Anion transport blockers inhibit DL-2-amino-4-phosphonobutyrate responses induced by quisqualate in the rat cerebral cortex

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1 Depolarizing responses to DL-2-amino-4-phosphonobutyrate (AP4) and related amino acids have been studied in the rat cerebral cortex slice following the application of quisqualate (Quis).

2 Before exposure to Quis, 500 μ M DL-AP4 had little or no effect. However, following a single application of 40 μ M Quis for 2 min, DL-AP4 produced depolarizing responses. With repeated applications of DL-AP4, there was a decline in response amplitude. A second application of Quis restored the depolarizing potency of DL-AP4 to a level above that for the first DL-AP4 response after the first Quis application. With a sequence of alternate applications of Quis and DL-AP4, the amplitude of DL-AP4 responses became maximal after the second Quis application. Responses to DL-AP4 could also be induced by the application of 1 μ M Quis for 60 min, but were smaller in amplitude.

3 Responses to the normally inactive amino acids L-cysteine (Cys), L-cystathionine (CTN) and L- α -amino adipate (AA) were also induced once Quis was applied. These responses were also maximized after a second application of Quis, except those to L-Cys, which failed to reach a plateau after three Quis applications.

4 The co-application of DL-AP4 with the first Quis application depressed the subsequent mean DL-AP4 response by 47%. Re-application of Quis restored the amplitude of DL-AP4 responses to levels comparable to control. L- α -AA also suppressed the induction of DL-AP4 responses, when co-applied with the first Quis exposure, reducing mean response amplitude by 98%. Unlike DL-AP4, however, the effect with L- α -AA persisted so that DL-AP4 responses were significantly suppressed compared to control, even after further applications of Quis.

5 The effects of the anion transport blockers, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and 4-acetoamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) on the induction process and the DL-AP4 responses themselves were examined. DIDS (100 μ M) significantly inhibited the DL-AP4 responses, and to a lesser extent the induction of the responses by 40 μ M Quis (2 min), while SITS (300 μ M) only inhibited the DL-AP4 responses. However, the induction of responses by 1 μ M Quis (60 min) was significantly affected by this concentration of SITS.

6 DIDS (100 μ M) had no effect on responses to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), but selectively potentiated those to Quis. Examination of the full concentration-response curve for Quis revealed that, while the $R_{\max}$ remained constant, the Hill slope was increased and the EC_{50} was decreased in the presence of DIDS. SITS (300 μ M), however, antagonized responses to AMPA, and had little effect on responses to Quis except at the highest concentration of Quis tested (20 μ M), where a potentiation was observed, suggesting that it is a non-NMDA receptor antagonist.

7 These observations indicate that the production of depolarizing responses to a number of amino acids, including DL-AP4, in the cerebral cortex is mediated via an anion transport mechanism sensitive to DIDS and SITS, and that the exchange of DL-AP4 for a sequestered excitatory amino acid receptor agonist, probably Quis, could underlie the production of these responses. Indeed, Quis is apparently sequestered via a similar process. However, the involvement of such a process in the induction of these responses remains inconclusive.

Keywords: DL-2-Amino-4-phosphonobutyrate; quisqualate; anion transport; cerebral cortex

Introduction

There is evidence for a novel interaction between the excitatory amino acid (EAA) receptor agonist quisqualate (Quis) and L-2-amino-4-phosphonobutyrate (AP4). Before exposure to Quis, L-AP4 has little or no direct action on the electrophysiological properties of the constituents of hippocampal and neocortical slices. However, the application of AP4 subsequent to Quis in these tissues produces a depolarizing response (Harris *et al.*, 1987; Sheardown, 1988) and/or suppression of synaptic responses (Robinson *et al.*, 1986; Harris *et al.*, 1987). Since α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) do not induce responses to AP4, this effect is

unlikely to depend on the activation of any of the three ionotropic EAA receptor subtypes for which these compounds are selective agonists (Watkins & Evans, 1981; Monaghan *et al.*, 1989) during the exposure to Quis. However, since the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione, but not NMDA antagonists, blocks the L-AP4 response in the cortex (Sheardown, 1988; Lodge & Zeman, 1989), the production of these responses would appear to be dependent on the activation of non-NMDA rather than NMDA receptors.

