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Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes

(quisqualate/inositol trisphosphate)

STEVEN R. GLAUM, JAMES A. HOLZWARTH, AND RICHARD J. MILLER*

Department of Pharmacological and Physiological Sciences, University of Chicago, 947 East 58th Street, Chicago, IL 60637

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ABSTRACT **We measured changes in the molar concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in individual astrocytes in culture produced by the glutamate analog quisqualate (QA) and related substances by using fura-2 digital fluorescence microscopy. In cells cultured from the cortex, hippocampus, and cerebellum, the QA analog α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA; 10 μM) produced a slow increase in $[Ca^{2+}]_i$ that was modest in amplitude (≈200 nM). These effects were completely abolished by 10 μM 6-nitro-7-cyano-quinoxaline-2,3-dione (CNQX). In cerebellar astrocytes, similar effects were produced by QA. However, in cortical and hippocampal astrocytes, the response to QA was much more complex. In these cells, QA produced an initial $[Ca^{2+}]_i$ spike that was followed by a sustained influx of Ca^{2+} ("plateau"). In the absence of extracellular Ca^{2+}, this plateau was abolished but the spike remained. CNQX did not block the spike and only slightly reduced the size of the plateau in some cells. Ni^{2+} (10 μM) but not nimodipine (10 μM) reduced the amplitude of the plateau. Pretreatment with 100 nM phorbol 12-myristate 13-acetate for 15 min abolished the spike but not the plateau portion of the QA response. Treatment with pertussis toxin at 250 ng/ml for 12–16 hr failed to alter the response. In some instances, the latency of the QA response differed considerably for individual cells in a group. It appeared that the response began in one cell and then spread to neighboring cells. Thus, QA appears to trigger a complex response in some astrocytes consisting of Ca^{2+} mobilization from intracellular stores and also Ca^{2+} influx resulting from the activation of AMPA-sensitive and -insensitive pathways.**

Two distinct classes of glutamate receptor have been identified in neurons (1). One category of glutamate receptor ("ionotropic") directly regulates the gating of a variety of ion channels. The glutamate analogues *N*-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) are archetypal agonists of this family of glutamate receptors (2). A second category of glutamate receptors ("metabotropic") are linked to guanine nucleotide-binding proteins (G proteins), which regulate phospholipase C and the subsequent production of the intracellular messengers inositol trisphosphate ($InsP_3$) and diacylglycerol (1, 3–6). Quisqualate (QA) has been shown to activate both metabotropic and AMPA-sensitive ionotropic receptors (7). Neuronal glutamate receptors appear to mediate fast synaptic transmission, synaptic plasticity, and neuronal degeneration associated with hypoxia and ischemia (8–10). Many of these responses appear to involve increases in neuronal molar concentrations of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) (11–13).

It is well established that astrocytes possess high-affinity and high-capacity glutamate uptake mechanisms (14–16). In addition, receptors for glutamate have recently been shown to be present on astrocytes (17, 18). Thus, kainate-induced membrane currents (19) and glutamate-induced $InsP_3$ synthesis have been demonstrated in astrocytes in culture (20, 21), although responses to NMDA have not (19). It is likely that changes in $[Ca^{2+}]_i$ are important in the regulation of astrocyte function as they are in neurons. We now demonstrate that activation of glutamate receptors in astrocytes induces complex changes in $[Ca^{2+}]_i$ that may involve an unusual type of astrocyte communication network.

MATERIALS AND METHODS

Cell Culture. Astrocytes were prepared from 3- to 5-day-old newborn rat brains by a modification of the procedure of Booher and Sensenbrenner (22). Briefly, hippocampus, cortex, and cerebellum were each isolated, finely chopped, and treated with 0.25% trypsin (GIBCO) for 20 min. Tissues were separately dissociated by trituration and allowed to settle. The supernatant from the triturated tissue was centrifuged at 150 × *g* for 5 min, the pellet was resuspended, and cells were plated on 60-mm culture dishes (3×10^5 cells per plate) in Dulbecco's modified Eagle's medium (DMEM; Hazleton Research Products, Reston, VA) containing 10% (vol/vol) fetal bovine serum (GIBCO). The following day, the medium was removed, and coverslips were washed with Hanks' balanced salt solution (Hazleton Research Products) and returned to culture in DMEM containing 10% (vol/vol) horse serum (GIBCO). After 5 days, astrocytes were incubated for 10 min with 0.25% trypsin to detach the cells, which were collected and replated on 15-mm glass coverslips. Experiments were performed on these secondary cultures between 10 and 20 days in culture.

