0026-895X/92/040603-06$03.00/0

MOLECULAR PHARMACOLOGY, 41:603-608



*ACCELERATED COMMUNICATION*

# Calcium Directly Permeates Kainate/α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors in Cultured Cerebellar Purkinje Neurons

JAMES R. BRORSON, DAVID BLEAKMAN, PAUL S. CHARD, and RICHARD J. MILLER

*Department of Pharmacological and Physiological Sciences (J.R.B., D.B., P.S.C., R.J.M.) and Department of Neurology (J.R.B.), The University of Chicago, Chicago, Illinois 60637*

Received November 26, 1991; Accepted January 28, 1992

**SUMMARY**

In cultures of rat cerebellar neurons that were enriched in Purkinje cells, the non-*N*-methyl-D-aspartate glutamate receptor agonist kainate (KA) stimulated $Ca^{2+}$ influx into all neurons in $Na^+$-containing solutions. A large $Ca^{2+}$ influx was also observed in most neurons when KA was applied in $Na^+$-free solutions, even when the cells were voltage-clamped at negative potentials. KA also stimulated $Co^{2+}$ uptake into both Purkinje and non-Purkinje neurons. The KA-induced $Ca^{2+}$ influx was insensitive to pharmacological antagonists of voltage-sensitive $Ca^{2+}$ channels and antagonists of *N*-methyl-D-aspartate receptors. Thus, different types of cerebellar neurons possess KA-gated ionophores that are permeable to $Ca^{2+}$. This $Ca^{2+}$ conductance may play an important role in glutamate-mediated physiological and pathological events in the cerebellum.

Changes in neuronal $[Ca^{2+}]_i$ mediate many of the important effects of excitatory amino acids (1). These include physiological changes such as long term potentiation and pathological changes such as excitotoxicity (2, 3). Activation of metabotropic glutamate receptors can produce mobilization of $Ca^{2+}$ from intracellular stores. Activation of NMDA receptors results in a large influx of $Ca^{2+}$, primarily through the $Ca^{2+}$-permeable ionophore that is opened by this receptor. Finally, activation of KA/AMPA receptors can also produce $Ca^{2+}$ influx (1). It has been generally thought that this influx results from cell depolarization and movement of $Ca^{2+}$ through voltage-sensitive $Ca^{2+}$ channels. However, some observations have not altogether supported this view and have apparently demonstrated a direct flux of $Ca^{2+}$ through KA/AMPA-gated channels in neurons (4–8) and astrocytes (9, 10). In support of this contention, recent molecular biological studies have shown that, depending on the subunits used, KA/AMPA receptors expressed in oocytes can exhibit either little or substantial $Ca^{2+}$ permeability (11, 12). We now demonstrate, in cultures of cerebellar neurons enriched in Purkinje cells, that $Ca^{2+}$-permeable KA/AMPA receptors are actually found in the majority of neurons and that these receptors can act as the major route for $Ca^{2+}$ entry.

This work was supported by National Institutes of Health Grants DA-02121 and MH-40165 and Digestive Diseases Research Core Center Award DK42086 to R.J.M. J.R.B. was supported by a Howard Hughes Medical Institute Physician Research Fellowship, D.B. by a Fulbright Fellowship, and P.S.C. by National Institutes of Health Training Grant PHS NRSA2T32 GM07151.

## Materials and Methods

Experiments were performed on cultures of cerebellar neurons taken from day 16 embryonic rats (13). Purkinje neurons represent about 50% of the neurons present in these cultures, and granule cells are virtually absent. $[Ca^{2+}]_i$ was measured by either loading cells with fura-2 acetoxymethyl ester or using combined whole-cell patch-clamp fura-2-based microfluorimetry.

**Cell culture.** The culture method, using neurons plated on glass coverslips inverted over a feeding layer of astrocytes, is described in detail in Ref. 13. For the present study, glass coverslips were treated by overnight exposure to the vapor of 3-aminopropyl triethoxysilane in an evacuated dessicator, followed by exposure to 10% glutaraldehyde for 15 min, rinsing in distilled water, and autoclaving. Rats were cared for in accordance with University of Chicago Animal Care Committee protocols. Maternal rats were anesthetized irreversibly with ether before dissection. Neurons were used for physiological experiments at 12–39 days *in vitro*; no correlation of responses with time *in vitro* was recognized.

**$[Ca^{2+}]_i$ measurements and electrophysiology.** Cells were incubated for 30 min at 37° with fura-2 acetoxymethyl ester (5 μM) in a buffered solution containing (in mM) NaCl, 143; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; and glucose, 10 (pH adjusted to 7.5 with NaOH).

**ABBREVIATIONS:** $[Ca^{2+}]_i$, intracellular free calcium concentration; KA, kainate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDG, *N*-methyl-D-glucamine; NMDA, *N*-methyl-D-aspartate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, aminophosphovalerate; TTX, tetrodotoxin.

