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Cellular uptake disguises action of L-glutamate on N-methyl-D-aspartate receptors

With an appendix: Diffusion of transported amino acids into brain slices

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1 Pharmacological properties of the guanosine 3'5'-cyclic monophosphate (cyclic GMP) responses to excitatory amino acids and their analogues were compared in slices and dissociated cells from the developing rat cerebellum maintained *in vitro*. The intention was to determine the extent to which cellular uptake might influence the apparent properties of receptor-mediated actions of these compounds.

2 In slices, the potencies of the weakly (or non-) transported analogues, N-methyl-D-aspartate (NMDA) and kainate (KA) (EC_{50} = 40 μM each) were higher than those of the transported amino acids, D- and L-aspartate (EC_{50} = 250 μM and 300 μM) and D- and L-glutamate (EC_{50} = 540 μM and 480 μM). Quisqualate (up to 300 μM) failed to increase cyclic GMP levels significantly. The sensitivity of agonist responses to the NMDA receptor antagonist, DL-2-amino-5-phosphonovalerate (APV), was in the order NMDA > L-aspartate > L-glutamate, KA.

3 In dissociated cells, L-glutamate was 280 fold more potent (calculated EC_{50} = 1.7 μM). L- and D-aspartate (calculated EC_{50} = 13 μM) and D-glutamate (EC_{50} = 130 μM) were also more effective than in slices. The potencies of NMDA and KA were essentially unchanged. Responses to NMDA, L-glutamate and L-aspartate under these conditions were equally sensitive to inhibition by APV but the response to KA remained relatively resistant to this antagonist.

4 The implications of these results are that, in slices, cellular uptake is responsible for (i) the dose-response curves to L-glutamate, L- and D-aspartate bearing little or no relationship to the true (or relative) potencies of these amino acids; (ii) the potency of APV towards the actions of transported agonists acting at NMDA receptors being reduced and (iii) a differential sensitivity to APV of responses to L-glutamate and L-aspartate being created, the consequence being that a potent action of L-glutamate on NMDA receptors is disguised.

5 These conclusions are supported by theoretical considerations relating to the diffusion of transported amino acids into brain slices, as elaborated in the Appendix.

Introduction

The acidic amino acid analogues, N-methyl-D-aspartate (NMDA), quisqualate (QA) and kainate (KA) are potent neuronal excitants in the vertebrate central nervous system and their actions are thought to be mediated through separate receptors. The identity of the natural ligands for these receptors remains uncertain but the continuing favourites are the neurotransmitter candidates, L-glutamate (L-Glu) and L-aspartate (L-Asp). Studies with a range of antagonists have led to the suggestion that, while neither compound acts exclusively on one receptor type, L-Asp acts mainly at NMDA receptors and L-Glu at non-NMDA (QA and KA) receptors when these amino acids are administered by iontophoresis or in the medium bathing *in vitro* preparations (Watkins & Evans, 1981; Davies *et al.*, 1982; McLennan, 1983).

One factor which complicates the interpretation of antagonist effects when mixed agonists such as L-Asp and L-Glu are used, is the presence – as is usual – of more than one receptor type on each neurone (Watkins, 1981; Mayer & Westbrook, 1984). Another potentially complicating factor arises because L-Glu and L-

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Asp are substrates for carrier-mediated uptake processes which transport the amino acids from the interstitial environment into cellular sites (Balcar & Johnston, 1972). The operation of these mechanisms is usually blamed, with experimental justification (Curtis *et al.*, 1970; Johnston *et al.*, 1980), for the low apparent potencies of exogenously-applied L-Glu and L-Asp. By influencing the concentration profiles of transported amino acids within tissues, cellular uptake mechanisms could also affect the sensitivity of their excitatory actions to antagonists (Curtis, 1976; Peet *et al.*, 1983).

The present experiments were performed in order to evaluate the importance of cellular uptake on the observed properties of receptor-mediated responses to excitatory amino acids and their analogues. To do so, pharmacological features of the guanosine 3',5'-cyclic monophosphate (cyclic GMP) responses to L-Glu, L-Asp, NMDA, KA and QA (Garthwaite, 1982) have been compared in cerebellar slices, in which access to receptors would depend on diffusion through interstitial spaces, and in dispersed cerebellar cells, where this barrier would be circumvented.

Methods

Slices and dispersed cells were prepared from the cerebella of 8 day old rats (Wistar origin, either sex). The animals were treated with the mitotic inhibitor, hydroxyurea (2 mg g^{-1}, i.p.) 2 days before use in order to delete the numerous, but non-responsive, proliferating cells (Garthwaite & Balázs, 1978; 1981). The cerebella were chopped at 0.4 mm intervals in both sagittal and coronal planes with a McIlwain chopper (Mickle Laboratory Engineering Co.).

Cell suspensions were obtained by mild trypsinization of the tissue slices followed by trituration in the presence of trypsin inhibitor using the method of Wilkin *et al.*, (1976) with modifications described by Dutton *et al.*, (1981). The cells were counted by means of a Coulter Counter or haemacytometer and incubated at 37°C in 1 ml volumes containing 18 to 30×10^6 cells. Such high cell concentrations are needed, for reasons that are unclear at present, to maximize the cyclic GMP responses to excitatory amino acids (see Garthwaite & Balázs, 1981). The incubation medium was equilibrated with air and contained (mM): NaCl 128, KCl 5, $CaCl_2$ 2, NaH_2PO_4 5, $MgSO_4$ 1.2, Tris-HCl 10 and glucose 11, supplemented with 1% bovine serum albumin, pH 7.4. In preliminary experiments, several alternative incubation solutions were tested, including the Krebs-Henseleit medium gassed with CO_2/O_2 used for slices (see below) and the above Tris/phosphate-buffered medium gassed with O_2. The results were essentially the same as those obtained with the ungassed, air-equilibrated medium finally adopted. However, responses from cells incubated in the gassed solutions tended to be lower (possibly because of O_2 toxicity) and the gassing of small volumes for relatively long periods of time sometimes caused serious problems of evaporation. In the air-equilibrated medium, intracellular Na^+ and K^+ were maintained at physiological levels for at least 2 h (Garthwaite & Balázs, 1981).