Fura-2 Imaging of $[Ca^{2+}]_i$. Digital fluorescent microscopy was performed on astrocytes by monitoring fura-2 fluorescence. Coverslips containing cultured astrocytes were washed twice with Hepes-buffered Hanks' balanced salt solution (HHBSS, pH 7.45, which contained 137 mM NaCl, 5.4 mM KCl, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 1.26 mM $CaCl_2$, 0.64 mM $KH_2PO_4 \cdot 7H_2O$, 3 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes. Cells were incubated in 5 μM fura-2 AM (Molecular Probes) in HHBSS for 30–60 min at 37°C. Coverslips were then washed twice in HHBSS and further incubated for 30 min. Loading and completeness of deesterification was judged by stabile fluorescent ratios and the manipulation of $[Ca^{2+}]_i$ with the Ca^{2+} ionophore ionomycin. Indicator concentrations were estimated (by comparison of mean brightness with a few cells injected with the free

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Abbreviations: $[Ca^{2+}]_i$, cytosolic molar calcium concentration; QA, quisqualate; $InsP_3$, inositol trisphosphate; AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate; NMDA, *N*-methyl-D-aspartate; CNQX, 6-nitro-7-cyano-quinoxaline-2,3-dione; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; G protein, guanine nucleotide-binding protein; ANOVA, analysis of variance.
*To whom reprint requests should be addressed.

acid form of fura-2) to be 70–250 μM. Coverslips were mounted in a laminar flow chamber (flow rate, 2–3 ml/min) over an inverted-stage Nikon microscope equipped with a ×20 fluorescence objective lens (UV-F; 0.75 numerical aperature) and continuously perfused with HHBSS. All drugs were dissolved and delivered in the perfusate.

Digital fluorescent microscopy was used to determine the spatial distribution of $[Ca^{2+}]_i$. Briefly, cells were alternately illuminated with 340-nm and 380-nm light from a Hg source (Oriel, Stratford, CT). Emitted light was passed through a 480-nm barrier filter into an image-intensified SIT camera (Dage–MTI, Michigan City, IN). Images were then digitized as 256 × 256 pixel by 8-bit arrays by a computerized imaging system (Magiscan; Joyce–Loebl, Garden City, NY). Ratios of sequential 340/380-nm excitation image pairs were compared to a standard curve for free Ca^{2+} constructed from shallow solutions of known Ca^{2+} and fura-2 concentration, as described for this apparatus (23). Numerical values reported are an average of >50 adjacent pixels. Exposure time for each wavelength was 500–750 msec, with cells exposed to UV light only during data collection. The decrease in mean 360-nm excitation was <5% over the course of the experiment. Because of the long periods of data acquisition used to produce a ratio image (1.5–3 sec), we underestimated the peak amplitude of the change in $[Ca^{2+}]_i$ in these experiments.

For Ca^{2+}-free experiments, Ca^{2+} was omitted and 20 μM EGTA was added to the HHBSS. Cells were perfused with Ca^{2+}-free HHBSS for 2 min prior to agonist application, a treatment previously shown in our laboratory to block $[Ca^{2+}]_i$ responses to depolarizing stimuli in neuronal cultures (24). In coverslips treated with pertussis toxin (PTX), 250 ng of PTX was added per ml of the culture medium, and cells were incubated overnight (12–16 hr). Posttreatment agonist responses in Ca^{2+}-free and Ca^{2+}-containing medium for each cell were compared to untreated sister cultures. Values were pooled by tissue source and compared by using two-way analysis of variance (ANOVA). $[Ca^{2+}]_i$ spike responses were calculated from the initial peak obtained with agonist treatment. Plateau responses were calculated from the mean $[Ca^{2+}]_i$ values observed during agonist application after the initial spike.

QA, AMPA, 6-nitro-7-cyano-quinoxaline-2,3-dione (CNQX; Cambridge Research Biochemicals, Valley Stream, NY) phorbol 12-myristate 13-acetate (PMA; Sigma), and PTX (List Biological Laboratories, Campbell, CA) were prepared as 100× stock solutions and kept frozen until use. Fura-2 AM and fura-2 free acid (Molecular Probes) were prepared as 1 mM stock solutions and kept frozen until use. All other compounds were purchased from standard commercial sources.

