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Elevated Extracellular K^+ Concentrations Inhibit *N*-Methyl-D-Aspartate-Induced Ca^{2+} Influx and Excitotoxicity

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ABSTRACT

Although extracellular $[K^+]$ ($[K^+]_E$) is highly elevated during brain ischemia, in vitro studies aimed at explaining the mechanisms of excitotoxicity have been conducted at low $[K^+]_E$. Whether high $[K^+]_E$ affects excitotoxicity has not been formally addressed. Therefore this study, using digital fluorescence microscopy, tested how the elevation of $[K^+]_E$ from 5.6 to 60 mM affects *N*-methyl-D-aspartate (NMDA)-induced Ca^{2+} and Na^+ influx, plasma membrane (PM) potential, mitochondrial Ca^{2+} load, and viability of primary cultures of rat cerebellar granule cells. High $[K^+]_E$ curtailed the NMDA-induced Ca^{2+} and Na^+ influx and mitochondrial Ca^{2+} overload, and prevented neuronal death. Surprisingly, the inhibitory effect of high $[K^+]_E$ on the NMDA-induced Ca^{2+} influx could not be linked to depolarization of the PM. Apparently, the PM of cerebellar granule cells exposed to NMDA was more depolarized at low than at high $[K^+]_E$, probably because the NMDA-induced Na^+ influx was greatly enhanced when the extracellular $[Na^+]/[K^+]$ ratio was increased. When this ratio was small, i.e., at high $[K^+]_E$, the NMDA-induced increase in cytoplasmic $[Na^+]$ was suppressed, preventing Ca^{2+} influx via the reverse operation of the Na^+/Ca^{2+} exchanger, which may explain the inhibitory effect of high $[K^+]_E$ on NMDA-induced Ca^{2+} influx and excitotoxicity.

Excitotoxicity has been linked causally with glutamate-elicited Ca^{2+} influx (Hartley et al., 1993; Eimerl and Schramm, 1994) and Ca-dependent mitochondrial depolarization (Budd and Nicholls, 1996; Schinder et al., 1996; White and Reynolds, 1996; Stout et al., 1998). Activation of ionotropic glutamate receptors depolarizes the plasma membrane (PM) and opens several Ca-permeable pathways: ionic channels of glutamate receptors (Mayer and Westbrook, 1987), voltage-sensitive Ca^{2+} channels (Reichling and MacDermott, 1993), and the Ca^{2+} influx resulting from the operation of the plasma membrane Na^+/Ca^{2+} exchanger in the reverse mode (Kiedrowski et al., 1994; Hoyt et al., 1998; Kiedrowski, 1999). In vitro experiments aimed at defining the pathway of the excitotoxic Ca^{2+} entry have shown that Ca^{2+} influx via *N*-methyl-D-aspartate (NMDA) receptors mediates glutamate excitotoxicity (Choi et al., 1988; Manev et al., 1989; Tymianski et al., 1993). Those experiments were conducted on cultured neurons incubated in media containing Na^+ and K^+ concentrations characteristic of neurons under resting conditions. It is well established, however, that within a few minutes of brain ischemia, extracellular concentrations of K^+ ($[K^+]_E$) reach 60 mM or higher, and extracellular Na^+ concentrations ($[Na^+]_E$) decrease to about 60 mM (Somjen, 1979; Hansen et al., 1980; Hansen, 1985; Erecinska and Silver, 1994). This implies that the Na^+ and the K^+ concentration gradients across the PM (Na/K gradient) are profoundly destabilized when the glutamate excitotoxicity in vivo is executed. Yet, how destabilization of the Na/K gradient affects the mechanisms of glutamate excitotoxicity has never been formally studied. One can suspect that the role of the Na/K gradient may be important in excitotoxicity because depolarization of the PM strongly affects the NMDA-induced Ca^{2+} influx (1999). Therefore, the present study was designed to determine how the elevation of $[K^+]_E$ combined with the decrease of $[Na^+]_E$ affect excitotoxicity.

Experimental Procedures

Neuronal Cultures. Primary cultures of cerebellar granule cells (CGCs) were prepared from 8-day-old Sprague-Dawley rats and were plated, using basal Eagle's medium supplemented with 25 mM KCl, 10% bovine fetal serum, 2 mM glutamine, and 50 μg/ml gentamycin,

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ABBREVIATIONS: PM, plasma membrane; CaDF, electrochemical force for Ca^{2+} influx; CGCs, cerebellar granule cells; CM, conditioned medium; $DiBAC_4(3)$, bis(1,3-dibutylbarbituric acid)trimethine oxonol; E_m, plasma membrane potential; F_{334}/F_{380}, ratio of fluorescence intensities emitted after 334 nm and 380 nm excitation; $[K^+]_E$ and $[Na^+]_E$, extracellular concentration of K^+ and Na^+, respectively; $[Na^+]_C$, $[K^+]_C$ and $[Ca^{2+}]_C$, cytoplasmic concentration of Na^+, K^+, and Ca^{2+}, respectively; NaCaX, Na^+/Ca^{2+} exchanger; nF_{488}, normalized fluorescence intensity emitted after excitation at 488 nm; NMDA, *N*-methyl-D-aspartate; SBFI, Na^+-binding benzofuran isophthalate; R/NaCaX, reverse operation of the Na^+/Ca^{2+} exchanger.

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as described in Kiedrowski (1999). Cultures at 8 to 11 days in vitro were used for the experiments.