For the slice experiments, the chopped tissues were taken through exactly the same dissociation procedure, including trypsin treatment, but instead of being dispersed, were incubated as intact slices at 37°C in a Krebs-Henseleit medium containing (mM): NaCl 118, KCl 4.7, $CaCl_{22}$, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4$ 1.19 and glucose 11, continuously gassed with 5% CO_2 in O_2, as described by Garthwaite & Balázs (1978).

The cells and slices were allowed a preincubation period of 1 h or more. At predetermined times after addition of test compounds, aliquots of cells or slices were withdrawn and boiled for 2–5 min in 3 or more volumes of 50 mM Tris, 4 mM EDTA buffer, pH 7.6. Cyclic GMP levels in the supernatant solutions obtained after sonication and centrifugation were measured by radioimmunoassay as described previously (Garthwaite & Balázs, 1978; Garthwaite & Gilligan, 1984). Proteins were measured by the automated method of Lowry *et al.* (1951).

Compounds

L-Glutamic acid (sodium salt), D-glutamic acid, L-aspartic acid, D-aspartic acid, kainic acid, quisqualic acid and bovine serum albumin (essentially fatty acid-free) were obtained from Sigma; γ-D-glutamylaminomethylsulphonic acid and L-2-amino-4-phosphonobutyric acid were from Tocris Chemicals; γ-D-glutamylglycine was from Merseyside Laboratories; DL-2-amino-4-phosphonobutyric acid was from Calbiochem and N-methyl-D-aspartic acid and DL-2-amino-5-phosphonovaleric acid were gifts from Dr J.F. Collins (City of London Polytechnic).

Results

In the slices, the time courses of cyclic GMP accumulation in response to the excitants were similar to those reported previously for slices which had neither been pretreated with hydroxyurea (used in the present study to deplete dividing cells *in vivo*) nor treated with trypsin (Garthwaite, 1982); peak elevations occurred after 1–2 min depending on the agonist tested (see legend to Figure 1). Concentration-response curves (Figure 1a) showed that KA and NMDA were apparently more potent than L-glutamate (L-Glu) and

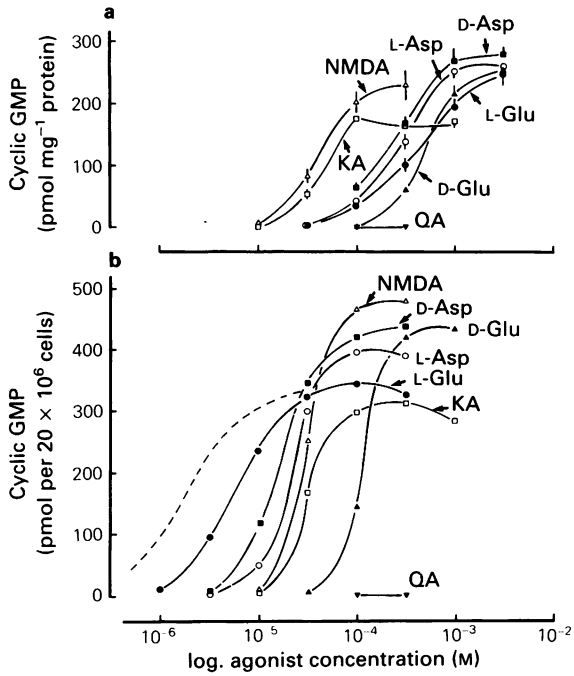

Figure 1 Cyclic GMP accumulation in slices (a) and dispersed cells (b) from 8 day old rat cerebellum in response to NMDA (△), KA (□), L-Glu (●), L-Asp (○), D-Glu (▲) and D-Asp (■). In (a), the responses (pmol-mg^{-1} protein) were measured 1 min (for NMDA and KA) and 2 min (for the others) after addition of the agonists, these being the times required for peak accumulations to develop. Each point is the mean of 4 determinations; bars are ± s.e.mean. In (b), the points are the means of duplicate or triplicate estimations, each estimation being made on 2 to 3 × 10^6 cells from a suspension prepared from the cerebella of 10 to 30 rats. The results with all agonists were checked in at least one further experiment. Time courses (covering 0–2 min exposure) were carried out for each concentration (see examples in Figure 2); the data plotted in (b) represent peak responses. In the case of QA (▼), cyclic GMP levels were followed for 10 min (a) or 2 min (b) without significant changes being detected.

L-aspartate (L-Asp); EC_{50} values were estimated to be 40 μM (for KA), 40 μM (NMDA), 300 μM (L-Asp) and 480 μM (L-Glu). These are close to estimates made previously from non-trypsinized slices (Garthwaite, 1982) but maximum responses (about 250 pmol mg^{-1} protein) and basal levels (1 pmol mg^{-1} protein) were about 80% higher in the present experiments because the slices were rid of the non-responsive dividing cells (Garthwaite & Balázs, 1978). The EC_{50} values for D-Asp and D-Glu (250 μM and 540 μM respectively) were similar to those of their L-isomers. Cyclic GMP levels were not changed significantly in the presence of QA (100 and 300 μM) for up to 10 min.

