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ACCELERATED COMMUNICATION

Reduction of Desensitization of a Glutamate Ionotropic Receptor by Antagonists

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SUMMARY

The glutamate receptor channel subtype that responds to both quisqualate (QA) and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) was expressed in *Xenopus* oocytes injected with rat cerebral cortex mRNA. Voltage-clamp current responses to QA, AMPA, and glutamate (GLU) exhibited a rapid increase followed by a decrease to a desensitized steady state (DS). Perfusion with high agonist concentrations produced smaller DS responses than perfusion with low concentrations. During the DS, the current was increased by lowering of the concentration of agonist or by application of low concentrations of a competitive antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX). This paradoxical increase of the agonist-induced currents during the DS was also observed in cultured Purkinje cells with another competitive antagonist, 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX). Dose-response curves obtained in oocytes were bell shaped, with a negative slope for high concentrations of QA. DNQX shifted these bell-shaped curves to the right. Together, these results indicate that the agonists are able to reversibly inhibit the AMPA receptor. The classical desensitization model of Katz and Thesleff [*J. Physiol. (Lond.)* **138**:63–80 (1957)] cannot account for our observations.

Excitatory synaptic transmission between nerve cells in the mammalian brain is mediated by the activation of at least two subtypes of GLU receptor channel complexes, the *N*-methyl-D-aspartic acid and the AMPA receptors (1, 2). In neurons, rapid activation of the AMPA receptors induces a large, rapidly inactivating current (3–7) and a DS that has been shown to occur after the application of only 2–3 μM GLU (6). In the present study, we have investigated the electrophysiological properties of the AMPA receptor channel that is expressed in *Xenopus* oocytes after injection of poly(A)$^+$ from rat cerebral cortex, as well as those of the AMPA receptor channel of cerebellar Purkinje cells in culture. In both preparations, agonist application induced a rapidly inactivating current followed by a DS. Moreover, we observed a pronounced transient increase of the current, a "hump," during washout. Similar humps had already been observed in recordings obtained from neurons (4, 7), but this phenomenon had never been fully investigated.

We report here the results of experiments done to understand the mechanisms producing the hump, as well as other paradoxical behavior of the response at the DS; the current was 1) increased by lowering of the concentration of agonist, 2) decreased by raising of agonist concentration, and 3) increased when low concentrations of a competitive antagonist were added. Our results indicate that the AMPA receptor is reversibly inhibited by QA, AMPA, and GLU.

Materials and Methods

Drugs were from Tocris Neuramin (UK).

Oocyte preparation. Total RNA was prepared from fresh cerebral cortex of 16-day-old rats, by using the guanidinium thiocyanate single-step method (8). Poly(A)$^+$ RNA was selected by using an oligo (dT) column. Fully grown oocytes in their follicular sacs were injected with 50 ng of poly(A)$^+$ RNA. After 3 days of incubation at 18°, the oocytes were manually defolliculated 2 hr before recording.

Recordings. Recordings were made using the two-electrode voltage-clamp method, with 3 M KCl-filled microelectrodes with resistances of 1 MΩ. The recorded currents have been filtered by a single time constant at 15 Hz. The storage and recording medium contained (in mM) NaCl, 82.5; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 1; HEPES, 1; and Na_2HPO_4, 1; pH 7.5. Magnesium was included in all experiments to block *N*-

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ABBREVIATIONS: GLU, glutamate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DS, desensitized steady state; QA, quisqualate; I_{QA}, inward current induced by quisqualate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

methyl-D-aspartate receptors (9). All experiments were done at 22°. The flow rate in the 100-μl recording chamber was about 300 μl/sec. The maximal peak responses to 100 μM QA were obtained in 100 msec. Change of solution was made by switching between inlet tubes, with no interruption of flow.

Thirteen control oocytes, not injected with poly(A)$^+$ RNA, were tested for endogenous responses to the drugs. In three of these, an outward K^+-dependent current was produced when QA, AMPA, GLU, or DNQX was superfused at concentrations above 100 μM. This current eventually disappeared during the experiments, suggesting that it was due to the removal of follicular cells with time. In four oocytes, small inward currents were produced by similar concentrations of agonists; in the rest, no effects were seen.

