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RESEARCH PAPER

Antiepileptic effects of two Rho-kinase inhibitors, Y-27632 and fasudil, in mice

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Background and purpose: Rho/Rho-kinase signalling is involved in many cellular events, including some in the CNS. However, the role of this pathway in epilepsy has not yet been assessed. Therefore, we determined the effects of two Rho-kinase inhibitors, Y-27632 and fasudil, on seizures induced by pentylenetetrazole (PTZ) or maximal electroconvulsive shock (MES).
Experimental approach: Effects of Y-27632 (5–10 mg kg^{-1}) and fasudil (5–25 mg kg^{-1}) on duration of myoclonic jerks, clonic and tonic convulsions, tonic hindlimb extensions and percentage of tonic convulsion index, as well as recovery latency for righting reflex were investigated in mice stimulated with PTZ (65 mg kg^{-1}) or MES (50 Hz, 50 mA and 0.4 s). These inhibitors were also tested on a model of kindling induced by PTZ (35 mg kg^{-1}, for 11 days). Membrane and cytosolic levels of RhoA protein were measured in brain homogenates from kindled mice.
Key results: Y-27632 and fasudil diminished onset of myoclonic jerks, clonic convulsions and tonic hindlimb extensions in mice given PTZ. These inhibitors suppressed the percentage of tonic convulsion index and recovery latency for righting reflex in the mice excited with MES. Western blotting demonstrated that Rho translocation to plasma membrane increased in the brain homogenates obtained from PTZ-kindled mice. However, the Rho-kinase inhibitors at the given doses did not change motor coordination of the mice.
Conclusions and implications: Rho/Rho-kinase signalling may play a role in epilepsy induced by PTZ and MES. Furthermore, Rho-kinase inhibitors could be novel important antiepileptic agents.
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Keywords: epilepsy; fasudil; Rho/Rho-kinase signalling; seizure; Y-27632

Abbreviations: MES, maximal electroconvulsive shock; PTZ, pentylenetetrazole; Y-27632, (+)-(*R*)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate

Introduction

Epilepsy is a heterogeneous syndrome characterized by recurrent and spontaneous seizures. Approximately 1% of the population in the world suffers from epilepsy. However, 20–30% of the patients are refractory to therapies using currently available antiepileptic drugs (Provini *et al.*, 1999; Patsalos, 2000; Jiang *et al.*, 2007). Epileptogenesis involves histological, biochemical and physiological alterations that, over time, alter the balance between excitatory and inhibitory neurotransmission in multiple brain structures (Sasa, 2006). The excessive enhancement of excitatory neurotransmission and/or the reduction of inhibitory pathways, as well as the regulation of some signal transductions may cause seizures (Gale, 1993; Tsuda *et al.*, 1997; Zilles *et al.*, 1999). For instance, a decrease in calcium/calmodulin-dependent protein kinase II (CaMK-II) activity has been shown to occur with the development of spontaneous recurrent epileptiform discharges in the hippocampal neuronal culture model of acquired epilepsy. Moreover, altered calcium homoeostasis has been implicated in epileptogenesis (Carter *et al.*, 2006).

Recently, a novel signal-transduction pathway, namely Rho/Rho-kinase signalling, has been proposed to be involved in diverse cellular events throughout the body, including somein the CNS. This pathway may be involved in pain phenomena (Ramer *et al.*, 2004; Tatsumi *et al.*, 2005; Büyükafşar *et al.*, 2006a). Activation of lysophosphatidic acid receptors, which are coupled with Rho signalling, is crucial in the initiation of neuropathic pain (Inoue *et al.*, 2004). It has been reported that suppression of Rho-kinase activity may enhance axonal regeneration (Borisoff *et al.*, 2003). Moreover, Rho-kinase inhibition may be beneficial in the treatment of cerebral vasospasm after subarachnoid haemorrhage (Janjua and Mayer, 2003). Several other functions have also been attributed to Rho/Rho-kinase signalling within the CNS, including central regulation of