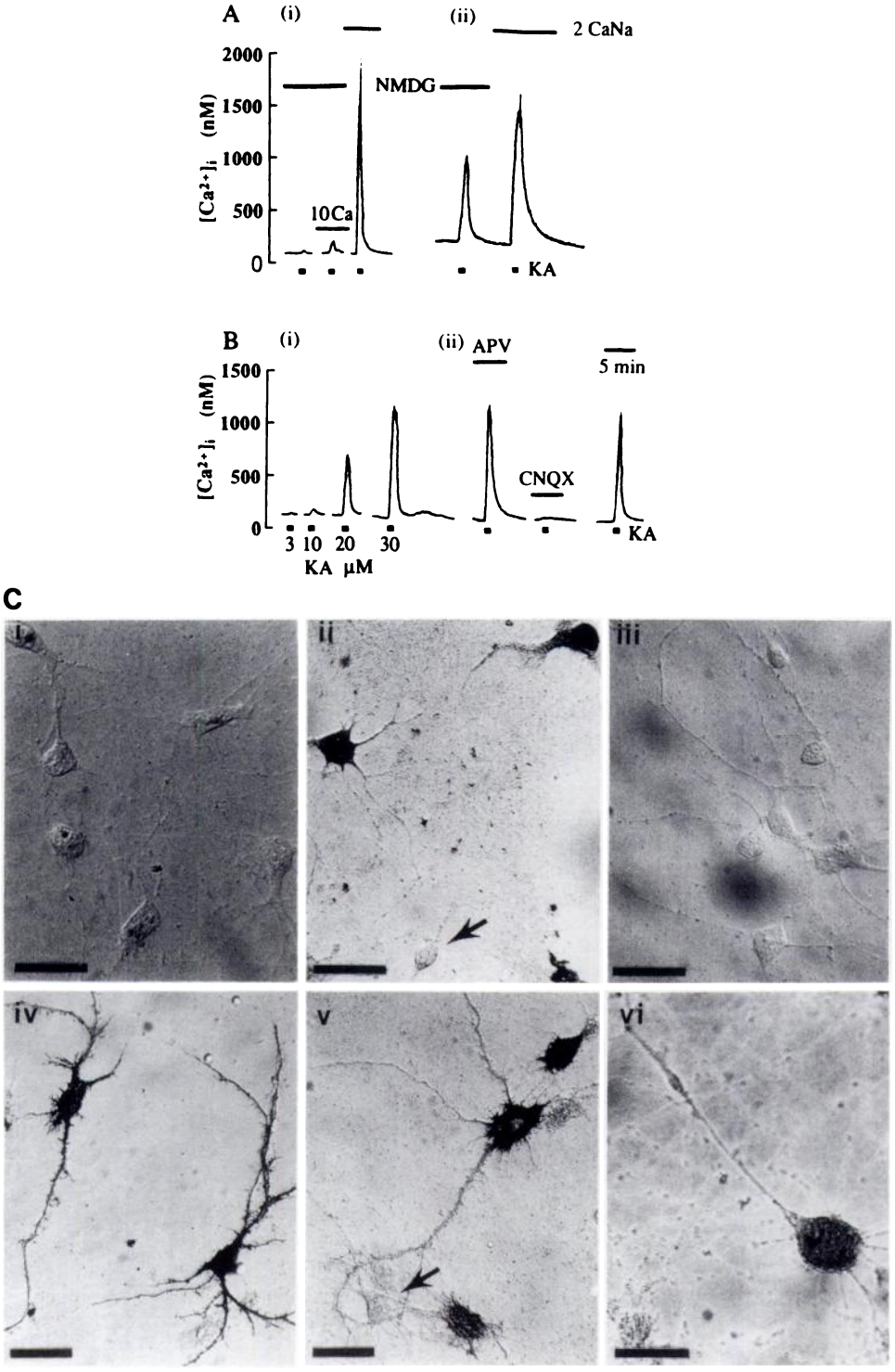


**Fig. 1.** A, KA-induced $[Ca^{2+}]_i$ responses in $Na^+$-containing and $Na^+$-free solutions. The effects of 30-sec applications of KA (30 μM) on $[Ca^{2+}]_i$ measured in single cerebellar neurons are shown in a cell that did not respond with an increase in $[Ca^{2+}]_i$ in $Na^+$-free solutions (*i*) and in a cell in which KA induced an increase in $[Ca^{2+}]_i$ both in $Na^+$-free and in $Na^+$-containing solutions (*ii*). Extracellular solutions contained 2 mM $Ca^{2+}$ and 140 mM NMDG (2Ca/NMDG), except where denoted otherwise. B, *i*, KA (3–30 μM) produced concentration-dependent increases in $[Ca^{2+}]_i$ in a responding cell in $Na^+$-free (2Ca/NMDG) solutions. *ii*, The rise in $[Ca^{2+}]_i$ induced by KA in $Na^+$-free solution was not reduced by APV (50 μM) but was blocked by CNQX (10 μM). C, $Co^{2+}$-uptake staining. Neurons were exposed to KA (100 μM) in the presence of 5 mM $Co^{2+}$, 0.5 μM TTX, and 50 μM APV, and were stained with silver enhancement for $Co^{2+}$ uptake (14). Cells treated with control solutions (*i*) or with KA in the presence of 20 μM CNQX (*iii*) failed to stain. Most of the KA-treated neurons (*ii, iv, v,* and *vi*) stained darkly, whereas some neurons on the same coverslips remained unstained (*arrows* in *ii* and *v*). *i, ii,* and *iii*, cells at 5 days *in vitro* treated in parallel; older cells (*iv* and *v*, 19 days *in vitro; vi*, 35 days *in vitro*) displayed more developed morphologies, with similar $Co^{2+}$-uptake staining. *vi*, $Co^{2+}$-uptake-stained neuron with morphology typical of the cultured Purkinje cells. *Scale bars*, each 30 μm.

Cells were then rinsed and incubated for an additional 30 min at 37° in a fura-2-free solution. Fura-2 fluorescence was determined as previously described (14). Background fluorescence was measured from a cell-free region of the coverslip. The aforementioned solution was used as a $Na^+$-containing solution. $Na^+$-free solutions contained (in mM) NMDG, 140; KCl, 5; $CaCl_2$, 2; HEPES, 10; and glucose, 10 (pH adjusted to 7.4 with HCl). All solutions contained TTX (0.5 μM).