Cerebellar slices were cultured by means of the roller-tube technique, as previously described (10). Whole-cell recordings of cultured Purkinje cells were performed as detailed in Ref. 11. Briefly, for electrophysiological studies, 10–40-day-old cultures were perfused at a flow rate of 1 ml/min with a solution containing (in mM) Na^+, 155; K^+, 5; Cl^-, 162; Ca^{2+}, 2; Mg^{2+}, 1; HEPES, 10; D-glucose, 10; and tetrodotoxine, 1 μM. All drugs were prepared in the same solution and applied with a U-tube microperfusing system. Recordings were performed at room temperature (18–22°), using the whole-cell configuration of the patch-clamp technique (12). Pipettes had a tip resistance of 1–3 MΩ and were filled with the following internal solution (in mM): CsCl, 120; NaCl, 5; $CaCl_2$, 0.5; $MgCl_2$, 1; EGTA, 5; and HEPES, 10.

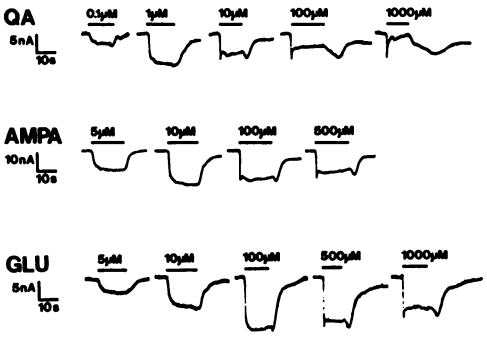

Fig. 1. Current responses to QA, AMPA, and GLU from the same oocyte, which was voltage clamped to −60 mV. Agonists were rapidly perfused into the chamber, at the concentrations shown, during the time indicated by the *bars*. Note that the peak responses were obtained at different concentrations for each of the agonists.

Results

Oocytes injected with poly(A)$^+$ RNA from rat cerebral cortex expressed all subtypes of the GLU receptors, but the metabotropic QA receptor (13, 14) was seldom expressed and, when detected in some batches of oocytes, was suppressed by injection of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid at an expected final intracellular concentration of about 100 μM (14). The ionotropic QA responses in both kinds of oocytes were identical and were identified by their activation with both QA and AMPA and their reversal potential near −10 mV. The majority of experiments were done using QA as agonist, but the voltage-clamp current responses to GLU and AMPA were very similar, as illustrated in Fig. 1. At QA doses of >10 μM, a fast inward current (I_{QA}) was elicited and decayed rapidly to a lower constant level, producing an initial I_{QA} peak. This decreased level of I_{QA} was taken to be the DS. After establishment of the DS, agonist washout resulted in a transient current increase, the hump.

The whole-cell currents induced in cultured Purkinje cells by QA displayed similar characteristics. The application of 100 μM QA resulted in a peak response (time to peak, 40 msec) that rapidly decreased to the DS, and a hump was observed at the washout (Fig. 2A). The voltage dependences of the peak, the DS, and the hump of the QA response were identical. The current-voltage relationships of the DS and the hump were both linear, with a reversal potential close to 0 mV (Fig. 2B), indicating that the hump current was indeed mediated by the AMPA receptor.

The duration of QA application had little influence on the magnitude of the hump, as shown in Fig. 3A. Perfusion of the oocytes with a 1 μM QA medium, after the DS was established at 100 μM, increased I_{QA} to levels nearly equal to the initial peak (Fig. 3B). These results, taken together, showed that the desensitization may be removed by a decrease in agonist concentration.

To test this apparent reversal of the desensitization, various combinations of low and high QA concentrations were tested. Starting from a nondesensitizing concentration of QA and then switching to 100 μM produced the initial peak and the DS. However, as has been observed also in neurons (4), I_{QA} at the

