Amino Acids (2002) 23: 325–329
DOI 10.1007/s00726-001-0145-z

Amino
Acids
Printed in Austria

Effects of cytoskeletal modifications on Ca^{2+} influx after cerebral ischemia

K. B. Fink[1], **M. Paehr**[1], **P. C. Djoufack**[1], **C. Weissbrich**[1], **J. Bösel**[2], and **M. Endres**[2]

[1] Department of Pharmacology, University of Bonn, Bonn and
[2] Department of Neurology, Charité Hospital, Humboldt-University of Berlin, Berlin, Germany

Received June 29, 2001
Accepted August 6, 2001
Published online August 9, 2002;

Summary. The fungal toxin cytochalasin D as well as endogenous gelsolin depolymerize filamentous actin which may induce dynamic uncoupling of membrane ion channels. *In vitro* application of cytochalasin D reduced NMDA-induced [^{3}H]noradrenaline release from mouse brain neocortical slices by 38%. In gsn deficient neocortical synaptosomes $[Ca^{2+}]_i$ increase in response to K^+ (30 mM) depolarization was 33% higher than in wild-type. After transient focal cerebral ischemia K^+-induced $[Ca^{2+}]_i$ increase in neocortical synaptosomes was 56% lower than in synaptosomes prepared from the non-ischemic contralateral hemisphere. After *in vivo* pretreatment with cytochalasin D 10 min before MCA occlusion K^+-induced $[Ca^{2+}]_i$ increase in synaptosomes *in vitro* prepared 1 h after reperfusion from the ischemic hemisphere was only 25% lower than in contralateral synaptosomes, while cytochalasin D pretreatment *in vivo* did not reduce K^+-induced $[Ca^{2+}]_i$ increase *in vitro*. Hence, presynaptic Ca^{2+} influx and subsequently neuronal vulnerability are attenuated by increased and are aggravated by decreased F-actin depolymerization.

Keywords: Voltage-gated Calcium channels – F-actin – Stroke – Cytochalasin D

Introduction

Cytoskeletal proteins such as actin are continuously modified to allow structural changes in cell shape and functional changes of plasmamembrane proteins linked to the cytoskeleton (Stossel, 1993).

During cerebral ischemia large amounts of glutamate are released both exocytotically and non-exocytotically by inversely running glutamate uptake transporters. Glutamate released into the synaptic cleft activates glutamate receptors. Among these N-methyl-D-aspartate (NMDA) receptor activation is considered most relevant in terms of Ca^{2+} excitotoxicity. Since during ischemia the Ca^{2+} ATPase and Na^+/Ca^{2+} exchanger work insufficiently Ca^{2+} homeostasis is lost and cytosolic Ca^{2+} concentration rises substantially (Rossi et al., 2000; Obrenovitch et al., 2000; Zipfel et al., 1999).

Activity of both ligand-gated as well as voltage-gated Ca^{2+} channels can be modified by dynamic changes in the actin cytoskeleton, consistent with a model in which actin filaments compartmentalize channel-regulatory proteins (Rosenmund and Westbrook, 1993; Johnson and Byerly, 1993). Cytochalasin D, an actin disrupting fungal toxin, depolymerizes actin in a reversible manner (Stevenson and Begg, 1994). Pharmacological increase in actin depolymerization by cytochalasin D would accelerate Ca^{2+} channel rundown thus limiting further Ca^{2+} influx following cerebral ischemia. We have previously shown that cytochalasin D reduces infarct volume and improves neurological outcome after transient focal cerebral ischemia (Endres et al., 1999). On the other hand, a decreased actin depolymerization rate as observed in gelsolin-deficient cells slows down Ca^{2+} channel rundown and increases Ca^{2+} influx (Furukawa et al., 1997). This mechanism may account for increased neuronal vulnerability and increased infarct volume after transient focal cerebral ischemia in gelsolin deficient mice (Endres et al., 1999; Zipfel et al., 1999). Here, we report that cytochalasin D administered before cerebral ischemia preserves functionally active voltage-gated Ca^{2+} channels in presynaptic nerve terminals.

