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# Concanavalin A enhances quisqualate-induced calcium influx in cerebellar granule cells in culture

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**Abstract**. In primary cultures of cerebellar granule cells kainate produced marked influx of $^{45}Ca^{2+}$, partially sensitive to the N-methyl-D-aspartate (NMDA) antagonist, 3-(±)-2-carboxypiperazin-4-yl)- propyl-1-phosphonic acid (CPP), indicating involvement of an NMDA receptor-sensitive component that may be secondary to kainate-induced glutamate release. Sodium removal partially inhibited kainate's effect. Quisqualate also produced influx of $^{45}Ca^{2+}$, but with lower efficacy and higher potency than kainate. This action of quisqualate was unaffected by CPP and by sodium removal. Preincubation of cells with the plant lectin concanavalin A (Con A), but not with its succinyl derivative, enhanced quisqualate- induced calcium influx, and to a lesser extent kainate's effect. Inclusion of quisqualate in preincubation medium antagonized Con A potentiation of quisqualate response. Also Con A was ineffective when included in the incubation medium only, without preincubation. Preincubation of rat brain cortical membranes with Con A but not with succinyl Con A increased the binding of the AMPA receptor agonist, [$^3$H]α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ([$^3$H]AMPA). The results suggest that Con A enhancement of quisqualate response possibly involves the modification of an AMPA recognition site and requires preincubation in the absence of an agonist (here quisqualate).

**Key words:** concanavalin A, AMPA receptors, glutamate, quisqualate, kainate, $^{45}Ca^{2+}$ influx, cerebellar granule cells

## INTRODUCTION

Traditionally, ionotropic glutamatergic receptors are distinguished by their different sensitivity to N-methyl-D-aspartate (NMDA), kainate and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or quisqualate (Wroblewski and Danysz 1989). Ionic channels coupled to these receptors are permeable to sodium, however, they differ in their permeability to $^{45}Ca^{2+}$. Activation of NMDA receptors produces massive $^{45}Ca^{2+}$ influx (Wroblewski et al. 1985, MacDermott et al. 1986), which has been implicated in toxicity and related pathology (Choi 1988). Kainate *in vivo* also increases calcium influx as shown by microdialysis experiments (Lazarewicz et al. 1986). However, *in vitro* its effect at low concentrations is dependent on the activation of voltage dependent calcium channels, at least in striatal neurons (Murphy and Miller 1989a). Similarly, some authors suggested that quisqualate does not produce calcium influx directly, but only as a result of sodium influx-induced depolarization (Murphy and Miller 1989b). Recently, due to cloning of ionotropic glutamate receptor subunits those controversies have been clarified (Hollmann and Heinemann 1994). Thus, it is now widely accepted that in the case of AMPA receptors (also activated by kainate), which are pentameric assemblies, the presence of GluR2 subunit limits $Ca^{2+}$ permeability due to presence of positively charged arginine in the protein region forming the ion channel wall (Burnashev et al. 1992, Hollmann and Heinemann 1994).

Electrophysiological studies indicate that AMPA receptors undergo characteristic response decay called desensitization (Wong and Mayer 1993). It can be seen as internal switch off, preventing receptor overstimulation (including $Ca^{2+}$ overload). Desensitization can be regulated by various exogenous agents, e.g. by cyclothiazide and the plant lectin, concanavalin A (Con A), (Sharon and Liss 1989, Wong and Mayer 1993). Con A has been shown to inhibit desensitization of glutamate responses as first demonstrated by Mathers and Usherwood (1976) in invertebrate preparation. Also in cultured mammalian hippocampal neurons it blocks desensitization of inward current produced by a glutamate agonist, quisqualate, but failed to affect kainate responses (Mayer and Vyklicky 1989).

The present study was undertaken to test whether Con A does affect kainate- and quisqualate-mediated calcium influx in a different experimental system, such as primary cultures of cerebellar granule cells. We also tested whether Con A changes binding characteristics of [$^3$H]AMPA in rat brain cortical membranes.