RESULTS

AMPA Induces Ca^{2+} Influx into Astrocytes. The glutamate analog QA can activate both ionotropic and metabotropic glutamate receptors in neurons (7). However, the QA analog AMPA only activates the former type of receptor (7). To assess the effect of stimulating this receptor type alone, we first examined the effects of AMPA on $[Ca^{2+}]_i$ in astrocytes cultured separately from the cerebellum, hippocampus, and cortex. In all three culture types, addition of 10 μM AMPA induced a reversible increase in $[Ca^{2+}]_i$ in some cells. The effect of AMPA was consistently abolished in Ca^{2+}-free medium or blocked by 10 μM CNQX, which has been shown to block QA/kainate ionotropic responses in neurons (Fig. 1). Responses to AMPA were observed in ≈25% of the hippocampal and cerebellar astrocytes examined but were less frequently noted (≈5%) in cortical astrocyte cultures (Fig. 1). In cerebellar astrocytes, the population of cells responding to QA and the characteristics of the response were indistinguishable from AMPA responses (Fig. 1). Thus, astrocytes cultured from all three parts of the brain appear to possess a subpopulation of cells with ionotropic-like QA receptors that can mediate modest increases in $[Ca^{2+}]_i$ resulting from Ca^{2+} influx.

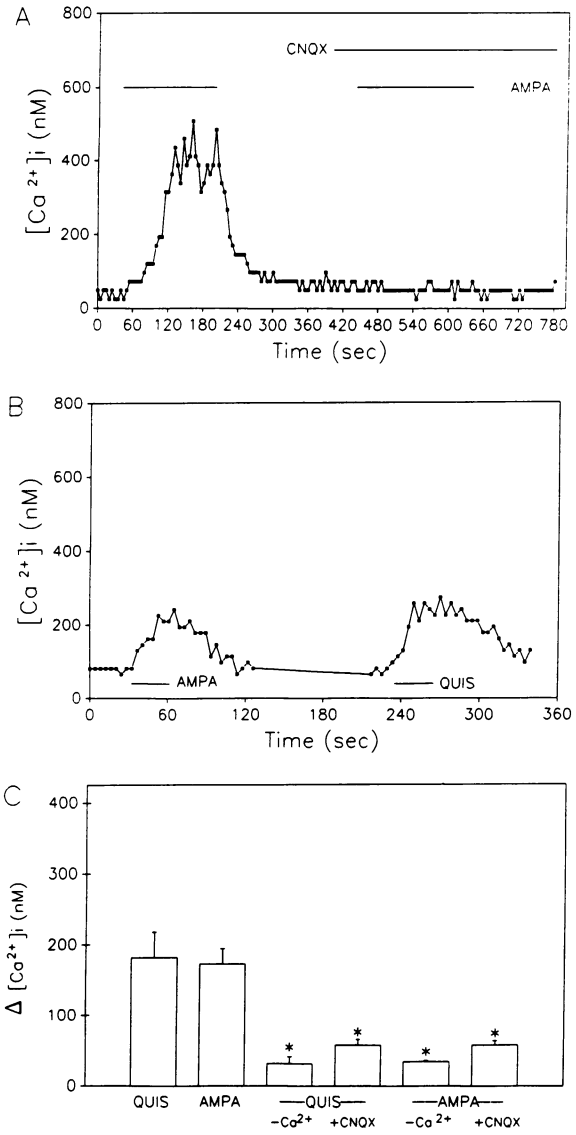

FIG. 1. Bath application of 10 μM AMPA produced an increase in $[Ca^{2+}]_i$ in single cortical (13 of 210), hippocampal (166 of 672), and cerebellar (46 of 189) cells. (*A*) Typical AMPA response in a single cortical astrocyte. AMPA responses in cortical, hippocampal, and cerebellar astrocytes were completely blocked by 10 μM CNQX. (*B*) Representative trace illustrating similarity of 10 μM AMPA and 10 μM QA responses in cerebellar astrocytes. (*C*) Summary of AMPA and QA (Quis) responses in cerebellar astrocytes (left to right, n = 24, 22, 11, 9, 11, and 12). Basal $[Ca^{2+}]_i$ was similar in cells derived from the cortex, hippocampus, or cerebellum (58.1 ± 1.3 nM, n = 210; 62.1 ± 1.1 nM, n = 672; 62.4 ± 2.7 nM, n = 189, respectively). *, $P < 0.01$ (ANOVA).