In the cell suspensions, the highest levels of cyclic GMP were present 15 s–1 min after addition of the agonists; for illustration a family of progress curves for different concentrations of L-Glu and L-Asp are shown in Figure 2. Maximum accumulations of cyclic GMP (300–500 pmol/2 × 10^7 cells) were 50 to 100% higher than in slices (2 × 10^7 cells contain about 1 mg protein; Gordon & Balázs, 1983). While the EC_{50} values for KA and NMDA (30 μM each) were very similar to the estimates made for slices, L-Glu and L-Asp were much more potent in the dispersed cells (Figure 1b). On the basis of initial concentrations, EC_{50} values were 5.6 μM for L-Glu and 20 μM for L-Asp. However, the concentrations of these amino acids in the medium would become progressively reduced with time because cells able to accumulate them are present in the suspensions (Gordon & Balázs, 1983). The concentrations at the peaks of the responses to L-Glu were computed from the integrated form of the Michaelis-Menton equation (Roberts, 1977) using kinetic constants derived from measurements of

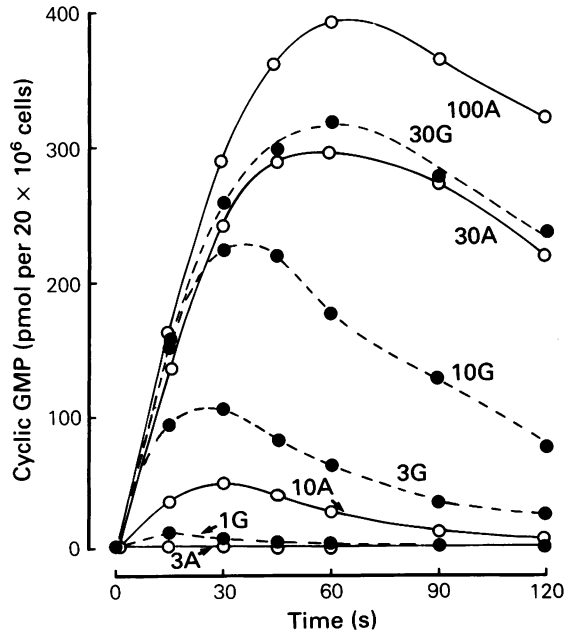

Figure 2 Time-dependence of cyclic GMP accumulation in cerebellar cell suspensions following addition of L-Glu (G; broken lines) or L-Asp (A; solid lines) to give the concentrations (in μM) indicated on each curve. Points are as described in the legend to Figure 1.

the rate of L-Glu uptake in equivalent cell suspensions (Gordon & Balázs, 1983). Based on these calculated final concentrations, the EC_{50} for L-Glu reduces to 1.7 μM (see Figure 1b, broken line). Assuming that similar uptake kinetics apply to L-Asp (Gordon & Balázs, 1983), the EC_{50} for this agonist would be 13 μM. D-Asp was approximately equipotent with its L-isomer but D-Glu (EC_{50} = 130 μM) was much less effective than L-Glu. As in slices, QA failed to increase cyclic GMP levels significantly.

The NMDA receptor antagonist, DL-2-amino-5-phosphonovalerate (APV) was tested over a range of concentrations against reponses to L-Glu, L-Asp, NMDA and KA in close to maximally-effective concentrations. In slices, the response to NMDA was antagonized more effectively than that to L-Asp; responses to L-Glu and KA were least affected, being less than 40% reduced even at a high (300 μM) APV concentration (Figure 3a). When examined on dissociated cells, however, responses to L-Glu and L-Asp were reduced with increasing APV concentrations approximately in parallel with the response to NMDA (Figure 3b) so that the actions of all three agonists were inhibited by more than 90% at 100 μM APV. The response to KA, in contrast, remained as insensitive to APV as it was in slices. Concentrations of APV causing 50% inhibition (IC_{50} values), estimated after subtraction of unstimulated cyclic GMP levels and replotting the data in Figure 3b on log-probit graph paper, were: 18 μM for NMDA, 25 μM for L-Glu and L-Asp and > 300 μM for KA.

Other antagonists were also examined for their ability to block responses to L-Glu (30 μM) and KA (100 μM) in the cell suspensions. γ-D-Glutamylglycine (tested over the range 30 μM to 1 mM) inhibited L-Glu-induced increases in cyclic GMP with 50% reduction occurring at 180 μM and >90% at 1 mM, without affecting KA responses. DL- and L-2-Amino-4-phosphonobutyric acid (10 to 300 μM), γ-D-glutamylaminomethylsulphonate (30 μM to 1 mM) and glutamic acid diethyl ester (30–300 μM) did not affect responses to either L-Glu or KA by more than 5% but the latter compound at a concentration of 1 mM caused a 20% inhibition of the L-Glu response without affecting the response to KA.

Discussion

The observations made on the slices, that L-Glu, L-Asp and their D-isomers were similarly effective and all less potent than NMDA and KA, and that the antagonist APV inhibited responses in the order NMDA > L-Asp > L-Glu, KA, are in general agreement with