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blood pressure (Ito *et al*., 2003), hypothermia (Tsushima *et al*., 2003), axonal growth (Borisoff *et al*., 2003), neurotransmitter release (Narita *et al*., 2003; Sasaki, 2003), opening of tight junctions between brain endothelial cells (Stamatovic *et al*., 2003), formation of branched dendrites (Leemhuis *et al*., 2004), regulation of the level of amyloidogenic $A_{\beta 42}$ (Zhou *et al*., 2003), modulation of long-term potentiation (O'Kane *et al*., 2004), extension and retraction of neurites (Sakisaka *et al*., 2004) and long-term spatial memory (Dash *et al*., 2004). Consequently, Rho kinase has been reported to be a promising drug target for neurological disorders (Mueller *et al*., 2005).

It has been reported recently that RhoA is activated in the cortex and hippocampus after traumatic brain injury and kainic acid-induced seizures (Dubreuil *et al*., 2006). However, the possible involvement of Rho/Rho-kinase signalling has yet to be investigated in ictogenesis and epileptogenesis, which are induced by maximal electroconvulsive shock (MES), acute and chronic (kindling) administration of pentylenetetrazole (PTZ) that blocks GABA-mediated Cl^- influx and consequently leads to neuronal membrane depolarization (Mortazavi *et al*., 2005). For that reason, two Rho-kinase inhibitors, fasudil and Y-27632, were tested on MES, PTZ-induced seizures and the development of kindling in mice. Furthermore, RhoA translocation from cytosol to the plasma membrane was determined in the brain homogenates of the kindled mice by western blot analysis.

Materials and methods

Animals

All animal procedures conformed to the National Institutes of Health (NIH) guidelines and were approved by the local ethics committee of Mersin University Medical Faculty. Male, inbred Swiss albino mice (2–3 months old) weighing 25–30 g obtained from the Medical Sciences Research Centre at the University of Çukurova were used in the experiments. The mice were housed 7–10 per cage in an environmentally controlled room under a 12:12-h light–dark cycle. They were allowed food and water *ad libitum*. Experiments were conducted between 0900 and 1500 hours. All mice used for the experiments were naive to the PTZ, MES, fasudil, Y-27632 or intraperitoneal injection.

Acute PTZ experiments

A group of animals were injected with a single dose of PTZ ($65\,mg\,kg^{-1}$) to investigate if the two Rho-kinase inhibitors, fasudil and Y-27632, changed the onset of PTZ seizures. Fasudil, Y-27632 or saline was given intraperitoneally 30 min before the PTZ injection. Each mouse was then observed for a 15-min period to measure the onset of the first myoclonic jerk, the onset of the first clonic convulsion and the occurrence of tonic hindlimb extension. Some of the animals died after tonic hindlimb extension, which is an expected outcome of acute PTZ injection. After the observation period, all animals were killed by halothane anaesthesia.

PTZ kindling experiments

Another group of mice were tested for PTZ kindling. Mice were injected with a sub-convulsive dose of PTZ ($35\,mg\,kg^{-1}$, i.p.) (on Mondays, Wednesdays and Fridays) of each week for a total of 11 injections. After each PTZ injection, mice were observed for 30 min and the occurrence of convulsive activity was recorded and classified using the scoring system of Fischer and Kittner (1998) as described below. After 30 min, the mice were then injected with either fasudil ($25\,mg\,kg^{-1}$, i.p.) or Y-27632 ($5\,mg\,kg^{-1}$, i.p.) and returned to their home cages until the next injection. Control mice for fasudil and Y-27632 received saline. An animal undergoing a stage 5 convulsion was considered to be fully kindled and was not further tested. The seizure stage rating scale was as follows:

Stage 0: no evidence of convulsive activity;
Stage 1: ear and facial twitching, head nodding;
Stage 2: myoclonic jerks;
Stage 3: forelimb clonuses with full rearing;
Stage 4: generalized clonic convulsions with loss of righting reflex, rearing, jumping and falling down; and
Stage 5: clonic-tonic convulsions with tonic hindlimb extensions.