Combined whole-cell patch-clamp fura-2-based microfluorimetry was performed as previously described in full (14). Cells were held at −80 mV. $Na^+$-containing solutions were as described above. $Na^+$-free solutions contained (in mM) NMDG, 140; $CaCl_2$, 2; HEPES, 10; and glucose, 10 (pH adjusted to 7.4 with HCl). An internal pipette solution contained (in mM) potassium gluconate, 145; $MgCl_2$, 1; HEPES, 10; fura-2 pentapotassium salt, 0.1; di-trisphosphocreatinine, 14; and Mg-ATP, 3.6; with creatinine phosphokinase, 50 units/ml (pH adjusted to 7.1 with KOH). Background fluorescence was measured upon obtaining a gigaseal. Whole-cell patch-clamp was performed as described previously (13). Cells were accepted for study if a stable seal with a whole-cell resistance of at least 200 MΩ formed and the resting $[Ca^{2+}]_i$ was less than 200 nM. Patch pipettes of 1.5–4 MΩ were used, producing series resistances of 5–10 MΩ. Capacitive compensation and 30–60% series resistance corrections were used in voltage-clamp experiments.

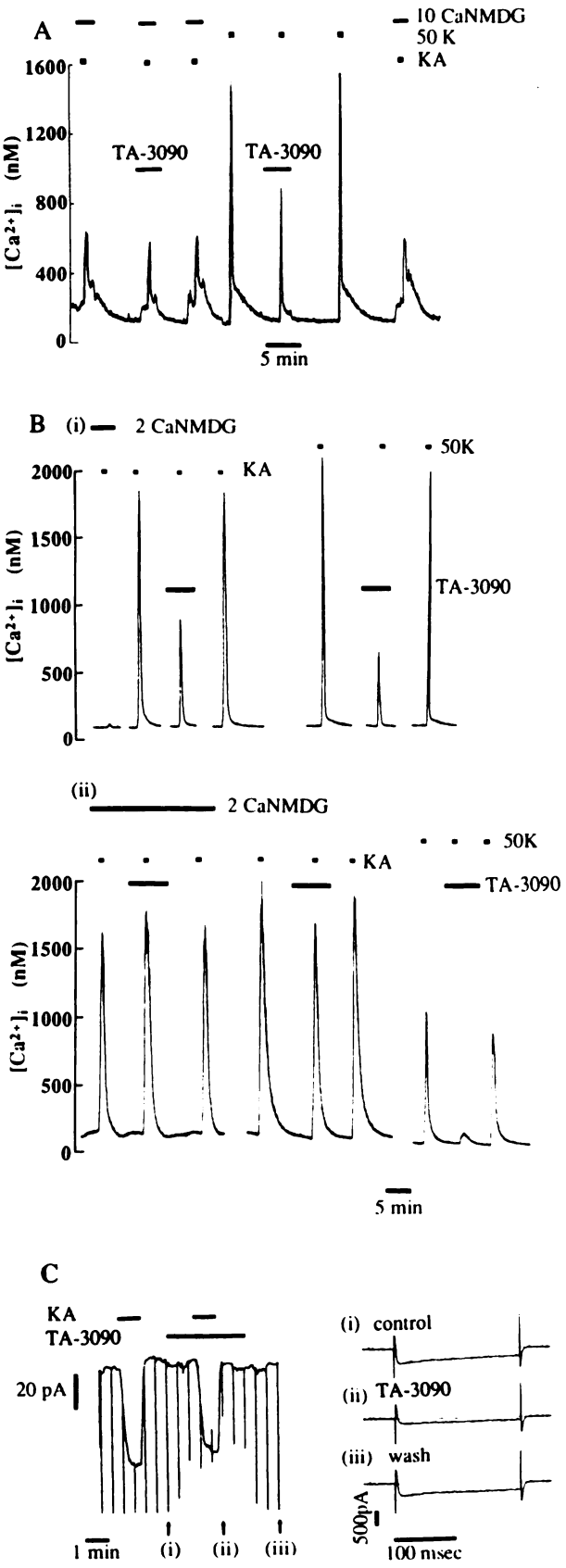


**Fig. 2.** Effects of the $Ca^{2+}$ channel antagonist TA-3090 on KA-induced $Ca^{2+}$ influx. A, In $Na^+$-free medium, the rise in $[Ca^{2+}]_i$ induced by KA (30 $\mu$M) was not affected by the voltage-gated $Ca^{2+}$ channel antagonist TA-3090 (clentiazem) (50 $\mu$M). However, the 50 mM $K^+$ (50 K)-induced rise in $[Ca^{2+}]_i$ was significantly reduced. B, *i*, In a neuron that did not respond to KA in $Na^+$-free solution, the size of the KA response in $Na^+$-containing solution could be significantly reduced by application of TA-3090, as could the 50 mM $K^+$-induced $[Ca^{2+}]_i$ rise. *ii*, In contrast, in a neuron responding to KA in $Na^+$-free solution, the rise in $[Ca^{2+}]_i$ was unaffected by TA-3090, and in $Na^+$-containing medium it was diminished by only a small amount, whereas the 50 mM $K^+$-induced rises in $[Ca^{2+}]_i$ were markedly reduced by TA-3090. (Extracellular solutions in A and B contained 2 mM $Ca^{2+}$ and 140 mM $Na^+$, except as indicated.) C, *Left*, in a whole-cell voltage-clamped cell, held at −80 mV, KA (30 $\mu$M) induced a 50-pA inward current in $Na^+$-free solution (2Ca/NMDG), which was little affected by TA-3090 (50 $\mu$M). *Right*, simultaneous recordings of the voltage-gated $Ca^{2+}$ currents with periodic depolarizations to 0 mV (represented in the *left trace*, filtered at 1 Hz, by downward deflections (at items marked i, ii, and iii) showed a reversible inhibition of the voltage-gated current of >50%.