Media. Experimental solutions were based on Locke's buffer that contained 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.4, adjusted with Tris. The desired concentrations of K^+ or Li^+ in these experimental solutions were achieved by an equimolar substitution of Na^+ with K^+ or Li^+. CGCs were exposed to these solutions at 37°C using a static bath. To apply a new solution, the previous solution was aspirated and the cells were washed with the new solution several times. $[K^+]_E$ was never decreased below 5.6 mM to prevent inhibition of Na^+/K^+ ATPase due to lack of extracellular K^+. Glutamate (1 mM unless indicated otherwise) or NMDA (300 μM) was applied in Mg-free Locke's containing 10 μM glycine. Depolarizing pulses of 60 mM K^+ were delivered in the presence of Mg^{2+} (1 mM) and MK-801 (10 μM) to prevent Ca^{2+} influx caused by activation of NMDA receptors by endogenous glutamate. Mitochondrial depolarization was carried out by application of Locke's buffer containing 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3 μg/ml oligomycin (Budd and Nicholls, 1996).

Viability Test. After excitotoxic challenge the cells were returned to a conditioned basal Eagle's medium (CM), i.e., the medium (containing 10% bovine fetal serum and 25 mM K^+), in which the cells were cultured. The CM was supplemented with 10 μM propidium iodide, and the viability of CGCs was quantified after approximately 24 h by detecting the propidium iodide fluorescence as described in Kiedrowski (1999) and illustrated in Fig. 1.

Simultaneous Assay of Cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_C$) and Plasma Membrane Potential (E_m). $[Ca^{2+}]_C$ and E_m were monitored in single CGCs loaded with 4 μM fura-2 acetoxymethyl ester and exposed to 100 nM bis(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$], as recently described (Kiedrowski, 1999). Briefly, the fluorescences of fura-2 and $DiBAC_4(3)$ were monitored at 37°C using the Attofluor digital imaging system (Atto Instruments, Rockville, MD), a Zeiss Axiovert 10 microscope, and a Zeiss Achrostigmat objective (40×, NA 1.30). The images of fluorescence emitted at over 520 nm after excitation at 334 nm (F_{334}) and 380 nm (F_{380}) for fura-2, and at 488 nm (F_{488}) for $DiBAC_4(3)$, were saved every 10 to 20 s. The $DiBAC_4(3)$ fluorescence measured in regions of interest in the peripheral parts of neuronal somata just underneath the PM was normalized and was used as a relative index of E_m. The fura-2 F_{334}/F_{380} ratio measured in the central part of somata was used as an index of $[Ca^{2+}]_C$ and was calibrated in situ. It must be stressed, however, that changes in the fura-2 F_{334}/F_{380} ratio do not always reflect $[Ca^{2+}]_C$ changes, i.e., when the fluorescent properties of fura-2 are affected by the NMDA-induced changes in cytoplasmic pH (Kiedrowski, 1999).

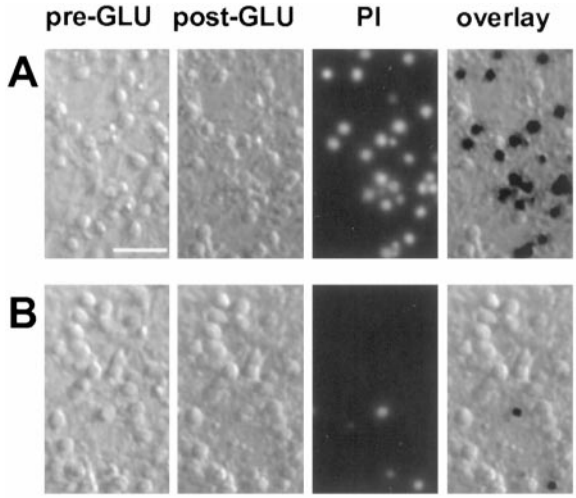

Fig. 1. Glutamate is neurotoxic at 5.6 mM $[K^+]_E$ but not at 60 mM $[K^+]_E$. CGCs were exposed to glutamate (1 mM glutamate plus 10 μM glycine and Mg-free medium) for 90 min at 5.6 mM $[K^+]_E$ (A) or 60 mM $[K^+]_E$ (B). Oblique illumination images of cells before the exposure (pre-GLU), 24 h after the exposure (post-GLU), the propidium iodide fluorescence image 24 h after the exposure (PI), and the overlay image that was obtained by subtracting digitally the image "PI" from the image "post-Glu" are shown. Scale bar, 30 μm. The combined data from three independent experiments are presented in Table 1.

Assay of Cytoplasmic $[Na^+]$ ($[Na^+]_C$). CGCs were loaded for 60 min at 37°C with 20 μM Na^+-binding benzofuran isophthalate acetoxymethyl ester (SBFI AM) dissolved in CM. The stock concentration of SBFI AM was 5 mM in dimethyl sulfoxide. SBFI fluorescence was monitored at 37°C using the same excitation and emission settings as described above for fura-2. The F_{334}/F_{380} ratio was calibrated for $[Na^+]_C$ in situ at the end of the experiments using five to six Na^+ concentrations in a range from 0 to 163 mM. The buffers used to calibrate $[Na^+]_C$ contained 5 μM gramicidin D and were prepared by appropriate mixing of high-concentration solutions of Na^+ described previously (Kiedrowski, 1999) with an analogous Na-free solution in which all Na^+ was substituted with K^+. $[Na^+]_C$ values were calculated using a nonlinear least-squares fit of the data to the Michaelis-Menten equation as described by Kasner and Ganz (1992).

$^{45}Ca^{2+}$ Uptake. Uptake of $^{45}Ca^{2+}$ was measured as described previously (Kiedrowski, 1999).

Materials. Fura-2 AM, SBFI AM, and $DiBAC_4(3)$ were obtained from Molecular Probes (Eugene, OR), MK-801 [(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine] and CPP [*R*-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid] were purchased from Research Biochemicals Inc. (Natick, MA) or Tocris (Ballwin, MO), and $^{45}Ca^{2+}$ was obtained from Amersham (Arlington Heights, IL). The culture media and all other chemicals were purchased from Sigma (St. Louis, MO).

