0026-895X/89/050671-10$02.00/0

MOLECULAR PHARMACOLOGY, 35:671-680

Two Distinct Quisqualate Receptors Regulate Ca^{2+} Homeostasis in Hippocampal Neurons *In Vitro*

SHAWN N. MURPHY and RICHARD J. MILLER

Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

Received November 23, 1988; Accepted February 13, 1989

SUMMARY

Addition of quisqualate to mouse hippocampal neurons *in vitro* elicited two types of changes in $[Ca^{2+}]_i$ as assessed by fura-2-based microfluorimetry. The first was a transient spike or group of oscillations and the second was a long lasting "plateau" response. The long-lasting response was abolished on removal of either Ca^{2+} or Na^+ from the external medium or by blocking voltage-sensitive Ca^{2+} channels. Furthermore, the novel glutamate antagonist 6-nitro-7-cyano-quinoxaline-2,3-dione was a competitive inhibitor of this response. In contrast, none of these manipulations abolished the transient $[Ca^{2+}]_i$ spike. Transient $[Ca^{2+}]_i$ spikes or oscillations could also be produced by the α_1-adrenergic agonist phenylephrine. Production of such an α_1-response reduced the size of a subsequently elicited quisqualate response. However production of transient $[Ca^{2+}]_i$ spikes with caffeine did not alter the size of the quisqualate-induced spike. We conclude that hippocampal neurons possess two different types of quisqualate receptors. The first mediates quisqualate-induced depolarization and the second mediates Ca^{2+} mobilization from intracellular stores.

Many neurotransmitters are able to activate more than a single type of receptor and, therefore, produce a variety of synaptic responses that occur over a wide range of time courses. Rapid synaptic responses tend to be produced by receptors that directly gate ion channels. On the other hand, slow synaptic responses often require the participation of intracellular second messenger systems. For example, the actions of GABA and acetylcholine at GABA-A (1) and nicotinic receptors (2), respectively, produce rapid responses, whereas their actions at GABA-B (1) and muscarinic receptors (3–5) frequently produce slower responses that are mediated by G proteins and diffusible second messengers (6–11). This categorization is not absolute, however, because we are now aware of situations in which receptors gate ion channels without requiring a traditional second messenger but still requiring a G protein (12, 13). Thus, another possible way of categorizing receptors would be into two classes. The first of these would consist of multisubunit ionophores (14) and the second of single protein subunits that are linked to G proteins (15, 16).

Because glutamate is probably the most widely used excitatory neurotransmitter in the central nervous system, a detailed knowledge of the glutamate receptor system is essential for our understanding of neuronal communication in the brain. Data accumulated over the past few years have clearly shown that glutamate can activate a variety of receptors for which NMDA, kainic acid, and quisqualic acid are archetypal agonists (17, 18). Activation of these receptors directly activates a group of closely related ionophores that are relatively specific for monovalent cations (19, 20) and, in the case of NMDA, for Ca^{2+} as well (21, 22). However, other studies in the literature have also revealed that excitatory amino acids can provoke the synthesis of IP_3 in preparations of central neurons (23–26) or in cell free systems derived from them (27). The major function of IP_3 is thought to be the mobilization of Ca^{2+} from specific intracellular storage sites (28), although other actions may also occur (8, 29). Thus, it has been proposed that at least one type of glutamate receptor is linked to this second messenger system rather than to an ion channel (30, 31). Because receptors that stimulate IP_3 production are known to be linked to phospholipase C by means of G proteins (28), these observations would indicate that, as with other neurotransmitters, glutamate receptors may fall into the two genral categories discussed above. However the phospholipase C enzymes that produce IP_3 from phosphatidylinositol bisphosphate can be activated by high $[Ca^{2+}]_i$ (32, 33). Because activation of glutamate-linked ion channels can produce considerable Ca^{2+} influx by a variety of

This research was supported by Public Health Service Grants DA-02121, DA-02575, and MH-40165 and grants from Miles and Marion Laboratories. S.M. was also supported by GM-07151.

ABBREVIATIONS: GABA, γ-aminobutyric acid; IP_3, inositol trisphosphate; NMDA, *N*-methyl-D-aspartate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole proponate; G protein, guanine nucleotide-binding protein; CMF-EMEM, Ca^{2+}/Mg^{2+}-free Earle's minimum essential medium buffered with HEPES.

mechanisms (21, 22, 34) it is not completely clear which instances of glutamate-induced IP_3 synthesis are due to the direct activation of phospholipase C and which are due to the secondary activation of the enzyme subsequent to glutamate-induced Ca^{2+} influx. Furthermore, even if some glutamate-induced IP_3 synthesis is produced directly, it is not clear whether this actually leads to Ca^{2+} mobilization in central neurons and, if so, under what conditions. Indeed, although a variety of agents have been shown to stimulate IP_3 synthesis in neuronal tissue (35, 36), there are very few studies actually demonstrating that these agonists mobilize Ca^{2+} from intracellular stores in neurons (36–39).

In the case of glutamate receptors the action of quisqualate has most consistently been shown to stimulate IP_3 synthesis (23, 24, 27). Furthermore, in oocytes injected with mRNA prepared from brain, quisqualate often activates a Cl^- current that can be shown to be Ca^{2+} dependent and the source of the Ca^{2+} seems to be intracellular (40, 41). Similar quisqualate-activated Ca^{2+}-dependent currents may also exist in invertebrate preparations (42, 43). We have previously been able to demonstrate that quisqualate was indeed able to mobilize Ca^{2+} from intracellular stores in hippocampal neurons *in vitro* (37). In the present report we further characterize this system and also demonstrate that hippocampal neurons possess two different types of quisqualate receptors, which have different pharmacological profiles and which mediate different cellular effects. However, activation of either receptor can lead to characteristic changes in hippocampal neuron Ca^{2+} homeostasis.

Methods

Embryonic hippocampal cells were cultured as described below. Pregnant mice (C57BL/6, Harlan Sprague-Dawley) were sacrificed by cervical dislocation on the 17th day of gestation. The dissection followed that described by Hemmendinger *et al.* (44). The dissection and dissociation were carried out entirely in CMF-EMEM. Pieces (1–2 mm) of tissue were washed three times to remove meningial fibroblasts and other small debris. The tissues were incubated in 0.1% trypsin (porcine; GIBCO, Grand Island, NY) for 25 min, which was then inactivated with 20% horse serum. The inactivated trypsin solution was removed and the tissue (still in pieces) was washed three times with the CMF-EMEM. The tissue was dissociated by gentle flushing through fine-bore Pasteur pipettes. Dissociated cells were counted to accommodate a density of 1200 cells/mm^2 and were plated on coverglasses (No. 1, 25 mm round) that had been coated overnight with polylysine (2 μg/ml, Sigma Chemical Co., St. Louis, MO) washed, and coated with laminin (6 μg/ml dissolved in CMF-EMEM; Collaborative Research, Waltham, MA) for 6 hr. Cultures were grown in Dulbecco's modified Eagle medium supplemented with 10% horse serum (heat inactivated; GIBCO), penicillin (5 μg/ml; GIBCO), and streptomycin (5 μg/ml; GIBCO). The medium was changed after 12 hr and once a week thereafter using Dulbecco's modified Eagle medium with horse serum. In cultures ≥12 days *in vitro* glial growth was controlled by treatment from days 6 to 9 with 10 μM cytosine arabinoside (Upjohn, Kalamazoo, MI). Experiments were performed on cells from 3–17 days in culture, unless otherwise noted.

All measurements were made on single cells. A total of 161 cells were used in these experiments. Glial cells were distinguishable as large flat cells that formed a thin layer over the coverslip and neurons could be distinguished on morphological grounds (45). Cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, was determined by using a microspectrofluorimeter, which has been previously described (46), to monitor the Ca^{2+}-sensitive, fluorescent chelator fura-2. Neurons were loaded with the dye by incubation in 5 μM fura-2 acetoxymethyl ester for 1 hr at 37° in HEPES-buffered Hanks' balanced salt solution. After the incubation, the cells were washed twice in HEPES-Hanks' solution and incubated for an additional 30 min. Complete hydrolysis of the fura-2 ester to fura-2 was confirmed by comparing excitation spectra run on individual dye-loaded cells (22).

