Separation of Quisqualate- and Kainate-Selective Glutamate Receptors in Cultured Neurons from the Rat Superior Colliculus

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The aim of the present study was to identify and characterize the receptors and ionic channels mediating the compound response of tectal neurons to exogenous L-glutamate (Glu). Particular attention was paid to the question of whether separate receptors and channels exist for quisqualate (QA) and kainate (KA) and, if so, whether binding to one of these receptors would modify the response elicited through the other.

Neurons were dissociated from the superficial gray layer of the superior colliculus from E21 or P1 rats. Between days 14 and 21 *in vitro*, responsiveness of tectal neurons to Glu and related substances was tested by recording the whole-cell currents induced by rapid superfusion with drug-containing salt solutions.

Our experiments showed that tectal neurons express at least 3 distinct types of receptors for acidic amino acids. KA-activated currents ($I_{(KA)}$) differ from QA-activated currents ($I_{(QA)}$) in their dose-response characteristics, desensitization patterns, selective blockade with kynurenic acid and suppression by elevated $[Ca^{2+}]_o$. $I_{(KA)}$, but not $I_{(QA)}$, is significantly reduced by low levels of $[Cl^-]_o$, and the $[Cl^-]_o$ -dependent shift of the reversal potential for $I_{(KA)}$ suggests that KA promotes a conductance decrease for Cl^- . Such an effect has been ascribed to APB-receptors, but L-2-amino-4-phosphonobutyrate (APB) itself failed to induce current responses in tectal neurons. KA was without effect when administered together, and in equimolar concentrations, with QA. The block of $I_{(KA)}$ was, however, surmounted by applying KA at considerably higher concentrations. It is concluded that QA acts as a low-affinity competitive antagonist at the KA site and as a high-affinity agonist at its own receptor. The response to the endogenous ligand Glu reflects properties of all receptors. QA and KA receptors account for 20–30% (QA) and 49–82% (KA) of the compound current elicited with 100 μM Glu. These results indicate that binding of Glu does not, in contrast to QA, produce any significant suppression of the KA-receptor-mediated current component.

The density of binding sites for L-glutamate (Glu) is known to be high in the superficial gray layer of the mammalian superior colliculus (SC) (Greenamyre et al., 1984; Monaghan and Cot-

man, 1985), but it has not been clarified to what extent particular acidic amino acid receptors (AARs) may contribute to glutamatergic synaptic transmission in this area. Moreover, the physiological effects of endogenous and exogenous acidic amino acids have remained unexplored. A tissue culture preparation of SC would provide a suitable means of addressing such issues, since neurons obtained from the target area of glutamatergic visual afferents (Fosse et al., 1986; Anderson et al., 1987) can be isolated, maintained in culture, and submitted to patch-clamp recording (Grantyn et al., 1987). A previous paper dealt with the N-methyl-D-aspartate (NMDA)-selective subclass of AARs and its link to a proton site that activates a transient Na^+ current (Grantyn and Lux, 1988). Here, we ask the following: Which of the previously described non-NMDA receptors are expressed by cultured neurons from the superficial gray layer? By analyzing the current responses to established Glu agonists, we hoped to reveal receptor and channel properties that could then be used to estimate the specific function of quisqualate (QA)-, kainate (KA)-, or L-2-amino-4-phosphonobutyrate (APB)-selective receptors in visual signal transmission.

Four AAR subtypes (A1–A4) have been distinguished by the selective capacity of NMDA, QA, KA, and APB to displace Glu from Na^+ -insensitive binding sites on synaptic membranes (Foster and Fagg, 1984; Mayer and Westbrook, 1987). The validity of this classification scheme is supported by peculiarities of receptor distribution (Monaghan et al., 1983; Greenamyre et al., 1984; Olson et al., 1987; Represa et al., 1987), differences in ionic requirements for ligand binding (Beaumont et al., 1979; Honoré et al., 1982; Fagg et al., 1983; Fagg and Lanthorn, 1985), neuropharmacological profiles (Biscoe et al., 1976; McLennan and Lodge, 1979; Davies and Watkins, 1981; Anis et al., 1983; Davies and Watkins, 1985; Huettner and Baughman, 1986; Miljkovic and MacDonald, 1986; Wong et al., 1986), and second-messenger effects (Sladeczek et al., 1985; Baudry et al., 1986; Nicoletti et al., 1986; McCaslin and Morgan, 1987; Novelli et al., 1987; Sugiyama et al., 1987).

As for electrophysiological studies, recent research has largely focused on NMDA receptors since reliable tools are available for identification of NMDA-activated receptors and channels (see Ascher and Nowak, 1987; Kemp et al., 1987; MacDermott and Dale, 1987; Mayer and Westbrook, 1987, for recent reviews). A physiological action of APB was demonstrated in On-bipolar cells of the mudpuppy retina, where this Glu agonist suppressed the response to light and induced hyperpolarization (Slaughter and Miller, 1981), possibly by decreasing a Cl^- conductance (Miller and Dacheux, 1976). However, APB-induced conductance changes have not yet been explored with voltage-clamp methods. In fact, it seems questionable that mammalian central neurons express synaptic APB receptors (Kessler et al.,

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Figure 1. Photomicrographs showing the superficial gray layer (SG) of rat superior colliculus and a dissociated cell culture derived from this layer. *A*, Frontal section through the mesencephalon of an E21 rat fetus. Staining with toluidine blue. Arrowhead indicates the level of cut. *B*, Dissociated cell culture after 2 weeks *in vitro*.