QA Induces $[Ca^{2+}]_i$ Mobilization and Ca^{2+} Influx. In contrast to the modest effects of QA observed in cerebellar astrocytes, large complex responses to this agonist were observed in astrocytes from the cortex or hippocampus. Upon addition of QA, a $[Ca^{2+}]_i$ spike was observed in approximately half of the cells examined (Fig. 2*A*), followed by a sustained "plateau" response that declined slowly after the removal of the agonist. However, several variations on this basic theme were also observed. In both cortical and hippocampal astrocytes, the rapid spike occasionally fused with the sustained plateau (16.6% and 19.8% of responding

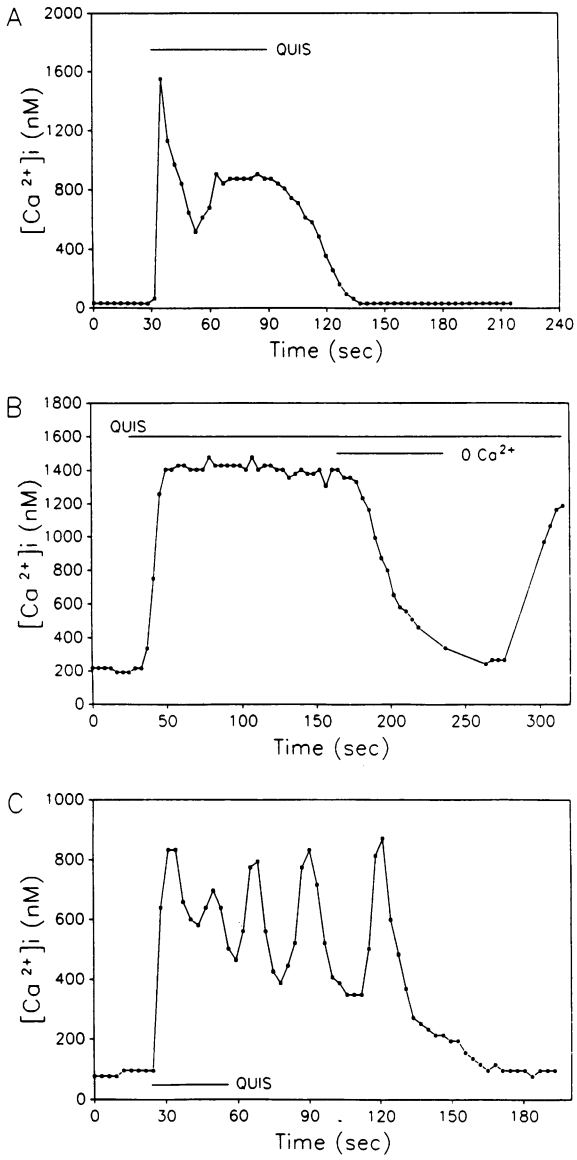

FIG. 2. $[Ca^{2+}]_i$ responses to 10 μM QA (Quis) in single hippocampal astrocytes were observed in ≈50% of the cells examined (n = 672). (*A*) Most frequently observed QA response (82% of responding cells) was biphasic, consisting of an initial spike followed by a sustained plateau that relaxed with agonist washout. (*B*) In the remaining cells, the spike phase fused into the plateau. During sustained agonist perfusion, removing extracellular Ca^{2+} rapidly reduced the level of $[Ca^{2+}]_i$. (*C*) Oscillations in $[Ca^{2+}]_i$ were frequently observed in response to QA. Oscillation frequency during the plateau phase ranged widely from cell to cell.

cells, respectively), as illustrated by Fig. 2*B*. In the majority of cells, oscillations in $[Ca^{2+}]_i$ of widely varying magnitude could be clearly distinguished on top of the plateau portion of the response (Fig. 2*C*). Furthermore, as we shall discuss below, QA responses in hippocampal and cortical astrocytes were both spatially and temporally heterogeneous. It is interesting to note that the plateau portion of the response could be maintained for the entire period of time the agonist was present (up to 6 min), relaxing slowly after the washout.