## METHODS

### $^{45}Ca^{2+}$ influx

Primary cultures of cerebellar granule cells were prepared from 8-day old Sprague Dawley rats (Zivic Miller) as described previously (Wroblewski et al. 1985). The uptake of $^{45}Ca^{2+}$ was studied in 8-9 day old cultures (37°C). Culture dishes were washed twice with 1 ml of Locke's solution (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose and 10 mM Hepes, pH = 7.4), and left for 10 min to adapt to the buffer. Then the medium was replaced with buffer containing 10 μM Con A or control Locke's solution. Next, dishes were washed once with 1 ml of buffer and 1 ml of buffer containing 1 μCi of $^{45}CaCl_2$ was added (New England Nuclear, specific activity 15.4 mCi/mg) and appropriate concentrations of agonists. Incubations were continued for 10 min and then stopped by washing with ice cold buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM Hepes and 2 mM EGTA). The cells were then dissolved in 0.5 M NaOH and aliquots were used for measuring both radioactivity and protein concentration (Lowry et al. 1951).

### Receptor binding

Brain membranes were prepared from male Sprague Dawley rats (Zivic Miller, 200-250 g) as described previously (Danysz et al. 1989). Final

pellets were frozen for at least 12 h at -60°C. The frozen membranes were added to binding buffer (30 mM tris-acetate buffer containing 10 mM KSCN and 2.5 mM $CaCl_2$, pH = 7.4), thawed at room temperature, incubated for 20 min at 37°C and then homogenized with polytron and centrifuged (20,000g x 10 min). The washing was repeated 2 more times. Then membranes were incubated for 20 min in 37°C, with or without 10 μM Con A (counted as a monomer). This was followed by two additional washings after which membranes were used for binding experiments. Specific binding of [$^3$H]AMPA (specific activity 17.8 Ci/mmol, final ligand concentration 20 nM) was determined in the presence of 1 mM AMPA. In all cases, incubations were initiated by adding membranes (protein content 0.2-0.4 mg) to final volume of 0.5 ml. Incubations were continued for 60 min at 4°C and were terminated by filtration through GF/C filters (PhD cell harvester). Filters were washed two times with 5 ml of ice cold buffer, dried and placed in the scintillation vials with 4 ml of scintillation fluid.

### Statistical methods

Dose response experiments were analyzed by nonlinear regression, from which the $EC_{50}$ or $IC_{50}$ values were derived. The results from a representative experiment performed in triplicate are shown (mean ± SE). Student's *t* test was used for pairwise comparison.

### Chemicals

Radioisotopes were purchased from New England Nuclear (Boston, MA, USA), AMPA, 6-cyjano-7-nitroquinoxaline-2,3-dione (CNQX, Honore et al. 1988), and 3-(±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, Harris et al. 1986) from Tocris Neuramin (Buckhurst Hill, England), quisqualate and (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate ((+)MK-801) from Research Biochemicals Incorporated (Natick, MA, USA) and all other chemicals from Sigma (St. Louis, MO, USA).

## RESULTS

Kainate produced marked influx of $^{45}Ca^{2+}$ which was reduced by 50% in the presence of NMDA receptor antagonist CPP (Fig. 1). This indicates that probably part of kainate's effect was due to enhancement of endogenous glutamate in the synaptic cleft and then activation of NMDA. This is supported by the fact that $EC_{50}$ of kainate action in the absence and presence of CPP was different (90.0 ± 7.0 and 130 ± 6.0 μM respectively, $P<0,05$, Student's *t* test). In contrast, quisqualate induced $^{45}Ca^{2+}$ influx in a CPP insensitive manner. Quisqualate was more potent ($EC_{50}$=3.9 ± 0.3 μM) and less efficacious than kainate (Fig. 1). In the presence of CPP both kainate and quisqualate effects reached plateau after 3-5 min of incubation (not shown). Ibotenate in the presence of 1 μM (+)MK-801 (to block NMDA receptors) failed to increase calcium influx in the experimental design used. Kainate- and quisqualate-induced calcium influx was antagonized by CNQX, an AMPA receptor antagonist, with respective $IC_{50}$ values of 0.81 ± 0.11 and 1.4 ± 0.13 μM (Fig. 2).