Intracellular solutions for current measurements and I/V curves contained (in mM) NMDG fluoride, 145; and BAPTA, 10 (pH adjusted to 7.1 with NMDG and HF). ATP was omitted to allow rundown of the voltage-gated $Ca^{2+}$ currents. External solutions contained (in mM) NaCl, 140; $CaCl_2$, 2; $MgCl_2$, 1: KCl, 3; HEPES, 10; and glucose, 10 (pH adjusted to 7.4 with NaOH). $Na^+$-free solutions contained (in mM) NMDG, 145; $CaCl_2$, 2; HEPES, 10; and glucose, 10 (pH adjusted to 7.4 with HCl). $Mg^{2+}$ and $K^+$ were omitted, to eliminate non-$Ca^{2+}$ conductances. All external solutions contained TTX (0.5 $\mu$M) and bicuculline (20 $\mu$M). Perfusion of the recording chamber was at a rate of 1–1.5 ml/min; figures have been corrected for a perfusion delay of approximately 20 sec. All experiments were performed at room temperature.

For I/V relationships, currents were measured during 200-msec depolarizations to potentials ranging up to +40 mV, by 10-mV steps, from holding potentials of −80 mV or −100 mV. Currents measured in control solution were subtracted from those measured during the plateau phase of KA application, to obtain the KA-induced currents. Voltage-gated $Ca^{2+}$ currents were eliminated by the inclusion of 100 $\mu$M $Cd^{2+}$ in the external solution and by measuring currents after the inactivation of the low-threshold currents.

Activation of $Cl^-$ conductances was blocked by using TTX (0.5 $\mu$M) and bicuculline (20 $\mu$M) in all recording solutions and by including 10 mM BAPTA in the pipette solution to prevent the activation of $Ca^{2+}$-activated $Cl^-$ channels. The inward rectification of the measured currents in $Na^+$-free (but $Cl^-$-containing) external solutions suggests that the $Cl^-$ channels did not contribute significantly to the measured KA-induced inward currents in $Na^+$-free solutions. In addition, replacement of external $Cl^-$ with methanesulfonate did not affect the magnitude of the inward currents generated by 30 $\mu$M KA ($n$ = 3).

**Cytochemistry.** For $Co^{2+}$-uptake staining, we followed the method of Pruss *et al.* (15), with certain modifications. KA stimulation was at 37° for 20 min in 5 mM $CoCl_2$; $Co^{2+}$ was precipitated in 1.2% $(NH_4)_2S$ and cells were fixed for 10 min at 37° in 3% paraformaldehyde in phosphate-buffered saline. Silver enhancement was done at 55° with the coverslips in an inverted position, to reduce background. Immunofluorescent staining was performed after silver enhancement, using a monoclonal antibody to calbindin-D-28K (Sigma), by following the method described in Ref. 13.

## Results and Discussion

KA acts primarily by opening $Na^+$-permeable channels, causing depolarization (but see also Ref. 16). In normal physiological medium, KA (30 $\mu$M; 30-sec application) produced large rises in $[Ca^{2+}]_i$ in all neurons examined ($\Delta[Ca^{2+}]_i$ = 810 ± 69 nM, mean ± standard error, $n$ = 53). If this $[Ca^{2+}]_i$ rise is due to $Na^+$-dependent depolarization of cells and influx of $Ca^{2+}$ through voltage-sensitive $Ca^{2+}$ channels, it should not occur if the external $Na^+$ is replaced by a nonpermeable cation such as NMDG. This prediction was tested. In some neurons, $Ca^{2+}$ influx was virtually absent under these conditions, even when the external calcium concentration was raised to 10 mM (Fig.