1987a, b). Binding of QA or KA is signaled by activation of a mixed cationic conductance (Mayer and Westbrook, 1984; Ishida and Neyton, 1985; Hals et al., 1986; Kiskin et al., 1986; Vlachová et al., 1987). Thus far, it has proved to be difficult to separate QA- and KA-activated conductance changes, although single-channel studies with QA and KA application (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987) revealed differences in the average level of unitary conductance (higher with QA) and probability of channel opening (higher with KA). The latter observations support the belief that electrophysiological criteria may eventually be established to distinguish QA- from KA-receptor mediated effects and to assess the contribution made by each of these receptors to the compound response elicited by exogenous and endogenous acidic amino acids.

Materials and Methods

Dissection and culture. Superior colliculi were dissected from the brains of 21-d-old rat fetuses or P1 postnatal rat pups and collected in cooled calcium/magnesium-free balanced salt solution with Hanks' salts (HBSS). At this age, the superficial gray layer of the rat SC has a depth of 330–350 μm and distinct margins. Care was taken to place the cut within the confines of this layer, removing tissue pieces of 200–300 μm thick only. Histological examination verified the correctness of the dissection (Fig. 1*A*).

The tissue was incubated for 15 min at room temperature in a solution of 1% trypsin with 0.5% deoxyribonuclease (Worthington) in HBSS, washed 3 times with HBSS, and transferred into a solution of 0.1% DNase in HEPES-buffered minimum essential medium with Hanks' salts (MEM-HEPES). Cells were dissociated by passage through a series of siliconized Pasteur pipettes with fire-polished narrowed tips, centrifuged (10 min at $65 \times g$), and resuspended and grown in MEM-bicar-

Figure 2. Current responses to receptor-selective Glu agonists. Response to NMDA (*A*), QA (*B*), and KA (*C*) at indicated concentrations. Bar marks the period of agonist application. At left of each upper trace, holding voltage (V_h). Recordings from 3 different neurons. Standard solutions, except *A*, where Mg^{2+} was omitted.

bonate supplemented with 5% horse serum and 5% fetal calf serum (Gibco). Plastic dishes, 35 mm (Nunc), were coated with a mixture of 0.05% collagen obtained from calf skin (Sigma Type III) and 0.002% poly (D-lysine) (Sigma). A glass ring was inserted in the center of each dish, and the cells placed inside the ring area of about 1 cm². The plating density was generally not less than 200,000 cells/cm². Neurons grew on top of a confluent glial layer in humidified air containing 5% CO₂ at 37°C (Fig. 1*B*). Glial mitosis was controlled by addition of cytosine-β-D-arabino-furanoside (Sigma) at a concentration of 10⁻⁵ M for 12 hr on the 4th or 5th day *in vitro* (div). The life span of these high-density mixed cultures ranged from 3 to 6 weeks. GAD immunohistochemistry (S. Warton and R. Grantyn, unpublished observations) indicated that in cultures obtained from E21 rat embryos GAD-positive neurons constitute about 35% of the total cell population. They form extensive terminal networks that release GABA from div 2 on. Therefore, TTX (1 μM) and bicuculline methiodide (100 μM) were added to suppress synaptic activity during the experiments.

Electrophysiological recordings. Glass micropipettes were prepared from thin-walled Duran glass (Schott Rohrglas, Main, FRG) following the procedure of Hamill et al. (1981). Electrodes were coated with Sylgard (Dow Corning, Seneffe, Belgium) and had resistances of about 10⁷ Ω. They were filled with a standard solution containing (in mM): *N*-methylglucamine, 120; TEA-Cl, 20; EGTA, 8; MgCl₂, 1.0; CaCl₂, 0.2; glucose, 10; HEPES, 10; ATP, 2; cAMP, 0.25. This composition of the internal solution has several advantages. First, *N*-methylglucamine penetrates very little through Glu-activated channels. Second, the absence of potassium and the addition of TEA-Cl ensures complete block of K⁺ conductances. Third, the presence of EGTA, ATP, and cAMP reduces the run-down of voltage- and transmitter-activated currents. However, as compared with other commonly used internal solutions, our standard intra- and extracellular solutions rendered reversal potentials for $I_{(QA)}$ and $I_{(KA)}$ more positive. In some cases, 60 mM NaCl or 120 mM CsCl were therefore added at the expense of *N*-methylglucamine to reverse $I_{(QA)}$ and $I_{(KA)}$ within a more negative range of membrane voltages (Figs. 7*C*, 10*C*).