Phorbol Esters Block $[Ca^{2+}]_i$ Mobilization but Not Influx. In the absence of external Ca^{2+}, QA still produced a rapid increase in $[Ca^{2+}]_i$ in responsive cortical and hippocampal astrocytes (Fig. 3). In a few cells, two or three successive spikes were observed. When cells were returned to Ca^{2+}-containing medium for a minimum of 5 min after an initial challenge with QA, then, on withdrawing Ca^{2+} a second time, a further response of similar magnitude could reliably be obtained. These spike responses presumably represent Ca^{2+} mobilization from intracellular stores subsequent to QA-induced $InsP_3$ synthesis. In all cells examined, these responses were maintained after pretreatment with PTX (Fig. 3). However, as is often the case for agonist-mediated $InsP_3$ responses, a 15-min preincubation of cells with the phorbol ester PMA abolished the response to QA in Ca^{2+}-free medium completely (Fig. 3).

Unlike the spike portion of the response, the plateau portion of the QA response clearly resulted from Ca^{2+} influx. Removal of the extracellular Ca^{2+} or addition of Ni^{2+} rapidly and reversibly reduced $[Ca^{2+}]_i$ levels in the continuing presence of

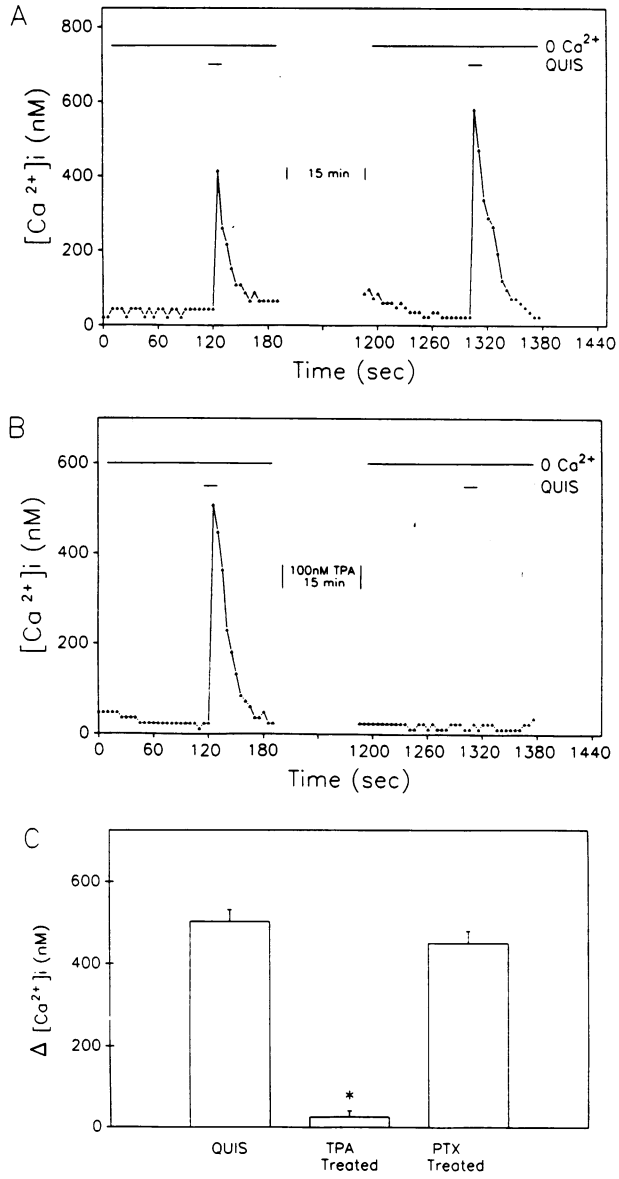

FIG. 3. Bath application of 10 μM QA (Quis) increased $[Ca^{2+}]_i$ in cortical and hippocampal astrocytes in the absence of extracellular Ca^{2+}. (*A*) Control response to repeat QA application in a single hippocampal astrocyte. Exposure for 5–15 min to Ca^{2+}-containing medium was universally sufficient to reload intracellular Ca^{2+} stores. (*B*) Treatment with the phorbol ester PMA (TPA) completely blocked the $[Ca^{2+}]_i$ response to QA in Ca^{2+}-free medium in both cortical and (as illustrated) hippocampal astrocytes. (*C*) Comparison of the effects of PMA treatment as in *B* and PTX treatment at 250 ng/ml for 12–16 hr on the QA response in hippocampal astrocytes in the absence of extracellular Ca^{2+}. QA-responding cells in PTX-treated coverslips were identified as described (left to right, n = 52, 14, and 15). *, $P < 0.01$ (ANOVA).