The coverslips containing the loaded and washed cells were mounted in an inverted flow-through chamber maintained at 25°. Solutions in the chamber were completely exchanged every 7 sec. This prevented significant accumulation of endogenous glutamate in the bath. The cells were usually maintained in a bath of HEPES-buffered Hanks' balanced salt solution (pH 7.45), which contains, in mM: NaCl, 137; KCl, 5.4; $MgSO_4$, 0.41; $MgCl_2$, 0.49; $CaCl_2$, 1.26; KH_2PO_4, 0.44; $Na_2HPO_4 \cdot 7H_2O$, 0.64; $NaHCO_3$, 3; glucose 5.5; HEPES, 20. Exceptions were Na^+-free experiments, in which equisomolar amounts of *N*-methyl-D-glucamine were substituted for Na^+, and Ca^{2+}-free experiments, in which Ca^{2+} was removed and 20μM EGTA was added to the medium. Washing the cells with our Ca^{2+}-free solutions for >1.5 min completely inhibited depolarization or agonist-induced Ca^{2+} influx as shown (37), supporting the existence of <50 nM Ca^{2+} in these solutions.

Fura-2 fluorescence from individual cells was monitored with a microspectrofluorimeter operating in the epi-illumination mode (46). Cells were alternately excited with 340 and 380 nm light at 60 Hz. Emissions from each wavelength were averaged and stored every second. Each wavelength was inspected for drug autofluorescence and changes in dye fluorescence. Fura-2 fluorescence typically decayed 10–20% in 1 hr, which represented mostly dye leakage from cells when compared with nonirradiated controls.

Estimations of $[Ca^{2+}]_i$ were made following background correction using the equation $K(R - R_{min})/R_{max} - R)$ in which K is a constant, R is the 340/380 nm fluorescence ratio, and R_{min} and R_{max} are the ratios in the absence of Ca^{2+} and presence of saturating concentrations of Ca^{2+}, respectively. The constants for calibration were obtained using calibration solutions or whole cell calibration as described in Ref. 34. Briefly, using calibration solutions a calibration curve was determined from the fura-2 pentapotassium salt in calibration buffer (pH = 7.05, 37°, containing, in mM: KCl, 120; NaCl, 5; KH_2CO_4, 1; $NaHCO_3$, 5; HEPES, 20; EGTA, 20) that contained CaEGTA and K_2EGTA in ratios that were calculated, using the stability constant 4.22×10^6 M^{-1} (47), to give Ca^{2+} concentrations ranging from 0 to 2000 nM. The standard curve was fit to the above equation.

Whole-cell calibration constants were determined by measuring fura-2 fluorescence from cells with ≥30 μM $[Ca^{2+}]_i$ and cells with $[Ca^{2+}]_i$ approaching 0 nM (34). K was calculated as described using the K_D for fura-2 of 224 nM (48). The constants R_{max}, R_{min}, and K obtained using the two calibration methods differed in two ways. First, the R_{max} was about twice as large when the calibration solutions were used, compared with the cell calibration. Second, it was difficult to obtain a consistent K using the cell calibration. The latter consideration favored using the calibration solution constants to quantify and display the data.

Dose-response relationships were fit with the equation $[Ca^{2+}]_i = (MCE \times A)/(EC_{50} + A)$ where A is the concentration of agonist and MCE (maximum calculated effect) and EC_{50} are varied to provide the best fit. On all graphs, illustrated error bars represent standard errors.

Fura-2 was purchased from Molecular Probes, Inc. (Eugene, OR). Quisqualate, AMPA, and ibotenate were purchased from Cambridge Research Biochemicals and found to be <1% glutamate using thin layer chromatography. CNQX was purchased from Tocris Neuramin, Essex, U.K. All other drugs were purchased from Sigma or Cambridge Research Biochemicals Ltd. Valley Stream, N.Y.

Results

Characteristics of the quisqualate response. Fig. 1A demonstrates that quisqualate produced two very different but characteristic effects on $[Ca^{2+}]_i$ in hippocampal neurons. In this experiment, the cell was first depolarized with 50 mM K^+, leading to a large rise in $[Ca^{2+}]_i$. This response is due to the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels. In the

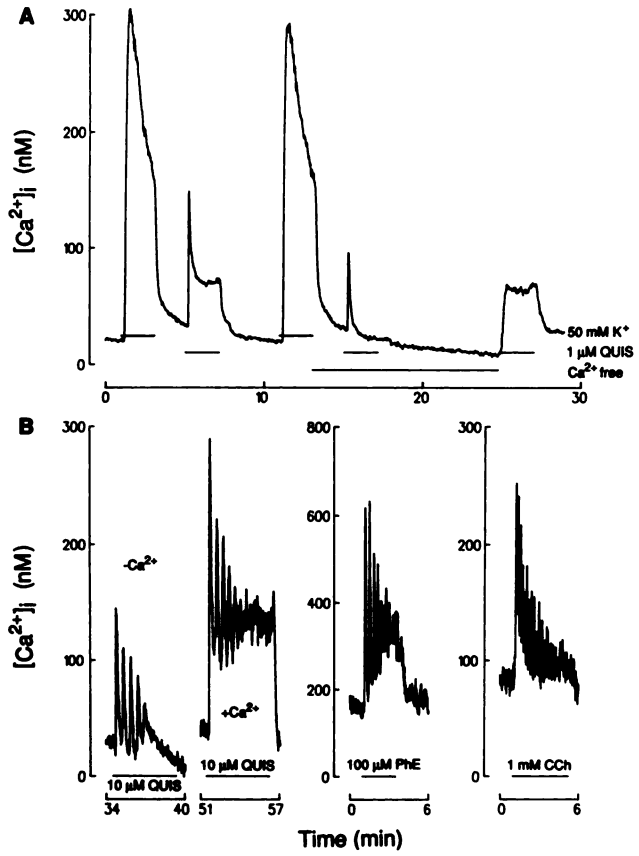

Fig. 1. Quisqualate (*QUIS*) produced two types of changes in $[Ca^{2+}]_i$ in hippocampal neurons. A, In normal medium, quisqualate (1 μM) produced a response consisting of a transient spike superimposed on top of a more maintained plateau response. On removal of external Ca^{2+}, only the transient spike was observed. The sustained plateau was produced once again when external Ca^{2+} was readmitted. B, In Ca^{2+}-free medium, quisqualate sometimes produced a series of oscillations rather than a single transient spike. When Ca^{2+} was present these could be observed on top of the more sustained plateau response, as in A. Also shown are oscillatory responses obtained with phenylephrine (*PhE*) and carbachol (*CCh*) in a hippocampal and striatal neuron, respectively.

absence of external Ca^{2+}, these K^+ depolarizations do not result in increases in $[Ca^{2+}]_i$ (37). We next added quisqualate (1 μM) and obtained a response that was clearly biphasic, consisting of a rapid spike followed by a maintained plateau. Following the production of another response to 50 mM K^+, we removed all external Ca^{2+} and added quisqualate once more. This time, only the rapid spike response was observed. When Ca^{2+} was added back to the external medium and quisqualate was added again, only the plateau response was now observed. These results suggest that quisqualate can provoke two types of responses. The first is a transient response that is independent of Ca^{2+}_o and the second is a more sustained response that requires Ca^{2+}_o. It should also be noted that, although quisqualate normally produced a single transient in Ca^{2+}-free medium, it sometimes produced a more complex response consisting of a series of oscillations of $[Ca^{2+}]_i$. Such a response can be observed in Fig. 1B. Furthermore, in Ca^{2+}-containing medium, quisqualate still produced the same pattern of oscillations but these were now superimposed on top of the plateau response (Fig. 1B).

This pattern of response suggests that quisqualate-induced Ca^{2+} transients observed in the absence of Ca^{2+}_o may be the result of quisqualate-induced IP_3 synthesis. It is interesting to note that, in nonneuronal cells, agonists that stimulate IP_3 synthesis also often produce oscillations in $[Ca^{2+}]_i$. For example, in a variety of tissues including brain, α_1-adrenergic agonists such as phenylephrine have frequently been shown to stimulate IP_3 synthesis and to cause Ca^{2+} mobilization (28, 35). We, therefore, compared the effects of phenylephrine with those of quisqualate. Phenylephrine also produced $[Ca^{2+}]_i$ transients in Ca^{2+}-free medium. As with quisqualate, these consisted either of single spikes or a series of oscillations (Fig. 1B). Indeed, similar responses could be produced with IP_3-stimulating agents in neurons from various parts of the brain. A response to carbachol in a striatal neuron is also illustrated (Fig. 1B).

One preliminary indication that the two types of quisqualate responses observed were mediated by different receptors was that they were not invariably found in the same cell. Thus, as we shall further demonstrate, we did observe neurons in which we were able to obtain only the transient or plateau response but not both.