Whole-cell membrane currents were recorded using an amplifier equipped with a 10¹⁰ Ω feedback resistor (Meyer and Renz, Munich, FRG). Data were stored at 5 kHz on a FM tape recorder (Racal) and displayed on a chart-recorder (Rikadenki), usually at a speed of 2.5 mm/sec.

Superfusion system and solutions. After removal of culture dishes from the incubator, the growth medium was changed for a standard salt solution containing (in mM): NaCl, 120; KCl, 3; CaCl₂, 1.5; MgCl₂, 1; glucose, 10; sucrose, 7.5; HEPES, 10, pH 7.3. In some experiments, Na⁺ was replaced by *N*-methylglucamine or choline, and Cl⁻ by isethionate

Figure 3. Dose-response characteristics for $I_{(QA)}$ (*A*, *B*) and $I_{(KA)}$ (*C*). Current amplitudes were measured 20 sec after start of application and plotted against agonist concentration. In *A*–*C*, data points are from 3 different neurons. V_h indicated at the right of each curve. Curves fit free-hand.

or gluconate. Osmolarity was balanced with sucrose. All solutions were applied at a pH of 7.3 and a temperature of 22°C. Individual dishes were not used for more than 2 hr. Cells chosen for recording were maintained under continuous flow of standard or test solutions. This arrangement minimized access of unidentified substances that might be released by the culture itself. All test solutions were freshly prepared by dissolving the appropriate amount of amino acids and their antagonists in the standard salt solution. In the NMDA-containing solution, Mg^{2+} was omitted. All results apply to NMDA in the absence of added glycine. In experiments with various concentrations of Na⁺, Ca²⁺, or Cl⁻, a stock solution was prepared to have exactly the same amount of agonist in each barrel. A fast-switch 6-barrel superfusion system allowed for rapid exchange of fluids via a common outflow pipette (see Konnerth et al., 1987). The exchange time was very reproducible and fell within the range of 100–150 msec. The pipette (200 μm diameter) was placed about 100 μm from the voltage-clamped neuron. Less ramified cells with soma diameters of about 20 μm were chosen for recording (Fig. 1*B*).

NMDA (10–400 μM), QA (0.1–200 μM), KA (1–400 μM), and APB (1–100 μM) were applied in a stepwise manner for a period of 30 sec at a frequency below per 2 min. As a rule, currents were recorded at holding voltages (V_h) between –90 and +30 mV. We tested a total of 130 neurons. Thirty of these were exposed to both QA and KA. To avoid variability in results, cultures were used for electrophysiological measurements during one week only, between div 14 and 21.

Results

Current responses to different Glu agonists

All tectal neurons were found to be responsive to NMDA, QA, and KA; no cells responded to APB. To facilitate comparison of the current responses to the effective agonists, representative examples are presented together in Figure 2. In this paper we shall consider the responses to QA and KA only. The currents elicited by these agonists differ in time course and amplitude (Fig. 2, *B*, *C*). With higher agonist concentrations, $I_{(QA)}$ had a biphasic configuration, while $I_{(KA)}$ remained steady throughout the application period. The maximal amplitudes of $I_{(KA)}$ exceeded the maximal amplitudes of $I_{(QA)}$ by about 10 times. Nonetheless, the noise increase was often smaller during application of KA (see also Fig. 4).

Prolonged application of high agonist concentrations and large amplitudes of $I_{(QA)}$ and $I_{(KA)}$ could lead to secondary effects causing an Off-response. The present study did not aim at identifying

Figure 4. Recovery of $I_{(KA)}$ (B) but not $I_{(QA)}$ (A) when a regular application cycle (per 2 min) was interrupted for 20 min.

the probably complex nature of the aftercurrents, which were particularly prominent following large $I_{(KA)}$ (Fig. 4B, middle trace). In any case, the aftercurrents did not result from incomplete wash-out of the agonist.

Concentration and time dependence of $I_{(QA)}$ and $I_{(KA)}$

Figure 3 presents the dose-response curves for $I_{(QA)}$ (A, B) and $I_{(KA)}$ (C). In each experiment ($n = 10$), one and the same cell was exposed to 5 different agonist concentrations. The concentrations of QA required to activate and to saturate the current were much lower than those of KA. Half-effective concentrations (EC_{50}) ranged from 0.25 to 0.65 μM for QA and from 26 to 33 μM for KA, the respective averages being 0.5 and 30 μM . These estimates apply to current amplitudes measured 20 sec after the start of agonist application. The EC_{50} for the peak of QA-activated currents were, on average, lower than those for the steady state (range, 0.1–0.31 μM). The decrease of $I_{(QA)}$ with concentrations above 2.5 μM reflects the stronger attenuation of the plateau compared with the peak current (Fig. 2B). Such behavior is indicative of receptor "desensitization" (Katz and Thesleff, 1957; Magazanik and Vyskočil, 1973; Dudel, 1975; Feltz and Trautman, 1982).

