arrows.
I_r = the current through the agonist-activated channels.
I_s = the current through the resting somatic conductance.
R_i = axoplasmic resistance.
R_w = extracellular resistance through the grease seal.
$R_{tot} = R_w + R_i + 1/g_a$.
V = potential difference between the two chambers.
V_{max} = the value of V when $g_r = G_r$.

The values of all these parameters except g_r, V and the currents remain constant. Our task is to eliminate the currents from the circuit equations so as to express V as a function of g_r and the constant parameters.

The voltage difference across the somatic membrane through the resting somatic membrane conductance (see Figure 1) is given by

$$V_1 - V_2 = E_s - I_s/g_s \tag{13}$$

and the voltage difference through the receptor-activated channels by

$$V_1 - V_2 = E_r - I_r/g_r. \tag{14}$$

Furthermore

$$I_r + I_s = I. \tag{15}$$

Eliminating I_s and I_r from equations (13)–(15) and rearranging gives

$$V_1 - V_2 = \frac{g_s E_s + g_r E_r - I}{g_s + g_r}. \tag{16}$$

The same voltage difference along the pathway which passes through the axonal membrane is given by

$$V_1 - V_2 = IR_w + E_a + I/g_a + IR_i. \tag{17}$$

Setting the right-hand sides of equation (16) and (17) equal to each other and rearranging gives

$$I = \frac{g_s(E_s - E_a) + g_r(E_r - E_a)}{R_{tot}(g_s + g_r) + 1} \tag{18}$$

where $R_{tot} = R_w + R_i + 1/g_a$.

Since $V = IR_w$ (see Figure 1) and E_a can be assumed equal to E_s (as without receptor activation, I = 0), from equation (18) we have

$$V = \frac{R_w g_r(E_r - E_a)}{R_{tot}(g_s + g_r) + 1}. \tag{19}$$

When the maximum response is obtained (at high agonist concentration), $g_r = G_r$ and $V = V_{max}$ so that

$$V_{max} = \frac{R_w G_r(E_r - E_a)}{R_{tot}(g_s + G_r) + 1}. \tag{20}$$

Dividing equation (19) by (20) gives

$$\frac{V}{V_{max}} = \frac{g_r}{G_r} \frac{(R_{tot}(g_s + G_r) + 1)}{(R_{tot}(g_s + g_r) + 1)}. \tag{21}$$

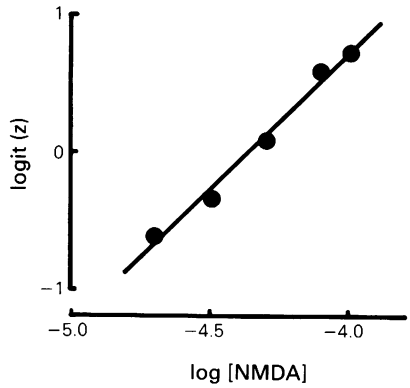

Figure 2 Hill plot of the voltage change induced by bath application of N-methyl-D-aspartate (NMDA) in mouse cortical slice. Data of Burton *et al.* (1988). z = fraction of maximal response. Line drawn from linear regression equation, logit (z) = 8.59 + 1.97 log [NMDA].

Let $z = V/V_{max}$, the normalised voltage measured for different agonist and antagonist concentrations. Then $0 \leqslant z \leqslant 1$.

Let $y = g_r/G_r$, the fraction of channels opened, $0 \leqslant y \leqslant 1$. Substituting for z and y in equation (21) and rearranging gives

$$y = (B - 1)\frac{z}{B - z} \tag{22}$$

where B is a constant given by

$$B = 1 + \frac{g_s + 1/R_{tot}}{G_r}. \tag{23}$$

Equation (22) thus gives the required relationship between the measured voltage (z) and the receptor-activated conductance (y).

The Hill plot

By the law of mass action, equation (1) relates the equilibrium concentrations of the agonist and the bound and unbound forms of the receptor. The conductance of the receptor-activated channels can be assumed to be proportional to C_nR, the number of agonist-bound receptors. Thus the normalised conductance, y, can be taken as equal to the fraction of receptors which are activated, and equations (2) and (3) are valid.