Results and Discussion

High $[K^+]_E$ Inhibits Excitotoxicity. To test the impact of high $[K^+]_E$ on excitotoxicity, CGCs were exposed for 90 min at 37°C to glutamate (1 mM glutamate, 10 μM glycine, and Mg-free medium) or NMDA (300 μM NMDA, 10 μM glycine, and Mg-free medium) at 5.6 or 60 mM $[K^+]_E$, and neuronal viability was assessed after 24 h (for details see *Experimental Procedures*). At 60 mM $[K^+]_E$, neither glutamate nor NMDA was neurotoxic (Fig. 1 and Table 1); moreover the glutamate excitotoxicity induced at 5.6 mM $[K^+]_E$ was completely prevented if 10 μM MK-801 or 100 μM CPP, a noncompetitive or competitive inhibitor of NMDA receptors, respectively, was present in the medium during the exposure (data not shown). The data indicate that 60 mM $[K^+]_E$ prevents the excitotoxicity resulting from NMDA receptor activation.

High $[K^+]_E$ Inhibits NMDA-Induced Ca^{2+} Influx. When $[K^+]_E$ was increased from 5.6 to 60 mM, the cytoplasmic $^{45}Ca^{2+}$ accumulation elicited by glutamate or NMDA was inhibited in a dose-dependent manner (Fig. 2A). This inhibition of $^{45}Ca^{2+}$ accumulation by high $[K^+]_E$ could not be explained by improved Ca^{2+} extrusion, because the rate of $^{45}Ca^{2+}$ efflux from CGCs preloaded with $^{45}Ca^{2+}$ was the same regardless of $[K^+]_E$ (Fig. 2B). These data indicate that high $[K^+]_E$ inhibits the NMDA-induced Ca^{2+} influx.

High $[K^+]_E$ Prevents Mitochondrial Ca^{2+} Overload in Neurons Exposed to Glutamate. Glutamate excitotoxicity has been linked causally to Ca^{2+} influx (Hartley et al., 1993; Eimerl and Schramm, 1994), and the consequent Ca^{2+} influx-dependent mitochondrial depolarization (Budd and Nicholls, 1996; Khodorov et al., 1996; Schinder et al., 1996;

White and Reynolds, 1996; Stout et al., 1998). To test whether high $[K^+]_E$ modifies mitochondrial buffering of Ca^{2+} in CGCs exposed to glutamate, how mitochondrial depolarization with 10 μM CCCP plus 3 μg/ml oligomycin (Budd and Nicholls, 1996) affects $[Ca^{2+}]_C$ was studied. CCCP plus oligomycin were expected to depolarize mitochondria, causing a release of the Ca^{2+} stored in the mitochondrial matrix, which can be detected as a rapid increase in $[Ca^{2+}]_C$.

$[Ca^{2+}]_C$ was monitored in CGCs exposed for 15 min to glutamate (1 mM glutamate plus 10 μM glycine and Mg-free Locke's) at $[K^+]_E$ of 5.6 or 60 mM. It was observed that 10 min after the glutamate application, $[Ca^{2+}]_C$ stabilized at the same plateau level regardless of $[K^+]_E$: 528 ± 15 nM (n = 97) at 5.6 mM $[K^+]_E$ and 553 ± 21 nM (n = 98) at 60 mM $[K^+]_E$. When CCCP plus oligomycin were then added to the same CGCs, $[Ca^{2+}]_C$ increased from the above-indicated plateau level to a level at which fura-2 became saturated with Ca^{2+} (over 2 μM) in as many as 69 of 97 neurons exposed to glutamate at 5.6 mM $[K^+]_E$, but in only 19 of 98 neurons exposed to glutamate at 60 mM $[K^+]_E$. These results indicate that at 60 mM $[K^+]_E$, the glutamate-induced Ca^{2+} influx across the PM is so much decreased that Ca^{2+} homeostasis can be maintained without overloading mitochondria with Ca^{2+}.

At High $[K^+]_E$ NMDA Induces Less Pronounced Depolarization of PM than at Low $[K^+]_E$ Depolarization of the PM curtails the NMDA-induced Ca^{2+} influx by affecting the electrochemical driving force for Ca^{2+} influx (CaDF), defined as the difference between the PM potential (E_m) and the Ca^{2+} equilibrium potential (E_{Ca}) (Kiedrowski, 1999). Therefore, one may envision that the mechanism of inhibition of the NMDA-induced Ca^{2+} influx by high $[K^+]_E$ involves a decrease in the CaDF due to PM depolarization. This explanation would only be valid, however, if the PM depolarization induced by NMDA at 60 mM $[K^+]_E$ were indeed greater than at 5.6 mM $[K^+]_E$. To test whether this is the case, the effects of $[K^+]_E$ on E_m and $[Ca^{2+}]_C$ were studied by monitoring the fluorescences of $DiBAC_4(3)$ and fura-2, respectively, in single CGCs exposed to NMDA.