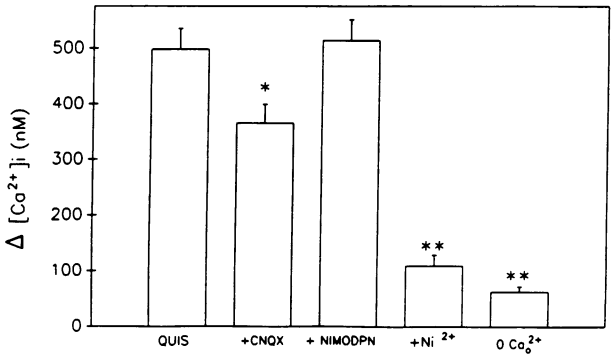

FIG. 4. Effects of antagonist treatment on the mean, sustained increase in $[Ca^{2+}]_i$ observed during continuous bath application of 10 μM QA (Quis). CNQX (10 μM) and nimodipine (NIMODPN; 10 μM) were added to the perfusate prior to and during QA application (e.g., see Fig. 1*A*). Ca^{2+}-free medium and 10 μM Ni^{2+} were added after the start of perfusion with QA (e.g., see Fig. 2*B*) (left to right, n = 32, 16, 23, 5, and 10). *, $P < 0.05$; **, $P < 0.01$ (ANOVA).

QA (Fig. 2*B*). Interestingly, the dihydropyridine Ca^{2+} channel blocker nimodipine failed to block this influx. An absence of $[Ca^{2+}]_i$ responses after perfusion with 50 mM K^+ further suggested the lack of L-type Ca^{2+} channels in astrocytes in these cultures. Fig. 4 summarizes these results for responsive hippocampal astrocytes. Responses of cortical astrocytes to these treatments were nearly identical. The characteristic plateau phase and associated oscillations induced by QA in a given cell were well preserved after the spike portion of the response had been abolished by PMA treatment (Fig. 5). Although post-PMA QA plateaus were reduced 5–34% by CNQX in some cells (n = 7), CNQX was never observed to abolish these responses. Thus, they must result from a second QA-sensitive/AMPA-insensitive influx pathway.

$[Ca^{2+}]_i$ Increases Exhibit a Differential Latency. Large clusters of adjoining cells were often imaged together. In both cortical and hippocampal cultures, QA-sensitive cells often responded with variable latency after addition of the agonist. When viewed in sequence, these images sometimes suggested that the increase in $[Ca^{2+}]_i$ was initiated by an individual cell or small group and then spread out in a "wave-like" manner to other cells in the vicinity, as illustrated in Fig. 6 (see also refs. 25 and 26). Such responses were not observed after stimulation with AMPA.