In agreement with previous studies on other central neurons (Ishida and Neyton, 1985; Kiskin et al., 1986; Vyklický et al., 1986), KA responses were little affected by a concentration increase beyond the dose giving maximal current amplitudes. However, regular repetition suppressed $I_{(KA)}$ even more than $I_{(QA)}$. Figure 4B shows that a break of 20 min and subsequent regular repetitions could lead to pronounced changes in the amplitude of $I_{(KA)}$, whereas $I_{(QA)}$ remained stable throughout the

Figure 5. Effects of KYN using equieffective (A) and equimolar (B) concentrations of QA and KA. In each experiment, QA- and KA-containing control and test solutions were prepared to contain the same amount of drug. A and B, Records from 2 different neurons. Experiments in 10 μM APV. Note block of $I_{(KA)}$ but not $I_{(QA)}$.

recording period (Fig. 4A). These results may reflect agonist-dependent differences in the time course of desensitization. Since many applications were necessary to complete an experiment, we decided to ignore the first responses to KA and to avoid longer interruptions in the application cycle.

Action of Glu antagonists

We intended to determine whether separate receptors account for the contrasting effects of QA and KA by using receptor-selective antagonists. In trying a number of known Glu antagonists, we found that, in tectal neurons, kynurenic acid (KYN) was a very selective and potent antagonist of KA, when NMDA receptors were blocked with D-2-amino-5-phosphonovaleric acid (APV) (Evans et al., 1982) or Mg^{2+} (Mayer et al., 1984). KYN blocked also $I_{(NMDA)}$ when this current was expressed in the absence of APV or Mg^{2+} (Fig. 12D), as described by others (Ganong et al., 1983; Elmslie and Yoshikami, 1985; Coleman and Miller, 1986; Huettner and Baughman, 1986). Figure 5 illustrates an experiment where KYN (in the presence of APV) blocked $I_{(KA)}$ without reducing $I_{(QA)}$. The blocking action of KYN was accomplished at concentrations as low as 30 times the EC_{50} for KA, whereas $I_{(QA)}$ remained unaffected by up to 200 times the average EC_{50} for QA. KYN was selective if used at equieffective (Fig. 5A) or equimolar (Fig. 5B) concentrations of QA and KA. Other established Glu antagonists turned out to be less potent. The results of tests with γ -D-glutamylaminomethylsulfonic acid (GAMS), γ -D-glutamylglycine (γ -DGG), and glutamic acid diethylester (GDEE) are summarized in Table 1. GAMS and γ -DGG were found to be selective antagonists for KA, and GDEE a selective but very weak antagonist for QA. We conclude

Figure 6. Effect of elevated $[Ca^{2+}]_o$ on $I_{(KA)}$ and $I_{(QA)}$. Experiments with 30 μM KA (A) and 1 μM QA (B, C). Note that $I_{(QA)}$ was totally unaffected by increasing $[Ca^{2+}]_o$ up to 10 mM. Records are from a single cell.

that separate receptors for QA and KA must exist in tectal neurons to generate the conductance changes demonstrated here.

Na⁺ and Ca²⁺ dependence of QA- and KA-activated currents

Further support for the idea of separate QA and KA receptors comes from our experiments on the dependence of $I_{(QA)}$ and $I_{(KA)}$ on extracellular Ca^{2+} concentrations. Differences in the reaction of $I_{(QA)}$ and $I_{(KA)}$ to variation of $[Ca^{2+}]_o$ could be expected, since elevation of $[Ca^{2+}]_o$ has been shown to induce a marked decrease of KA but not QA binding (Beaumont et al., 1979; Monaghan et al., 1983; Honoré et al., 1986). Figure 6 illustrates that $I_{(KA)}$ was indeed more prone to reduction by higher $[Ca^{2+}]_o$ than $I_{(QA)}$. However, channel block by Ca^{2+} and activation of a Ca^{2+} conductance could complicate the overall picture (Nowak and Ascher, 1985; Tachibana, 1985; Jahr and Stevens, 1987; Mayer and Westbrook, 1987). In fact, a reduction of $I_{(QA)}$ after increasing $[Ca^{2+}]_o$ was observed in 4 of 10 neurons. In these cells, QA opened a Ca^{2+} conductance, as indicated by a Ca^{2+} -dependent shift of $I_{(QA)}$ (7.8–9.1 mV by a 10-fold increase of $[Ca^{2+}]_o$) (Fig. 7, A, B). The reversal potential for $I_{(QA)}$ in the remaining 6/10

neurons and the reversal potential for $I_{(KA)}$ were unaffected by raising $[Ca^{2+}]_o$ (Fig. 7C).

Changing $[Na^+]_o$ influenced $I_{(QA)}$ and $I_{(KA)}$ in similar ways. $[Na^+]_o$ was varied by substituting *N*-methylglucamine for Na^+ (Fig. 8). The Na^+ -dependent shifts of $E_{(QA)}$ (B) and $E_{(KA)}$ (D) were nearly the same. On average, a 10-fold decrease of $[Na^+]_o$ moved $E_{(QA)}$ 27 mV towards more negative voltages ($n = 5$; range, 18–31 mV). The respective value for $E_{(KA)}$ was 27 mV ($n = 5$; range, 23–30 mV). These numbers probably represent underestimates, since more positive reversal potentials would result from free-hand curve fitting.