In control experiments, increasing $[K^+]_E$ from 5.6 to 60 mM caused a typical $[Ca^{2+}]_C$ transient associated with an increase in $DiBAC_4(3)$ fluorescence intensity (Fig. 3A), representing depolarization of the PM (Kiedrowski, 1999). By contrast, in CGCs exposed to NMDA (300 μM NMDA plus 10 μM glycine and Mg-free medium), a switch in $[K^+]_E$ from 60 to 5.6 mM induced a further increase in $DiBAC_4(3)$ fluorescence intensity and a short-lasting increase in $[Ca^{2+}]_C$ (Fig. 3B). On the other hand, when $[K^+]_E$ was increased from 5.6 to 60 mM, the $DiBAC_4(3)$ fluorescence intensity started to decrease, and $[Ca^{2+}]_C$ dropped temporarily (Fig. 3 C). Within 2 min after the change in $[K^+]_E$, $[Ca^{2+}]_C$ stabilized at a similar level regardless of $[K^+]_E$ (Fig. 3, B and C). The rate of increase in the $DiBAC_4(3)$ fluorescence was faster, and the peak increase in $[Ca^{2+}]_C$ was larger, when NMDA was applied with 60 mM $[K^+]_E$ (Fig. 3B) than with 5.6 mM $[K^+]_E$ (Fig. 3C). Very likely, a greater fraction of voltage-gated Ca^{2+} channels contributed Ca^{2+} influx when NMDA and 60 mM $[K^+]_E$ were simultaneously applied. The short-lasting changes in $[Ca^{2+}]_C$ observed when $[K^+]_E$ values were changed during the neuronal exposure to NMDA (Fig. 3, B and C) might be explained by transient changes in the CaDF, which, however, needs to be tested further.

The effect of $[K^+]_E$ on E_{Ca} can be analyzed using the Nernst equation:

$$E_{Ca} = RT/2F \times \ln[Ca^{2+}]_E/[Ca^{2+}]_C,$$

where R, T, and F have their usual meanings, and $[Ca^{2+}]_E$ represents extracellular $[Ca^{2+}]$.

Because of the large volume of the extracellular medium in vitro, it can be assumed that $[Ca^{2+}]_E$ is a constant. Thus the only parameter that affects E_{Ca} is $[Ca^{2+}]_C$. Because $[Ca^{2+}]_C$ stabilized at the same levels regardless of $[K^+]_E$, it appears that the E_{Ca} component of the CaDF at each $[K^+]_E$ stabilized

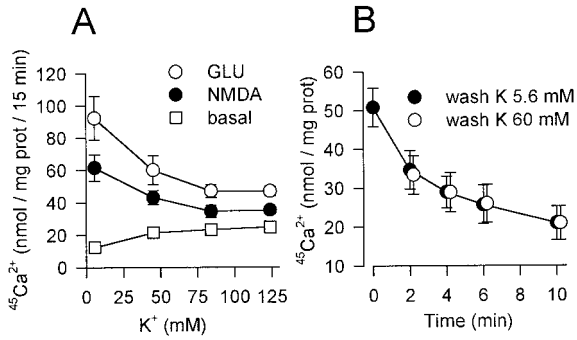

Fig. 2. High $[K^+]_E$ inhibits NMDA- or glutamate-elicited $^{45}Ca^{2+}$ uptake (A) but does not affect $^{45}Ca^{2+}$ extrusion (B). Presented are the means ± S.E.M. from three independent experiments. A, $^{45}Ca^{2+}$ uptake was stimulated by 300 μM NMDA or 100 μM glutamate (GLU) applied in Mg-free Locke's buffer supplemented with 10 μM glycine. Basal $^{45}Ca^{2+}$ uptake was measured in the presence of 10 μM MK-801 to inhibit the Ca^{2+} influx resulting from the activation of NMDA receptors by endogenous glutamate. B, CGCs were incubated for 5 min at 37°C with 20 μM glutamate applied with Mg-free Locke's buffer containing $^{45}Ca^{2+}$ (1 μCi) and 10 μM glycine, then the cells were washed with a nonradioactive Locke's buffer containing either 5.6 or 60 mM K^+, and the amounts of $^{45}Ca^{2+}$ trapped in the cells at the indicated time intervals were determined.

TABLE 1

High $[K^+]_E$ concentrations protect CGCs against NMDA or glutamate excitotoxicity

CGCs were exposed for 90 min to Locke's buffer containing the indicated concentrations of K^+ and 300 μM NMDA or 1 mM glutamate (GLU). During the exposure to NMDA or GLU, the Locke's buffer was Mg-free and contained 10 μM glycine. Neuronal viability was determined after 20 to 24 h using the approach illustrated in Fig. 1. The combined data from three to six independent experiments are presented. * $P < .01$ compared with the respective exposures at 60 mM K^+ (one-way ANOVA followed by Newman-Keuls test).

Exposure	No. Experiments	No. Dead Cells	No. Surviving Cells	Viability
				% ± *S.E.M.*
K^+, 5.6 mM	6	7	895	99 ± 0.8
K^+, 60 mM	6	8	1061	99 ± 0.6
K^+, 5.6 mM, + NMDA	3	257	209	48 ± 13.8*
K^+, 60 mM, + NMDA	3	26	537	96 ± 1.6
K^+, 5.6 mM, + GLU	3	283	67	20 ± 2.3*
K^+, 60 mM, + GLU	3	20	407	95 ± 1.1

at the same level. On the other hand, the $DiBAC_4(3)$ fluorescence data seem to indicate that the PM of CGCs exposed to NMDA was, paradoxically, more depolarized at low than at high $[K^+]_E$. Considering that the $DiBAC_4(3)$ fluorescence is an indirect index of E_m, the changes of this fluorescence in terms of E_m have to be interpreted cautiously. A mechanism by which an increase in the extracellular $[Na^+]/[K^+]$ ratio may depolarize the PM in CGCs exposed to NMDA, as well as alternative interpretations of the changes in $DiBAC_4(3)$ fluorescence intensity, are discussed below.