Combining equations (3) and (22) and rearranging gives

$$\frac{z}{1 - z} = \frac{B}{B - 1}\frac{[C]^n}{K_C}. \tag{24}$$

Substituting for B from equation (23) and taking logarithms leads to

$$\log\frac{z}{1 - z} = \log\left(1 + \frac{G_r}{g_s + 1/R_{tot}}\right) - \log K_C + n\log [C]. \tag{25}$$

This equation differs from equation (5) only by an additive constant. Thus if the mass action model is adequate a Hill plot (i.e. log (z/(1 − z)) versus log [C]) will yield a straight line with a slope of n. The intercept will no longer give the value of the dissociation constant because of the extra term in equation (25). It will still be possible in a given experiment to use the intercept to compare the affinity of different agonists for the same receptor, since the additive constant is specific for the preparation and recording system and also for the receptor (because of the term G_r).

The modified Gaddum-Schild plot

Rearranging equation (12) and taking logarithms we obtain

$$\log (DR^n - 1) = pK_A + m\log [A]. \tag{26}$$

Having determined n (the number of agonist molecules needed at the receptor site) from a Hill plot based on equation (5) or (25) we may now make modified Gaddum-Schild plots from equation (26), i.e. by raising to the power n the dose ratios obtained for different concentrations of antagonist and then plotting log $(DR^n - 1)$ against log [A]. The slope of this plot will give the value of m, the number of antagonist molecules needed simultaneously at the receptor site.

The stoichiometry of the NMDA receptor

In this section we shall attempt to use the quantitative data on amino acid agonists and antagonists reported by Burton *et al.* (1988) in order to deduce the characteristics of the stoichiometry of amino acid receptors. For this analysis it will be important to note that individual application of both agonists and antagonists were continued until their respective effects had reached a maximum amplitude. This is not stated explicitly in the original paper by Burton *et al.* (1988), but the durations of agonist superfusion (2 min) and antagonist application (15–30 min) were carefully selected on the basis of preliminary experiments to confirm the attainment of maximum response.

Table 1 Data from Burton *et al.* (1988)

Agonist	*Hill slope* ± *s.e.*	n	*Modified Schild slope* ± *s.e.* *Kynurenate*	n	(DL)-*AP5*	n
Quinolinate	1.95 ± 0.18	6	2.08 ± 0.13	6	1.54 ± 0.052	5
NMDA	2.28 ± 0.10	6	2.24 ± 0.07	7	1.83 ± 0.08	4

NMDA = N-methyl-D-aspartate; DL-AP5 = DL-2-amino-5-phosphonopentanoic acid.

It was also noted by Burton *et al.* (1988) that in order to prevent desensitization or toxicity the concentration-range of some agonists was limited and as a result a clear maximum on the concentration-response curve was not always produced. As this would affect the following analysis, we have restricted consideration only to those concentration-response curves which did reach a clear maximum level.

In Figure 2 is shown a Hill plot of data from Burton *et al.* (1988) for the action of NMDA in mouse cerebral cortex. A linear regression analysis yielded a slope of 1.97 ± 0.22 (s.e.). The average Hill slopes obtained for both NMDA and quinolinate are given in Table 1. Neither value is statistically different from 2 but both are different from either 1 or 3, with $P < 0.001$ for NMDA and $P < 0.01$ for quinolinate. Thus these data are consistent with a receptor-agonist interaction in which 2 molecules of agonist are required to bind simultaneously for receptor activation.

On the basis of this finding, modified Gaddum-Schild plots were constructed from the data of Burton *et al.* (1988) with n = 2 in equation (26). A representative result is shown in Figure 3 for the antagonist kynurenate against the agonist quinolinate. In this experiment a regression analysis yielded a slope of 2.19 ± 0.10 (s.e.). The average slope values are given in Table 1 for the antagonists kynurenate and 2-amino-5-phosphonopentanoic acid (AP5) against the agonists quinolinate and NMDA. For kynurenate neither of the average slopes was significantly different from 2. Hence these data are consistent with the view that 2 molecules of kynurenate are required at the receptor site.

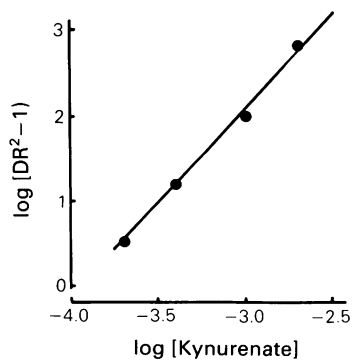

Figure 3 Modified Gaddum-Schild plot of data obtained from mouse cortical slice by Burton *et al.* (1988) of antagonism of quinolinic acid by kynurenic acid. Line drawn from regression equation, $\log (DR^2 - 1) = 8.65 + 2.19 \log$ [kynurenate].