Both L-AP4 and Quis displace Cl^- -dependent L-[³H]-glutamate and DL-[³H]-AP4 binding, with inhibition constants in the 0.1–10 μ M range (Butcher *et al.*, 1983; Foster & Fagg, 1984). The Cl^- -dependent L-[³H]-glutamate binding is heterogeneous, composed of at least two components with different sensitivities to Quis. The relatively Quis-sensitive component

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and DL-[³H]-AP4 binding have very similar pharmacological profiles, indicating that they probably represent the same site (Werling *et al.*, 1983). These binding correlates have been suggested to represent Cl⁻-dependent L-glutamate sequestration (Pin *et al.*, 1984), mediated via an anion transport mechanism sensitive to 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (Recasens *et al.*, 1987; Zaczek *et al.*, 1987a). These uptake/transport sites differ from the Cl⁻-dependent L-[³H]-glutamate binding sites which represent the metabotropic L-glutamate receptor (Cha *et al.*, 1990), when their pharmacological profiles are compared. The Quis effect could, therefore, represent an electrophysiological manifestation of this sequestration process, as proposed by Harris *et al.* (1987), with sequestered Quis being released via a heterogeneous exchange with L-AP4. An alternative explanation is that Quis 'sensitizes' neuronal tissue to AP4 as proposed by Whittemore & Koerner (1991). Since agonists selective for receptors coupled to phosphoinositide (PI) turnover are also able to induce responses to AP4 (Whittemore & Cotman, 1991), the induction of these responses by Quis could result from the stimulation of PI turnover via its agonist action at the metabotropic L-glutamate receptor.

Methods

Male Wistar rats (200–300 g) were killed by decapitation and their brains rapidly removed. Wedges of anterior cingulate cortex 500 µm thick were prepared from coronal slices (corresponding to plates 13–22, Paxinos & Watson, 1986) with a Vibroslice (Camden Instruments) as a modification to the method of Harrison & Simmonds (1985). The wedges were set up in two-compartment baths so that the cortical tissue projected through a greased gap in a two piece barrier separating the two compartments. The majority of the cortex was in one compartment and the underlying white matter was in the other. The compartments were perfused independently with Krebs medium of the following composition (mM): NaCl 118, NaHCO₃ 25, KCl 2.1, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1, glucose 11; and gassed with 95% O₂/5% CO₂. The flow rate was approximately 2 ml min⁻¹ and the temperature maintained at 21–23°C. All drugs were dissolved in the Krebs medium and perfused through the cortical compartment only. The d.c. potential difference between the two compartments was recorded continuously using Ag/AgCl bath electrodes, amplified and displayed on a pen recorder. Negativity induced in the cortical compartment was interpreted as a depolarization of the neuronal tissue passing through the barrier. Responses were measured at their peak amplitude.

In experiments where responses to DL-AP4 and related amino acids were induced by Quis, all drugs were applied for 2 min, except where stated. These amino acids were selected for study on their ability to displace either Cl⁻-dependent L-[³H]-glutamate or [³H]-AP4 but not AMPA, KA and NMDA receptor binding (Simon *et al.*, 1976; Butcher *et al.*, 1983; Werling *et al.*, 1983; Kessler *et al.*, 1987; Murphy *et al.*, 1987; Pullan *et al.*, 1987; Olverman *et al.*, 1988). L-α-Amino adipate (AA), L-cysteine (Cys) and L-cystathionine (CTN), fulfilled this criterion, although the first two are weakly active at NMDA receptor binding sites. Paradigms for the examination of the effects of L-α-AA, of DL-AP4 itself, and of the anion transport blockers 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and 4-acetoamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) on the induction by Quis, and the DL-AP4 responses themselves, are detailed in the results. In a few experiments, the influence of DIDS and SITS on responses to AMPA and Quis was also examined. Concentrations of 2.5 and 5 µM AMPA; and 5, 10 and 20 µM Quis were perfused for 2 min to produce depolarizing responses which are on the lower halves of the linear portions of their respective DR curves. Either DIDS or SITS was then perfused for 30 min prior to and during the re-determination