Materials and methods

[^{3}H]Noradrenaline release in neocortical slices

Neocortical slices (0.3 mm thick, 1.5 mm diameter) were prepared from the neocortex of C57Bl/6 mice and incubated for 30 min

with 50 nM [^{3}H]noradrenaline (specific activity 42.0 Ci/mmol). Slices were layered on Whatman GF/C filters in chambers and superfused at 0.6 ml/min with Krebs-Henseleit buffer. Tritium overflow was evoked by adding NMDA 300 μM for 2 min after 40 min superfusion. The superfusate was continuously collected in 5-min fractions and the tritium content determined by liquid scintillation counting. Tritium efflux was calculated as the fraction of tritium content in the slice at the beginning of the respective collection period. Basal tritium efflux was assumed to decline linearly during fraction collection. Stimulation-evoked tritium overflow was calculated by subtracting basal efflux from total tritium overflow.

$[Ca^{2+}]_i$ measurement in neocortical synaptosomes

Synaptosomes were prepared from the ischemic neocortical MCA territory of either hemisphere of gelsolin null (gsn−/−) or wild-type mice according to Gleitz et al. (1996) with slight modifications. Briefly, cerebral cortex was homogenized with a Potter-Elvehjem glass homogenizer (900 rpm) in 40 vol (w/v) of 0.32 M sucrose. The homogenate was centrifuged (10 min, 1000×g, 4°C) and the supernatant frozen and stored at −80°C. After thawing at 37°C, the supernatant was centrifuged (10 min, 12,000×g, 4°C) and the synaptosomal pellet resuspended in a Ca^{2+} free buffer (pH7.4) containing 133 mM NaCl, 4.8 mM KCl, 10 mM HEPES, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$ and 10 mM glucose. $[Ca^{2+}]_i$ was measured by fluorescence ratiometry (λ_{ex} = 340/380 nm, λ_{em} = 510 nm) according to Grynkiewicz et al. (1985) and modified by Meder et al. (1997). The synaptosomal suspension was incubated with fura-2/AM (5 μM) for 35 min at 37°C. Fura-2 loaded synaptosomes were washed and kept on ice until use. Aliquots (200 μl) of the washed synaptosomal suspension containing 136 ± 7 μg/ml of protein were diluted with 1.8 ml Ca^{2+} free buffer plus 1.3 mM $CaCl_2$, placed in a quartz cuvette in a spectrofluorometer at 37°C and incubated for 6 min before K^+ was elevated by 30 mM for membrane depolarization. For calibration at the end of each measurement Triton X-100 was added to yield maximum and EGTA for minimum Ca^{2+} concentrations. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985).

Gelsolin null mice

Littermates from gsn+/− × gsn+/− crosses from mixed 129/SV × C57Bl/6 backgrounds were used as described previously (Witke et al., 1995; Furukawa et al., 1997; Endres et al., 1999).

Drug administration

For *in vivo* experiments cytochalasin D (Aldrich, Milwaukee, WI) was dissolved in 1% DMSO in phosphate buffered saline. Two μl of the solution containing 1 μg cytochalasin D or vehicle were injected intracerebroventricularly (bregma −0.9 mm lateral, −0.1 mm posterior, −3.1 mm deep) 10 min before MCA occlusion using a Hamilton syringe. For *in vitro* experiments 1 μM cytochalasin D was dissolved in 1‰ DMSO in Krebs-Henseleit buffer.