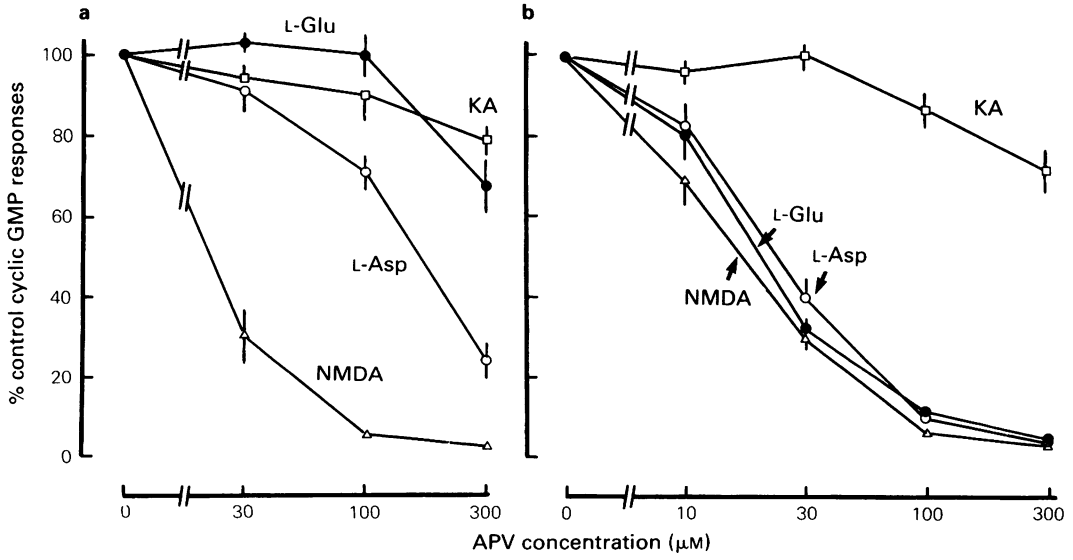

Figure 3 Concentration-dependence of the effects of APV on cyclic GMP responses to NMDA (Δ), KA (□), L-Glu (●) and L-Asp (○) in slices (a) and dispersed cells (b) from rat cerebellum. Agonist concentrations in (a) were 100 μM (NMDA and KA) and 1 mM (L-Glu and L-Asp) and in (b), 100 μM (NMDA, KA and L-Asp) and 30 μM (L-Glu). APV was added 5 min (a) or 1 min (b) before addition of the agonists. Each point is the mean of 4 separate determinations, vertical lines show ± s.e.mean.

numerous previous results from iontophoretic experiments on spinal and supraspinal neurones (see Watkins & Evans, 1981; McLennan, 1983; Peet *et al.*, 1983) and from electrophysiological and biochemical experiments in which the compounds have been applied via the bathing medium to isolated preparations of spinal cord (Curtis *et al.*, 1961; Biscoe *et al.*, 1976; Davies *et al.*, 1982) and brain (Constanti *et al.*, 1980; Teichberg *et al.*, 1981; Baudry *et al.*, 1983; Fagni *et al.*, 1983). The one notable difference in the present experiments was the failure of QA to produce significant responses (as also found with the isolated cells) but this appears to be a peculiarity of the immature tissue since QA (3 to 100 μM) induces substantial accumulations of cyclic GMP in slices of adult cerebellum (unpublished results). Also, KA is somewhat more potent in the adult relative to the immature tissue (Garthwaite, 1982).

The potencies of NMDA and KA were very similar in cell suspensions and slices, as was expected since KA is not actively taken up into brain tissue (Johnston *et al.*, 1979) and NMDA is transported only at a low rate by a low affinity process (Skerritt & Johnston, 1981). The transported amino acids L- and D-Asp and L-and D-Glu were all more potent in the cell suspensions. Based on inverse ratios of EC_{50} values, L-Glu was 280 fold, L- and D-Asp, 23 fold and D-Glu, 4 fold more potent than in slices. Furthermore, responses to L-Glu, L-Asp and NMDA were equally sensitive to inhibition by APV, suggesting that all three agonists were activating the same receptors to increase cyclic GMP levels. Binding characteristics of NMDA receptors on rat brain membranes have recently been investigated using radiolabelled D-APV as ligand (Olverman *et al.*, 1984) with results very comparable to these in terms of both relative and absolute potencies of agonists and antagonists, indicating identity between the receptor sites recognized under the two different experimental situations. The pharmacological properties of NMDA receptors assessed on the basis of cyclic GMP responses in the cell suspensions therefore appear to have a surprisingly close relationship to the binding properties of the receptor. If the EC_{50}s of the agonists are taken to approximate to their K_D, therefore, K_i values appropriate to the antagonist IC_{50} values are 2–4 μM for APV (depending on the agonist) and 20 μM for γ-D-glutamylglycine (against L-Glu response), assuming competitive inhibition (calculated according to Cheng & Prusoff, 1973). K_i values of 1.7 μM for DL-APV and 40 μM for γ-D-glutamyglycine were reported in the binding experiments (Olverman *et al.*, 1984).

Assuming, then, that the results from the cell suspensions correspond more closely to the true pharmacological properties of NMDA receptors and that the only difference, in effect, between dispersed cells and slices lies in the influence of cellular uptake on the access of agonists to receptors, as shown by the closely similar behaviour of NMDA and KA in the two preparations, the implications are that, in slices, cellular uptake can (a) distort dose-response curves to transported agonists to such an extent that they bear little or no relation to true dose-response curves; (b) reduce the potency of antagonists towards responses to transported agonists and (c) create a differential sensitivity to antagonists of responses to two agonists (L-Glu and L-Asp) acting at the same receptor.

To examine the credibility of these conclusions, a model which allows the concentration profiles of transported amino acids in brain slices to be described quantitatively has been elaborated (see Appendix). The diffusion model considered appropriate to the slices used in the present experiments predicts, for any amino acid which is transported at the same rate as L-Glu and whose true maximum response (in the absence of cellular uptake) occurs at concentrations $< 100\,\mu$M, that an external concentration of near 1 mM would be required to attain a measured maximum tissue response (apparent EC_{50} about 300 μM) irrespective of the true potency of the amino acid. As a consequence of both this and the predicted domination of tissue responses by cells receiving supramaximal interstitial concentrations, responses to a less potent transported agonist (L-Asp) would be expected to be blocked more readily by a competitive antagonist than responses to a more potent agonist acting at the same receptors (L-Glu); both should be more resistant than responses to a non-transported agonist. The influence of uptake, therefore, is sufficient to explain the disparities between slices and cell suspensions with respect to agonist potencies and the selectivity of APV. It is mooted that the same considerations would apply to iontophoretic experiments, thus explaining the similarities between results using this method and results from bath-application of agonists and antagonists to isolated preparations (see above).