Preincubation of cells for 10 minutes with 10 μM Con A potentiated the effect of kainate (Fig. 3). Similarly, calcium influx produced by quisqualate

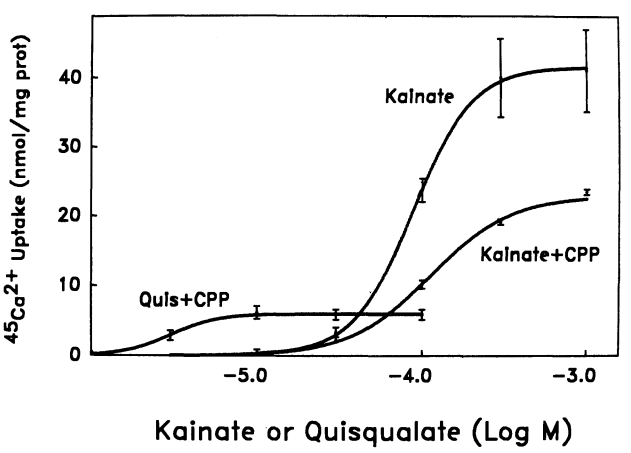


Fig. 1. Effect of CPP on kainate-induced $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granule cells. In the case of quisqualate only the curve with added CPP is shown since it was nearly identical to the curve without CPP added. Values are mean ± SE of a representative experiment performed in triplicate.

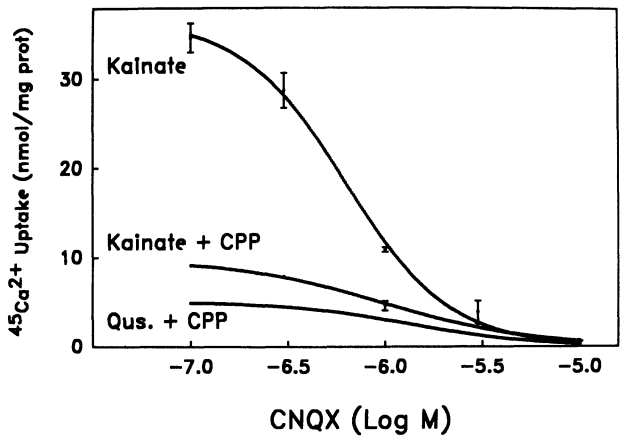


Fig. 2. Effect of non-NMDA receptor antagonist CNQX on kainate- and quisqualate-induced $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granule cells. In the case of quisqualate only the curve with added CPP is shown since it was nearly identical to the curve without CPP added. Values are mean ± SE of a representative experiment performed in triplicate.

in the same conditions was potentiated by preincubation with Con A (Fig. 4). However, when quisqualate was included in the preincubation medium together with Con A, the enhancement to subsequently given quisqualate was not observed (Fig. 4). Preincubation with quisqualate alone slightly in-

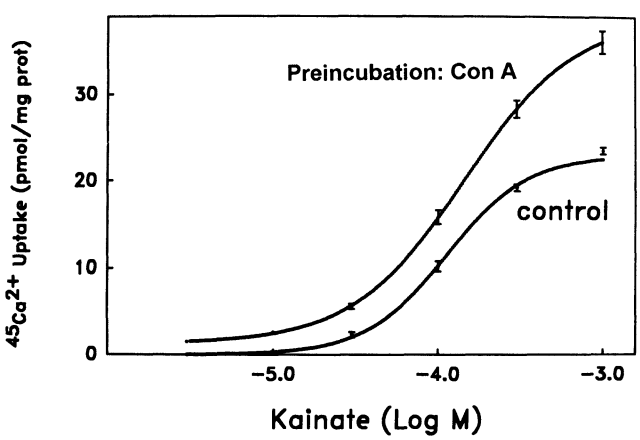


Fig. 3. Preincubation with Con A enhances kainate-mediated $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granule cells. Cells were preincubated for 10 with the control buffer, or buffer containing Con A. The medium was then replaced with buffer containing $^{45}Ca^{2+}$ and appropriate concentration of kainate. Values are mean ± SE of a representative experiment performed in triplicate.