of agonist responses, bridging the same response range as the control DR lines. The slopes of DR lines were calculated by linear regression analysis. From parallel shifts of the DR lines, equi-effective dose-ratios for each agonist could be calculated. In these experiments, AMPA and Quis DR lines were obtained from the same slices so that a direct comparison of the two AMPA receptor agonists could be made. In addition, the effect of DIDS on the full concentration-response curve to Quis was examined. One concentration-response curve was obtained from one experiment, either in the absence or in the presence of DIDS to eliminate the problem of desensitization. The curves were compared by measuring all responses relative to an initial response to 20 µM Quis = 1 recorded in the absence of DIDS. These initial responses to Quis were also compared to a response to 5 µM AMPA obtained under the same conditions. This analysis did not reveal any significant difference (*P* > 0.1, Student's *t* test) in the sensitivities of control and drug applied slices to AMPA and Quis. Each full concentration-response curve was fitted using the log concentration-response relationship:

$$y = R_{\max}/1 + (EC_{50}/x)^H$$

where *x* = agonists concentration producing response amplitude *y*, *R*_{max} = maximal response, *EC*₅₀ = agonist concentration producing half maximal response, *H* = Hill coefficient.

DIDS and SITS were dissolved directly into the Krebs medium just before use. All other drugs were made up as stock solutions at 10 mM and diluted in the Krebs medium just before use. Drugs were purchased from the following sources: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, DL-2-amino-4-phosphonobutyrate and quisqualate from Tocris; L-α-amino adipate, L-cysteine, L-cystathionine, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid and 4-acetoamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid from Sigma.

Results

Induction of responses to amino acid analogues

Prior to the application of Quis, 500 µM DL-AP4 had little or no effect on the baseline (Figure 1). In some slices small depolarizations were observed, but these never exceeded 0.04 when measured relative to the subsequent 40 µM Quis response = 1. This concentration of Quis was used in order to maximize the amplitude of DL-AP4 depolarizing responses. It was just above the *EC*₅₀ value for Quis (22.3 µM, cf. Figure 8 and Table 4), consistent with a previous study (24.0 µM, Horne & Simmonds, 1989). Four consecutive DL-AP4 applications subsequent to Quis produced responses of progressively diminishing amplitude (Figure 1 and Table 1). A second application of Quis restored the DL-AP4 response amplitude to a level above that found initially after the first Quis application. These results clearly indicate that the induction of DL-AP4 responses in cortical tissue is readily reversible. However, whether this loss of responsiveness is time- or use-dependent was not determined. In addition, this loss of responsiveness is also readily counteracted by a further application of Quis, indicating that the mechanism underlying the induction of responsiveness of cortical tissue to DL-AP4 can undergo reactivation. This loss of responsiveness precluded a more detailed examination of DL-AP4 responses involving the construction of concentration-effect curves.

In a second set of experiments Quis and DL-AP4 or one of the selected amino acids were applied alternately (Figures 2 and 3). Prior to the application of Quis, 50 µM L-Cys, 500 µM L-α-AA (Figure 2) and 100 µM CTN (data not shown) had little or no effect on the baseline. This is consistent with binding data (Simon *et al.*, 1976; Kessler *et al.*, 1987; Murphy *et al.*, 1987; Pullan *et al.*, 1987; Olverman *et al.*, 1988), which indicated that these compounds are relatively inactive at AMPA, KA, and NMDA receptors. However, once the

Figure 1 A continuous recording of d.c. potential showing the induction of depolarizing responses to 500 μ M DL-AP4 by 40 μ M Quis (Q) and their subsequent decline in amplitude upon repeated applications of DL-AP4. Both Quis and DL-AP4 were perfused for 2 min. For abbreviations in this and subsequent figures, see text.