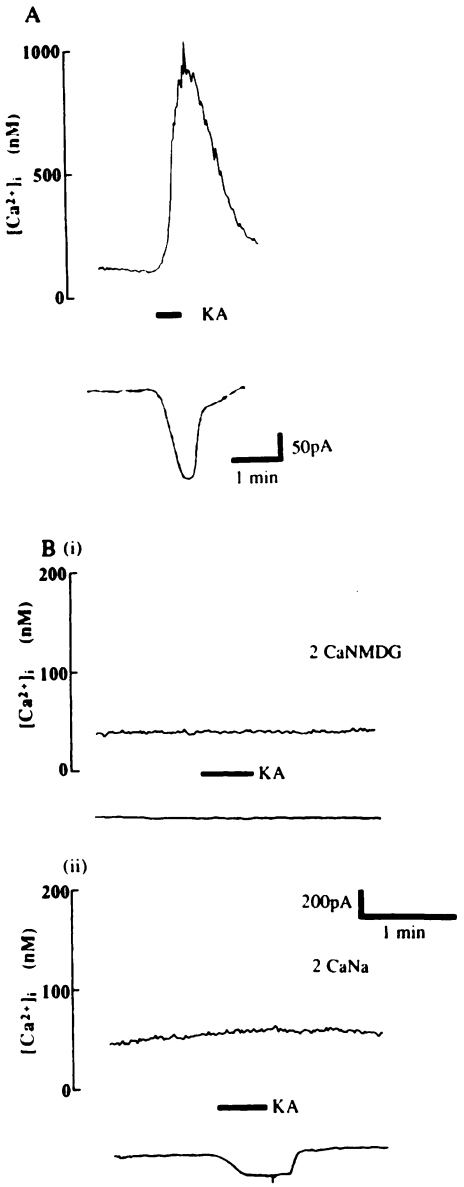


**Fig. 3.** Simultaneous voltage-clamp and $[Ca^{2+}]_i$ microfluorimetric recordings and effect of KA. A, In a cerebellar neuron under whole-cell voltage-clamp at −80 mV, KA (30 μM) induced an inward current (*bottom*) and a simultaneous increase in $[Ca^{2+}]_i$ (*top*) in a $Na^+$-free solution (2Ca/NMDG). B, *i*, In a nonresponding cell, neither an inward current nor an increase in $[Ca^{2+}]_i$ was induced by KA in $Na^+$-free solution. *ii*, In the same cell, KA in $Na^+$-containing solution induced an inward current but no associated increase in $[Ca^{2+}]_i$.

1A). However, in the majority of cells studied, a substantial $Ca^{2+}$ influx was observed even under these circumstances; 42 of 54 (77%) cells responded in a 2 mM $Ca^{2+}$/140 mM NMDG (2Ca/NMDG) solution to 30 μM KA, with the $\Delta[Ca^{2+}]_i$ being 705 ± 81 nM ($n$ = 42) in the responding cells (defined as those with $\Delta[Ca^{2+}]_i$ of >50 nM). In 15 cells examined with a 30 μM KA application in both $Na^+$-containing and $Na^+$-free solutions, the $\Delta[Ca^{2+}]_i$ in $Na^+$-free solution was 59 ± 6% of the $\Delta[Ca^{2+}]_i$ in $Na^+$-containing solution. The concentration dependence showed a threshold of between 3 μM and 10 μM KA (Fig. 1B). Similar results were also obtained with the glutamate analog AMPA in $Na^+$-free solutions (five cells exposed to 30 μM AMPA, with $\Delta[Ca^{2+}]_i$ of 430 ± 158 nM, which was 46 ± 10% of the response to 30 μM KA in the same cells). The effects of KA in either $Na^+$-containing or $Na^+$-free media were not reduced by the NMDA receptor antagonist APV (50 μM) but could be completely blocked by the non-NMDA antagonist CNQX (10 μM) (Fig. 1B). These data suggest that activation of KA/AMPA receptors in these cells can produce $Ca^{2+}$ influx in the absence of $Na^+$-dependent depolarization. A reasonable explanation for this phenomenon is that the KA/AMPA receptors activated are $Ca^{2+}$ permeable.

$Ca^{2+}$-permeable KA/AMPA receptors constructed in oocytes are very nonselective with respect to cation permeability and are quite permeable to $Co^{2+}$ (17), which does not permeate voltage-sensitive $Ca^{2+}$ channels (18), NMDA receptors (19), or $Ca^{2+}$-impermeable KA/AMPA receptors (20). Using a histochemical silver staining method to identify $Co^{2+}$ uptake, Pruss *et al.* (15) have shown that stimulation with KA causes $Co^{2+}$ influx through $Ca^{2+}$-permeable KA/AMPA receptors in neurons. Following this method, we found that 65 ± 6% of the neurons in culture showed $Co^{2+}$-uptake staining after incubation with 100 μM KA (Fig. 1C). This effect was blocked by 20 μM CNQX but not by 50 μM APV. $Co^{2+}$-uptake staining could also be induced by 30 μM KA and inhibited by 10 μM CNQX. The morphology of neurons stained for $Co^{2+}$ uptake suggested that Purkinje cells were included in this population but that other types of neurons with non-Purkinje cell-like morphology were also stained. This impression was confirmed by double-staining of neurons with immunofluorescence for calbindin-D-28K, which is a specific marker for Purkinje cells among cerebellar neurons, and for $Co^{2+}$ uptake. Both calbindin-positive and calbindin-negative cells stained for $Co^{2+}$ uptake; some calbindin-positive cells were negative for $Co^{2+}$ uptake as well (data not shown).

Cerebellar Purkinje cells possess large intracellular $Ca^{2+}$ stores that can be mobilized by caffeine (13). These stores are probably involved in the phenomenon of $Ca^{2+}$-induced $Ca^{2+}$ release. Thus, it seemed possible that intracellular $Ca^{2+}$ stores may contribute to the large KA-stimulated rise in $[Ca^{2+}]_i$ in $Na^+$-free solutions, either solely or by amplification of a small $Ca^{2+}$ influx. However, no increase in $[Ca^{2+}]_i$ was induced by KA in $Ca^{2+}$-free solutions ($n$ = 13 cells). In addition, in cells that showed no response to caffeine (10 mM) substantial increases in $[Ca^{2+}]_i$ were, nevertheless, induced by KA ($\Delta[Ca^{2+}]_i$ = 816 ± 261 nM, $n$ = 4).