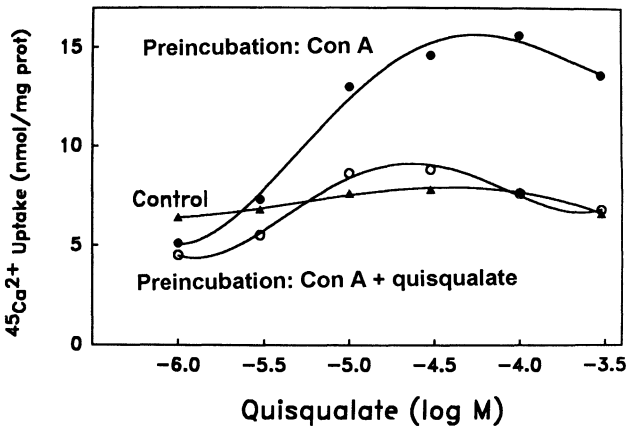


Fig. 4. Preincubation with Con A enhances quisqualate-mediated $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granule cells. Cells were preincubated for 10 min with control buffer (open circles), 10 M Con A (filled circles) or quisqualate + Con A (filled triangles). The medium was then replaced with buffer containing $^{45}Ca^{2+}$ and appropriate concentration of quisqualate. Values are means of a representative experiment performed in triplicate.

hibited subsequent stimulation with the same agonist (not shown). Applying Con A in the incubation medium instead of preincubation failed to affect quisqualate action (Table I). Preincubation with succinyl Con A failed to potentiate quisqualate or AMPA action (Table I).

High potassium (50 mM) mediated calcium influx was not affected by the pretreatment with Con A (not shown) when 1 μM MK-801 was used to block NMDA receptors. Substitution of sodium ions with N-methyl-D-glucamine failed to change quisqualate's action, however kainate's effect was strongly inhibited in the absence of CPP and less effectively in its presence (Fig. 5). Pretreatment of cells for 12 h with pertussis toxin did not affect either kainate- or quisqualate-produced calcium influx (Fig. 5). Binding experiments revealed that preincubation of brain cortical membranes with 10 μM Con A for 10 min followed by two washings increased [$^3$H]AMPA binding, from 0.41 ± 0.009 to 0.57 ± 0.013 pmol/mg protein ($P$<0.05). The same treatment with succinyl Con A was without effect (0.44 ± 0.021 pmol/mg protein). In saturation experiments (constant concentration of [$^3$H]AMPA -

TABLE I

Effect of incubation (10 min) or preincubation (10 min) with Con A or Succinyl Con A on quisqualate- and AMPA-induced $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granular cells. Agonists (quisqualate, AMPA) were added together with $^{45}Ca^{2+}$ - incubation. Values are results of representative experiment performed in triplicate (mean ± SE, $^{45}Ca^{2+}$ uptake nmol/mg protein). Experiment was replicated two times with similar results

| Preincubation | Buffer | Succ. Con A | Con A | Buffer |
|---|---|---|---|---|
| Incubation | Buffer | Buffer | Buffer | Con A |
| Control | 4.6 ± 0.1 | 4.6 ± 0.26 | 8 ± 1.2 | 5.7 ± 0.5 |
| Quisqualate 100 μM | 10.8 ± 0.5 | 9.4 ± 0.8 | 19.6 ± 0.2* | 11.0 ± 0.4 |
| AMPA 1 mM | 16.4 ± 1.6 | 16.2 ± 1.1 | 24.4 ± 0.9* | 21.4 ± 1.9 |

*$P<0.05$ vs. respective "Buffer-Buffer" group (Student $t$ test)