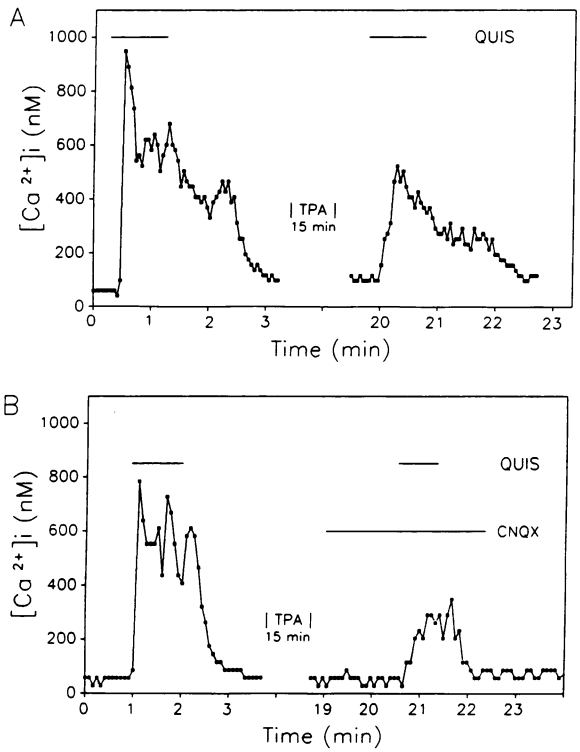

FIG. 5. Effects of phorbol ester treatment on 10 μM QA (Quis)-induced $[Ca^{2+}]_i$ responses in single astrocytes. (*A*) Incubation (15 min) with PMA (TPA) (100 nM) blocked the initial $[Ca^{2+}]_i$ spike resulting from bath-applied QA without blocking the sustained plateau in a single hippocampal astrocyte. (*B*) Individual cortical astrocyte response to QA treated as in *A* illustrates that the post-PMA $[Ca^{2+}]_i$ plateau was attenuated in this cell but not completely blocked by 10 μM CNQX.

DISCUSSION

These studies demonstrate that, as in the case of central neurons, several types of QA-specific glutamate receptors appear to exist on astrocytes. Similar observations to ours have apparently been made in two other laboratories and have been reported recently in abstract form (25–27). Our observations suggest the presence of QA/AMPA-specific ionotropic receptors on 5–25% of the cells in cultures derived from

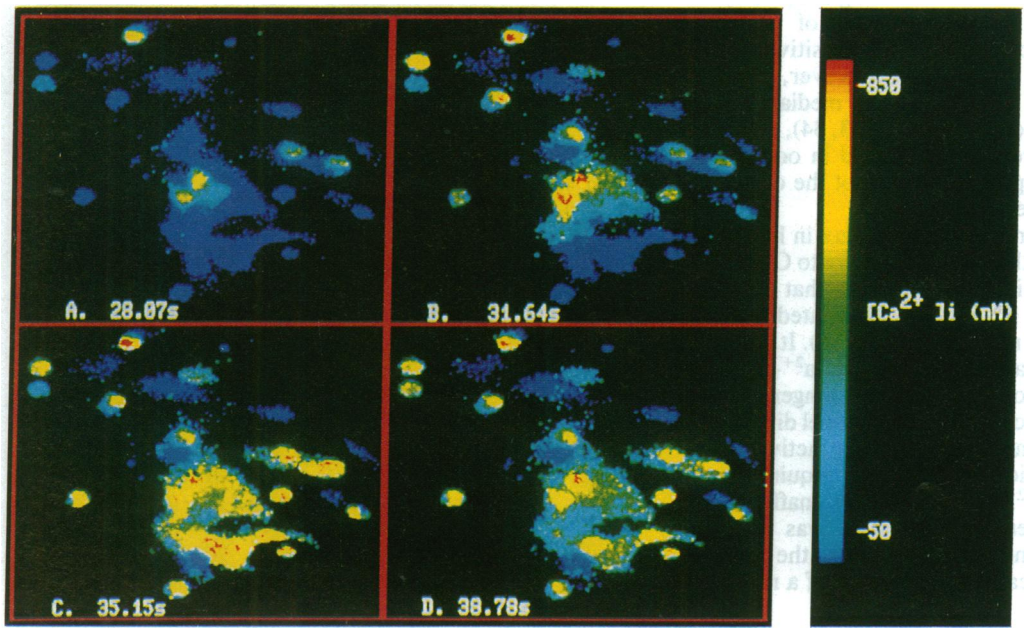

FIG. 6. Pseudocolor sequence illustrating spreading $[Ca^{2+}]_i$ response to bath-applied 10 μM QA in a cluster of hippocampal astrocytes. (*Left A*) Initial QA responses appear in discrete cells. QA was applied in the perfusate at t = 15 sec for 15 sec (t = 15–30). (*Left B*) $[Ca^{2+}]_i$ continues to rise in regions adjacent to initiating cells. (*Left C*) Peak of $[Ca^{2+}]_i$ response. Because of the long data acquisition times (≈3 sec), the calibrated $[Ca^{2+}]_i$ scale underestimates the peak of the $[Ca^{2+}]_i$ response. (*Left D*) Response begins to recover with washout of QA. Pseudocolor images were produced as described in *Materials and Methods*. (*Right*) Color calibration scale for $[Ca^{2+}]_i$.