Focal cerebral ischemia

Adult C57Bl/6 mice, 18–23 g were anesthetized with 1.5% halothane for introduction and maintained with 1.0% halothane in 70% N_2O and 30% O_2. Focal cerebral ischemia was induced by introducing a silicone-coated 8–0 monofilament into the internal carotid artery and advancing it (Endres et al., 1998) in order to occlude the middle cerebral artery. After two hours the filament was completely withdrawn in order to reperfuse the middle cerebral artery (MCA) territory. Regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry using a flexible skull probe as described (Huang et al., 1994, Hara et al., 1997). In randomly selected animals the left femoral artery was cannulated to measure blood pressure, PaO_2 and $PaCO_2$. Rectal temperature was controlled and maintained at approximately 37°C with a temperature feedback-control unit and a heating lamp during the monitoring period until 1 h after reperfusion. No differences in any of the physiological parameters were observed between groups during the experiment. The ischemia protocol was approved by the state committee for animal welfare.

Data analysis

Values are presented as mean and standard error of mean (SEM). Statistical comparisons were performed by unpaired student's *t*-test or one-way ANOVA followed by student's *t*-test. P values <0.05 were considered statistically significant.

Results

NMDA-induced tritium overflow in neocortical clices

Slices were preincubated with [^{3}H]noradrenaline and superfused with Mg^{2+}-free Krebs-Henseleit buffer. Exposure to NMDA 300 μM for 2 min induced a reversible increase in tritium efflux reflecting [^{3}H]noradrenaline release (14.0% ± 0.5% of total tissue tritium corresponding to 4.9 ± 0.3 nCi). NMDA-evoked [^{3}H]noradrenaline release was reduced by 1 μM cytochalasine D by 38% (Fig. 1) whereas the basal efflux remained unchanged. Vehicle (i.e. 1‰ DMSO) had no effect on NMDA-evoked evoked [^{3}H]noradrenaline release.

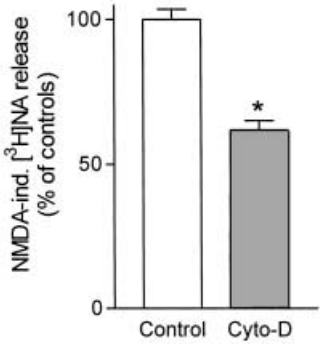

Fig. 1. Effect of cytochalasin D (*Cyto D*) on NMDA (300 µM)-induced [^{3}H]noradrenaline release from C57Bl/6 mouse brain neocortical slices superfused with Mg^{2+}-free medium. NMDA was added for 2 min after 40 min of superfusion. Cytochalasin D 1 μM was present in the medium from the 30th min to the end of the experiment. [^{3}H]noradrenaline release is expressed as percentage of that in controls superfused in the presence of the vehicle DMSO 1‰. Means ± SEM of 8 experiments; *p < 0.0001, compared to controls

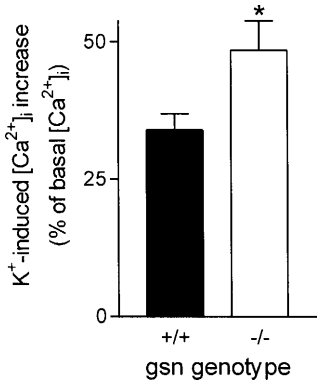

Fig. 2. Effect of gelsolin deficiency (*gsn*−/−) on K^+(30 mM)-induced $[Ca^{2+}]_i$ increase in fura-2-loaded mouse neocortical synaptosomes. For depolarization K^+ concentration in the incubation buffer was elevated by 30 mM. The K^+-induced $[Ca^{2+}]_i$ increase is presented as percentage of basal cytosolic Ca^{2+} concentrations. Means ± SEM of 8 experiments; *$p < 0.03$, compared to wild-type