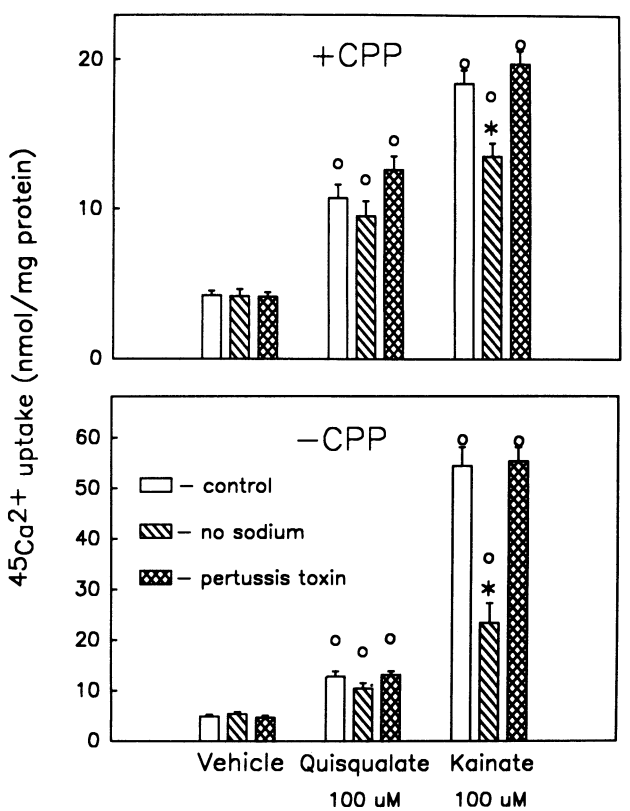


Fig. 5. Effect of pretreatment with pertussis toxin (for 12 h, 1 g/ml) or substitution of $Na^+$ with N-methyl-D-glucamine on kainate- and quisqualate-induced $^{45}Ca^{2+}$ influx in primary cultures of cerebellar granule cells. Values are mean ± SE of a representative experiment performed in triplicate. * $P<0.05$ as compared to respective control group (Student $t$ test); o $P<0.05$ as compared to respective vehicle group (Student $t$ test)

20 nM added with increasing concentrations of cold AMPA) a non-linear Scatchard plot was obtained (see Hall et al. 1992). However, two site model could not be applied due to high variability of contribution of both sites in separate experiments.

## DISCUSSION

In the present study a portion of kainate-induced calcium influx was the result of NMDA receptor stimulation, as evidenced by the partial attenuation by the competitive NMDA antagonist CPP. The activation of NMDA receptors seems to be a secondary effect since kainate is known to release endogenous glutamate in slices (Ferkany and Coyle 1983) and in primary cultures of cerebellar granule cells (Gallo et al. 1982, Ulivi et al. 1989). The present results show also that calcium influx related to NMDA receptor stimulation (due to glutamate release) is much more sensitive to $Na^+$ removal than direct kainate action. In fact, some reports suggest that kainate-mediated release of glutamate is sodium-dependent and may result from reversal of glutamate carrier by high intracellular sodium concentration (Ulivi et al. 1989). In concontrast, in cerebellar granule cells calcium influx produced by quisqualate was completely independent from the activation of NMDA receptors, which is in line with data indicating negligible effect of this agonist on [$^3$H]aspartate (Gallo et al. 1989) and endogenous glutamate release (Ulivi et al. 1989).