If other receptors able to be activated by L-Glu and L-Asp were also present, this could clearly provide a further complication; their presence has been proposed previously as a possible explanation for the differential effects of NMDA receptor antagonists against responses to these amino acids (Watkins, 1981). This is unlikely to constitute a significant complication in the present experiments, firstly because QA receptors appear to be effectively absent and secondly, since the actions of maximally-, or near maximally-effective concentrations of L-Glu and L-Asp could be completely inhibited by concentrations of APV which left the response to KA largely sustained, it is reasonable to suppose that neither of these endogenous amino acids behave as agonists for the site acted on by KA. Assuming this to be a distinct excitatory receptor type, a compound other than L-Glu or L-Asp is likely to be the natural agonist at these receptors.

Appendix

Diffusion of transported amino acids into brain slices

The purpose of the following calculations is to examine the likely effects of carrier-mediated cellular uptake of, in particular, L-Glu and L-Asp, on the ability of these amino acids to diffuse from the medium bathing brain slices to receptor sites on cell membranes within the tissue. Special emphasis is given to the estimation of how the interstitial concentrations of the amino acids at different depths from the slice surface would vary with external concentration, in order to provide a basis for predicting the effects of cellular uptake on the apparent pharmacological properties of receptor-mediated responses to L-Glu and L-Asp.

In principle, the problem of supplying cells with an amino acid by a route from which it is constantly being removed by cellular uptake processes is analogous to the problem of supplying oxygen to an oxygen-consuming tissue. The formulations described by Hill (1929) for the diffusion of oxygen through tissues have therefore been adopted in a modified form.

Equations

The slices used experimentally are deemed to approximate to long cylinders of radius (r_o) = 0.2 mm (length approx. 10 r_o). Under steady state conditions, the concentration of amino acid in the extracellular (interstitial) fluid (C_e) at any point distance r from the axis of a cylindrical tissue bathed in the amino acid, is given by the equation:

$$C_e = \frac{vr^2}{4D'} + B\log r + E \qquad (1)$$

where v is the rate of uptake, D′ a diffusion coefficient (defined below) and B and E are constants whose values depend on the boundary conditions adopted. If the amino acid, present in the medium at a concentration, C_o, can penetrate the whole tissue, equation (1) simplifies to:

$$C_e = C_o - \frac{v}{4D'}(r_o^2 - r^2) \qquad (2)$$

If the amino acid fails to penetrate the whole tissue and its concentration falls to zero when $r = r_1$, then:

$$C_o = \frac{vr_o^2}{4D'}\left(1 - \frac{r_1^2}{r_o^2} - \frac{r_1^2}{r_o^2}\log\frac{r_o^2}{r_1^2}\right) \qquad (3)$$

There is no explicit solution for r_1 but the equation can be solved by constructing a curve for $(1 - r_1^2/r_o^2 - (r_1^2/r_o^2)\log(r_o^2/r_1^2))$ as a function of r_1. The constants B and E are then: $B = -vr_1^2/2D'$ and $E = B(0.5 - \log r_1)$.

Constants

(i) v. For a carrier-mediated uptake mechanism which obeys Michaelis-Menton kinetics, the rate of uptake, v, is given by the usual expression $V_{max}C_e(C_e + K_t)^{-1}$. K_t values for the transport of L-Glu and L-Asp into various preparations of cerebellar cells have been estimated to be 1–4 μM (Gordon & Balázs, 1983). As the main concern here is with concentrations $> 10\,K_t$ (say > 30 μM), v can be taken to be independent of C_e and approximate to V_{max}. The V_{max} for uptake of L-[^{3}H]-Glu into cerebellar cell suspensions from hydroxyurea-treated rats has been calculated to be 10 nmol mg^{-1} protein min^{-1} (Gordon & Balázs, 1983) or 1 μmol ml^{-1} min^{-1} assuming a cell volume of 1 ml per 100 mg protein. (Superficially, it might be thought more appropriate to determine V_{max} directly in the slices rather than to calculate a value from measurements made on isolated cells. However, when a rate of cellular uptake is similar to, or exceeds, the rate of diffusion through the extracellular space of a tissue, the derived kinetic constants will be biased towards describing extracellular diffusion rather than transmembrane transport (cf. Lund-Andersen & Kjeldsen, 1976). Using the above estimates, L-Glu, at concentrations $< K_t$, would be taken up with a rate

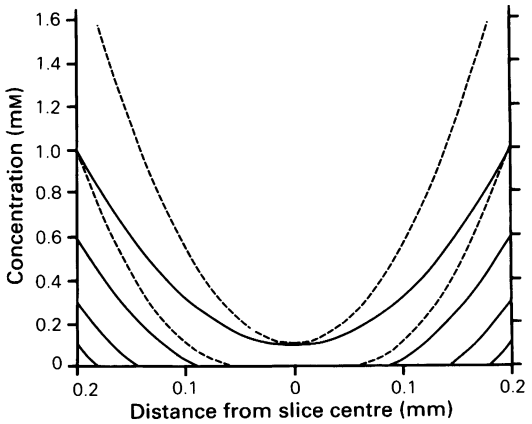

Figure 4 Concentration profiles of a transported amino acid across the cross section of a cylindrical slice (solid lines) of radius 0.2 mm, calculated from equations (2) and (3), for external amino acid concentrations of 1, 0.6, 0.3 and 0.1 mM. The broken lines correspond to a planar slice (0.4 mm thick, 40% extracellular space) exposed on both sides to external concentrations of 1.9 mM (upper curve) and 1 mM.

constant of about 300 min^{-1}, a value greatly in excess of the rate constant for extracellular diffusion in brain slices (e.g. 0.3 min^{-1}; Lund-Andersen & Kjeldsen, 1976). Any attempt to derive V_{max} in brain slices is therefore likely to lead to a gross underestimation).