Table 1 Sequential responses to quisqualate (Quis) 40 μ M and DL-2-amino-4-phosphonobutyrate (DL-AP4) 500 μ M

Quis ₁	AP4 ₁	AP4 ₂	AP4 ₃	AP4 ₄	Quis ₂	AP4 ₅	AP4 ₆
1.00	0.61 ± 0.042	0.53 ± 0.024	0.33 ^a ± 0.021	0.27 ^a ± 0.022	1.06 ^b ± 0.018	0.74 ^a ± 0.035	0.64 ^c ± 0.036

Response amplitude was measured relative to the initial response to Quis = 1. Values are mean $\pm$ s.e.mean from 16 experiments.

^a $P < 0.001$ compared to AP4₁ response, ^b $P < 0.01$ compared to Quis₁ response; ^c $P < 0.01$ compared to AP4₅ response (paired *t* test).

tissue had been exposed to Quis all three compounds produced depolarizing responses. The mean amplitudes for these responses in the sequence of alternating applications with Quis are shown in Figure 3. The amplitude of these amino acid responses became maximal after two Quis applications, except for those to L-Cys, which failed to plateau after three Quis applications. A second application of these amino acids after the third Quis response produced a response of comparable amplitude to the first. These results indicate that Quis is able to induce responses to a number of amino acids, that are highly selective ligands for Cl⁻-dependent L-[³H]-glutamate and L-[³H]-AP4 binding sites, other than DL-AP4.

Responses to 500 μ M DL-AP4 were also induced following perfusion of 1 μ M Quis for 60 min (Figure 4a). However, these responses were of smaller amplitude than those initially induced by 40 μ M Quis for 2 min (Table 2). The subsequent application of 40 μ M Quis resulted in a larger response to DL-AP4 comparable in amplitude to that of responses in the previous experiments, where alternate applications of 40 μ M Quis and DL-AP4 were used (Table 2). The fact that a low concentration of Quis, applied for a relatively long period of time, is able to induce responses to DL-AP4 indicates that this effect is not solely dependent on the level of depolariza-

tion produced by Quis, but on a combination of concentration and time course of application.

The effect of DL-AP4 and L- α -AA on induction by Quis

In an attempt to determine whether the sites underlying the production of the responses to DL-AP4 also underlie the induction process as predicted by the exchange hypothesis, the influence of DL-AP4 and L- α -AA on the induction of DL-AP4 responses by Quis was studied using a paradigm based on the design in Figure 3. DL-AP4 and L- α -AA (both 500 μ M) were perfused for 10 min before and then during the first application of 40 μ M Quis. There then followed a 60 min re-equilibration period in control Krebs medium. Responses to alternate applications of 500 μ M DL-AP4 and 40 μ M Quis were obtained and compared to the data in Figure 3 (recalculated relative to the third Quis responses = 1) as shown in Table 2. DL-AP4 partially suppressed the induction of responses by Quis in a readily reversible manner (Figure 5a), while L- α -AA suppressed induction almost completely, an effect that showed only a small reversal after several Quis applications (Figure 5b). The persistent suppression of DL-AP4 responses by L- α -AA applied for a relatively short

Figure 2 Continuous recordings of d.c. potential showing the induction of depolarizing responses to (a) 50 μM L-Cys and (b) 500 μM L- α -AA by 40 μM Quis (Q) and their subsequent enhancement upon repeated applications of Quis. Both Quis and L-Cys or L- α -AA were perfused for 2 min.

period of time during induction indicated that there was a slow recovery for its effect on induction. This could explain the relatively small responses obtained for L- α -AA in Figure 3, as the L- α -AA applied before the Quis could be blocking the induction process. In addition, since the same concentration of L- α -AA and DL-AP4 were used in these experiments, and these compounds are almost equipotent as displacers of Cl^- -dependent L-glutamate/AP4 binding sites (Butcher *et al.*, 1983; Werling *et al.*, 1983), the data would suggest that it is unlikely that these sites are involved in the induction of responses. However, without either comparing the DL-AP4 concentration-effect curve for control induction with those for induction in the presence of DL-AP4 or L- α -AA, or using different concentrations of DL-AP4 and L- α -AA during the induction process, it is impossible to measure the difference in potency with respect to the inhibition of induction.