Quisqualate-induced Ca^{2+} influx. It is well established that quisqualate can activate an ion channel that is selective for monovalent cations and consequently produces cell depolarization (49, 50). This depolarization could lead to the opening of voltage-sensitive Ca^{2+} channels and Ca^{2+} entry via this route. Indeed, kainate has been shown to raise neuronal $[Ca^{2+}]_i$ in this way (21, 34). That this is also true for quisqualate can be seen in the following series of experiments. We examined the effect of substituting Na^+_o by the organic cation *N*-methyl-D-glucamine. This substitution completely blocked the ability of quisqualate to produce the sustained plateau response that was dependent on the presence of Ca^{2+}_o but had no effect on the ability of quisqualate or glutamate to produce the $[Ca^{2+}]_i$ transient that was independent of Ca^{2+}_o (Fig. 2, A and B). These results suggest that the plateau but not the transient responses are the result of quisqualate-induced, Na^+-dependent depolarization.

We next examined the effects of blocking voltage-sensitive Ca^{2+} channels. We have previously demonstrated that Ca^{2+} channels in neurons can be completely blocked by a combination of depolarization of the cell in Ca^{2+}-free medium (voltage dependent inactivation) and the drug nitrendipine (34, 51). Fig. 3A illustrates results from a cell that only produced a plateau response to quisqualate in Ca^{2+}-containing medium. It can be observed that multiple responses to quisqualate addition could be obtained. Fig. 3B also shows responses to quisqualate in another cell, followed by a second challenge obtained after blocking voltage sensitive Ca^{2+} channels. In this case, the response to quisqualate was virtually completely blocked. Results summarizing several such experiments are shown in the accompanying histogram (Fig. 3C). Thus, the plateau response to quisqualate presumably results from quisqualate-dependent Na^+-mediated depolarization and subsequent Ca^{2+} entry through voltage-sensitive Ca^{2+} channels.

Pharmacology of the quisqualate response. We have previously demonstrated that the quisqualate analogue AMPA, which produces neuronal depolarization, does not produce Ca^{2+} mobilization (37). We attempted to identify additional pharmacological tools that would clearly distinguish between the two types of quisqualate responses. Fig. 4A illustrates the concentration-dependent effects of quisqualate in a cell that

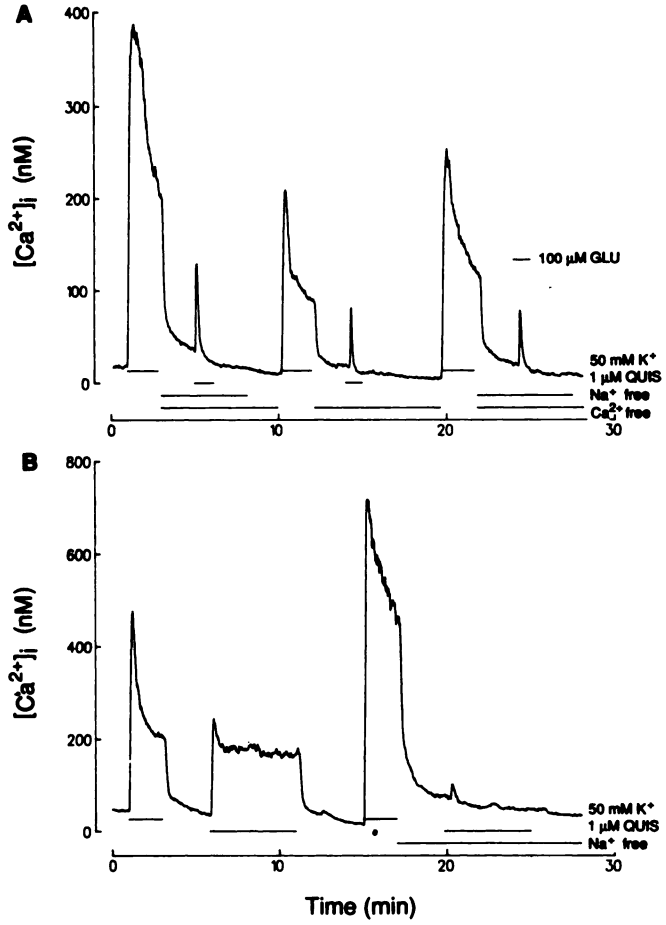

Fig. 2. Quisqualate (*QUIS*)-dependent Ca^{2+} mobilization is independent of extracellular sodium. A, Ca^{2+} mobilization in Ca^{2+}-free medium in the absence and presence of external Na^+. Glutamate (*GLU*) also caused Ca^{2+} mobilization in Na^+-free medium. In the cell illustrated in B, using Ca^{2+}-containing medium, the addition of quisqualate resulted predominantly in Ca^{2+} influx rather than mobilization. The removal of external Na^+ nearly totally abolished the response to quisqualate.

predominantly produced plateau responses in Ca^{2+}-containing medium. The dose-response relationship is summarized in Fig. 4B. Recently, a new group of excitatory amino acid receptor antagonists have been developed that are reportedly specific for quisqualate and kainate receptors in comparison with those for NMDA (52, 53). Fig. 4C illustrates the effects of one of these antagonists, known as CNQX, on quisqualate-induced Ca^{2+} influx. Quisqualate at 10 μM produced a response that was considerably smaller in the presence of 10 μM CNQX and then recovered following washout of the antagonist. Using the same cell, it is clear that the response to 1 μM quisqualate was even further reduced. On the other hand, 10 μM CNQX had no effect on Ca^{2+} influx induced by 50 mM K^+. Fig. 4D shows that 10 μM CNQX shifted the quisqualate dose-response curve to the right, suggesting that it acted as a competitive inhibitor of this type of quisqualate response Fig. 4D *inset* shows that CNQX produced a dose-dependent inhibition of quisqualate-induced Ca^{2+} influx. We calculated a K_e value of 250 nM. In contrast to these results, Fig. 4F demonstrates that 10 μM CNQX did not alter the dose-response curve for quisqualate-induced intracellular Ca^{2+} mobilization. However, at 100 μM, CNQX was frequently observed to produce a small increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium, indicating that it may have weak agonist effects at a Ca^{2+} mobilizing receptor. An experiment distinguishing the two quisqualate responses with CNQX can be seen in Fig. 4E using a cell that had both transient and plateau responses to quisqualate. In this case, a response to quisqualate was first produced in Ca^{2+}-free medium. Ca^{2+} was then replaced and 10 μM CNQX also added. On addition of quisqualate again, only a transient response was produced. However, if Ca^{2+} was retained in the medium and the CNQX was washed out, a challenge with quisqualate now produced both the transient and sustained plateau responses superimposed upon one another. Thus, CNQX in addition to AMPA completely distinguishes between the transient and sustained quisqualate responses. These observations prove that the two responses are unquestionably mediated by the activation of two different receptor types. We have not yet found an antagonist that selectively blocks the receptor-mediated quisqualate-dependent Ca^{2+} mobilization. This includes 2-amino-4-phosphonobutanate, which has been reported to block quisqualate-induced IP_3 synthesis in some cases (e.g., Ref. 23).

In addition to quisqualate, other agonists that activated the Ca^{2+} mobilization response included glutamate and ibotenate, as we have reported elsewhere (37). However, several other excitatory amino acids including homocysteic acid and quinolinic acid had no effect. Of particular interest, we also observed

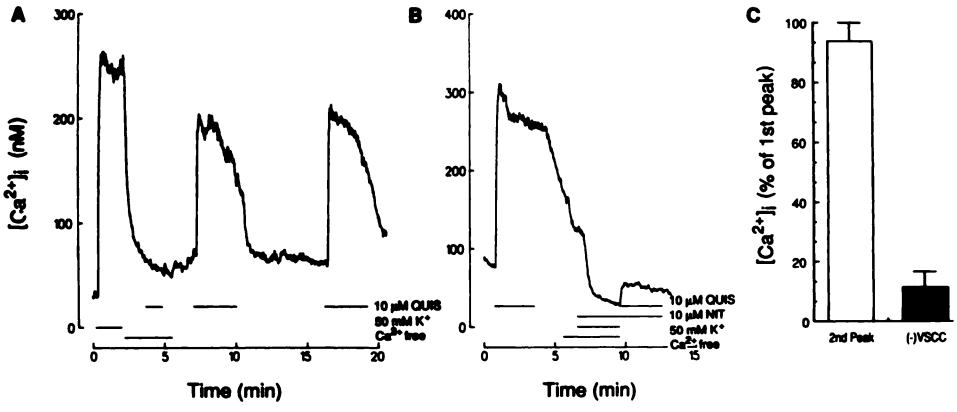

Fig. 3. Role of voltage-sensitive Ca^{2+} channels (*VSCC*) in mediating quisqualate-induced Ca^{2+} influx. A, Multiple additions of quisqualate (*QUIS*) to a cell demonstrate that multiple responses of comparable size can be obtained in succession. Note that this cell showed very little quisqualate-induced Ca^{2+} mobilization. B, Effect of quisqualate on a cell before and after blocking of voltage-sensitive Ca^{2+} channels by predepolarization in Ca^{2+}-free medium in the presence of nitrendipine (*NIT*). This procedure greatly reduced the effect of quisqualate. C, Summary of the results of several experiments of the type described in B. The size of second quisqualate-induced responses under normal conditions (*2nd peak*) are compared with those obtained after voltage-sensitive Ca^{2+} channels were blocked ($n = 4$).