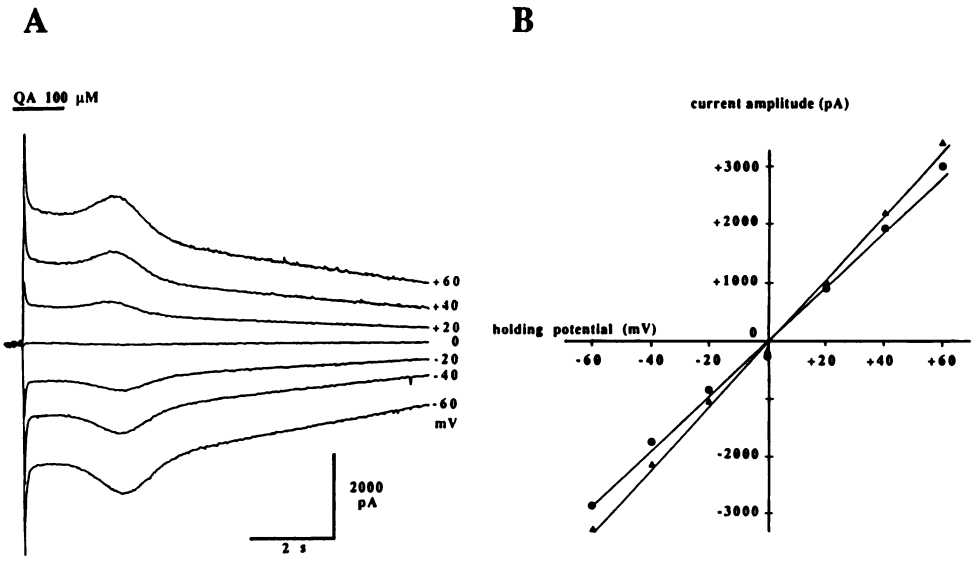

Fig. 2. Voltage dependence of whole-cell responses of a cultured Purkinje cell to QA (100 μM) applied with a U-tube perfusing system. A, Membrane currents induced by QA at various membrane potentials, as indicated to the *right* of each trace. Note that the initial peak, the DS, and the hump currents were also observed at positive holding potentials. Latency (300 msec) between the beginning of the application bar and the onset of the peak is inherent to the U-tube perfusing system. Time to peak was 40 msec. B, Plot of the current induced by QA as a function of the holding potential. The currents were measured at the DS (●) and at the maximum of the hump (▲).

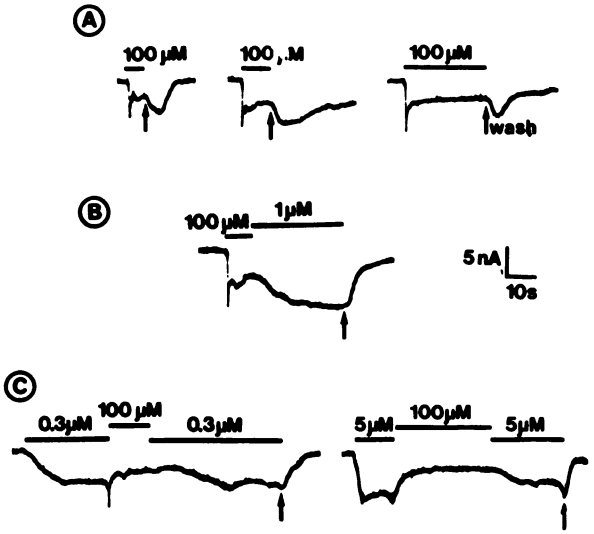

Fig. 3. Changing steady state I_{QA} by changing [QA]. All I_{QA} recordings are from the same poly $(A)^+$ RNA-injected oocyte. *Bars*, times of application of QA, at the concentrations indicated. A, The duration of QA application did not change the magnitude of the hump current at washout. B, The DS I_{QA} was increased by changing to lower [QA], and no hump appeared at washout. C, *Left*, preapplication of a low [QA] followed by application of high [QA] produced a peak and a decreased DS I_{QA}. Returning to low [QA] increased the DS I_{QA}, and no hump was produced at washout. *Right*, with 5 μM preapplied QA producing an I_{QA} with little desensitization, high [QA] no longer produced a peak, and only a decrease in I_{QA} was obtained.