At High $[K^+]_E$ NMDA-Induced Na^+ Influx Is Greatly Decreased. Because the NMDA-induced depolarization of the PM is caused by Na^+ influx (Hösli et al., 1973), it is possible that the counterintuitive effect of $[K^+]_E$ on E_m in CGCs exposed to NMDA, i.e., the greater depolarization of the PM at lower $[K^+]_E$ (Fig. 3B), might be the result of a greater Na^+ influx. To test this hypothesis, the effects of $[K^+]_E$ on $[Na^+]_C$ in CGCs were studied under control conditions and following exposure to NMDA. As shown in Fig. 4A, under control conditions (no NMDA added), changes in $[K^+]_E$ had only minor effects on $[Na^+]_C$: an increase of $[K^+]_E$ from 5.6 to 35.6 mM caused a transient increase in $[Na^+]_C$ from a basal level of about 2 to 3 mM to 8 ± 0.8 mM, followed by a drop and stabilization at 6 ± 0.5 mM. Upon a subsequent increase of $[K^+]_E$ to 65.6 mM, $[Na^+]_C$ dropped to 4 ± 0.4 mM. When the same CGCs were then exposed to NMDA at different $[K^+]_E$, there was an inverse relationship between $[K^+]_E$ and $[Na^+]_C$: at 65.6 mM $[K^+]_E$, $[Na^+]_C$ increased to 26 ± 2.1 mM; when $[K^+]_E$ was decreased to 35.6 mM, $[Na^+]_C$ increased to 48 ± 4.3 mM; $[Na^+]_C$ increased further, to as much as 89 ± 12.4 mM, when $[K^+]_E$ was decreased to the 5.6 mM level (Fig. 4A). The decrease in $[Na^+]_C$ that was induced by application of high $[K^+]_E$ in CGCs exposed to NMDA was promptly reverted by inhibiting Na^+/K^+ ATPase with 1 mM ouabain (Fig. 4B). This indicates that Na^+/K^+ ATPase activity is involved in lowering $[Na^+]_C$ when $[K^+]_E$ is increased. The fact that at 5.6 mM $[K^+]_E$, NMDA exposure excessively elevates $[Na^+]_C$ in CGCs (Fig. 4A) implies that under these conditions the NMDA-induced Na^+ influx exceeds the rate of Na^+ extrusion by Na^+/K^+ ATPase; indeed Na^+/K^+ ATPase is saturated when $[Na^+]_C$ exceeds 30 mM (Collins et al., 1992). Very likely, this enormous, NMDA-induced elevation in $[Na^+]_C$ in a neuronal culture is an experimental artifact caused by the great excess of extracellular over cytoplasmic volume; because of that, the NMDA-induced Na^+ influx does not lead to any significant drop in $[Na^+]_E$. By contrast, the extracellular space is only about 20% of brain volume (Nicholson and Sykova, 1998), and therefore the Na^+ influx during brain ischemia is associated with a significant drop in $[Na^+]_E$, a limiting factor for Na^+ influx.

The increase in $DiBAC_4(3)$ fluorescence intensity upon a switch from 60 to 5.6 mM $[K^+]_E$ in CGCs exposed to NMDA (Fig. 3B) may indicate that the depolarizing effect of the NMDA-induced Na^+ influx overcomes the hyperpolarizing effect of decreasing $[K^+]_E$ from 60 to 5.6 mM. A calculation of how much the PM might depolarize was not attempted because this study monitored $[Na^+]_C$ in the bulk of the cytoplasm, whereas $[Na^+]_C$ in the immediate vicinity of the PM, which remains unknown, may differ significantly (Wendt-Gallitelli et al., 1993).

It should be considered that $DiBAC_4(3)$ fluorescence might be affected by other factors than E_m changes. For example, the NMDA-induced Na^+ fluxes across the plasma membrane might affect the electric potentials of membranes of cytoplasmic organelles, which might affect $DiBAC_4(3)$ distribution within the cytoplasm and $DiBAC_4(3)$ fluorescence. Given the fact that $DiBAC_4(3)$ fluorescence intensity is greatly enhanced upon binding to membranes, the changes in the membrane surface caused by swelling or shrinkage may also affect the $DiBAC_4(3)$ fluorescence intensity. Whether these possibilities are relevant to the paradoxical increase in the $DiBAC_4(3)$ fluorescence intensity elicited by low $[K^+]_E$ in CGCs exposed to NMDA remains at present unclear.

Can the Inhibitory Effect of High $[K^+]_E$ on NMDA-Induced Ca^{2+} Influx Be Related to Plasma Membrane Na^+/Ca^{2+} Exchange Operation? Because an increase in $[K^+]_E$ decreases $[Na^+]_C$ in neurons exposed to NMDA (Fig. 4,