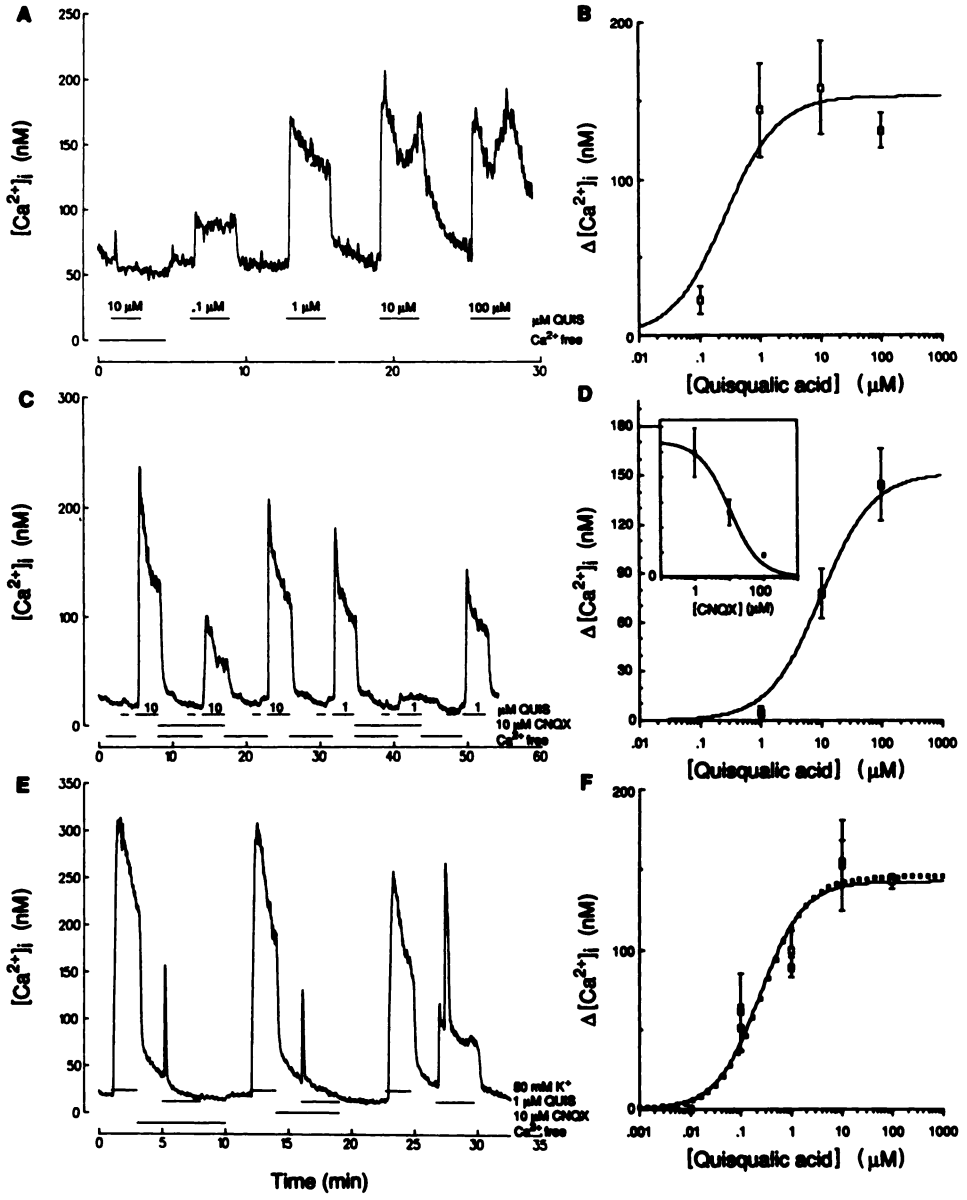

Fig. 4. A, Dose-dependent effects of quisqualate (*QUIS*) on $[Ca^{2+}]_i$. This is an example of a cell that showed little quisqualate-induced Ca^{2+} mobilization and, therefore, effects predominantly represent Ca^{2+} influx. The small contribution of Ca^{2+} mobilization can be observed during the initial addition of quisqualate (10 μM) in Ca^{2+}-free medium. B, Dose-response relationship for quisqualate-induced Ca^{2+} influx. EC_{50} = 263 nM. Maximum calculated effect = 153 nM ($6 \geq n \geq 3$). C, Effect of CNQX on quisqualate-induced Ca^{2+} influx. *Trace* shows the effect of 10 μM CNQX on Ca^{2+} influx induced by various concentrations of quisqualate. In this cell there was little contribution from quisqualate-induced Ca^{2+} mobilization. D, Dose-response curve for quisqualate-induced Ca^{2+} influx in the presence of 10 μM CNQX. Dose-response curve is shifted to the right in a parallel manner (EC_{50} = 9.77 μM), assuming no change in maximal effect. Comparison of curves in B and D enabled us to calculated a K_e value of 250 nM for CNQX action at the quisqualate receptor responsible for activating Ca^{2+} influx. *Inset* in *D* illustrates the dose-dependent inhibition of Ca^{2+} influx induced by 10 μM quisqualate produced by CNQX (IC_{50} = 10.3 μM) (n = 3). E, In this cell, which exhibited both quisqualate-induced Ca^{2+} influx and Ca^{2+} mobilization, CNQX clearly blocked influx but not mobilization. F, Dose-response curves for quisqualate-induced Ca^{2+} mobilization in the absence (□) and the presence (■) of 10 μM CNQX. Theoretical curves constructed described as in Methods in the absence of CNQX (○) and the presence (——) of 10 μM CNQX show that there was no effect of this concentration of CNQX. In the absence of CNQX the EC_{50} for quisqualate-induced Ca^{2+} mobilization was 277 nM, maximum calculated effect = 146 nM, and in the presence of CNQX the EC_{50} was 239 nM, assuming no change in maximal effect ($23 \geq n \geq 3$).

that aspartate was completely ineffective even at concentrations as high as 3 mM (Table 1).

Quisqualate-induced Ca^{2+} mobilization. It can be seen that, in several of the experiments presented here, we always elicited a 50 mM K^+-induced Ca^{2+} influx, producing a large rise in $[Ca^{2+}]_i$, before releasing intracellular Ca^{2+} stores with quisqualate. The reason for this is as follows. Fig. 5A demonstrates that a quisqualate-induced Ca^{2+} transient in Ca^{2+}-free medium could be obtained after elevating $[Ca^{2+}]_i$ by a previous 50 mM K^+ depolarization. However, a second quisqualate application without a previous 50 mM K^+ depolarization was ineffective. The Ca^{2+} transient returned when quisqualate was again preceded by elevated $[Ca^{2+}]_i$. Thus, it seems that loading the cytoplasm with Ca^{2+} by depolarization in Ca^{2+}-containing medium allows the quisqualate-sensitive intracellular stores to be rapidly refilled. However, these stores are not always refilled by a 2-min exposure to normal $Ca^{2+}{}_o$ without elevating $[Ca^{2+}]_i$. This refilling of stores can also be achieved through quisqualate-induced Ca^{2+} influx, as can be seen in Fig. 5B. Ca^{2+} influx was first produced using 10 μM quisqualate. $Ca^{2+}{}_o$ was next removed. Now quisqualate produced a single transient response, a second challenge being ineffective. However, following Ca^{2+} influx induced by a second addition of quisqualate in Ca^{2+}-containing medium, another quisqualate-induced transient could be produced upon $Ca^{2+}{}_o$ removal. The lack of the second quisqualate-induced response is probably due to the depletion of quisqualate-sensitive intracellular stores. We wondered whether the use of such a cytoplasmic Ca^{2+}-loading procedure was always necessary for the loading of intracellular stores or whether the quisqualate-sensitive stores would reload under nondepolarizing conditions if sufficient time elapsed in Ca^{2+} containing medium. Clearly, in Fig. 5, A and B, this was not the case, at least over the time course employed. Sometimes, however, refilling was observed even in the absence of a depolarization-induced Ca^{2+} influx. Such an example can be seen in Fig. 5C. Here, a second quisqualate-induced Ca^{2+} transient was produced after waiting for a time in 5 mM K^+/Ca^{2+}-containing medium, without depolarization-induced cytoplasmic Ca^{2+}

TABLE 1

Pharmacology of quisqualate-induced Ca^{2+} mobilization in hippocampal neurons

IC_{50} is versus 1 μM quisqualic acid.