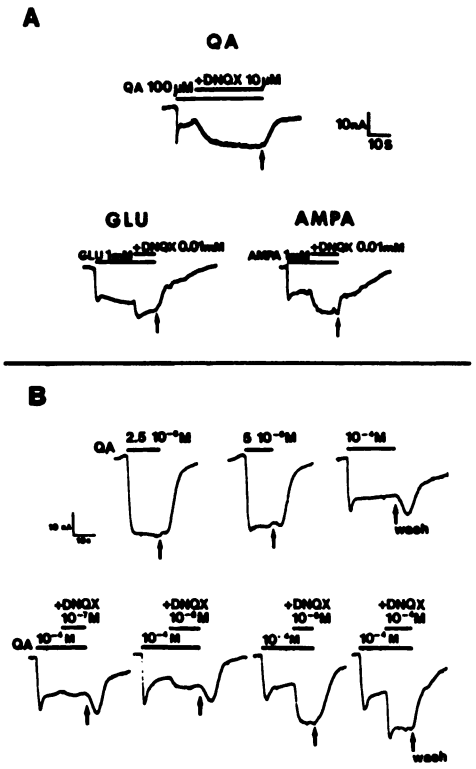

Fig. 4. Effects of the competitive inhibitor DNQX on I_{QA}. A, Low concentrations of DNQX added to high concentrations of QA, AMPA, or GLU increased the DS I_{QA}. *Bars*, times of application. Note that GLU and AMPA were used in the millimolar range, due to their lower potencies (see Fig. 1). B, DNQX removed the QA inactivation. All I_{QA} responses are from the same poly $(A)^+$ RNA-injected oocyte, which was voltage clamped to −60 mV. *Bars*, times of application of QA and addition of DNQX, at the concentrations noted. Note that 1) in the *upper traces* the initial I_{QA} is smaller for 10^{-4} M QA than for 10^{-5} M, showing that I_{QA} inactivation became dominant and 2) when 10^{-4} M DNQX was added to 10^{-4} M QA, the rapid disinactivation was followed by a second inactivation, which was probably due to a DNQX block of the activating sites. This effect of DNQX has not yet been studied in greater detail.

DS was smaller at the higher concentration (Fig. 3C). This indicated that at concentration of QA below 1 μM all receptors were not activated or inactivated, allowing the appearance of a peak when switching to 100 μM QA (Fig. 3C, *left*). At 5 μM QA, nearly all receptors were activated, because switching to 100 μM did not produce a pronounced peak (Fig. 3C, *right*). However, not all receptor channels were inactivated at 5 μM QA, because 100 μM QA further decreased I_{QA}. Taken together, these results suggested that I_{QA} was reversibly inactivated by the occupancy of additional sites by QA.

The effect of decreased AMPA receptor occupancy by the agonist was tested using the competitive antagonist DNQX (15). At 500 μM, DNQX alone produced no change in the voltage-clamp current of the oocyte but completely blocked the response to 100 μM QA. However, when the DS was obtained with 100 μM QA, AMPA, or GLU, the current was increased when 10 μM DNQX was added (Fig. 4A). This increased I_{QA} produced by DNQX depended on its concentration, as shown in Fig. 4B. The I_{QA} established at the DS with 100 μM QA was only partially relieved by addition of 0.1 μM DNQX, and the hump was present at washout. When 10 μM DNQX was added, I_{QA} increased to about the magnitude obtained with 2.5 μM QA alone, and no hump was produced by washout. When 100 μM DNQX was added to 100 μM QA, a rapid increase in I_{QA} to even greater levels was produced, followed by an inhibition (see legend to Fig. 4B).

A similar increase of the DS current was observed in Purkinje cells when increasing concentrations of CNQX were added to 50 μM QA (Fig. 5). The hump at the washout concomitantly decreased and finally disappeared at 5 μM CNQX.

In oocytes, dose-response curves for I_{QA} were made for QA alone and with two concentrations of DNQX. The curves were roughly bell shaped, and the presence of DNQX shifted them to the right without major changes in form (Fig. 6). Therefore, the level of the DS response is controlled by the occupancy of the receptor by QA. The Hill coefficient for activation calculated from the QA dose-response curve was 1.7, in agreement with other reports (4, 6), and was 1 for inactivation.

Discussion

In the present study, fast perfusion systems allowing changes of solution in less than 1 msec were not used, and the kinetics of the fast desensitization occuring in less than 10 msec were not studied. In the following discussion, we assume that changes in steady state current reflect changes in the level of desensitization.

The bell-shaped dose-response curve for AMPA receptor agonists has already been observed in injected oocytes by Verdoorn and Dingledine (16). Curves with a negative slope at high concentrations of agonist were also observed in neurons (4, 7, 17). In this report, we show that the bell-shaped dose-response curve obtained from the recordings of oocytes is shifted to the right by addition of DNQX. We also show that the current recorded at the DS in oocytes or in Purkinje cells in culture increases after a simple decrease of agonist concentration. Lowering of the agonist concentration or addition of a