The effect of the anion transport blockers DIDS and SITS

A similar paradigm based on the design in Figure 3 was used to study the influence of DIDS on Quis-induced DL-AP4 responses (Figure 6a). In a similar sequence of alternating applications of Quis and DL-AP4, medium containing 100 μM DIDS was perfused 20 min before and during the first Quis and third DL-AP4 applications, and 60 min allowed for re-equilibration with normal Krebs medium. The amplitude of the DL-AP4 responses was measured relative to the third Quis response = 1, and compared with the data in Figure 3 (Tables 2 and 3). DIDS had no effect on the baseline, but produced a suppression of the first DL-AP4 response following the priming application of Quis, and almost completely abolished the responses once they had been induced. There was a significant recovery of the DL-AP4 responses after their

Figure 3 The mean $\pm$ s.e. mean amplitude of depolarizing responses to alternate applications of 500 μ M DL-AP4 ($n = 9$) (a), 50 μ M L-Cys ($n = 6$) (b), 100 μ M L-CTN ($n = 3-4$) (c) and 500 μ M L- α -AA ($n = 6$) (d) with 40 μ M Quis measured relative to the first priming Quis response = 1. Open columns, Quis; hatched column, DL-AP4; stippled columns, L-Cys; checkered column, L-CTN; solid columns, L- α -AA. * $P < 0.05$; ** $P < 0.01$ (paired t test) when compared with the preceding response to the same compound.

Figure 4 Continuous recordings of d.c. potential showing (a) the induction of depolarizing responses to 500 μ M DL-AP4 by 1 μ M quisqualate (Q) and (b) the effect of 300 μ M SITS on this induction. Quis 1 μ M was perfused for 60 min, 40 μ M Quis and 500 μ M DL-AP4 for 2 min. SITS 300 μ M was perfused for the time period indicated by the bar above the record.

depression by DIDS, but the mean amplitude of the response was only 52% of that prior to the application of DIDS. This is less than would be predicted for the control experiments (Figure 3) as shown in Table 3, probably indicating that there is a very slow recovery from the effects of DIDS in these experiments, casting doubt on the interpretation of the effect of DIDS on the induction of DL-AP4 responses. This is consistent with previous observations which have indicated that the effects of DIDS are difficult to reverse (White & Miller, 1979).

As a result, a second anion transport blocker, SITS, which is known to be reversible (White & Miller, 1979), was used. In a design like that for DIDS, 300 μ M SITS was applied 20 min before and during the first Quis and third DL-AP4 applications in an alternating sequence, and 60 min allowed for re-equilibration with control medium (Figure 6b). SITS had no effect on baseline, and unlike DIDS had no effect on the induction of DL-AP4 responses by 40 μ M Quis (Table 2), although it blocked the induced DL-AP4 responses in a reversible manner (Figure 6b and Table 3). This finding supports the suggestion that the poor washout of DIDS is responsible for the apparent suppression of induction in the DIDS experiments. Therefore, the apparent lack of effect of the anion transport blockers on the induction of DL-AP4 responses by Quis indicates that a process other than anion exchange underlies induction, when 40 μ M Quis is used. Binding studies show that Quis inhibits Na^+ -dependent L-[^3H]-glutamate binding to whole brain synaptosomes (Ogita & Yoneda, 1986) and Na^+ -dependent D-[^3H]-aspartate binding to brain slices (Anderson *et al.*, 1990), with approximated IC_{50} s in the 10–100 μ M range. Therefore, with 40 μ M Quis, there may well be sufficient sequestration by Na^+ -dependent transport to provide an anion transport blocker-resistant source of Quis for heterogeneous exchange with AP4. A study of uptake of Quis by hippocampal tissue reveals that negligible amounts of Quis are sequestered via a Na^+ -dependent mechanism at a concentration of 16 μ M (Harrison & Kilpatrick, 1991). Therefore, the experiments with SITS were repeated using 1 μ M Quis applied for 60 min as the sensitiz-