Another group of kindled mice were injected with a single dose of either fasudil ($25\,mg\,kg^{-1}$) or Y-27632 ($5–10\,mg\,kg^{-1}$) to test if acute administration of these Rho-kinase inhibitors could change the seizure susceptibility in kindled mice. At the end of the kindling protocol, all animals were killed by cervical dislocation for the measurement of RhoA translocation in the brain by western blotting.

MES experiments

To investigate the possible effects of Rho-kinase inhibitors, fasudil and Y-27632, on the tonic hindlimb extensions induced by MES, a group of mice was exposed to an electroshock (50 Hz, 50 mA and 0.4 s; UGO Basile, ECT Unit 7801, Comerio VA, Italy) by ear-clip electrodes. Fasudil, Y-27632 or saline was injected 30 min before electroshock. Immediately after the electroshock, each mouse was put into a Plexiglas chamber to observe the duration of tonic hindlimb extension and the recovery latency for righting reflex for 10 min. After the observation period, all animals were killed by halothane anaesthesia.

Rota-rod performance test

A separate group of mice received either $25\,mg\,kg^{-1}$ of fasudil or $10\,mg\,kg^{-1}$ of Y-27632 and was examined in a rota-rod performance test to see if there was any failure of motor coordination induced by Rho-kinase inhibitors.

Whole-brain western blot analysis

Control and kindled mice were killed by cervical dislocation. The brain with two hemispheres was isolated and homogenized with a lysis buffer solution in the following: Tris-HCl (pH 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulphonyl fluoride 10 μM, leupeptin $10\,\mu g\,mL^{-1}$, pepstatin $1\,\mu g\,mL^{-1}$ and benzamidine 1 mM. The homogenate was centrifuged at 10 000 *g* for

10 min at 4 °C, and the supernatant was removed. It was then re-centrifuged at 38 000 *g* for 90 min; the supernatant was removed and kept as the cytosolic fraction. The pellet (cell membranes) was re-suspended with the lysis buffer. Both fractions were used for protein analysis (with Bradford method). Equal amounts of proteins were loaded in wells, separated by electrophoresis on 10% polyacrylamide-sodium dodecyl sulphate gels and then transferred to a nitrocellulose membrane overnight. The membrane was blocked with the blocking agent of enhanced chemiluminescence (ECL advance) kit (Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05% Tween-20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against RhoA (monoclonal IgG; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at 1:1000 dilution (overnight) followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1:2000; Santa Cruz Biotechnology Inc). The blots were then detected with the advanced chemiluminescence detection kit (Amersham Biosciences) and visualized on a commercial X-ray film.

Statistical analysis

Data were expressed as means ± s.e.mean. One-way ANOVA followed by a *post hoc* least significant difference (LSD) test was used to analyse the data. A repeated measure (group × day) ANOVA was used to analyse the PTZ-kindling data. Student's *t*-test was used to analyse western blot data. Significance was set at $P<0.05$.

Drugs

Pentylenetetrazole was purchased from Sigma Chemical Co (St Louis, MO, USA). Fasudil and (+)-(*R*)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) were purchased from TOCRIS Bioscience (Bristol, UK). Fasudil (5 or 25 mg kg^{-1}), Y-27632 (5 or 10 mg kg^{-1}) and PTZ (35 or 65 mg kg^{-1}) were dissolved in 0.9% NaCl (saline) and injected intraperitoneally in a volume of 0.1 mL per 10 g body weight. Control animals received saline. The doses of the Rho-kinase inhibitors, fasudil and Y-27632, were chosen based on our previous reports in which antinociceptive effects of these inhibitors were investigated (Büyükaf°ar *et al*., 2006a).