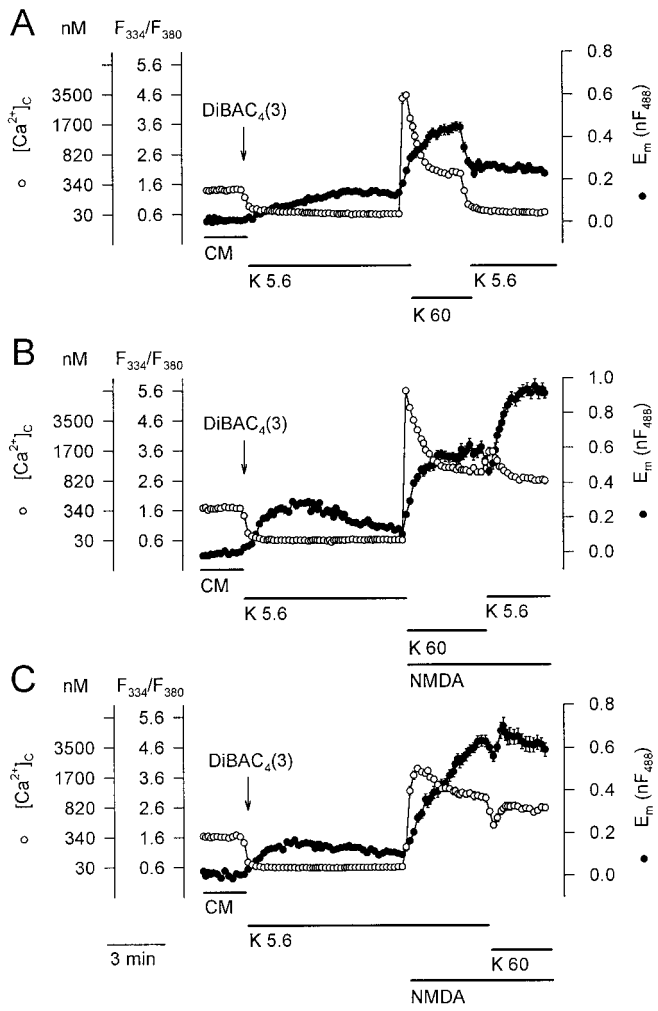

Fig. 3. CGCs exposed to NMDA are more depolarized at low than at high $[K^+]_E$. $[Ca^{2+}]_C$ and E_m were simultaneously monitored in CGCs exposed to the indicated $[K^+]_E$. Initially CGCs were incubated with CM without $DiBAC_4(3)$, then CM was replaced with Locke's buffer containing 100 nM $DiBAC_4(3)$ (arrows) and 5.6 mM K^+ (K5.6). $DiBAC_4(3)$ (100 nM) was present in the extracellular medium at all times thereafter. Data are the means ± S.E.M. from 15 to 24 neurons in representative experiments that were repeated at least three times with similar results. The S.E.M. are small and therefore in most cases are obscured by the circles representing the data points. A, control, CGCs exposed to the indicated increase in $[K^+]_E$ alone; 60 mM $[K^+]_E$ (K60) was applied together with 10 μM MK-801 to prevent the activation of NMDA receptors by endogenous glutamate. B and C, CGCs treated with the indicated $[K^+]_E$ and exposed to NMDA.

A and B), it must be considered that high $[K^+]_E$ may inhibit the Ca^{2+} influx resulting from the reverse operation of the plasmallemal Na^+/Ca^{2+} exchanger (R/NaCaX). Because half-maximal activation of the R/NaCaX occurs at 38 mM $[Na^+]_C$ (Hilgemann, 1989), one may expect that at 60 mM $[K^+]_E$, when $[Na^+]_C$ is 27 mM, the R/NaCaX will transport less Ca^{2+} than at 60 mM $[K^+]_E$, when $[Na^+]_C$ is 80 mM (Fig. 4B).

If high $[K^+]_E$ indeed inhibits the NMDA-induced Ca^{2+} influx by preventing $[Na^+]_C$ from reaching the R/NaCaX activating levels, one might expect that 60 mM $[K^+]_E$ should not affect the NMDA-induced Ca^{2+} influx in neurons in which the R/NaCaX has already been activated by high $[Na^+]_C$. This turns out to be the case, because as shown in Fig. 5A, 60 mM $[K^+]_E$ failed to affect $^{45}Ca^{2+}$ accumulation in CGCs that were pre-exposed to NMDA at 5.6 mM $[K^+]_E$ (see experiment 3 in Fig. 5A), conditions under which $[Na^+]_C$ is elevated to 80 mM (Fig. 4B). This result suggests that 60 mM $[K^+]_E$ does not inhibit the Ca^{2+} influx directly via the NMDA receptor channels, but does so indirectly by preventing $[Na^+]_C$ from reaching the R/NaCaX activating levels.

To further test the presumption that the R/NaCaX operation may be modified at high $[K^+]_E$, the effects of K^+ on the NMDA-induced Ca^{2+} influx were compared with the effects of Li^+, a well known inhibitor of Ca^{2+} influx via the R/NaCaX (Hilgemann, 1989; Hume et al., 1991). It was observed that Li^+ mimicked the inhibitory effect of K^+ on the NMDA-induced $^{45}Ca^{2+}$ influx (Fig. 5B, left). As expected, upon substitution of Na^+ with Li^+ under control conditions (no NMDA receptor activation) the PM failed to acutely depolarize (data not shown), and therefore, in contrast to K^+, Li^+ did not affect the basal $^{45}Ca^{2+}$ influx (Fig. 5B, right).

The inhibitory effect of Li^+ on the NMDA-induced $^{45}Ca^{2+}$ uptake can be explained in the following manner. Li^+ can permeate NMDA-receptor channels (Tsuzuki et al., 1994) but, in contrast to Na^+ or K^+, is not pumped effectively across the PM by Na^+/K^+ ATPase (Hemsworth et al., 1997; Kiedrowski, 1999). As a result, cytoplasmic $[Li^+]$ increases and Li^+ displaces Na^+ from its binding site at the cytoplasmic surface of the NaCaX, which inhibits the R/NaCaX-mediated Ca^{2+} influx; apparently, high cytoplasmic $[Li^+]$ or $[K^+]$ inhibits R/NaCaX via the same mechanism.

It has to be emphasized, however, that an alternative or concurrent explanation of the mechanism by which extracellular K^+ or Li^+ may affect NaCaX function is possible. An