Fig. 2 presents experiments further demonstrating that KA causes $Ca^{2+}$ influx that does not involve voltage-sensitive $Ca^{2+}$ channels. Depolarization of neurons with 50 mM $K^+$ produced a substantial $Ca^{2+}$ influx in all neurons ($\Delta[Ca^{2+}]_i$ = 923 ± 94 nM, $n$ = 28). We have previously shown that the organic $Ca^{2+}$ channel antagonist TA-3090 (clentiazem) is an effective but nonselective blocker of high-threshold $Ca^{2+}$ currents in neurons (21). In the present experiments, 50 μM TA-3090 effectively inhibited both $Ca^{2+}$ currents (Fig. 2A) and 50 mM $K^+$-induced $Ca^{2+}$ influxes (reduction of peak $[Ca^{2+}]_i$ of 66 ± 13%, $n$ = 10) in cerebellar neurons. In contrast, when KA-induced $Ca^{2+}$ influxes were examined in 2Ca/NMDG solutions, TA-3090 was ineffective (−6 ± 5%, $n$ = 7) (Fig. 2B). Similarly, under voltage-clamp conditions in 2Ca/NMDG solutions, KA-induced inward currents were unaffected by TA-3090 (Fig. 2C) ($n$ = 4). We also wished to obtain an indication of how much $Ca^{2+}$ entered neurons via voltage-sensitive $Ca^{2+}$ channels, relative to KA/AMPA receptors, in a more physiological medium containing $Na^+$. When cells were examined that only exhibited $Ca^{2+}$ influx when stimulated with KA in $Na^+$-containing media, TA-3090 blocked a proportion of

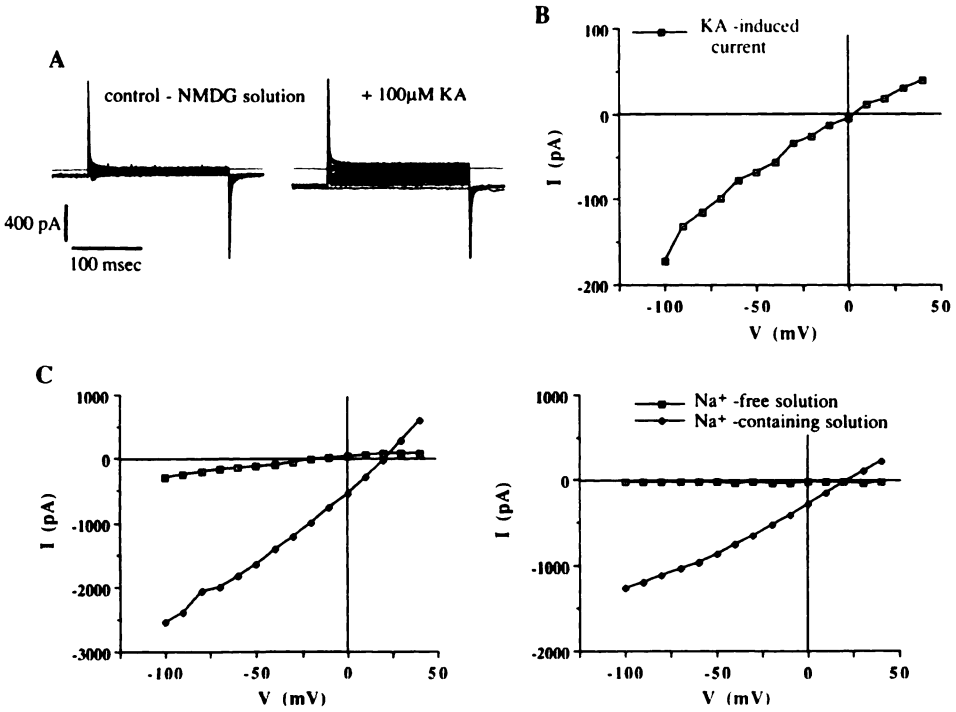


**Fig. 4.** Current-voltage characteristics of the KA-induced conductance. A, Current traces in control and 100 μM KA-containing $Na^+$-free solutions showed that KA induced an inward current at negative potentials. Whole-cell voltage-clamped cells were depolarized from a holding potential of −100 mV to test potentials stepping by 10 mV from −100 mV to +40 mV. No leak subtractions were performed. (External solution, 10 mM/NMDG). B, The KA-induced current-voltage relationship, obtained by subtraction of the currents shown in A, showed inward rectification. The small outward currents found at positive potentials decreased with time in the whole-cell mode and were thought to represent efflux of residual intracellular $K^+$. C, In the same cell (*left*), KA-induced inward currents were about 10-fold greater in $Na^+$-containing (2 mM $Ca^{2+}$/$Na^+$) than in $Na^+$-free solution (2 mM $Ca^{2+}$/NMDG). In contrast (*right*), a nonresponding cell showed no KA-induced inward currents in $Na^+$-free solution (2 mM $Ca^{2+}$/NMDG, 30 μM KA), despite a large inward current in $Na^+$-containing solution.

this $Ca^{2+}$ influx (41 ± 5%, $n$ = 5), as it did the 50 mM $K^+$-induced $Ca^{2+}$ influx in the same cells (70 ± 12%, $n$ = 4). However, in "responding" cells (those with a $Ca^{2+}$ influx when stimulated with KA in $Na^+$-free solutions), TA-3090 produced substantially less inhibition of the KA responses in $Na^+$-containing solutions (13 ± 3%, $n$ = 3), compared with the nonresponding cells ($p$ = 0.01, Student's $t$ test), while still reducing the 50 mM $K^+$-induced increase in $[Ca^{2+}]_i$ to the same extent (63 ± 10%, $n$ = 6; $p$ = 0.675, compared with nonresponding cells). These data are consistent with the idea that, when neurons possess $Ca^{2+}$-permeable KA/AMPA receptors, KA-stimulated $Ca^{2+}$ influx occurs substantially via this pathway even in the presence of $Na^+$.