Dependence of $I_{(QA)}$ and $I_{(KA)}$ on $[Cl^-]_o$

Binding of QA and KA to synaptic receptors is generally considered to be independent of extracellular Cl^- concentrations (Foster and Fagg, 1984). On the other hand, it has been reported (Fagg et al., 1982; Mena et al., 1986) that QA acts as a potent displacer for APB at presumed extrasynaptic Cl^- -dependent sites which may be uptake sites for Glu (Pin et al., 1984; Fagg and Lanthorn, 1985; Bridges et al., 1986; Kessler et al., 1987a). We

Figure 7. Whole-cell I - V relations and reversal potentials for $I_{(QA)}$ (A, B) and $I_{(KA)}$ (C) at different $[Ca^{2+}]_o$. Standard intra- and extracellular solutions, with osmolarity balanced with sucrose. Note Ca^{2+} -dependent change of $E_{(QA)}$ (A) but not $E_{(KA)}$ (C). Data points in A and B are fitted with linear-regression lines ($r = 0.995$ for B). A, Electrode filled with *N*-methylglucamine; C, electrode filled with *N*-methylglucamine/ $NaCl$.

Figure 8. Whole-cell I - V relations and reversal potentials for $I_{(QA)}$ (A, B) and $I_{(KA)}$ (C, D) at various $[Na^+]_o$. An intracellular solution containing 20 mM Na^+ and 100 mM N-methylglucamine faced with different extracellular concentrations of Na^+ . N-methylglucamine was substituted for Na^+ . QA, 1 μ M (A), and KA, 30 μ M (C), in the presence of 10 μ M APV. Curves represent the regression lines of best fit ($r = 0.999$ for B, $r = 0.997$ for D).

found it worthwhile to reevaluate the effect of various $[Cl^-]_o$ levels on both $I_{(KA)}$ and $I_{(QA)}$. $[Cl^-]_o$ was changed by substituting isethionate or gluconate for Cl^- . Surprisingly, low $[Cl^-]_o$ reduced $I_{(KA)}$ by up to 50% (Fig. 9, C, D). The time to peak of $I_{(QA)}$ was prolonged, but the steady-state amplitude of $I_{(QA)}$ was unaffected (Fig. 9, A, B).

To discriminate between the significance of $[Cl^-]_o$ for binding and conductance, we examined the Cl^- dependence of $E_{(KA)}$ (Fig. 10). In all neurons tested ($n = 14$), $E_{(KA)}$ was found to be more negative at lower $[Cl^-]_o$, as should be the case if a background gCl^- were decreased during KA application. This effect was observed using either isethionate (Fig. 10A) or gluconate (Fig. 10C) as a substitute for extracellular Cl^- , irrespective of whether electrodes were filled with N-methylglucamine chloride (Fig. 10A) or CsCl (Fig. 10C). A 10-fold reduction of $[Cl^-]_o$ decreased $E_{(KA)}$ on average by 7.9 mV (range, 2.3–13.8 mV). Twelve other

Figure 9. Effect of $[Cl^-]_o$ on $I_{(QA)}$ and $I_{(KA)}$. A and C, Specimen recordings. B and D, Current amplitudes at 2 and 20 sec after start of agonist application. Note that the steady-state amplitude (at 20 sec) is reduced in $I_{(KA)}$ but not $I_{(QA)}$.

neurons tested similarly with 2 μ M QA did not display the $[Cl^-]_o$ -dependent shift of $E_{(QA)}$. It is interesting to note that in cells generating large $I_{(KA)}$, small (10–50 pA) outward currents were seen when KA was applied together with KYN.

This type of Cl^- dependence has been discussed elsewhere only in connection with APB, which by itself did not cause any current in tectal neurons. We conclude therefore that tectal neurons may express a receptor having the characteristics of the A4 type of AAR but preferring KA to APB as a ligand. However, this leaves open the question of whether KA binding (to its own or a different site) is also dependent on $[Cl^-]_o$. The slowing of $I_{(KA)}$ in low $[Cl^-]_o$ suggests that this may, indeed, be the case. In experiments on 3 neurons exposed to low (18 mM) Cl^- on both sides of the membrane, $I_{(KA)}$ was slowed and reduced by about 20% compared with the control in high (128 mM) $[Cl^-]_o$.