Quisqualate-induced influx of calcium seen in the present study is probably also a direct effect and not secondary to depolarisation or stimulation of metabotropic glutamate receptors (mGluR) coupled to phospholipase C. It is evidenced by the present experiments showing that removal of sodium did not change quisqualate response and ibotenate (an agonist of mGluR, Schoepp and Johnson 1989) failed to produce calcium influx (in presence of MK-801 to block NMDA receptors). Hence, in primary cultures of cerebellar granule cells quisqualate produces direct influx of calcium, as previously reported in the same experimental model (Bouchelouche et al. 1989, Halopainen et al. 1989). A similar phenomenon is also observed in synaptosomes from immature brains (Benavides et al. 1988). However, in striatal neurons no quisqualate-induced $Ca^{2+}$ influx has been observed (Murphy and Miller 1989b). In fact it is now accepted that the composition of AMPA receptor subunits that are unevenly expressed in various brain regions determines $Ca^{2+}$ permeability (Burnashev et al. 1992). Although the GluR2 subunit of AMPA receptors that limits $Ca^{2+}$ permeability is highly expressed in cerebellar granule cells (Day et al. 1995), it is likely that in primary cultures the non-edited ($Ca^{2+}$ permeable, as seen prenatally *in vivo*) form of this subunit dominates (Hollmann and Heinemann 1994). As evidenced by patch clamp experiments, desensitization of quisqualate response may be blocked by the lectin Con A, a protein which is known to interact with membrane glycoproteins (O'Dell and Christensen 1986, Mayer and Vyklicky 1989, Sharon and Liss 1989). The mechanism of action may be related to its ability to form tetramers since succinyl Con A, devoid of that feature, is ineffective in blocking desensitization in electrophysiological experiments and in other biological systems (Gunther et al. 1973, Mayer and Vyklicky 1989, Raulli et al. 1991). Similar results were obtained in the present study since succinyl Con A, in contrast to Con A, failed to enhance quisqualate action and modify [$^3$H]AMPA binding. Of course the process of desensitization due to its short time course cannot be studied by the methods used in the present study. However, the enhancement of quisqualate response in the present study may be interpreted as a consequence of desensitization inhibition. Present results indicate that kainate responses were also slightly enhanced by Con A. Similarly, electrophysiological studies indicate that kainate responses in dorsal root ganglion (at kainate receptors) are also potentiated by Con A, in contrast to hippocampal neurons where it acts at AMPA receptors (Heuttner 1989, Mayer and Vyklicky 1989, Wong and Mayer 1993).

When in preincubation medium quisqualate was included together with Con A, no potentiation was observed. Also no enhancement was observed if Con A was added together with quisqualate directly to incubation medium. Thus, Con A's effect may either require access to quisqualate recognition site, which in the above examples was limited by excess of the agonist, or alternatively it requires the presence of the receptor in the inactivated state. Interestingly, preincubation with AMPA antagonist CNQX does not seem to prevent Con A action - at least on quisqualate-stimulated PI hydrolysis - favoring the second possibility (see Raulli et al. 1991). Binding experiments (this study) also suggest that Con A modifies AMPA receptor recognition site since Con A increased [$^3$H]AMPA binding to the agonist recognition site. In contrast, cyclothiazide which also inhibits AMPA receptors desensitization decreases the affinity of [$^3$H]AMPA binding (Hall et al. 1993) which is in line with the electrophysiological studies showing that AMPA shows higher affinity to the desensitized state of the receptor (Patneau and Mayer 1991). Hence, the mode of cyclothiazide and Con A action seem to be very different. In fact Con A inhibits preferentially desensitization of kainate preferring receptors (dorsal root ganglion neurons) and less AMPA preferring receptors (hippocampal neurons) (Wong and Mayer 1993). The opposite holds true for cyclothiazide (ibid.). Since quisqualate shows poor receptor selectivity, the effect of Con A seen in the present study may involve both AMPA and kainate receptors (Hollmann and Heinemann 1994). However, by the experiment with AMPA (selective for

AMPA receptors, Hollmann and Heinemann 1994), a direct modulation of AMPA receptors by Con A is implicated.

In conclusion, present results support the view that not only kainate but also quisqualate may produce $Ca^{2+}$ influx independent from the voltage sensitive calcium channels. Moreover, the lectin Con A enhances quisqualate responses, which may be analogous to the process of desensitization seen in some electrophysiological studies. The mechanism of Con A action on quisqualate response probably involves modification of the agonist recognition site or requires an inactivated state of the receptor.

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