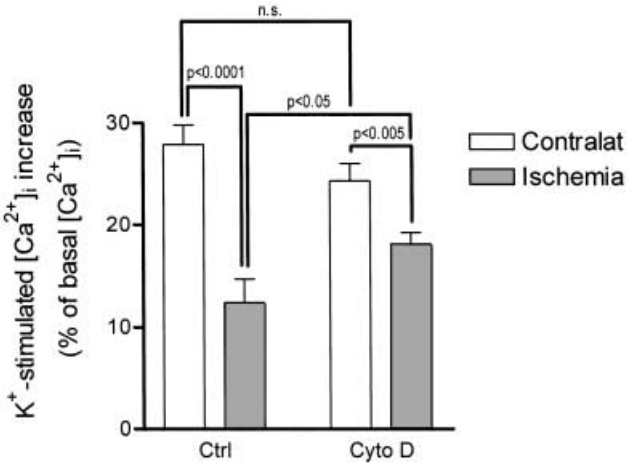

Fig. 3. Effect of ischemia on K^+ (30 mM)-induced increase in $[Ca^{2+}]_i$ in neocortical synaptosomes prepared from C57Bl/6 mice treated with cytochalasin D (1 μg intracerebroventricularly 10 min before ischemia) or vehicle DMSO 1% for control. Cytosolic Ca^{2+} concentrations were determined ratiometrically using fura-2/AM. Increase in $[Ca^{2+}]_i$ is presented in % of basal $[Ca^{2+}]_i$. *Contralat*, contralateral cortex; *ischemia*, ipsilateral cortex after 2 h ischemia and 1 h reperfusion. Results are given as means ± SEM of 3–5 experiments in duplicate. ANOVA followed by Student's t-test was applied

K^+-induced $[Ca^{2+}]_i$ increase in neocortical synaptosomes

The basal cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ immediately before membrane depolarization by K^+ elevation (30 mM) was 298 ± 16 nM in wild-type (gsn+/+) synaptosomes. Basal cytosolic Ca^{2+} concentrations did not differ between the genotypes. K^+ depolarization induced an increase in $[Ca^{2+}]_i$ by 36 ± 3.3% of basal $[Ca^{2+}]_i$ (Fig. 2).

K^+-induced $[Ca^{2+}]_i$ increase in neocortical synaptosomes after focal cerebral ischemia

In synaptosomes isolated from normal contralateral cortex neither basal cytosolic Ca^{2+} concentrations nor K^+ (30 mM)-induced $[Ca^{2+}]_i$ increase were affected by intracerebroventricular application of cytochalasin D 10 min before MCA occlusion (basal $[Ca^{2+}]_i$ with cytochalasin D 290 ± 10 nM; basal $[Ca^{2+}]_i$ without cytochalasin D 325 ± 18 nM; Fig. 3). Basal synaptosomal $[Ca^{2+}]_i$ was reduced following ischemia with or without cytochalasin D pretreatment, probably due to a reduced number of functionally intact terminals (248 ± 12 and 266 ± 20 nM, respectively). After 2 h ischemia followed by 1 h reperfusion, depolarization by 30 mM K^+ induced a 56% lower $[Ca^{2+}]_i$ increase in vehicle-treated ischemic synaptosomes as compared to vehicle-treated contralateral control synaptosomes. However, after cytochalasin D pretreatment K^+-induced $[Ca^{2+}]_i$ increase in synaptosomes from ischemic cortex was only 30% lower than in contralateral control synaptosomes (Fig. 3). K^+-induced $[Ca^{2+}]_i$ increase in cytochalasin D treated ischemic synaptosomes was 46% higher than in vehicle-treated ischemic synaptosomes.

Discussion

High concentrations of glutamate released onto NMDA receptors in concert with activation of voltage-gated Ca^{2+} channels lead to excessive Ca^{2+} influx during cerebral ischemia. Increased cytosolic Ca^{2+} concentrations activate Ca^{2+} dependent enzymes such as calpain or neuronal nitric oxide synthase, lead to activation of caspase 12 thus initializing the caspase cascade, or cause formation of free radicals and eventually cell death. Neuroprotection is a potentially successful strategy for stroke therapy although up to the present date no drug has been proven clinically effective and reasonably safe.