Simultaneous application of QA and KA

To test for possible convergence of QA and KA receptors, we investigated the effect of simultaneous agonist application (Fig. 11). If the receptors and channels activated by QA and KA were independent, and both ligands perfectly selective, $I_{(QA)}$ and $I_{(KA)}$

Table 1. Effects of 4 different Glu antagonists on $I_{(QA)}$ and $I_{(KA)}$

Antagonist	No. cells	QA			KA		
		Conc. QA (μ M)	Conc. antagonist (μ M)	$I_{(QA)}$ (% control)	Conc. KA (μ M)	Conc. antagonist (μ M)	$I_{(KA)}$ (% control)
KYN	14	1/30	10/1000	100/94.5	30	1000/3000	0
γ -DGG	14	1	10	100	30	300	34
GAMS	7	2	20/300	100/94	30	300	49
GDEE	5	1	100	91	30	3000	100

Results apply to steady-state amplitudes of $I_{(QA)}$ and $I_{(KA)}$ recorded in the presence of 10 μ M APV, 100 μ M bicuculline, and 1 μ M TTX at $V_h = -60$ mV. In any given cell, effects of antagonist were tested on both $I_{(QA)}$ and $I_{(KA)}$. At the indicated concentrations, antagonists did not elicit currents by themselves.

Figure 10. Whole-cell I - V relations and reversal potentials of $I_{(KA)}$ dependent on $[Cl^-]_o$. **A** and **B**, Substitution of isethionate for Cl^- . Electrode filled with N -methylglucamine. $[Cl^-]_o = 120$ mM. The levels of $[Cl^-]_o$ in **A** were, from top to bottom, 8, 38, 68, and 128 mM. Data points are fitted by linear-regression lines. For the line drawn in **B**, $r = 0.991$. **C** and **D**, Substitution of gluconate for Cl^- . Electrode filled with CsCl. $[Cl^-]_i = 115$ mM. **C**, I - V characteristics for 3 different $[Cl^-]_o$. **D**, Dependence of reversal potential for $I_{(KA)}$ on the logarithm of transmembrane Cl^- gradient. Data points fitted by linear-regression line, with $r = 0.968$. Both experiments in $10 \mu M$ APV.

should sum algebraically. This was not the case. As in retinal neurons (Ishida and Neyton, 1985; Aizenman et al., 1988), currents resulting from simultaneous application of both agonists were smaller than the sum of $I_{(QA)}$ and $I_{(KA)}$. With increasing doses of QA, the response of both QA and KA approached the amplitude level expected for QA application alone. That QA may, in fact, induce a complete block of $I_{(KA)}$ is indicated by the absence of a KYN-sensitive current fraction in the compound response to both agonists (Fig. 11*A*, *d*, *e*). At equimolar QA and KA concentrations, $I_{(KA)}$ is fully suppressed (Fig. 11*Ad*). The block of $I_{(KA)}$ by QA was, however, surmounted by increasing the concentrations of KA (Fig. 11, *B*, *C*). These results suggest that QA operates as a competitive antagonist of KA and support the previous notion (Monaghan and Cotman, 1982; Monaghan et al., 1983; Fagg and Matus, 1984; Honoré et al., 1986) that QA binds to a KA site. At this site, QA fails to induce a high-amplitude, nondesensitizing response, i.e., a response with the characteristic features of $I_{(KA)}$.

Contribution of NMDA, QA, and KA receptors to $I_{(Glu)}$

The experiments described above led to the conclusion that, in tectal neurons, NMDA, QA, and KA utilize separate receptors

Figure 11. Experiment showing the effect of simultaneous application of QA and KA. **A**, Demonstration of a KA-receptor-mediated current in the compound response to KA and QA. *a*, Control with KA alone; *b* and *d*, response to KA in the presence of 2 different concentrations of QA (application through a single barrel); *c* and *e*, Absence of a KYN-sensitive component when QA concentration equals KA concentration. Experiment in $10 \mu M$ APV. **B**, Experiment showing that the QA-induced block of $I_{(KA)}$ can be overcome by raising the concentration of KA. KA concentration indicated below each trace. **C**, Dose-response characteristics for $I_{(KA + QA)}$. Results from experiment shown in **B**. QA concentration, $50 \mu M$. Ordinate, normalized current amplitudes measured 20 sec after start of drug application.

or receptor subunits to induce ionic conductance changes. It is not known, however, whether any or all of these receptors are also involved in the current response to the endogenous ligand Glu. Comparing the maximal current amplitudes induced with saturating doses of QA, KA, and Glu, it was noticed that QA-activated currents were generally smaller than $I_{(Glu)}$, whereas $I_{(KA)}$ exceeded $I_{(Glu)}$ in amplitude by 2–3 times. It is therefore conceivable that KA-like effects are suppressed during $I_{(Glu)}$ as Glu mimics the blocking action of QA at the KA (A3) site. In this case, KYN would have little or no effect on $I_{(Glu)}$, provided that the NMDA-selective current component is already blocked with APV or Mg^{2+} . The block of the steady-state component of $I_{(NMDA)}$ was shown to be complete at $V_h = -90$ mV (Grantyn et al., 1987).