Results

The effects of fasudil and Y-27632 on the onset of myoclonic jerks induced by acute PTZ injection

The effects of fasudil and Y-27632 on the onset of myoclonic jerks are shown in Figure 1. At the dose of 25 mg kg^{-1}, fasudil significantly prolonged the onset time of myoclonic jerks induced by a single dose of PTZ (65 mg kg^{-1}, i.p.) when compared with the saline group ($P<0.05$). However, it did not change the onset time at a dose of 5 mg kg^{-1}. Furthermore, at the doses of 5 and 10 mg kg^{-1}, Y-27632 significantly prolonged the onset time of myoclonic jerks when compared with those observed in the saline group ($P<0.05$).

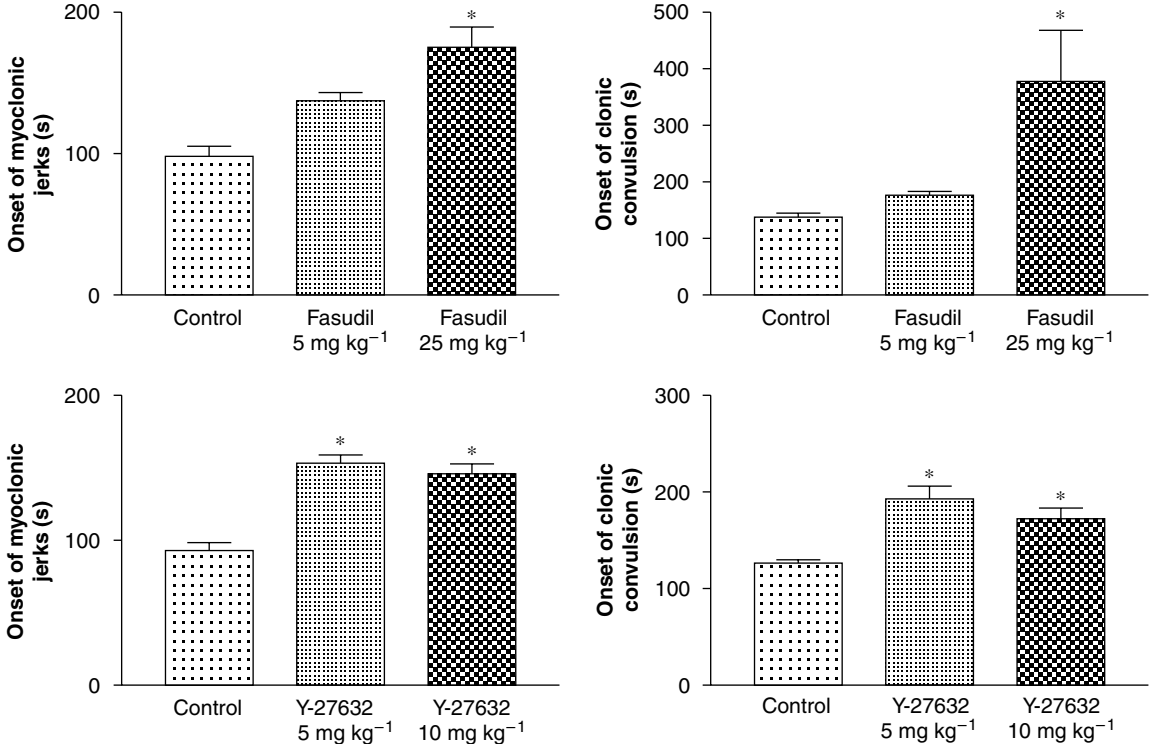

Figure 1 The effects of fasudil (5–25 mg kg^{-1}, $n=10$–11) and Y-27632 (5–10 mg kg^{-1}, $n=10$ for each group) on the onset of pentylenetetrazole (PTZ, 65 mg kg^{-1})-induced myoclonic jerks (left panels) and clonic convulsions (right panels). At the dose of 25 mg kg^{-1}, fasudil (upper graphs) significantly increased the onset time when compared with the saline group ($n=22$, $P<0.05$). However, 5 mg kg^{-1} fasudil did not change the onset time. Y-27632 (5 and 10 mg kg^{-1}; lower graphs) significantly increased the onset time when compared with the saline group ($n=22$, $P<0.05$). *$P<0.05$, different from control.