(ii) D'. The diffusion coefficient in equation (1) differs from the diffusion constant, k, defined by Hill (1929) for the case of oxygen because of the assumption that the diffusion path for L-Glu and L-Asp is restricted to the interstitial fluid spaces (i.e. that efflux from cells < influx). D' is therefore defined by the equation (cf. Keynes, 1954):

$$D' = \frac{V_e}{V_t}\frac{D}{\lambda^2} \quad (4)$$

where D is the diffusion constant for the amino acid in water, λ is the tortuosity and V_e/V_t the ratio of extracellular to total fluid volumes of the tissue. V_e has been measured in slices of cerebellum from normal 8 day old rats in relation to some other (unpublished) experiments. Using [^{14}C]-sucrose as a marker, the value was $3.5 \pm 0.12\,\mu l\,mg^{-1}$ protein (mean ± s.e.mean; $n = 16$). Since the hydroxyurea pretreatment would be expected to increase V_e somewhat, V_e/V_t has been set at 0.4. Using the values $D = 1.1 \times 10^{-5}$ $cm^2\,s^{-1}$ (Herz *et al.*, 1969) and $\lambda = 1.55$ (Nicholson & Phillips, 1981), $D' = 1.1 \times 10^{-4}\,cm^2\,min^{-1}$.

Results of calculations

Figure 4 (solid lines) shows examples of how the interstitial concentrations of L-Glu, or an amino acid transported at the same rate as L-Glu, varies across a cross-section of a cylindrical slice exposed to different external concentrations according to equation (1). When $C_o < 0.91$ mM, C_e falls to zero at distances from the slice surface $< r_o$ (0.2 mm), the gradients being about $-60\,\mu M$ for each 10 μm distance into the slice (down to $C_e \approx 0.1\,C_o$). An external concentration of 1 mM (upper solid curve, Figure 4) supplies the axis of the slice with a concentration close to 100 μM.

The percentage of the total slice which is supplied with various interstitial concentrations, in relation to C_o is illustrated in Figure 5a. The significance of these curves is firstly, that they allow predictions to be made of how a measured receptor-mediated response to a transported amino acid would vary in magnitude with respect to external concentration under conditions when the maximum response attainable corresponds to all the cells in the slice responding maximally (e.g. when tissue depolarizations or biochemical responses calculated with respect to whole slice protein/weight, are measured); secondly, they allow an appreciation of the likely effects of a competitive receptor antagonist.

The first point to emerge is that the external concentrations needed to elicit a maximum tissue response ($C_o$max) would vary little despite large differences in the concentrations needed to induce a maximum cellular response in the absence of cellular uptake ($C_e$max), except when $C_e$max is very high. Thus, if $C_e$max is 30 μM, 100 μM, 300 μM or 1 mM, $C_o$max would need to be 0.94 mM, 1.01 mM, 1.21 mM and 1.91 mM respectively. Apparent dose-response curves would only start to resemble true dose-response curves when the true threshold concentration is

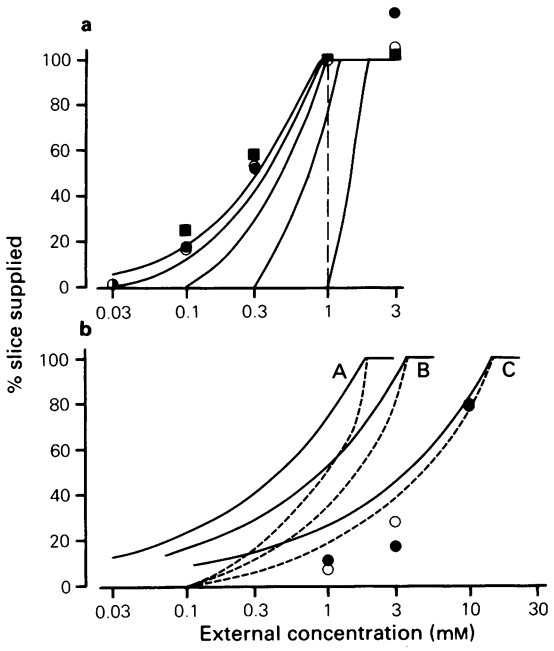

Figure 5 The curves in (a) show the calculated percentage of the cross-sectional area of a cylindrical slice (radius 0.2 mm) that is supplied with a transported amino acid in concentrations (from left to right) of >0, >0.03, >0.1, >0.3 and >1 mM, in relation to external concentration. The vertical broken line (corresponding to 1 mM on the abscissa scale) is included to correct for the optical illusion created by these curves. (b) Shows analogous curves for concentrations of >0 (solid lines) and >0.1 mM (broken lines) in planar slices containing an extracellular space of 40% (A) or 20% (B,C) exposed on both sides (A,B) or on one side only (C) to the amino acid. The points in (a) are the experimentally determined mean cyclic GMP responses to L-Glu (●), L-Asp (○) and D-Asp (■) in 8 day old rat cerebellar slices (see Results) expressed as % mean response at 1 mM external concentration (see text). In (b), the points are mean cyclic GMP responses in pial slices of adult cerebellum (from Garthwaite, 1982); as responses to concentrations >;10 mM were not measured in this study, the data have been expressed as % maximum response to NMDA (100 pmol mg^{-1} protein) in the same tissues.

greater than 1 mM and $C_e max > 10$ mM ($C_o max = 10.9$ mM when $C_e max = 10$ mM). Below these values, dose-response curves for agonists of low potency should be slightly steeper than those for more potent agonists but it is very doubtful that the potencies of agonists whose $C_e max < 100\ \mu M$ could be distinguished from each other experimentally. Viewed in a slightly different way, this means that in order to get the same response from two agonists transported at the same rate, the same external concentrations (producing the same interstitial concentrations) would be needed, even though the true potencies of the agonists may be very different.