Table 2 The influence of amino acid analogues and anion transport blockers on the induction of DL-2-amino-4-phosphonobutyrate (DL-AP4, 500 μ M) responses, by their application prior to and during the first quisqualate (Quis) exposure

	Quis ₁	AP4 ₁	Quis ₂	AP4 ₂
Quis 40 μ M (2 min) (<i>n</i> = 9)	0.904 $\pm$ 0.031	0.47 $\pm$ 0.046	0.973 $\pm$ 0.047	0.76 $\pm$ 0.077
+ DL-AP4 500 μ M (<i>n</i> = 5)	1.031 $\pm$ 0.119	0.25 $\pm$ 0.076**	0.926 $\pm$ 0.051	0.69 $\pm$ 0.082
+ L- α -AA 500 μ M (<i>n</i> = 5)	0.931 $\pm$ 0.052	0.01 $\pm$ 0.006***	0.880 $\pm$ 0.050	0.11 $\pm$ 0.050***
+ DIDS 100 μ M (<i>n</i> = 7)	1.140 $\pm$ 0.055**	0.22 $\pm$ 0.053***	1.014 $\pm$ 0.043	0.54 $\pm$ 0.069
+ SITS 300 μ M (<i>n</i> = 5)	0.993 $\pm$ 0.074	0.36 $\pm$ 0.103	0.900 $\pm$ 0.065	0.78 $\pm$ 0.117
Quis 1 μ M (60 min) (<i>n</i> = 7)	–	0.35 $\pm$ 0.055	0.987 $\pm$ 0.025	0.70 $\pm$ 0.096
+ SITS 300 μ M (<i>n</i> = 5)	–	0.14 $\pm$ 0.047*	0.966 $\pm$ 0.076	0.65 $\pm$ 0.095

Response amplitude was measured relative to the third Quis response = 1. Values are mean $\pm$ s.e.mean of the number of experiments in parentheses.

P* < 0.05, *P* < 0.01, ****P* < 0.005; compared to control (Student's *t* test).

Figure 5 Continuous recordings of d.c. potential showing the effects of (a) 500 μ M DL-AP4 and (b) 500 μ M L- α -AA on depolarizing responses to 500 μ M DL-AP4 and their induction by 40 μ M Quis (Q). All drugs were perfused for the time periods indicated by the bars above and below the records at the concentrations indicated in μ M.

Figure 6 Continuous recordings of d.c. potential showing the effects of (a) 100 μM DIDS and (b) 300 μM SITS on depolarizing responses to 500 μM DL-AP4 and their induction by 40 μM Quis (Q). Both DL-AP4 and Quis were perfused for 2 min. DIDS and SITS were perfused for the time period indicated by the bars above the record.

ing pulse, and 60 min allowed for the re-equilibration in control Krebs medium. As demonstrated in Figure 4b and Table 2, under these conditions 300 μM SITS was able to suppress the induction of DL-AP4 responses by 60%.

Since the production of DL-AP4 responses is apparently dependent on the activation of non-NMDA receptors, DIDS and SITS were tested against responses to AMPA and Quis. DIDS (100 μM) produced a leftward shift of the Quis DR line, with a dose-ratio range of 0.64–0.44 (1.6–2.2 fold leftward shift, Figure 7a), with no significant effects on responses to AMPA (Figure 7b). This enhancement of Quis responses is consistent with the significantly larger 40 μM Quis responses observed in the presence of DIDS in Table 2. Indeed, examination of the full concentration-response curve

for Quis in the absence and presence of 100 μM DIDS (Figure 8) indicate that, while R_{max} is unaffected, the EC_{50} and Hill slope of the concentration-response curve are decreased and increased, respectively, in the presence of DIDS (Table 4). On the other hand, SITS (300 μM) had no significant effect on responses to 5 and 10 μM Quis, and only potentiated those to 20 μM , with an increased amplitude of $36 \pm 10\%$ (Figure 7c). In addition, it antagonized responses to AMPA, shifting the DR line to the right 1.71 fold in a parallel manner (Figure 7d). This would suggest that as well as the DIDS-like potentiation of Quis responses, there is also an underlying non-NMDA receptor antagonism. Therefore, examination of the effect of SITS on the full Quis concentration-response curve was not performed. These results are