The effects of fasudil and Y-27632 on the onset of clonic convulsions induced by acute PTZ injection

The effects of fasudil and Y-27632 on the onset of clonic convulsions are shown in Figure 1. At the dose of 25 mg kg^{-1}, fasudil significantly prolonged the onset time of clonic convulsions induced by a single dose of PTZ (65 mg kg^{-1}, i.p.) when compared with the saline group ($P<0.05$). However, it did not change the onset time at the dose of 5 mg kg^{-1}. Moreover, at the doses of 5 and 10 mg kg^{-1}, Y-27632 significantly prolonged the onset time of clonic convulsions when compared with saline group ($P<0.05$).

The effects of fasudil and Y-27632 on tonic hindlimb extensions induced by acute PTZ injection

Seven out of ten mice in the PTZ-treated group had tonic hindlimb extensions and died after that. However, fasudil, in both doses, prevented the occurrence of tonic hindlimb extensions and death (data not shown). Similar to fasudil, Y-27632, in both doses, also prevented the occurrence of tonic hindlimb extensions and death (data not shown).

The effects of fasudil and Y-27632 on the duration of tonic hindlimb extensions and the recovery latency for righting reflex in MES series

Both fasudil (25 mg kg^{-1}) and Y-27632 (5 and 10 mg kg^{-1}) significantly decreased the duration of tonic hindlimb extensions induced by a single application of MES when compared with the group treated with saline (Figure 2, $P<0.05$). Moreover, the Rho-kinase inhibitors fasudil and Y-27632 also significantly decreased the recovery latency for righting reflex when compared with the saline group (Figure 2, $P<0.05$).

The effects of acute single administration of fasudil or Y-27632 on the onset of myoclonic jerks and clonic convulsions in PTZ-kindled mice

Neither fasudil (25 mg kg^{-1}) nor Y-27632 (5 and 10 mg kg^{-1}) changed the onset times of myoclonic jerks and clonic convulsions when compared with the saline group (Figure 3).

The effects of repeated administration of fasudil or Y-27632 on the development of PTZ kindling

The effects of repeated administration of fasudil or Y-27632 on the development of PTZ kindling are shown in Figure 4. PTZ injections by group interaction were found significant for saline, 25 mg kg^{-1} fasudil and 5 mg kg^{-1} Y-27632 groups ($P<0.05$). As shown in Figure 4, fasudil was not able to prevent the development of PTZ kindling, but it had a significant effect on the 2nd, 3rd and 4th days of the