The experimentally-determined cyclic GMP responses to L-Glu, L-Asp and D-Asp in slices (see Results), expressed as % response at 1 mM (the predicted $C_o max$ when $C_e max < 100\ \mu M$), have been plotted alongside the theoretical curves in Figure 5a. The experimental points accord sufficiently well with predictions should the true dose-response curves of these compounds cover the concentration range 0–100 μM (as found in the isolated cells) to indicate that the model adopted is appropriate.

The second point concerning Figure 5a relates to the relative contributions made by high and low interstitial concentrations to the total amount actually supplied. At the external concentrations needed to supply 50 to 100% of the tissue with a minimum concentration of 100 μM (i.e. to attain 50 to 100% of the maximum response when $C_e max = 100\ \mu M$), most of the supplied cells would receive the amino acid at concentrations higher than 100 μM. Thus, at an external concentration of 1 mM, 75% of the tissue would receive a concentration between 300 μM and 1 mM. The combination of these two factors, high interstitial concentrations and the inability of dose-response curves to distinguish between agonists differing in true potency, would have important repercussions on the effects of a competitive receptor antagonist. These can be appreciated by considering the relationship $K_i = IC_{50}(1 + C_e/K_D)^{-1}$ (Cheng & Prusoff, 1973). If two agonists acting at the same receptor, but with different affinities (expressed as K_D), are applied at the same concentration (in order to elicit the same response) and $C_e \gg$ both K_Ds, the ratio of IC_{50} values would be equal to the inverse ratio of the K_D values. Responses to the more potent agonist would therefore be more resistant to the antagonist than those to the less potent agonist; both would be expected to be more resistant than responses to a non-transported agonist whose interstitial concentrations should approximate to the external concentrations and whose measured potency should approximate to its true potency. The experimental observation that, in slices, APV inhibits responses in the order NMDA > L-Asp > L-Glu is entirely consistent, therefore, with all three agonists acting on the same receptor but with the true potency of L-Glu being higher than that of L-Asp (as observed in the isolated cells) and NMDA being effectively non-transported.

At a more microscopic level, the same factors would be expected to complicate iontophoretic experiments, viz. agonists that are able (because of uptake) to reach only a small area of neuronal cell membrane near the ejecting pipette would have to be applied in high concentration in order to produce the same increase in firing rate as a low concentration of a non-transported agonist of similar affinity free to diffuse widely over the cell surface (see also Curtis, 1976; Peet *et al.*, 1983). To obtain equal responses from two agonists transported at a high rate, the same concentrations would have to be applied. The actions of the non-transported agonist would then be more susceptible to the effects of a competitive antagonist than those of the transported agonists; the relative vulnerability of the latter, in turn, would depend on their affinities for the receptor. The resemblances between results from iontophoretic experiments and results from bath application to isolated preparations (see Discussion) suggest that this possibility merits consideration.

It is interesting to note, parenthetically, that if a receptor antagonist is also an uptake inhibitor, responses to the transported agonist of higher affinity might be potentiated at the same time as responses to the agonist of lower affinity are inhibited. The NMDA receptor antagonist, D-α-aminoadipate, (a substrate for the carrier, though relatively weak; Charles & Chang, 1981) has been reported to have just these opposing effects on the actions of L-Glu and L-Asp on Renshaw cells (Lodge *et al.*, 1978).

Limitations

The principal foible in this model is the use of a constant value for v. (The mathematics become prohibitively complicated when v follows neither zero order nor first order kinetics.) The treatment therefore (a) is not valid for substrate concentrations below $10\ K_t$ (30 μM), (b) does not take into account the possible presence of low affinity mechanisms and (c) ignores efflux from cells.

(a) As C_e falls below 30 μM, v will decrease but a proportionately greater amount of the interstitial concentration will be removed. The model is therefore likely to overestimate interstitial concentrations < 30 μM. Except at low external concentrations (< 100 μM), the proportion of slice calculated to receive concentrations less than 30 μM is small (Figure 5a).

(b) Low affinity acidic amino acid transport systems, for which L-Glu, L-Asp and D-Glu are substrates, have been detected in slices or homogenates of rat brain (Logan & Snyder, 1972; Davies & Johnston, 1976; Benjamin & Quastel, 1976). There is justifica-

tion, however, for considering high affinity uptake to be more important in the present study because (i) kinetic studies of the uptake of L-Glu at concentrations up to 0.2 mM revealed only a single (high affinity) process in cerebellar cell suspensions (Gordon & Balázs, 1983); (ii) the potency of D-Asp appeared to be influenced by uptake to the same extent as that of L-Asp (see Results) but, while both isomers share the same high affinity carrier (Gordon & Balázs, 1983), D-Asp is not a good substrate for the low affinity mechanism (Davies & Johnston, 1976). (The reduced potency of D-Glu in slices compared to cell suspensions could be a consequence of low affinity uptake (Benjamin & Quastel, 1976) or a weak interaction with the high affinity carrier (Davies & Johnston, 1976; Gordon & Balázs, 1983)). (iii) The operation of the high affinity system alone is sufficient to account for the discrepancies between slices and cell suspensions with respect to the potencies of L-Glu, L-Asp and D-Asp.

(c) It might be anticipated that if the rate of uptake exceeds the rate of intracellular metabolism (or sequestration) of a transported amino acid, carrier-mediated efflux could, after a certain time, contribute significantly to the interstitial concentrations and thus introduce a time-dependent component to the penetration of the amino acid (in addition to the time-dependence for the establishment of a steady state) that would invalidate the steady state assumption. Studies of the neurotoxicity of L-Glu in cerebellar slices, however, provide visible evidence that the concentration profiles depicted in Figures 4 and 5a are maintained for at least 2 h (Garthwaite & Gilligan, 1984). Similarly, autoradiographic studies of olfactory cortex slices incubated with 1 mM [^{3}H]-GABA indicate that the concentration gradients of this amino acid across the slice width that are present after 5 min incubation are still there after 3 h exposure (see Figure 1 of Brown *et al.*, 1980).