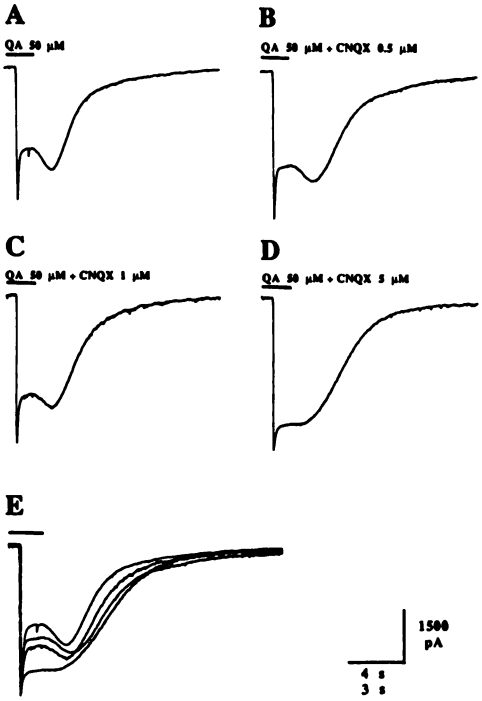

Fig. 5. Effect of increasing concentrations of CNQX on whole-cell responses of a cultured Purkinje cell to QA (50 μM) applied with a U-tube perfusing system. A, I_{QA} consisted of an initial peak followed by a desensitized state. The agonist washout resulted in a current hump. B, C, and D, Increasing concentrations of CNQX induced a larger I_{QA} at the DS and a concomitant disappearence of the hump associated with the washout of QA. E, Superimposition of the traces shown in A, B, C, and D. Holding potential, −60 mV. Calibration bar, 4 sec for A, B, C, and D, 3 sec for E.

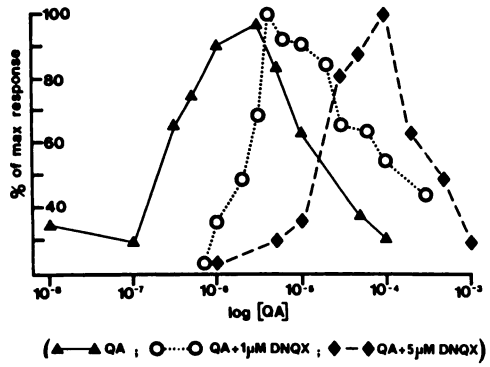

Fig. 6. QA dose-response curves. Data for the curves were the I_{QA} measured at least 5 sec after agonist perfusion began. Such measurements were averaged for each concentration from seven oocytes for QA only, three for QA plus 1 μM DNQX, and three for QA plus 5 μM DNQX. The averaged currents were then normalized and plotted. The competitive nature of DNQX inhibition is shown by the parallel shift of the curves to the right. The data for the 5 μM DNQX curve may have been affected by nonspecific effects of QA at concentrations of >500 μM (see Materials and Methods).

competitive antagonist has a similar effect; both experimental procedures increase the steady state current. These two experimental procedures have the same end results on the level of receptor occupancy by agonist, i.e., a decrease of occupancy. Our main novel observation is that a decrease of receptor occupancy by agonist during the DS is rapidly followed by a current increase, and this can account for the observed hump.

A negative slope in the dose-response curve for the nicotinic receptor has also been observed at high concentration of agonists. In this latter case, Colquhoun and Ogden (18) have shown that this characteristic results from an open-channel block by acetylcholine at negative membrane potentials. A block of the cationic channel of the AMPA receptor is very unlikely to occur with the anions GLU, QA, and AMPA. Moreover, our observations that DNQX and CNQX increase the steady state current and that the hump current is present at both negative and positive potentials rule out a possible channel block by the agonist.

The bell-shaped dose-response curve and the rapid reversal of desensitization by decreases in the receptor occupancy by the agonist cannot be explained by the classical desensitization models (19, 20). Our results could be fitted by a model in which the AMPA receptor channel can be opened by the binding of a single agonist molecule. However, the Hill coefficient of 2 suggests that the binding of a second agonist molecule increases the probability of opening, and thus we propose that desensitization proceeds more rapidly with two molecules of agonist bound to the receptor than with only one. In such a model, a decrease of receptor occupancy by the agonist (from two to one) will slow desensitization. Therefore, the proportion of opened receptor channels will increase because of the observed fast resensitization of the AMPA receptor (4, 6). This model is supported by our observation (not shown) that responses to QA were detected at doses as low as 1 nM. This could indicate that the receptor channel is opened after the binding of a single QA molecule.

Data concerning the molecular structure of the AMPA receptor channel have recently been obtained after the molecular cloning of a family of at least eight AMPA receptor subunits. The AMPA receptor has been shown to be a heterooligomer composed of functionally different subunits, any one of which responds to kainate, QA, and AMPA (21, 22). It is, thus, possible that the number of binding sites of the AMPA receptor is higher than two and that all the binding sites may not be equivalent. In view of these recent developments, another possible interpretation of our results would be that activation and desensitization of the AMPA receptor are not performed by the same binding sites.

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