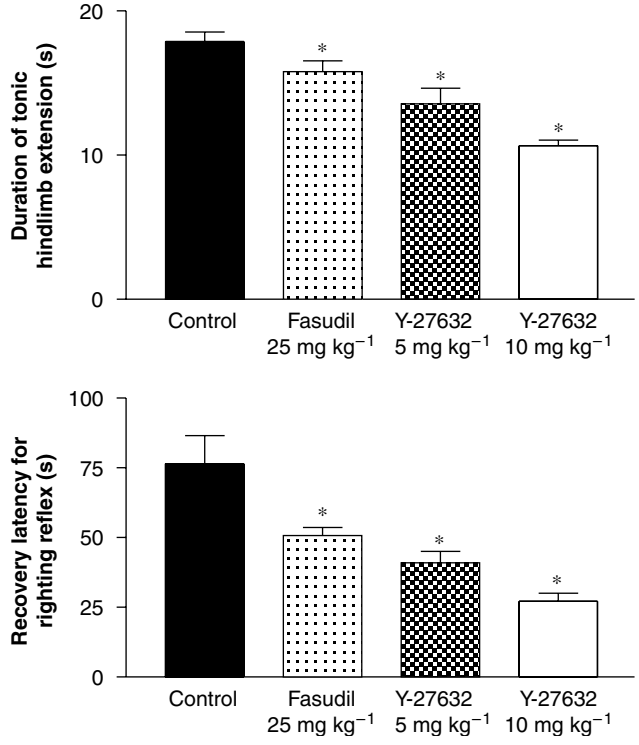

Figure 2 The effects of fasudil and Y-27632 on the duration of tonic hindlimb extensions and recovery latency for righting reflex immediately after tonic hindlimb extensions, which were induced by maximal electroconvulsive shock (50 Hz, 50 mA and 0.4 s). Both fasudil (25 mg kg^{-1}, $n=10$) and Y-27632 (5 and 10 mg kg^{-1}, $n=8$–9) significantly reduced the duration of tonic hindlimb extensions (upper graph) and recovery latency for righting reflex (lower graph) when compared with the saline group ($n=10$, $P<0.05$). *$P<0.05$, different from control.

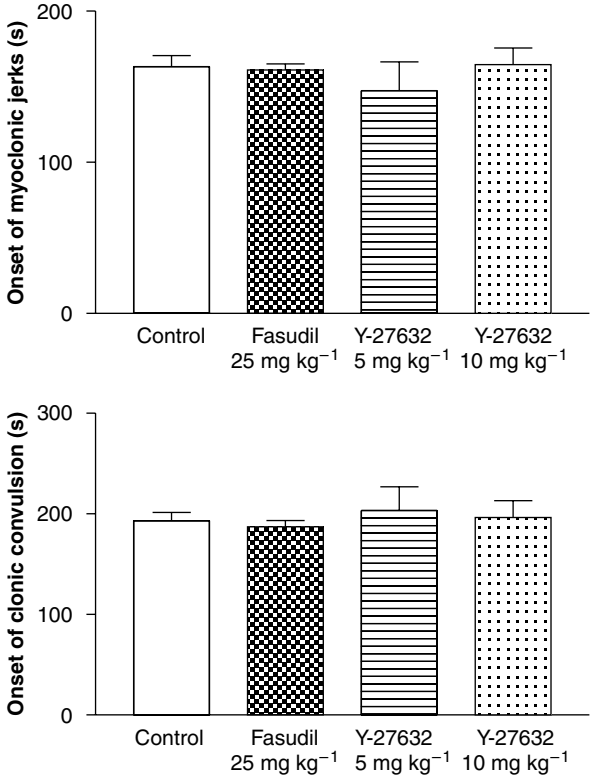

Figure 3 The effects of acute single dosing with fasudil or Y-27632 on the onset of myoclonic jerks and clonic convulsions in pentylenetetrazole (PTZ, 35 mg kg^{-1}, for 11 days, three times a week)-kindled mice. Neither fasudil (25 mg kg^{-1}, $n=10$) nor Y-27632 (5 and 10 mg kg^{-1}, $n=8$–10) changed the onset times of myoclonic jerks and clonic convulsions when compared with the saline group ($n=10$).