Another way to eliminate the contributions of voltage-gated $Ca^{2+}$ currents to $Ca^{2+}$ influx is to prevent depolarization of the cell membrane. Fig. 3A demonstrates that KA also produced clear $Ca^{2+}$ influx in $Na^+$-free medium into neurons that were voltage-clamped at −80 mV, a potential at which high-threshold $Ca^{2+}$ currents are not activated in these cells. In keeping with the other types of data presented in this study, 30 μM KA produced $Ca^{2+}$ influx, accompanied by a sustained inward current, in the majority of neurons voltage-clamped at −80 mV (five of seven cells; $\Delta[Ca^{2+}]_i$ ranged from 200 to 800 nM). However, there were also clearly cells that did not respond in this way. Fig. 3B demonstrates a cell not showing $Ca^{2+}$ influx in $Na^+$-free medium; in this cell, no inward current was observed. In these negative neurons, a large KA-induced current was still observed in $Na^+$-containing medium, without a concomitant increase in $[Ca^{2+}]_i$.

The properties of the KA-induced inward currents observed in $Na^+$- and NMDG-containing solutions were further examined under whole-cell voltage-clamp conditions. All neurons responded to KA in $Na^+$-containing medium. Because all neurons expressed KA receptors permeable to monovalent cations, measurements using $K^+$- or $Cs^+$-containing pipette solutions revealed large outwardly rectifying currents, even in $Na^+$-free external solutions. Therefore, in order to isolate the divalent cation permeability of KA receptors, we used an intracellular solution in which all cations were replaced with NMDG and 10 mM BAPTA was used to buffer the $Ca^{2+}$ influx. Under these conditions, 16 of 29 neurons (55%) responded to KA (30 or 100 μM) with inward currents of at least 50 pA in $Na^+$-free solution (2 Ca/NMDG). In cells of culture age 17 days *in vitro* and older, morphological identification of Purkinje neurons became possible (see Ref. 13). Of seven such cells identified *a priori* as Purkinje neurons, six responded to KA.

Further evidence against the contribution of voltage-gated $Ca^{2+}$ channels to the KA-induced inward current came from measurements in solutions containing 100 μM $Cd^{2+}$. This concentration of $Cd^{2+}$, which we found to be sufficient to block all noninactivating voltage-gated $Ca^{2+}$ currents, did not block the KA-induced inward current in 2Ca/NMDG. In five cells, the KA-induced currents in $Cd^{2+}$-containing solutions were 107 ± 7% of the currents in $Cd^{2+}$-free, 2Ca/NMDG solutions.

Current-voltage (I/V) relationships for the KA-induced currents were measured in both $Na^+$-containing and $Na^+$-free media, using 100 μM $Cd^{2+}$ to block the voltage-gated $Ca^{2+}$ currents. In $Na^+$-containing media, KA induced large inward currents throughout the negative voltage range ($n$ = 15), similar to results often previously reported for KA-induced currents (6, 22). In contrast, the I/V relationships of the KA-induced currents recorded in $Na^+$-free external solutions displayed inward rectification ($n$ = 22) (Fig. 4), as has been previously observed in a subset of hippocampal neurons (6, 22) and in oocytes injected with cerebellar messenger RNA (23). In cerebellar neurons, the I/V curves for the KA-induced currents recorded in NMDG shifted in the positive direction when the external $[Ca^{2+}]$ was raised from 2 mM to 10 mM, consistent with the opening of a $Ca^{2+}$-permeable channel (reversal potentials shifted by +14.5 ± 2.0 mV, $n$ = 11).

Even in those cells that responded to KA in $Na^+$-free solutions,

the KA-induced currents were much larger in $Na^+$-containing than in $Na^+$-free media, suggesting that the bulk of the current was through the traditional monovalent cation-permeable KA/AMPA receptors, rather than the divalent cation-permeable KA receptors. It might be thought that the $Ca^{2+}$ permeability measured was merely the small but non-zero divalent cationic permeability of the traditional KA receptor. However, the magnitudes of the KA-induced [$Ca^{2+}$] increases (Fig. 1A) or inward $Ca^{2+}$ currents (Fig. 4C) in $Na^+$-free solutions did not merely scale proportionately with the corresponding responses in $Na^+$-containing solutions, as would be expected if they were due to the same receptor populations. Instead, the KA responses in $Na^+$-free solutions were substantial in some cells and entirely absent in other cells. Thus, although all of the neurons express the $Na^+$-permeable KA receptors, some, in addition, express a fraction of $Ca^{2+}$-permeable KA receptors.

These data clearly demonstrate that KA can activate a substantial $Ca^{2+}$ influx in Purkinje cells and other cerebellar neurons that does not require cell depolarization and influx via voltage-sensitive $Ca^{2+}$ channels. It is likely that these $Ca^{2+}$-permeable, KA-activated channels are formed by the association of KA receptor binding subunits similar or identical to those shown to produce $Ca^{2+}$-permeable channels in oocytes (11, 12). What seems particularly significant is that this phenomenon does not occur in only a minority of neurons but occurs in the majority of cultured cells, where these receptors seem to coexist with traditional types of non-$Ca^{2+}$-permeable KA receptors. Moreover, judging by $Co^{2+}$-uptake staining data (15), these results are not confined to cell culture but are also observed in the mature rat brain. Indeed, several of the non-NMDA receptor subtypes have been shown by *in situ* hybridization to be particularly heavily expressed in cerebellar Purkinje cells in adult rat brain sections (24). This $Ca^{2+}$-permeable pathway may be of great significance in mediating excitatory amino acid-induced phenomena of various types, particularly in instances where NMDA receptors do not appear to operate.