Agonists	EC_{50}	Antagonists	IC_{50}
	μM		μM
Glutamic acid	30	L-glutamic acid diethyl ester	>5000
Aspartic acid	>3000	γ-D-glutamylaminomethylsulfonate	>1000
Homocysteic acid	>1000	DL-2-amino-4-phosphonobutanate	>1000
Quisqualic acid	0.225	CNQX	>100[a]
AMPA	>300	Pentobarbital	>1000
NMDA[b]	>300	NMDA[b]	>300
Ibotenic acid	100	Kainic acid	>300
Quinolinic acid	>3000		
Kainic acid	>300		

[a] Possible partial agonist effects ≥100 μM.
[b] Includes 10 μM glycine.

loading. Thus, quisqualate-sensitive intracellular stores can apparently reload in this way if the time elapsed is long enough for a particular cell. However, stores always reloaded quickly following a 2-min-depolarization-induced increase in $[Ca^{2+}]_i$ and so this procedure was used routinely so that experimental conditions could be standardized.

We investigated the nature of the intracellular stores mobilized by quisqualate. Fig. 6A illustrates the production of a transient response to quisqualate. After refilling, we next produced a response with the α_1-adrenergic agonist phenylephrine. A subsequent addition of quisqualate now produced a response only 36% as large as would be anticipated by taking the mean of the bordering quisqualate responses. When three sequential challenges with quisqualate were given to control cells, the responses to the second challenge were normally 93.8 ± 5.3% (mean ± SE; $n = 8$) of the mean of the first and third responses. Such results imply that phenylephrine and quisqualate share a common intracellular pool of Ca^{2+}. Looked at in another way, it is likely that all IP_3-generating systems interact at the level of Ca^{2+} mobilization.

We have previously demonstrated that neurons seem to possess two distinct types of intracellular Ca^{2+} stores (54). One

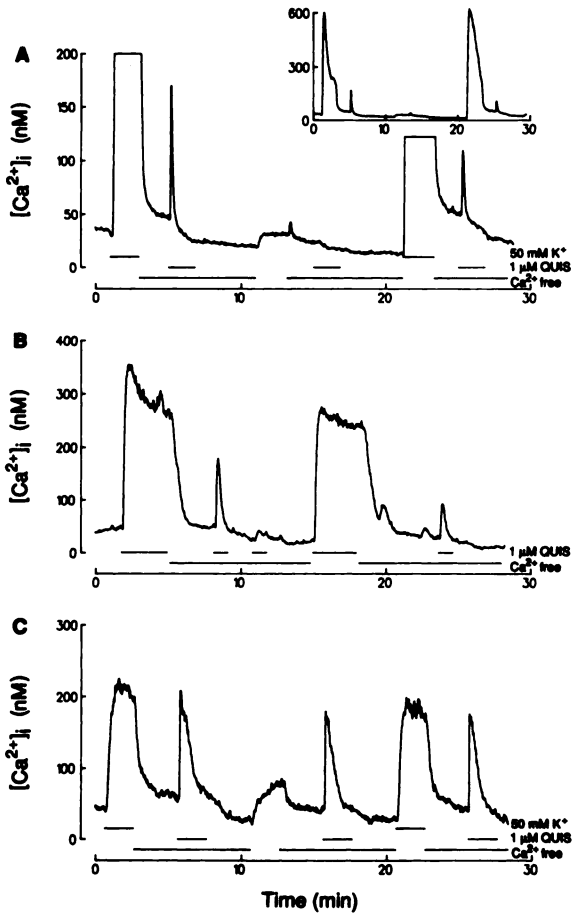

Fig. 5. Reloading of quisqualate (*QUIS*)-sensitive Ca^{2+} stores. A, A cell in which a response to quisqualate could be obtained only when preceded with depolarization-induced Ca^{2+} influx. *Inset*, experiment full scale. B, Reloading of quisqualate-sensitive stores using quisqualate-induced Ca^{2+} influx rather than 50 mM K^+. C, An example of a cell in which a second quisqualate response could be obtained after a sufficient waiting period even without reloading stores with depolarization as in A or B.

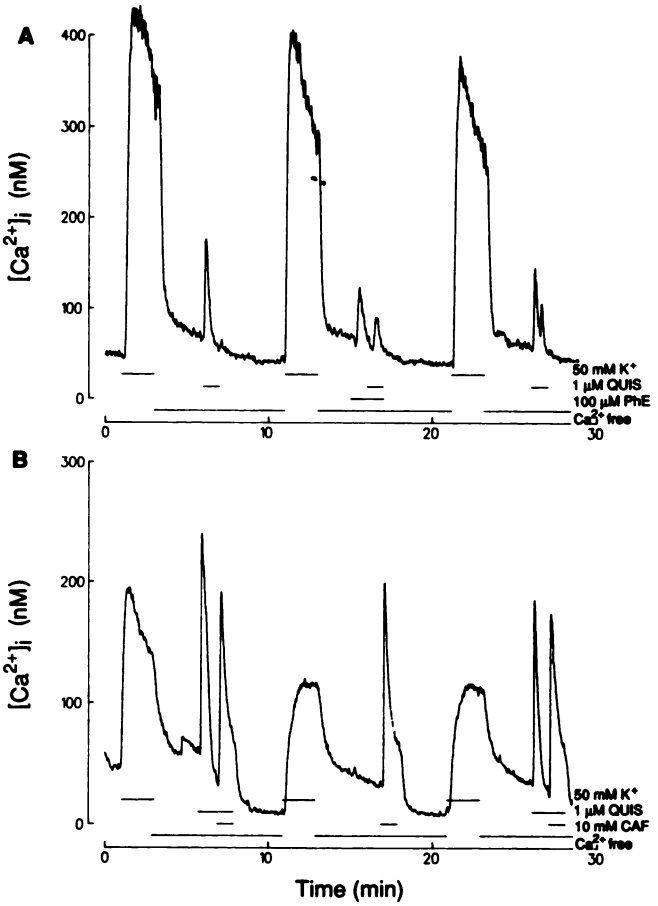

Fig. 6. Interactions between different types of Ca^{2+} stores. A, This experiment illustrates that the size of a quisqualate-induced Ca^{2+} mobilization response was reduced by prior application of the α_1-adrenergic agonist phenylephrine (*PhE*), which also produced Ca^{2+} mobilization. Following washout of the phenylephrine, the magnitude of the quisqualate response recovered. B, This experiment illustrates the lack of interaction between intracellular Ca^{2+} stores mobilized by quisqualate and those mobilized by caffeine (*CAF*). Prior application of quisqualate had no effect on the size of responses to caffeine.

of these is mobilized by IP_3-generating agents and the other can be released by high concentrations of methylxanthines such as caffeine. In sensory neurons, in any case, these two stores are clearly separated anatomically. In contrast to the interaction observed between quisqualate and phenylephrine, no interaction was observed between quisqualate and caffeine. Fig. 6B shows that caffeine-induced Ca^{2+} transients elicited in the absence of Ca^{2+}_o are of approximately the same magnitude whether or not a quisqualate response has been produced before caffeine addition. Similar results were obtained if the two agents were added the other way round. Thus, the two types of stores seem to be separate in hippocampal neurons, as we have previously reported for sensory neurons.

Ontogeny of the quisqualate response. The data presented so far clearly demonstrate that two separate types of quisqualate receptors exist in hippocampal neurons. This conclusion was further strengthened by studying the ontogeny of the two responses. Fig. 7A shows that, over an extended period of time *in vitro*, the Ca^{2+}-mobilizing response to quisqualate increased in frequency up until 13–17 days in culture. Following that time, the response frequency began to decline. Phenylephrine-induced responses also increased in frequency until 13–17 days *in vitro* but then remained constant. Because the frequencies did not change in parallel, they probably reflect changes in the number of receptors rather than the Ca^{2+} stores per se. However, the magnitude of both quisqualate and phenylephrine-induced Ca^{2+} mobilization responses did change in parallel, being greatest at the earliest times in culture and subsequently declining. Quisqualate-induced Ca^{2+} influx responses also seemed to increase in frequency during the first few days in culture. However, in contrast to quisqualate-induced Ca^{2+} mobilization, the responses remained stable at these high levels throughout the remainder of the culture period. Ca^{2+} influx elicited by depolarization with 50 mM K^+ was observed in virtually all neurons even at the earliest times *in vitro*. The magnitude of the depolarization- or quisqualate-induced Ca^{2+} influx was fairly constant over the entire culture period.