Figure 12 illustrates an experiment in which Mg^{2+} and KYN were combined to reveal the A3-mediated fraction of $I_{(Glu)}$. Comparing the current traces at -90 mV (Fig. 12, *B*, *C*, bottom row), it can be seen that with $50 \mu M$ Glu the contribution of A3 receptors is indeed small (10%). However, in experiments with $100 \mu M$ Glu, the KYN-sensitive component of $I_{(Glu)}$ increased to 50–80% ($n = 8$). Similar estimates were achieved using APV as a blocker at A1 receptors. These experiments indicate that KA receptors do contribute to the compound response to exogenous Glu, although at lower concentrations Glu binds preferentially to NMDA receptors.

As mentioned, KYN antagonizes not only $I_{(KA)}$, but also $I_{(NMDA)}$ (Ganong et al., 1983; Elmslie and Yoshikami, 1985; Coleman and Miller, 1986; Huettner and Baughman, 1986). This is confirmed by the results illustrated in Figure 12D. A larger portion of $I_{(Glu)}$ becomes sensitive to KYN when the Mg^{2+} block is released by depolarization. At $V_h = +30$ mV, the KYN-induced block of $I_{(Glu)}$ equals the combined effect of Mg^{2+} and KYN at -90 mV.

Discussion

Cultured tectal neurons—a substrate for KA?

When development of tectal neurons takes place *in situ*, Glu receptors should be functional by P14–21. Intracellular recording from neurons in the isolated rat superficial gray layer showed that nearly all neurons are able to generate EPSPs in response to stimulation of the brachium colliculi 3 weeks after birth (R. Grantyn and R. Schümann, unpublished observations). The pharmacological properties of these EPSPs have, however, remained unexplored. In the present study on cultured neurons, we allowed for 2–3 weeks of *in vitro* differentiation and found that all neurons from the superficial gray layer are responsive to NMDA, QA, and KA, but not to APB. Close analysis of the agonist-induced effects led us to conclude that 4 distinct receptors for acidic amino acids must exist on these cells. However, it is now well recognized that dissociation procedures, time *in vitro*, and culture composition may influence the likelihood of revealing one or the other receptor type (see O'Brien and Fischbach, 1986b, c, for a beautiful demonstration).

We show here that KA-activated currents are larger than $I_{(QA)}$ and $I_{(NMDA)}$, although binding studies and autoradiographic work on the adult SC has shown that the KA receptor density is very low in this region (London and Coyle, 1979; Unnerstall and Walmsley, 1983). Responsiveness to KA has been studied in rat dorsal root ganglion cells (DRG) of various age (Agrawal and Evans, 1986). These authors suggest that high responsiveness to KA might be a characteristic feature of neonatal neurons. Moreover, in immature DRG neurons, KA receptors are reported to occur on axons (Evans, 1980). Thus, it must be asked to what extent, if any, can KA receptors be expected to participate in generation of EPSPs.

Convincing evidence favoring a role of KA receptors in synaptic transmission exists for some retinal and hippocampal connections (Coleman et al., 1986; Cotman et al., 1986; de Montigny et al., 1987; Massey and Miller, 1987). Generally, non-NMDA receptors (both A2 and A3) are thought to be responsible for the initial fast EPSP. In contrast, slower peak and decay times were seen in EPSPs mediated by NMDA receptors (Dale and Roberts, 1985; Jahr and Jessel, 1985; Rothman and Samaie, 1985; Nelson et al., 1986; O'Brien and Fischbach, 1986c). In unidentified cortical neurons, stimulation of cultured cortico-tectal neurons evoked EPSPs with the characteristics of non-NMDA receptors (Huettner and Baughman, 1988). These EPSPs were blocked with KYN, although KYN failed to discriminate between QA and KA receptors. The high selectivity of KYN for KA receptors, as demonstrated in tectal and some retinal (Coleman et al., 1986) neurons, recommends this drug as a tool for further investigation of the role of KA receptors in primary visual pathways.

We cannot explain why most studies (Ganong et al., 1983; Elmslie and Yoshikami, 1985; Jahr and Jessel, 1985; Huettner and Baughman, 1986; see, however, Gallo et al., 1987) failed to demonstrate a selective block of KA receptors by KYN. A

Figure 12. Test to reveal NMDA and KA-receptor-mediated currents in the compound response to Glu. Recordings from a single cell at different holding voltages. Calibration bars for current apply to all records in one horizontal row. *A*, Current response to Glu in a solution without added Mg^{2+} . *B*, Same response in the presence of Mg^{2+} . Strong reduction of response at -90 mV. *C*, Same as *B*, but with addition of KYN. *D*, Fraction of the Mg^{2+} - and KYN-sensitive current as a percentage of the current amplitude with Glu alone. Amplitudes measured 20 sec after start of Glu application.

high EC_{50} for KA may be taken as an indication for the absence of distinct KA receptors in some other preparations (O'Brien and Fischbach, 1986a; O'Dell and Christensen, 1987). Our EC_{50} agree well with the estimates of Fagg and Matus (1984), who determined the K_i for Glu displacement in a postsynaptic density (PSD) preparation from rat brain. But these authors reported that KA acts exclusively at a site that binds QA with much higher affinity than KA (EC_{50} between 0.5 and 10 μM). They discussed the possibility that a specific KA receptor, as demonstrated by receptor autoradiography (Foster et al., 1981; Monaghan and Cotman, 1982; Unnerstall and Walmsley, 1983; Represa et al., 1987), may exist outside the PSD. As QA caused a complete block of $I_{(KA)}$, it seems unlikely that tectal neurons bear 2 classes of KA receptors capable of activating a cationic current. However, KA may, in addition to its own site (A3), affect a receptor that promotes a decrease of anionic conductance.