For AP5 the average slopes are somewhat less than 2, and the value for AP5 against quinolinic acid is significantly different from 2 ($P < 0.001$). However the racemic mixture of AP5 was used in these experiments and the partial agonist activity of L-AP5 may reduce the apparent dose ratios and thus yield a slope which is less than the true value of m.

Discussion

There are three main findings in this paper. Firstly, we have demonstrated that the equivalent circuit of the cortical slice preparation (Harrison & Simmonds, 1985) leads to equation (25), which allows the use of a Hill plot to determine the cooperativity of the agonist-receptor activation and to compare the affinity of different agonists for the same receptor. Secondly, we have shown how a modified Gaddum-Schild plot based on equation (26) can be used to determine the stoichiometry of the antagonist-receptor interaction and to provide a means of comparing different agonists and antagonists at stoichiometries other than 1:1. Finally, we have applied these results to the data of Burton *et al.* (1988) to obtain evidence for a stoichiometry of 2:1 for the interactions between agonists and NMDA-receptor sites (NMDA and quinolinic acid; Stone & Connick, 1985; Stone & Burton, 1988) in mouse neocortex.

A similar stoichiometry of 2 appears to obtain for the antagonism of NMDA and quinolinate by kynurenic acid (Stone & Burton, 1988) and probably for the action of the active isomer of AP5. It is not immediately obvious why two molecules of antagonist should be required to block activation of the receptor; intuitively one should be sufficient.

However, little is known about the structure of amino acid receptors or the degree to which conformational changes may be required for the binding of a first and second molecule of agonist. We therefore envisage a situation in which two molecules of agonist mutually enhance each other's binding to the receptor such that a single molecule of antagonist will be readily displaced. Two antagonist molecules however may mutually potentiate their binding so that the receptor is locked into a form relatively inaccessible to the agonists.

One argument which may be raised against this view is that competitive antagonists (such as AP5 or kynurenic acid) are traditionally considered as interacting with a receptor at the same site and in the same spatial orientation as the agonist. There has never been strong evidence that this is so and indeed recent evidence suggests that the competitive antagonists AP5 and AP7 interact with the receptor at sites different from the agonists (Fagg *et al.*, 1988).

The use of Hill and Gaddum-Schild analyses as outlined here may be applied to similar *in vitro* preparations, such as the hemisected spinal cord (Evans & Watkins, 1978), as long as there is reason to assume (as in the presence of tetrodotoxin) that the agonist is acting directly on the cells whose axons provide the intracellular current pathway through the seal (the pyramidal cells in the slice preparation, the motoneurones in the hemisected cord).

The authors' work is supported by the SERC and the Wellcome Trust.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BURTON, N.R., SMITH, D.A.S. & STONE, T.W. (1988). A quantitative analysis of some excitatory amino acid receptors in the mouse neocortex *in vitro*. *Br. J. Pharmacol.*, **93**, 693–701.

CLARK, A.J. (1926). The reaction between acetylcholine and muscle cells. *J. Physiol.*, **61**, 530–546.

COLQUHOUN, D. (1973). The relation between classical and cooperative models for drug action. In *Drug Receptors*. ed. Rang, H.P. London: Macmillan.

EVANS, R.H. & WATKINS, J.C. (1978). Specific antagonism of excitatory amino acids in the isolated spinal cord of the neonatal rat. *Eur. J. Pharmacol.*, **50**, 123–129.

FAGG, G.E., BAUD, J., HALL, R. & DINGWALL, J. (1988). Agonists and competitive antagonists bind to distinct sites on the NMDA receptor complex. *Neurochem. Internat.*, **12**, Suppl. 1, 25.

GADDUM, J.H. (1937). The quantitative effects of antagonistic drugs. *J. Physiol.*, **61**, 141–150.

HARRISON, N.L. & SIMMONDS, M.A. (1985). Quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex. *Br. J. Pharmacol.*, **84**, 381–391.

HILL, A.V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol.*, **40**, IV–VIII.

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

STONE, T.W. & BURTON, N.R. (1988). NMDA receptors and endogenous ligands in the vertebrate CNS. *Progr. Neurobiol.*, **30**, 333–368.

STONE, T.W. & CONNICK, J.H. (1985). Quinolinic acid and related kynurenines in the CNS. *Neuroscience*, **15**, 597–617.

(Received April 28, 1988
Accepted June 7, 1988)