Table 3 The influence of anion transport blockers on the production of DL-2-amino-4-phosphonobutyrate (DL-AP4) response induced by quisqualate (Quis)

DL-AP4 responses in relation to third Quis response			
	Prior Control	Subsequent Drug	Wash
Control (n = 9)	0.76 ± 0.077	0.81 ± 0.066	0.81 ± 0.081
DIDS 100 µM (n = 7)	0.54 ± 0.069	0.03* ± 0.011	0.28** ± 0.036
SITS 300 µM (n = 5)	0.78 ± 0.117	0.06* ± 0.015	0.77 ± 0.171

Response amplitude was measured relative to the third Quis response = 1. Values are mean ± s.e.mean of the number of experiments in parentheses, and are a continuation of those experiments shown under the conditions stated used with 40 µM Quis as the inducing pulse in Table 2.

* $P < 0.005$ compared to Control; ** $P < 0.05$ compared to DL-AP4 response prior to third Quis response (Student's *t* test).

Figure 7 Log concentration-response lines for the non-NMDA receptor agonists Quis and AMPA in the absence (□) and presence (■) of 100 µM DIDS (a and b) or 300 µM SITS (c and d). Each point is the mean ± s.e.mean of response values calculated relative to an initial response to 20 µM Quis = 1 in the absence of anion transport blocker from 5 individual experiments for both DIDS and SITS, with data for Quis and AMPA being collected from the same slice. **Figure 8** Full log concentration-response curves for Quis in the absence (■) and presence of 100 µM DIDS (◆), obtained from 6 and 4 experiments, respectively. Each point is the mean ± s.e.mean of response values calculated relative to the mean of two initial responses to 20 µM Quis = 1 obtained in the control conditions.

consistent with the proposed selective uptake of Quis over AMPA by a Cl^- -dependent anion transport/sequestration mechanism. However, there is a block of non-NMDA receptors by SITS, indicating that the suppression of the production of DL-AP4 responses by SITS may in part, at least, be the result of the antagonism of non-NMDA receptor activation, which probably underlies these responses, and if washout were incomplete, cast doubt on the suppression of induction using 1 µM Quis as the sensitizing pulse. Therefore, it is evident that the use of either of these drugs has its limitations, and that while the effect on the production of responses is clear for DIDS, and although it is evident that Quis could be sequestered by a mechanism sensitive to anion transport blockers, the effects on the induction by Quis remain inconclusive.

Discussion

The present study has clearly shown that Quis is able to sensitize the cerebral cortex to amino acids other than DL-AP4, so that they produce depolarizing responses. All these amino acids are highly selective for L-glutamate/AP4 binding sites, with an order of potency consistent with that found here, except for L-α-AA, but this probably reflects the ability of L-α-AA to inhibit strongly its own induction. In addition, the block of DL-AP4 responses by the anion transport

Table 4 Mean values ± s.e.mean of maximum response ($R_{\max}$), log EC_{50} and Hill slope coefficient from log concentration-response curves for quisqualate (Quis) in Figure 8, fitted to the equation given in the text, for the number of experiments given in parentheses

Condition	$R_{\max}$	log EC_{50}	Hill slope
Control (n = 6)	2.20 ± 0.154	-4.65 ± 0.080 [22.3 µM]	0.96 ± 0.070
DIDS 100 µM (n = 4)	1.97 ± 0.148	$-5.10 \pm 0.076^{**}$ [7.9 µM]	$1.50 \pm 0.217^*$

$R_{\max}$ is expressed relative to a response to 20 µM Quis obtained in the control condition, and the log EC_{50} in log [M].