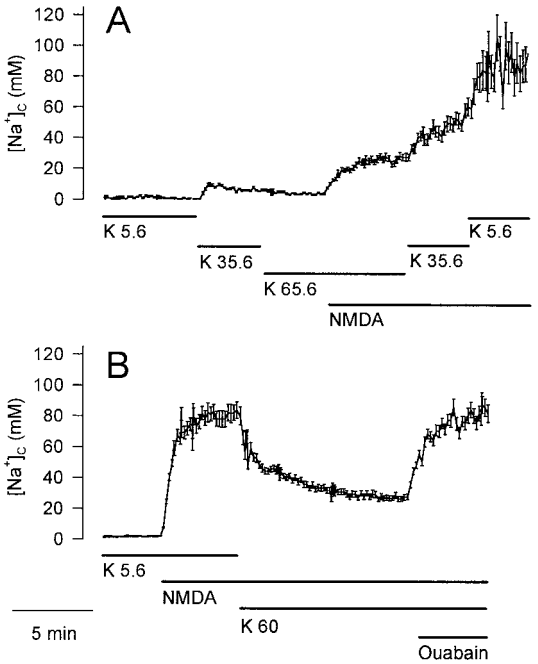

Fig. 4. Effects of $[K^+]_E$ on the NMDA-induced changes in $[Na^+]_C$ in CGCs. A, decreasing $[K^+]_E$ of CGCs exposed to NMDA leads to an increase in $[Na^+]_C$. $[Na^+]_C$ was monitored in CGCs that were exposed to the indicated $[K^+]_E$ changes first in the absence and then in the presence of NMDA, as indicated. Data are the means ± S.E. from 25 neurons monitored in a representative experiment. B, inhibition of Na^+/K^+ ATPase with 1 mM ouabain counteracts the $[K^+]_E$-dependent decrease in $[Na^+]_C$ in CGCs exposed to NMDA. Data are the means ± S.E. from 40 neurons monitored in a representative experiment. The time bar applies to A and B.

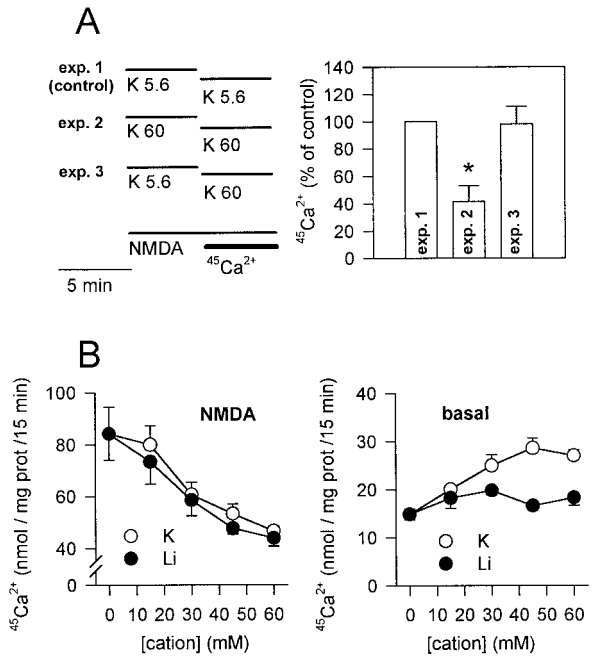

Fig. 5. Effects of high $[K^+]_E$ on the NMDA-induced Ca^{2+} influx may be linked to the inhibition of the reverse operation of the NaCaX. A, high $[K^+]_E$ fails to inhibit the NMDA-elicited Ca^{2+} influx in CGCs pre-exposed to NMDA at low $[K^+]_E$. CGCs were exposed to NMDA and to the indicated $[K^+]_E$ for 10 min; after the first 5 min, the medium was supplemented with 1 μCi $^{45}Ca^{2+}$ and $[K^+]_E$ was varied (left). The amount of $^{45}Ca^{2+}$ trapped in the cells during the second 5 min of exposure to NMDA is expressed as a percentage of control (exp. 1). $^{45}Ca^{2+}$ uptake data (right) are the means ± S.E. from three independent experiments. The background $^{45}Ca^{2+}$ accumulation, measured at 5.6 mM K^+ (no NMDA), was 19 ± 1.1 nmol/mg protein/5 min, and was subtracted from all the data. The control NMDA-induced $^{45}Ca^{2+}$ accumulation measured before the background subtraction was 48 ± 5.0 nmol/mg protein/5 min (exp. 1). $^*P < .05$, t test. Note that 60 mM K^+ failed to inhibit $^{45}Ca^{2+}$ accumulation in CGCs pre-exposed to NMDA at 5.6 mM K^+ (exp. 3), but not at 60 mM K^+ (exp. 2). B, Li^+ mimics the inhibitory effect of K^+ on the NMDA-induced $^{45}Ca^{2+}$ uptake (left), but, in contrast to K^+, fails to affect the basal $^{45}Ca^{2+}$ accumulation (right). CGCs were exposed for 15 min to NMDA, while $[K^+]$ or $[Li^+]$ in the extracellular medium were increased as indicated on the x-axis; $[Na^+]$ was appropriately decreased to maintain osmoticity. The initial $[K^+]$ in the extracellular medium (before the addition of extra K^+ or Li^+) was 5.6 mM, and therefore the final $[K^+]$ was greater by 5.6 mM than indicated on the x-axis. The Li^+ buffers contained the $[Li^+]$ indicated on the x-axis plus 5.6 mM K^+. The effects of K^+ or Li^+ on basal $^{45}Ca^{2+}$ accumulation were assessed in the presence of 1 mM Mg^{2+} plus 10 μM MK-801 (no NMDA or glycine added). Data are the means ± S.E. from five independent experiments.

increase in extracellular $[K^+]$ or $[Li^+]$ potently activates the Ca^{2+}/Ca^{2+} exchange mode of the NaCaX (Blaustein, 1977; Slaughter et al., 1983; DiPolo and Beaugé, 1990) via a mechanism that is still obscure. Because the Ca^{2+}/Ca^{2+} exchange does not result in a net Ca^{2+} influx, a switch of NaCaX operation from the Na^+/Ca^{2+} exchange mode to the Ca^{2+}/Ca^{2+} exchange mode would effectively prevent Ca^{2+} accumulation in the cytoplasm. Further work is necessary to explain how increases in extracellular $[K^+]$ or $[Li^+]$ may affect NaCaX function.