A pharmacological approach leading to attenuated Ca^{2+} influx appears to be feasible and we demonstrate here that the actin disrupting toxin cytochalasin D causes (1) decreased Ca^{2+} influx after NMDA receptor activation, (2) decreased Ca^{2+} influx after voltage-gated Ca^{2+} channel activation, and (3) decreased loss of Ca^{2+} channel function after cerebral ischemia. NMDA receptor-mediated [^{3}H]noradrenaline release

is completely dependent on extracellular Ca^{2+} and reflects Ca^{2+} influx via the NMDA receptor cation channel (Fink et al., 1989, 1996). Interference with the vesicular release mechanism is probably not the cause of the inhibitory effect of cytochalasin D on NMDA-evoked [^{3}H]noradrenaline release because actin disruption is supposed to facilitate exocytotic neurotransmitter release (Vitale et al., 1991). NMDA receptors mediating [^{3}H]noradrenaline release in the slice are located on noradrenergic variosity chains and/or on excitatory interneurons. Thus, cytochalasin D in this model most likely causes uncoupling (Rosenmund and Westbrook, 1993) mainly of presynaptic NMDA receptors.

K^+ depolarization-induced increase in cytosolic Ca^{2+} concentration in synaptosomes, i.e. torn off and resealed nerve terminals, does not involve intracellular Ca^{2+} stores but is mainly dependent on extracellular Ca^{2+}, reflecting Ca^{2+} influx via voltage-gated Ca^{2+} channels (Meder et al., 1997; Fink et al., 2000). The facilitatory effect of gelsolin deficiency on K^+-induced $[Ca^{2+}]_i$ increase indicates an increased net Ca^{2+} influx presumably through N or P/Q type voltage-gated Ca^{2+} channels which are found on presynaptic terminals (Fink et al., 1990; Pittaluga and Raiteri, 1991). This finding is consistent with the notion that voltage-gated Ca^{2+} channels are dynamically uncoupled from the actin cytoskeleton (Johnson and Byerly, 1993) by endogenous gelsolin resulting in a rather slowly occurring Ca^{2+} channel rundown. Absence of gelsolin leads to an increased Ca^{2+} influx.

Cytochalasin D treatment 10 min before MCA occlusion *in vivo* had no effect on K^+-induced $[Ca^{2+}]_i$ increase in contralateral control synaptosomes prepared in vitro 1 h after reperfusion whereas cytochalasin D in the superfusion buffer inhibited the NMDA receptor-mediated effect. Both results are consistent because the effect of cytochalasin D is reversible and can no longer be observed 1.5 h after application (Stevenson and Begg, 1994). In ischemia experiments, however, the measurements on synaptosomes were performed more than 4 h (i.e. 10 min before MCA occlusion +2 h MCA occlusion +1 h reperfusion +1 h synaptosomal preparation) after cytochalasin D administration *in vivo*. After this period a direct effect of cytochalasin D may no longer be expected and any effect would be downstream or mediated indirectly.

Ischemia caused a reduction in K^+-induced $[Ca^{2+}]_i$ increase by 56% compared to the non-ischemic contralateral hemisphere indicating a loss of functional nerve terminals due to the ischemic lesion. After cytochalasin D treatment the K^+-induced $[Ca^{2+}]_i$ increase in synaptosomes from the ischemic hemisphere was reduced by a lesser extent, i.e. by 30% only, compared to synaptosomes from the non-ischemic hemisphere. We conclude that cytochalasin D has neuroprotective effects and preserves nerve terminals during cerebral ischemia due to a reduced Ca^{2+} influx.

Acknowledgements

The authors thank D. J. Kwiatkowski for providing gsn null mice. The skilled technical assistance of H. Burisch is gratefully acknowledged. The study was supported by grants from the Deutsche Forschungsgemeinschaft (K.F., Fi600/2-1 and 7-1 and M.E., En343/1-1 and 6-1) and the Hermann and Lilly Schilling Foundation.

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Authors' address: Dr. Klaus B. Fink, Department of Pharmacology, University of Bonn, Reuterstrasse 2, D-53113 Bonn, Germany, Fax +49 228 73 5404, E-mail: finkk@uni-bonn.de