Discussion

The results presented in this study clearly demonstrate that two types of quisqualate-specific glutamate receptors exist on hippocampal neurons. Activation of both types of receptors leads to characteristic changes in $[Ca^{2+}]_i$. These changes may be important in mediating both the normal and pathophysiological effects of glutamate.

The first type of quisqualate receptor described produces a Na^+-dependent depolarization resulting in the opening of voltage-sensitive Ca^{2+} channels. This receptor can also be activated by the quisqualate analogue AMPA (50) and, as we demonstrate here, it can be blocked by the novel glutamate antagonist CNQX. This type of receptor probably mediates many of the fast synaptic currents activated by glutamate released during excitatory synaptic transmission in the brain (50, 56). Indeed, in normal physiological medium, CNQX blocks fast synaptic transmission at the Schaeffer collateral/CA_1 synapse in the hippocampus (56). CNQX apparently blocks both kainate and quisqualate receptors but is considerably more potent at the latter (52). Thus, it may be that quisqualate/AMPA/CNQX-specific receptors mediate fast synaptic transmission at this and a number of other central synapses. The changes in $[Ca^{2+}]_i$ produced on stimulation of glutamate receptors of this

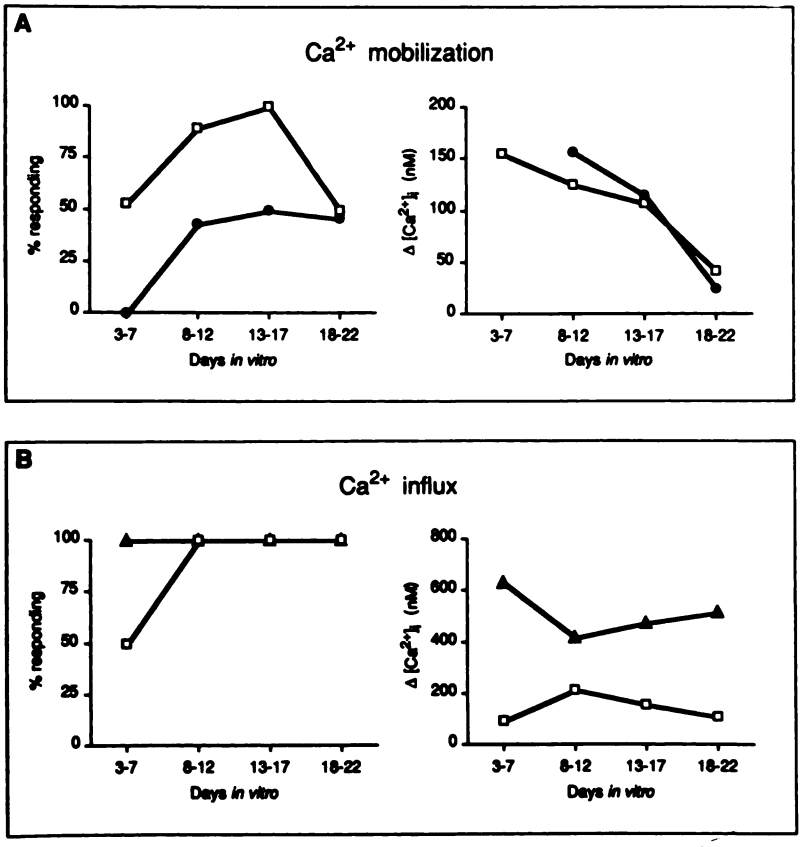

Fig. 7. Ontogeny of quisqualate-induced Ca^{2+} mobilization and Ca^{2+} influx. A, *Left*, the time course of development *in vitro* of the fraction of neurons exhibiting Ca^{2+} mobilization in response to quisqualate (□) and phenylephrine (■). *Right*, the magnitude of the response to the two agents. B, *Left*, the time course of development *in vitro* of the fraction of neurons exhibiting quisqualate-induced Ca^{2+} influx (□) and Ca^{2+} influx induced by 50 mM K^+ (▲). *Right*, The magnitude of the response. Only responses ≥5 nM $\Delta[Ca^{2+}]_i$ were included. A population of 139 cells were analyzed. Each *point* represents $n \geq 10$.

type may have a number of important consequences. Under normal conditions, the rise in $[Ca^{2+}]_i$ produced may activate or inhibit a number of Ca^{2+}-dependent ion channels that will alter the excitability of the receptive neuron. Furthermore, $[Ca^{2+}]_i$ is thought to be an important mediator of long term plastic changes that occur at many glutaminergic synapses (57–59). In some cases, such as the Schaeffer collateral/CA_1 synapse, the source of this Ca^{2+} appears to be influx via the NMDA-gated ionophore (59). However, in other instances the source of Ca^{2+} is unknown and could be supplied by quisqualate-induced Ca^{2+} influx or even quisqualate-induced Ca^{2+} mobilization (see below) (57, 60). In addition to mediating the normal physiological effects of glutamate, quisqualate receptor-activated Ca^{2+} influx may also play a role in glutamate-induced neurotoxicity (61). During periods of cerebral ischemia, abnormally large amounts of glutamate are released in the brain (62). Persistent activation of a combination of all the different types of glutamate receptors would produce long lasting increases in neuronal $[Ca^{2+}]_i$ and these have been shown to be profoundly toxic (63). Here again, NMDA-induced Ca^{2+} influx is thought to play the major role in this process in many cases (63). Moreover, it should be pointed out that quisqualate does have appreciable affinity for NMDA receptors (17, 18). Nevertheless, quisqualate-mediated neurotoxicity might be at least partially due to the mechanisms described in the present study and glutamate-induced neurotoxicity *in situ* may also be partially mediated in this way. One clear way to test this possibility would be to examine potential antineurotoxic effects of CNQX-like compounds in models of cerebral ischemia.

The second type of response we have described here and in another recent report (37) concerns the ability of quisqualate and glutamate to mobilize Ca^{2+} from intracellular stores. Indeed, in addition to the hippocampus, we have also observed this response in several other parts of the brain including the striatum, cortex, and cerebellum. This second type of quisqualate response is completely resistant to the blocking effects of CNQX and is clearly mediated by a different receptor from the one mediating depolarization and Ca^{2+} influx. One observation of particular interest is the complete inability of aspartate to activate this type of response. Thus, if both glutamate and aspartate are indeed endogenous neurotransmitters, this is probably the clearest difference in the pharmacology of these two compounds. In support of this notion, Verdoorn and Dingledine (55) have recently observed that, in frog oocytes injected with rat brain mRNA, aspartate was unable to produce the oscillating currents produced by quisqualate, which are thought to be dependent on the mobilization of intracellular Ca^{2+}.

Various pieces of evidence make it highly likely that this type of quisqualate response is mediated by quisqualate-stimulated IP_3 production. For example, the pharmacology of the response observed is in almost all respects identical to that of quisqualate-stimulated IP_3 production. However, there are a few exceptions. In particular 2-amino-4-phosphonobutanate has been reported to block quisqualate-stimulated IP_3 production, but not in all cases (23, 25, 27). A second noteworthy point is the apparent interaction between quisqualate-induced Ca^{2+} mobilization and that activated by phenylephrine. α_1-Adrenergic effects have been studied in many tissues including the nervous system and are almost invariably associated with the activation of phospholipase C and synthesis of IP_3 (28, 35). Thus, the ability of phenylephrine to reduce a subsequent response is consistent with the idea that both receptors lead to the mobilization of Ca^{2+} from some IP_3-sensitive pool and that this becomes substantially depleted following the addition of phenylephrine. A similar scenario presumably explains the inability to produce two quisqualate responses in a row unless refilling intervenes. However, other explanations for the phenylephrine/quisqualate interaction are possible, including protein kinase C-mediated effects at the receptor/G protein level (64, 65). In contrast to the interaction observed between quisqualate and phenylephrine, none was observed between quisqualate and caffeine. This is consistent with our previous proposal based on studies in sensory neurons that IP_3- and methylxanthine-sensitive Ca^{2+} stores in neurons were functionally and anatomically separate (54), a conclusion also recently suggested by Pfaffinger *et al.* (38), using bullfrog sympathetic neurons. The caffeine-sensitive intracellular Ca^{2+} store may participate in the phenomenon of Ca^{2+}-induced Ca^{2+} release (66, 67), a method of amplifying the size of incoming Ca^{2+} signals, whereas the IP_3-sensitive pool may be primarily concerned with mediating the effects of neurotransmitters. Naturally, these pools may interact in some circumstances. Indeed, a model has been proposed suggesting that such an interaction produces the oscillations in $[Ca^{2+}]_i$ reported here and elsewhere (68). It is interesting to note that quisqualate-activated Cl^- currents in oocytes are often seen to oscillate, presumably reflecting underlying oscillatory changes in $[Ca^{2+}]_i$ (41). Thus, it is possible that quisqualate-induced oscillations in neuronal $[Ca^{2+}]_i$ may produce similar oscillatory changes in neuronal excitability.