Consistent with the herein proposed causal role of the R/NaCaX in mediating the excitotoxic NMDA-induced Ca^{2+} influx and excitotoxicity, it was recently observed that KB-R7943 (2-[2-[4-(nitrobenzyloxy)phenyl]ethyl]-isothiourea), which preferentially inhibits the R/NaCaX (Iwamoto et al., 1996; Watano et al., 1996), protects hippocampal CA1 neurons from hypoxic/hypoglycemic injury (Schröder et al., 1999). By contrast, KB-R7943 failed to protect cortical neurons against the excitotoxicity elicited by a 10-min exposure to glutamate (Hoyt et al., 1998). Although it remains to be tested whether KB-R7943 protects CA1 neurons by inhibiting the R/NaCaX, it has to be emphasized that the contribution of the R/NaCaX to excitotoxicity may differ in different experimental models. For example, glutamate seems to induce less pronounced $[Na^+]_C$ elevations in cultured hippocampal or cortical neurons (Pinelis et al., 1994; Stout et al., 1996) than in CGCs (Fig. 4; see also Kiedrowski et al., 1994). It is therefore likely that the contribution of the R/NaCaX to the excitotoxic Ca^{2+} influx may be greater in CGCs than in cultured hippocampal or cortical neurons.

Although voltage-sensitive Ca^{2+} channels contribute to the NMDA-induced Ca^{2+} influx (Reichling and MacDermott, 1993), it is unlikely that an inhibition of Ca^{2+} influx via these channels might contribute significantly to the mechanism of inhibition of the NMDA-induced Ca^{2+} influx by high $[K^+]_E$. If this were the case, high $[K^+]_E$ would inhibit the basal $^{45}Ca^{2+}$ influx, which was not observed (Fig. 2A).

An activation of NMDA receptors leads not only to Na^+ and Ca^{2+} influx but also to K^+ efflux (Yu et al., 1999; Kiedrowski, 1999). High $[K^+]_E$ prevents an excessive K^+ efflux from the cytoplasm of neurons exposed to NMDA (Yu et al., 1999; Kiedrowski, 1999). Therefore, it has to be considered that an inhibition of K^+ efflux may also play a role in the high $[K^+]_E$-elicited protection against NMDA excitotoxicity. Recently, Yu et al. (1999) reported that an exposure to NMDA causes K^+ efflux and apoptosis in primary cortical cultures incubated in a medium containing reduced concentrations of Ca^{2+} (0.1 mM) and Na^+ (30 mM), where Na^+ is substituted with *N*-methyl-D-glucamine (NMG^+, 90 mM); both K^+ efflux and apoptosis were prevented by replacing 20 mM NMG^+ in the extracellular medium with 20 mM K^+, which might suggest that the NMDA-induced K^+ efflux plays a role in apoptosis. However, Khodorov et al. (1999) demonstrated that the NMDA-induced excitotoxicity in CGCs incubated in a Na-free medium, with Na^+ replaced with NMG^+, can be substantially prevented by substituting 20 mM NMG^+ with 20 mM Li^+, a maneuver that promotes K^+ efflux (Kiedrowski, 1999). Therefore, the hypothesis that the increased $[K^+]_E$ inhibits the NMDA-induced apoptosis because of the observed decrease in K^+ efflux (Yu et al., 1999) needs to be tested further.

In conclusion, the data presented in this report suggest that inhibition of the NMDA-induced Ca^{2+} influx by high $[K^+]_E$ cannot be explained in terms of a reduced CaDF. It appears that high $[K^+]_E$ inhibits the R/NaCaX-dependent component of the NMDA-induced Ca^{2+} influx by 1) preventing the increase in $[Na^+]_C$ to the levels at which the R/NaCaX is fully activated, or 2) switching the NaCaX to the electroneutral Ca^{2+}/Ca^{2+} exchange mode, or 3) a combination of these two mechanisms. An increase in the NMDA-receptor single channel activity observed in high $[Na^+]_C$ (Yu and Salter, 1998) may contribute to the enhanced NMDA-induced Ca^{2+} influx at low $[K^+]_E$ and high $[Na^+]_E$, provided that the Na^+ influx-dependent depolarization of the plasma membrane that normally occurs under such conditions (Hösli et al., 1973) is slight or is prevented. The fact that high $[K^+]_E$ prevents excessive elevation in $[Na^+]_C$ only as long as Na^+/K^+ ATPase is active suggests that the protective effect of the high $[K^+]_E$ against the NMDA-induced Ca^{2+} influx may be energy dependent, which is supported by preliminary experiments (L.K., data not shown).

Although it must be tested further how changes in the extracellular $[Na^+]/[K^+]$ ratio might affect Ca^{2+} fluxes in ischemic brain, one may speculate that the rapid restoration of low $[K^+]_E$ and high $[Na^+]_E$ during reperfusion (Hansen et al., 1980) might play a role in the mechanism of neuronal death.

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Erratum

In the article by Kiedrowski L, 1999 [Kiedrowski L (1999) Elevated extracellular K^+ concentrations inhibit *N*-methyl-D-aspartate-induced Ca^{2+} influx and excitotoxicity. *Mol Pharmacol* **56:**737–743], an error in proof processing resulted in an omission in the introductory remarks. The final reference in that section should read Kiedrowski, 1999 (page 737).

Also, on page 742, the sentence beginning "An increase in the NMDA-receptor. . ." and ending with ". . .or is prevented" should read as follows:

> An increase in the NMDA-receptor single channel activity observed at high $[Na^+]_c$ (Yu and Salter, 1998) may also contribute to the enhanced NMDA-induced Ca^{2+} influx at low $[K^+]_E$ and high $[Na^+]_E$.

We apologize for these errors and regret any confusion caused by them.