Ca^{2+} and Cl^- dependence of $I_{(KA)}$ —a fourth receptor?

The demonstration of a pronounced Cl^- dependence of $I_{(KA)}$ was an unexpected finding of the present study. A shift of $E_{(KA)}$ towards more negative membrane potentials by reduction of $[Cl^-]_o$ suggested that KA decreases a preexisting Cl^- conductance (g_{Cl^-}). With our standard intra- and extracellular solutions, this decrease of g_{Cl^-} would cause an apparent outward current at negative membrane potentials and mask the inward current carried by Na^+ . The amplitude of the outward component would depend on the background conductance for Cl^- —a variable not easily isolated. A decrease of g_{Cl^-} by Glu has been observed before (see Miller and Slaughter, 1986), but the identity of un-

derlying receptors (A4) has not been fully established. Here, we show that KA may be a ligand for the hypothetical A4 site. It is possible that NMDA is also an A4 agonist, because a noticeable outward current remained in some neurons when $I_{(NMDA)}$ was blocked with APV or Mg^{2+} (Grantyn et al., 1987, Fig. 1D).

The lack of a selective A4-agonist hindered the demonstration of a stimulating effect of higher $[Ca^{2+}]_o$ at this site (Fagg et al., 1982). In experiments with KA, currents were always reduced by elevation of $[Ca^{2+}]_o$, as predicted by previous studies of KA binding (Beaumont et al., 1979; Honoré et al., 1981; Monaghan et al., 1983) and KA-activated ionic conductance (Nowak and Ascher, 1985; Tachibana, 1985).

Possible configurations of acidic amino acid receptors and channels

There are at least 4 possibilities of receptor and channel arrangement. To explain the lower amplitudes of $I_{(QA)}$ (at saturating agonist concentrations), it could be assumed, first, that fewer receptors and channels were available for QA. The lower number of QA-selective receptors and channels might be a consequence of insufficient expression or rapid desensitization. Second, the number of available QA and KA receptors could be equal, but QA might activate ionic channels with lower opening probability or unitary conductance, e.g., have lower efficacy. Third, several receptors could converge to one and the same channel protein. These receptors must be able to promote different conductance states of the common ionic channel. A configuration such as this is in line with results of single-channel analysis (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987), which showed that in any given patch QA-, KA-, and NMDA-activated ionic channels coexisted, and each receptor-selective agonist activated all possible conductance levels. Fourth, it has been suggested that QA and KA may share not only their channels but also their receptors (Kiskin et al., 1986; O'Dell and Christensen, 1987). The state of receptors and channels would then be determined by the ligand or any modulatory factor that could make the receptor-channel complex more QA- or KA-like.

The first 2 configurations would imply that QA acts as a low-affinity competitive antagonist at KYN-sensitive KA receptors and a high-affinity agonist at its own, KYN-insensitive receptor. In this case, the QA-induced block should be overcome by higher concentrations of KA. This is the case in tectal neurons. The fourth configuration (shared channels and receptors) is unlikely, because (1) a selective blocking ligand exists for KA receptors, and (2) KA, but not QA, binding is reduced in high $[Ca^{2+}]_o$.

Unfortunately, we are not in the position to either affirm or reject the third possible configuration. Preliminary results from developing tectal neurons indicate that QA and KA sensitivity evolve together, whereas NMDA responses appear after some delay. However, the ratio of maximal $I_{(QA)}$ and $I_{(KA)}$ amplitudes varies largely from cell to cell, indicating that the number or affinity of QA and KA sites is not strictly matched. An uneven distribution of Glu receptors has also been demonstrated in neurons bearing functional synaptic connections (Dale and Roberts, 1985; O'Brien and Fischbach, 1986c; Trussel and Fischbach, 1987). NMDA or non-NMDA receptors could accumulate to give rise to receptor-selective unitary EPSPs (Dale and Roberts, 1985). As long as more specific arguments are not available, we prefer the most general configuration, the first listed above.

Note added in proof: Recently, new selective non-NMDA receptor antagonists became available from TOCRIS (England). We have tested the compounds 6,7-Dinitroquinoxaline-2,3-dione (DNQX) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) against half-effective concentrations of QA (0.5 μM) and KA (30 μM). It was found that 5 μM DNQX or CNQX reduce both $I_{(QA)}$ and $I_{(KA)}$ to about 10% of the control response. The IC_{50} of DNQX was about 0.5 μM under these conditions. DNQX was slightly more potent than CNQX. We thus confirm that DNQX and CNQX are effective non-NMDA receptor antagonists, which do not, however, discriminate between A2 and A3 receptor subtypes.

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