There are a considerable number of observations in the literature attesting to the ability of excitatory amino acids to stimulate or inhibit the synthesis of IP_3 in neuronal tissue (23–27, 30, 69). However, it has not been clear how many of these responses actually lead to increases in $[Ca^{2+}]_i$ and how many are the result of the secondary activation of phospholipase C subsequent to glutamate-induced increases in $[Ca^{2+}]_i$ produced by other means. Thus, phospholipase C can be activated by Ca^{2+} (32, 33) and $[Ca^{2+}]_i$ can be greatly increased as the result of glutamate-induced Ca^{2+} influx through the NMDA-linked ionophore or through voltage-sensitive Ca^{2+} channels (21, 22). In hippocampal neurons *in vitro*, it is clear that only quisqualate, glutamate, and ibotenate mobilize Ca^{2+} from intracellular stores, although all excitatory amino acids can increase $[Ca^{2+}]_i$ by other methods. Thus, it may be that in many cases NMDA- or kainate-stimulated IP_3 synthesis is the result rather than the cause of increases in $[Ca^{2+}]_i$.

Perhaps the most pressing question at this time is what the precise function of quisqualate-induced IP_3 synthesis in neurons might be. Under what physiological conditions does this system operate? There is virtually no evidence for the participation of the IP_3/Ca^{2+} system in a synaptic response, although there are several suggestions as to the participation of the diacylglycerol/protein kinase C arm of the same pathway (7, 61). Agonist-induced IP_3 synthesis is a G protein-mediated event (28), and in some cases quisqualate-induced IP_3 synthesis has been shown to be blocked by treatment of cells with pertussis toxin (69). Thus, it is extremely interesting to note that pertussis toxin blocks the induction of long term potentiation at the mossy fiber/CA_3 synapse in the hippocampus, although the toxin has no effect on long term potentiation at the Schaeffer collateral/CA_1 synapse (60). Because excitatory amino acids have been suggested as mediating long term poten-

tiation at the mossy fiber/CA_3 synapse, it may be that the IP_3/Ca^{2+} system plays a role in the production of this response (59). An observation that has been consistently made concerns the ontology of the quisqualate/IP_3 system studied biochemically or as in the present series of experiments. It is clear that, although the response does occur in mature animals, it occurs to a greater extent in young and embryonic animals at times when the greatest synaptogenesis seems to occur (25). This might also indicate a role for the quisqualate/IP_3/Ca^{2+} system not just in the regulation of synaptic transmission but also in the establishment and stabilization of synaptic contacts.

References

1. Bormann, J. Electrophysiology of GABA-A and GABA-B receptor subtypes. *Trends Neurosci* **11**:112–116 (1988).
2. Colquhoun, D., D. C. Ogden, and M. Mathie. Nicotinic acetylcholine receptors of nerve and muscle: functional aspects. *Trends Pharmacol. Sci.* **8**:465–472 (1987).
3. Brown, D. A. M-currents, in *Ion Channels* (T. Narahashi, ed.), Vol. 1. Plenum Publishing, New York, 55–94 (1988).
4. Cassell, J. F., and E.M. McLachlan. Muscarinic agonists block five different potassium conductances in guinea-pig sympathetic neurons. *Br. J. Pharmacol.* **91**:259–261 (1987).
5. McCormick, D. A., and D. A. Prince. Mechanisms of action of acetylcholine in the guinea-pig cerebral cortex *in vitro*. *J. Physiol. (Lond.)* **375**:169–194 (1986).
6. Pfaffinger, P. J. Muscarine and t-LHRH suppress M-currents by activating an IAP insensitive G-protein. *J. Neurosci.* **8**:3343–3353 (1988).
7. Brown, D. A., and P. R. Adams. Effects of phorbol dibutyrate on M-currents and M-current inhibition in bullfrog sympathetic neurons. *Cell Mol. Neurobiol.* **7**:255–269 (1987).
8. Dutar, P., and R. A. Nicoll. Stimulation of phosphatidyl inositol turnover may mediate the muscarinic suppression of the M-current in hippocampal pyramidal cells. *Neurosci. Lett.* **85**:89–94 (1988).
9. Scott, R. H., and A. C. Dolphin. Regulation of Ca^{2+} currents by a GTP analogue: potentiation of (−)-baclofen mediated inhibition. *Neurosci. Lett.* **69**:59–64 (1986).
10. Malenka, R. C., D. V. Madison, R. Andrade, and R. A. Nicoll. Phorbol esters mimick some cholinergic actions in hippocampal pyramidal neurons. *J. Neurosci.* **6**:475–480 (1986).
11. Wanke, E., A. Ferroni, A. Malgaroli, A. Ambrosini, T. Pozzan, and J. Meldolesi. A novel type of inhibition of voltage gated Ca^{2+} channels via muscarinic receptors in mammalian sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **84**:4313–4317 (1987).
12. Logothetis, D. E., D. Kim, J. K. Northup, E. J. Neer, and D. E. Clapham. Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K^+ channel. *Proc. Natl. Acad. Sci. USA* **85**:5814–5818 (1988).
13. Yatani, A., J. Codina, R. D. Skeura, L. Birnbaumer, and A. M. Brown. Reconstitution of somatostatin and muscarinic receptor mediated stimulation of K^+ channels by isolated G_K protein in clonal rat anterior pituitary cell membranes. *Mol. Endocrinol.* **1**:283–289 (1987).
14. Maelicke, A. Structural similarities between ion channel proteins. *Trends Biochem. Sci.* **13**:199–202 (1988).
15. Gilman, A. G. G-proteins: transducers of receptor generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
16. Lochrie, M. A., and M. I. Simon. G-protein multiplicity in eukaryotic signal transduction systems. *Biochemistry* **27**:4957–4965 (1988).
17. Shinozaki, H. Pharmacology of the glutamate receptor. *Prog. Neurobiol.* **30**:399–435 (1988).
18. Foster, A. C., and G. E. Fagg. Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. *Brain Res. Rev.* **7**:103–164 (1984).
19. Cull-Candy, S. G., J. R. Howe, and D. C. Ogden. Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurons. *J. Physiol. (Lond.)* **400**:189–222 (1988).
20. Vycklicky, L., J. Krusek, and C. Edwards. Differences in the pore sizes of the *N*-methyl-D-aspartate and kainate cation channels. *Neurosci. Lett.* **89**:313–318 (1988).
21. MacDermott, A., M. L. Mayer, G. L. Westbrook, S. J. Smith, and J. Barker. NMDA receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. *Nature (Lond.)* **321**:519–522 (1986).
22. Murphy, S. N., S. A. Thayer, and R. J. Miller. The effect of excitatory amino acids on intracellular Ca^{2+} in single mouse striatal neurons *in vitro*. *J. Neurosci.* **7**:4145–4158 (1987).
23. Schoepp, D. D., and B. G. Johnson. Excitatory amino acid agonist/antagonist interactions at 2-amino-4-phosphonobutyric acid-sensitive quisqualate receptors coupled to phosphoinositide hydrolysis in slices of rat hippocampus. *J. Neurochem.* **50**:1605–1613 (1988).
24. Godfrey, P. P., C. J. Wilkins, W. Tyler, and S. P. Watson. Stimulatory and inhibitory actions of excitatory amino acids on inositol phospholipid metabolism in rat cerebral cortex. *Br. J. Pharmacol.* **95**:131–138 (1988).
25. Nicoletti, F., J. L. Meek, M. J. Iadarola, D. M. Chuang, B. L. Roth, and E. Costa. Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J. Neurochem.* **46**:40–46 (1986).
26. Sladeczek, F., J. P. Pin, M. Recasens, J. Bockaert, and S. Weiss. Glutamate stimulates inositol phosphate formation in striatal neurons. *Nature (Lond.)* **317**:717–719 (1985).
27. Recasens, M., J. Guiramand, A. Nourigat, I. Sassetti, and G. Devilliers. A new quisqualate receptor subtype (SAA_2) responsible for the glutamate induced inositol phosphate formation in rat brain synaptoneursomes. *Neurochem. Int.*, in press.
28. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**:159–193 (1987).
29. Penner, R., G. Matthews, and E. Neher. Regulation of calcium influx by second messengers in rat mast cells. *Nature (Lond.)* **334**:499–504 (1988).
30. Nicoletti, F., M. J. Iadarola, J. T. Wroblewski, and E. Costa. Excitatory amino acid recognition sites coupled with inositol phospholipid hydrolysis: developmental changes and interaction with α_1-adrenoceptors. *Proc. Natl. Acad. Sci. USA* **83**:1931–1935 (1986).
31. Wroblewski, J. T., F. Nicoletti, E. Fadda, and E. Costa. Phencyclidine is a negative allosteric modulator of signal transduction at two subclasses of excitatory amino acid receptors. *Proc. Natl. Acad. Sci. USA* **84**:5068–5072 (1987).
32. Harden, T. K., P. T. Hawkins, L. Stephens, J. L. Boyer, and C. P. Downes. Phosphoinositide hydrolysis by guanosine-5-[γthio]-trisphosphate activated phospholipase C of turkey erythrocyte membranes. *Biochem. J.* **252**:583–593 (1988).
33. Orellana, S., P. A. Solski, and J. H. Brown. Guanosine 5′-*O*-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. *J. Biol. Chem.* **262**:1638–1643 (1987).
34. Murphy, S. N., and R. J. Miller. The regulation of Ca^{2+} influx into striatal neurons by kainic acid. *J. Pharmacol. Exp.Ther.*, in press.
35. Nahorski, S. R. Inositol polyphosphates and neuronal calcium homeostasis. *Trends Neurosci.* **11**:444–449 (1988).
36. Kudo, Y., A. Ogura, and T. Iijima. Stimulation of muscarinic receptors in hippocampal neurons induces characteristic increases in cytosolic free Ca^{2+} concentration. *Neurosci. Lett.* **85**:345–350 (1988).
37. Murphy, S. N., and R. J. Miller. A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **85**:8737–8741 (1988).
38. Pfaffinger, P. J., M. D. Leibowitz, E. M. Subers, N. M. Nathanson, W. Almers, and B. Hille. Agonists that suppress M-currents elicit phosphoinositide turnover and Ca^{2+} transients but these events do not explain M-current suppression. *Neuron* **1**:477–484 (1988).
39. Womack, M. D., A. B. MacDermott, and T. M. Jessell. Sensory transmitters regulate intracellular calcium in dorsal horn neurons. *Nature (Lond.)* **334**:351–353 (1988).
40. Sigal, E., and R. Bauer. Activation of protein kinase C differentially modulates neuronal Na^+, Ca^{2+} and γ-aminobutyrate type A channels. *Proc. Natl. Acad. Sci. USA* **85**:6192–6196 (1988).
41. Sugiyama, H., I. Ito, and C. Hirono. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature (Lond.)* **33**:531–533 (1987).
42. Miwa, A., and N. Kawai. Presynaptic glutamate receptor: possible involvement of a K^+ channel. *Brain. Res.* **385**:161–164 (1986).
43. Miwa, A., N. Kawai, and M. Ui. Pertussis toxin blocks presynaptic glutamate receptors: a novel "glutamate B" receptor in the lobster neuromuscular synapse. *Brain. Res.* **416**:162–165 (1987).
44. Hemmendinger, L. M., B. B. Garber, P. C. Hoffmann, and A. Heller. Selective association of embryonic murine mesencephalic dopamine neurons *in vitro*. *Brain Res.* **222**:417–422 (1981).
45. Panula, P., P. Emson, and J.-Y. Wu. Demonstration of enkephalin; substance P- and glutamate decarboxylase-like immunoreactivity in cultured cells divided from newborn rat striatum. *Histochemistry* **69**:169–179 (1980).
46. Thayer, S. A., M. Sturek, and R. J. Miller. Measurement of neuronal Ca^{2+} transients using simultaneous microfluorimetry and electrophysiology. *Pfluegers Arch. Eur. J. Physiol.* **412**:216–222 (1988).
47. Fabiato, A., and F. Fabiato. Calculator programs for computing the composition of solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)* **75**:463–505 (1979).
48. Scanlon, M., D. A. Williams, and F. S. Fay. A Ca^{2+} insensitive form of fura-2 associated with polymorphonuclear leukocytes. *J. Biol. Chem.* **262**:6308–6312 (1987).
49. Cull-Candy, S. G., and M. Usowicz. Patch clamp recording from single glutamate receptor channels. *Trends Pharmacol. Sci.* **8**:218–224 (1987).
50. Mayer, M. L., and G. L. Westbrook. The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.* **28**:197–278 (1987).
51. Thayer, S. A., L. D. Hirning, and R. J. Miller. Distribution of multiple types of Ca^{2+} channels in rat sympathetic neurons *in vitro*. *Mol. Pharmacol.* **32**:579–586 (1987).
52. Honoré, T., S. N. Davies, J. Drejer, E. J. Fletcher, P. Jacobsen, D. Lodge, and F. E. Nielsen. Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science (Wash. D. C.)* **241**:701–704 (1988).