Application to other slices

It might be of value to consider briefly the application of this diffusion model to slice preparations in more general use than the ones used here (i.e. from immature brain and cut in two directions). The main concern was to determine if the need for external concentrations of L-Glu as high as 10 to 20 mM to obtain maximal or submaximal responses could realistically be attributed to access limitations imposed by cellular uptake.

The brain slices in widest use should correspond more closely to a planar model than a cylindrical one. Figure 5b illustrates how planar slices (0.4 mm thick) would become supplied with a transported amino acid at different external concentrations according to equations (3) and (6) of Hill (1929), modified as above. Three different situations are considered and for each, concentrations >0 (solid curves, Figure 5b) and $>100\,\mu$M (broken curves) have been plotted.

(a) In curves A, the constants previously adopted for the cylindrical model have been applied to a plane which is exposed on both sides to the amino acid. The main difference is that approximately double the external concentrations would be needed to supply a given proportion of the slice with concentrations of 30–100 μM, e.g. $C_o$max should be increased from 1 mM to nearly 2 mM. (Some sample concentration profiles are included in Figure 4, broken lines.)

(b) Adult brain has a smaller extracellular space than the immature tissue (Vernadakis & Woodbury, 1965; del Cerro *et al.*, 1968). This will affect D′ (equation 4). Curves B represent a slice containing 20% extracellular space, corresponding to the value estimated for adult rat cerebellum *in vivo* (Nicholson & Phillips, 1981). These predict that, for a transported agonist displaying a $C_e$max of 30–100 μM, $C_o$max would need to be near 4 mM (apparent $EC_{50} = 1.5$ mM). Dose-response curves for L-Glu-induced sodium fluxes in striatal (Teichberg *et al.*, 1981) and hippocampal (Baudry *et al.*, 1983) slices match these curves well.

(c) The experimentally-determined dose-cyclic GMP response curves to L-Glu and L-Asp in surface (pial) slices of adult cerebellum (Garthwaite, 1982; see filled and open circles in Figure 5b) lie well to the right of curves B. In the light of evidence that the pial membranes constitute a barrier to the inward diffusion of L-Glu (Garthwaite & Gilligan, 1984) and GABA (Brown *et al.*, 1980), this is not unexpected. Curves C correspond to a slice (20% extracellular space) into which diffusion can only occur through one surface. This would have the effect of increasing $C_o$max about 4 fold, to 15 mM (apparent $EC_{50} = 4.5$ mM) for an agonist exhibiting a $C_e$max of 30–100 μM. These curves match the experimental points more closely; the deviations present at $C_o < 10$ mM would be expected if the majority of cells generating the response were located some distance from the cut edge of the slice, as is the case for Purkinje cells in surface cerebellar slices (Garthwaite & Gilligan, 1984). Curves C also resemble dose-response relationships for the depolarizing actions of L-Glu in pial slices of olfactory cortex (Brown & Galvan, 1979) and in the isolated, hemisected, spinal cord of the toad (Curtis *et al.*, 1961).

Experiments in which responses of single neurones to bath-applied amino acids have also employed millimolar concentrations (e.g. Okamoto & Quastel, 1973; Constanti *et al.*, 1980; Ishida & Fain, 1981). It would be predicted, however, that the sensitivity of neurones to externally-applied agonists would depend on their position in the slice. This has been noted by Segal (see Alger *et al.*, 1984) with respect to L-Glu-induced depolarization of CA1 pyramidal cells in

hippocampal slices but has been studied in more detail in the case of GABA-induced conductance changes in neurones of the olfactory cortex slice (Brown & Scholfield, 1984). Figure 6 depicts the external concentrations needed to supply a transported agonist at a concentration of 30 μM at different depths from the slice surface. From this, it would be anticipated that sensitivity would be highly depth-dependent near the slice surface; for example, about a 10 fold difference in external concentration would be required for a 40 to 60 μm difference in distance from the slice surface (depending on the slice model). Near the centre of the slice (or the opposite edge if applied on one side only), sensitivity should become relatively depth-independent. This form of curve fits accurately the experimental data of Brown & Scholfield (1984) who estimated a sensitivity gradient near the surface of the slices of $10\,C_o$ per 60 μm depth in order to produce a given conductance change.

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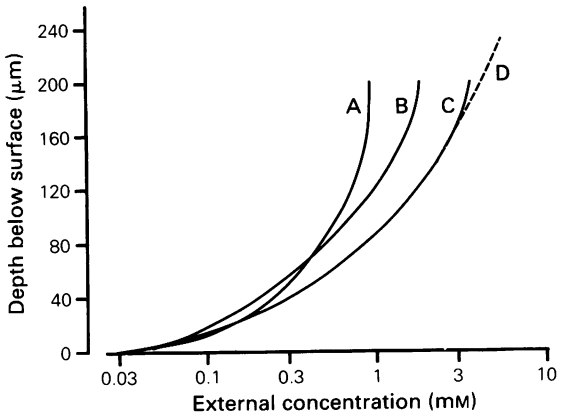

Figure 6 Calculated relationship between external concentration of a transported amino acid and the depth below the surface of a slice at which an interstitial concentration of 30 μM is found, for a cylindrical slice (radius = 0.2 mm) containing 40% extracellular space (A) and planar slices (0.4 mm thick) exposed on both sides (B,C) or on one side only (D) to the amino acid and containing 40% (B) or 20% (C,D) extracellular space.

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