cerebellum, cortex, or hippocampus, as would be predicted from previous electrophysiological studies (17, 19). Activation of these responses produced a gradual increase in the $[Ca^{2+}]_i$ resulting from Ca^{2+} influx. QA and AMPA responses in cerebellar astrocytes occurred on the same population of cells and were essentially identical, suggesting that QA responses in these cells were mediated via an AMPA-like ionotropic receptor. It is unlikely that this was simply due to depolarization-induced activation of voltage-sensitive Ca^{2+} channels in these cells, as produced by AMPA in neurons (7). Although astrocytes have been reported to possess both L- and T-type Ca^{2+} channels, L channels at any rate may only be present under specific culture conditions (e.g., supplementation with cAMP), which were not used in the present studies (28). T channels, if present, would presumably be inactivated at the normal resting potential of these cells. Moreover, perfusion of cells with K^+-rich solutions produced virtually no increase in $[Ca^{2+}]_i$, which also suggests the absence of voltage-sensitive Ca^{2+} channels. The most parsimonious explanation for the increases in $[Ca^{2+}]_i$ produced by AMPA is that they resulted from a limited permeability of the AMPA-gated ion channel to Ca^{2+}. We have demonstrated similar effects for kainate in striatal neurons (24).

In cultures derived from the hippocampus or cortex, activation of QA receptors initiated a complex response that appeared to consist of both Ca^{2+} influx and the release of internal Ca^{2+} stores. Although QA responses in some cells were partially blocked by CNQX during sustained agonist application, QA responses were observed in a far greater percentage of cells and were generally larger than AMPA responses. Therefore, unlike cerebellar astrocyte cultures, these cultures seem to express both CNQX-sensitive and -insensitive glutamate receptors.

We also found evidence of QA-induced Ca^{2+} mobilization in these cells, as would be predicted from previous studies that demonstrated QA-induced $InsP_3$ synthesis in astrocyte cultures (20, 21, 29, 30). Agonist-induced $InsP_3$ synthesis has been shown to be blocked by phorbol ester treatment in many cell types (31). QA-induced Ca^{2+} mobilization in cortical and hippocampal astrocytes was completely blocked by phorbol ester pretreatment. It is also interesting to note that we found these responses were resistant to PTX treatment. Indeed, QA-induced Ca^{2+} mobilization in hippocampal neurons is also PTX-insensitive in our hands (32). This is interesting as metabotropic glutamate responses induced in frog oocytes by brain messenger RNA (a significant proportion of which presumably results from astrocytes) are PTX sensitive (6). Why this discrepancy exists is unclear. However, both PTX-sensitive and -insensitive G proteins can mediate receptor-induced activation of phospholipase C (33, 34), and it is possible that metabotropic receptors induced in oocytes make use of an endogenous G protein that is not the one to which they are normally coupled.

The nature of the large QA-induced Ca^{2+} influx in hippocampal and cortical astrocytes that was insensitive to CNQX is less clear. It is a well-established observation that many $InsP_3$-associated increases in $[Ca^{2+}]_i$ are associated with Ca^{2+} influx subsequent to Ca^{2+} mobilization (35, 36). It could represent an $InsP_3$/inositol tetrakisphosphate- or Ca^{2+}-gated channel, a channel gated by another second messenger such as diacylglycerol, a "refilling" channel, or a channel directly gated by the receptor (35–40). In the present study, activation of the Ca^{2+} influx pathway did not appear to require the mobilization of intracellular Ca^{2+} stores, as it was unaffected by PMA pretreatment. Furthermore, as influx was maintained in the continued presence of the agonist, the most parsimonious explanation indicates the presence of a receptor-operated channel (40).

A particularly striking observation we have made is the apparent ability of QA-induced $[Ca^{2+}]_i$ increase to propagate from one cell to another. There are several possible explanations for this phenomenon. For example, it could be the result of differential sensitivity of the cells in a cluster to QA. However, another intriguing possibility is that it represents the presence of Ca^{2+} permeable gap junctions between cells in culture (41). Indeed, the gap junction protein connexin 43 has recently been detected in astrocytes in high concentrations (42). Considering that the properties of such cells appear to change very easily in culture (43), it should be of considerable interest to see whether the phenomenon of Ca^{2+} propagation actually occurs *in situ*.

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