**Acknowledgments**

We wish to thank Patricia Manzolillo for assistance with culturing and with $Co^{2+}$ uptake staining.

**References**

1. Mayer, M. L., and R. J. Miller. Excitatory amino acid receptors: regulation of neuronal $[Ca^{2+}]_i$ and other second messengers. *Trends Pharmacol. Sci.* **11**:254–260 (1990).
2. Meldrum, B., and J. Garthwaite. Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11**:379–387 (1990).
3. Collingridge, G. C., and W. Singer. Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol. Sci.* **11**:290–297 (1990).
4. Murphy, S. N., and R. J. Miller. Regulation of $Ca^{2+}$ influx into striatal neurons by kainic acid. *J. Pharmacol. Exp. Ther.* **249**:184–193 (1989).
5. Holopainen, I., M. O. K. Enkvist, and K. E. O. Akerman. Glutamate receptor agonists increase intracellular $Ca^{2+}$ independently of voltage gated $Ca^{2+}$ channels in rat cerebellar granule cells. *Neurosci. Lett.* **98**:57–62 (1989).
6. Iino, M., S. Ozawa, and K. Tsuzuki. Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. *J. Physiol. (Lond.)* **424**:151–165 (1990).
7. Ogura, A., K. Akita, and Y. Kudo. Non-NMDA receptor mediates cytoplasmic $Ca^{2+}$ elevation in cultured hippocampal neurones. *Neurosci. Res.* **9**:103–113 (1990).
8. Ogura, A., M. Nakazawa, and Y. Kudo. Further evidence for calcium permeability of non-NMDA receptor channels in hippocampal neurons. *Neurosci. Res.*, **12**:606–612 (1992).
9. Glaum, S. R., J. Holzwarth, and R. J. Miller. Metabotropic glutamate receptors activate $Ca^{2+}$ mobilization and $Ca^{2+}$ influx in astroglial cells. *Proc. Natl. Acad. Sci. USA* **87**:3454–3458 (1990).
10. Jensen, A. M., and S. Y. Chiu. Differential intracellular calcium responses to glutamate in type 1 and type 2 cultured rat brain astrocytes. *J. Neurosci.* **11**:1674–1684 (1991).
11. Hollman, M., M. Hartley, and S. Heinemann. $Ca^{2+}$ permeability of KA-AMPA gated glutamate channels depends on subunit composition. *Science (Washington D. C.)* **252**:851–853 (1991).
12. Miller, R. J. The revenge of the kainate receptor. *Trends Neurosci.* **14**:477–479 (1991).
13. Brorson, J. R., D. Bleakman, S. Gibbons, and R. J. Miller. The properties of intracellular $Ca^{2+}$ stores in cultured rat cerebellar neurons. *J. Neurosci.* **11**:4024–4043 (1991).
14. Thayer, S. A., M. Sturek, and R. J. Miller. Measurement of neuronal $Ca^{2+}$ transients using simultaneous microfluorimetry and electrophysiology. *Pfluegers Arch.* **412**:216–223 (1988).
15. Pruss, R. M., R. C. Akeson, M. M. Racke, and J. L. Wilburn. Agonist activated cobalt uptake identifies divalent cation-permeable kainate receptors on neurons and glial cells. *Neuron* **7**:509–518 (1991).
16. Gho, M., A. E. King, Y. Ben-Ari, and E. Cherubini. Kainate reduces two voltage-dependent potassium conductances in rat hippocampal neurons *in vitro*. *Brain Res.* **385**:411–414 (1986).
17. McGurk, J. F., R. S. Roginski, R. S. Zukin, and M. V. L. Bennett. Divalent ion permeability and pharmacology of cloned kainate/AMPA receptors expressed in oocytes. *Soc. Neurosci. Abstr.* **17**:335 (1991).
18. Hagiwara, S., and L. Byerly. Calcium channels. *Annu. Rev. Neurosci.* **4**:69–127 (1981).
19. Mayer, M. L., and G. L. Westbrook. Permeation and block of *N*-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. *J. Physiol. (Lond.)* **394**:501–527 (1987).
20. Gu, Y., and L. Y. Huang. Block of kainate receptor channels by $Ca^{2+}$ in isolated spinal trigeminal neurons of rat. *Neuron* **6**:777–784 (1991).
21. Bleakman, D., P. S. Chard, S. Foucart, and R. J. Miller. Block of neuronal $Ca^{2+}$ influx by the antiischemic agent TA3090. *J. Pharmacol. Exp. Ther.* **259**:430–438 (1991).
22. Ozawa, S., M. Iino, and K. Tsuzuki. Two types of kainate response in cultured rat hippocampal neurons. *J. Neurophysiol.* **66**:2–11 (1991).
23. Randle, J. C. R., P. Vernier, A.-M. Garrigues, and E. Brault. Properties of the kainate channel in rat brain in RNA injected *Xenopus* oocytes: ionic selectivity of blockage. *Mol. Cell. Biochem.* **80**:121–132 (1988).
24. Keinanen, K., W. Wisden, B. Sommer, P. Werner, A. Herb, T. A. Verdoorn, B. Sakmann, and P. H. Seeburg. A family of AMPA selective glutamate receptors. *Science (Washington D. C.)* **249**:556–560 (1990).

**Send reprint requests to:** Richard J. Miller, Ph.D., Department of Pharmacological and Physiological Sciences, The University of Chicago, 947 East 58th Street, Chicago, IL 60637.