53. Birch, P. J., C. J. Grossman, and A. G. Hayes. Kynurenate and FG9041 have both competitive and non-competitive actions at excitatory amino acid receptors. *Eur. J. Pharmacol.* **151**:313–318 (1988).
54. Thayer, S. A., T. M. Perney, and R. J. Miller. Regulation of Ca^{2+} homeostasis in sensory neurons by bradykinin. *J. Neurosci.* **8**:4089–4097 (1988).
55. Verdoorn, T. A., and R. Dingledine. Excitatory amino acid receptors expressed in *Xenopus* oocytes: agonist pharmacology. Mo. Pharmacol. **34**:298–307 (1988).
56. Blake, J. F., M. W. Brown, and G. L. Collingridge. CNQX blocks acidic amino acid induced depolarizations and synaptic components mediated by non-NMDA receptors in rat hippocampal slices. *Neurosci. Lett.* **89**:182–186 (1988).
57. Kano, M., and M. Kato. Quisqualate receptors are specifically involved in cerebellar synaptic plasticity. *Nature (Lond.)* **325**:276–279 (1987).
58. Lynch, G., J. Larson, J. S. Kelso, G. Barrionuevo, and F. Schottler. Intracellular injections of EGTA block induction of hippocampal long term potentiation. *Nature (Lond.)* **305**:719–721 (1983).
59. Nicoll, R. A., J. A. Kauer, and R. C. Malenka. The current excitement in long term potentiation. *Neuron* **1**:97–103 (1988).
60. Ito, I., D., Okada, and H. Sugiyama. Pertussis toxin suppresses long term potentiation of hippocampal mossy fiber synapses. *Neurosci. Lett.* **90**:181–185 (1988).
61. Silverstein, F. S., K. Chen, and M. V. Johnson. The glutamate analogue quisqualic acid is neurotoxic in striatum and hippocampus of immature rat brain. *Neurosci. Lett.* **71**:13–18 (1986).
62. Sanchez-Prieto, J., and P. Gonzalez. Occurrence of a large Ca^{2+} independent release of glutamate during anoxia in isolated nerve terminals (synaptosome). *J. Neurochem.* **50**:1322–1324 (1988).
63. Siesjo, B. K. Calcium, ischaemia and the death of brain cell. *Ann. N. Y. Acad. Sci.* **522**:638–662 (1988).
64. El-Fakahany, E. E., B. E. Alger, W. S. Lai, T. A. Pitler, P. F. Worley, and J. M. Baraban. Neuronal muscarinic responses: role of protein kinase C. *FASEB J.* **2**:2575–2583 (1988).
65. Katada, T., A. G. Gilman, Y. Watanabe, S. Bauer, and K. H. Jakobs. Protein kinase C phosphorylates the inhibitory guanine nucleotide binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151**:431–437 (1985).
66. Lipscombe, D., D. V. Madison, M. Poenie, H. Reuter, R. W. Tsien, and R. Y. Tsien. Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. *Neuron* **1**:355–365 (1988).
67. Thayer, S. A., L. D. Hirning, and R. J. Miller. The role of caffeine-sensitive calcium stores in the regulation of the intracellular free calcium concentration in rat sympathetic neurons *in vitro*. *Mol. Pharmacol.* **34**:664–673 (1988).
68. Berridge, M. J., P. H. Cobbold, and K. S. R. Cuthbertson. Spatial and temporal aspects of cell signalling. *Philos. Trans. R. Soc. Lond.-B Biol. Sci.*, **320**:325–343 (1988).
69. Nicoletti, F., J. T. Wroblewski, E. Fadda, and E. Costa. Pertussis toxin inhibits signal transduction at a specific metabolotropic glutamate receptor in primary cultures of cerebellar granule cells. *Neuropharmacology* **27**:551–556 (1988).

Send reprint requests to: Dr. Richard J. Miller, Department of Pharmacological and Physiological Sciences, University of Chicago, 947 E. 58th St., Chicago, IL 60637.