* $P < 0.05$; ** $P < 0.005$ compared to control (Student's *t* test).

blockers DIDS and SITS demonstrated in the present study is consistent with the sensitivity of Cl^- -dependent L-[^3H]-glutamate binding to these agents (Recasens *et al.*, 1987; Hollmann *et al.*, 1988). This evidence supports the idea that Cl^- -dependent L-[^3H]-glutamate/AP4 binding sites are the biochemical correlate of the sites involved in the production of responses to these amino acids. DIDS and SITS block a number of anion transport processes across neural cell membranes (Kimelberg, 1981), and the production of the Quis-induced DL-AP4 responses would appear superficially to be due to an anion exchange process similar to that described for the uptake of L-glutamate in glial cells by Waniewski & Martin (1983). However, Quis/AP4-sensitive L-[^3H]-glutamate binding represents a Cl^- -dependent Na^+ -independent uptake site located on neuronal membranes (Zaczek *et al.*, 1987b), and L-AP4 has little or no affinity for the Cl^- -dependent L-[^3H]-glutamate binding to astrocyte membranes that corresponds to glial uptake sites (Bridges *et al.*, 1987). Since these sites are believed to be exchange transport sites for amino acids, the exchange uptake of these amino acids for a neuroactive one could be responsible for the production of the depolarizing responses, lending support to the exchange hypothesis. In addition, the location of the processes underlying DL-AP4 responses would appear to be neuronal rather than glial. If the AP4 responses are the result of heterogeneous amino acid exchange, one would expect that the sequestered EAA agonist would be released close to AMPA receptor sites, so a neuronal location is not surprising.

The role these transport/L-glutamate/AP4 sites play in the induction of AP4 responses remains unresolved, primarily owing to the problems of poor reversibility for DIDS, and the antagonism of non-NMDA receptors by SITS. However, the proposed sequestration of Quis via these sites (Zaczek *et al.*, 1987b) is supported in the present study by the fact that responses to Quis rather than AMPA are relatively selectively enhanced by anion transport blockers, consistent with the >60 fold greater potency of Quis over AMPA to displace Ca^{2+} and Cl^- -dependent L-[^3H]-glutamate and DL-[^3H]AP4

binding (Butcher *et al.*, 1983; Werling *et al.*, 1983). This is circumstantial evidence indicating that the exchange hypothesis is possible, but there is no direct evidence that Quis has indeed been sequestered by the cortical tissue, although this has been demonstrated in the hippocampus (Harrison & Kilpatrick, 1991). However, the marked difference in the suppression of induction by DL-AP4 and L- α -AA points away from the exchange hypothesis, and the evidence that these amino acids are also potential antagonists of the metabotropic L-glutamate receptor (Schoepp & Johnson, 1989) suggests that PI turnover may also be involved in the induction process, supporting the sensitization hypothesis. Indeed, it has been established that agonists for receptors coupled to PI turnover are able to sensitize the responses to DL-AP4 (Whittemore & Cotman, 1991), and that calmodulin and gangliosides are able to modulate $\text{Cl}^-/\text{Ca}^{2+}$ -dependent L-[^3H]-glutamate binding (Yoneda *et al.*, 1989; Hollmann & Seifert, 1989), indicating that these sites are under the control of inducible intracellular mechanisms. Therefore, the inconclusive evidence concerning the effect of DIDS and SITS on the induction process, but the clear block of DL-AP4 responses suggest that the combination of the two hypotheses would provide the ultimate solution to this problem. Certainly, the number of Cl^- -dependent L-[^3H]-glutamate binding sites is known to increase following exposure to Quis (Kessler *et al.*, 1986), supporting the notion of a sensitized exchange uptake site being responsible. In addition, while Quis remains the prime candidate for the sequestered EAA released during exchange, another possibility is L-glutamate as it is known to be tightly sequestered into pools associated with a Cl^- -dependent, AP4-sensitive mechanism in the retina (Mitchell & Redburn, 1988).

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