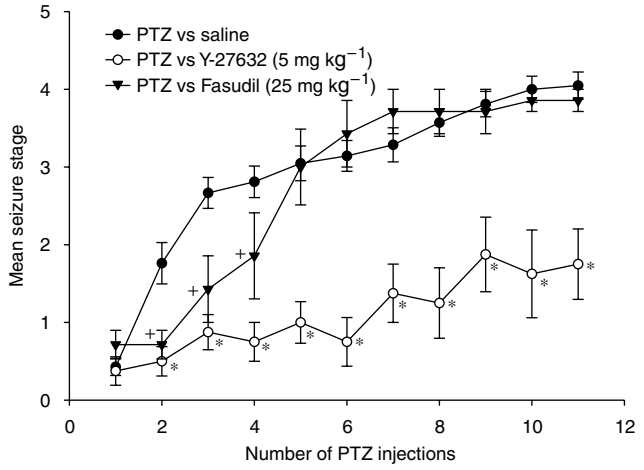

Figure 4 The effects of repeated administration of fasudil (25 mg kg^{-1}, $n=11$) or Y-27632 (5 mg kg^{-1}, $n=10$) on the development of pentylenetetrazole (PTZ) kindling (35 mg kg^{-1}, for 11 days, three times a week). Fasudil was not able to prevent the development of PTZ kindling, but it had a significant effect on the 2nd, 3rd and 4th days of the development by lowering the mean seizure stages when compared with the saline group ($n=10$, $P<0.05$). Unlike fasudil, Y-27632 had a significant effect on the development of PTZ kindling from the beginning of the second day (compared with the saline group) by lowering the mean seizure stages ($P<0.05$). $^{*,+}P<0.05$, different from control.

development by lowering the mean seizure stages when compared with the saline group ($P<0.05$). Unlike fasudil, Y-27632 was more potent and had a significant effect on the development of PTZ kindling from the beginning of the 2nd day, as compared with the saline group, by lowering the mean seizure stages ($P<0.05$).

Effect of PTZ kindling on RhoA translocation in whole-brain homogenates

Chronic PTZ administration (35 mg kg^{-1}, for 11 days and three times in a week) significantly increased RhoA translocation from cytosol to the plasma membrane as demonstrated by western blotting ($P<0.05$, Figure 5).

The effects of fasudil and Y-27632 on the rota-rod performance

Neither fasudil (25 mg kg^{-1}) nor Y-27632 (5 and 10 mg kg^{-1}) changed motor coordination evaluated by performance on the rota-rod, at 20 r.p.m. (Table 1).

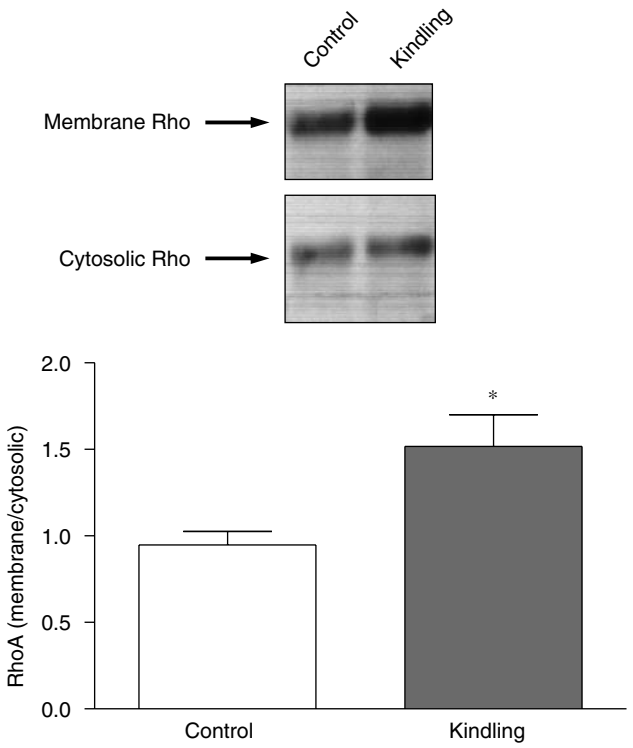

Figure 5 The effects of pentylenetetrazole kindling on RhoA translocation in whole-brain homogenates. Chronic PTZ administration (kindling; 35 mg kg^{-1}, for 11 days and three times in a week) significantly increased RhoA translocation from cytosol to the plasma membrane as demonstrated by western blotting when compared with the control group ($P<0.05$). Numbers of animals in each group were as follows: control, 8 and PTZ kindling, 10. $^{*}P<0.05$, different from control.

Table 1 Effects of Rho-kinase inhibitors on motor coordination

Groups	*Rota-rod performance (20 r.p.m.; s)*	n
Saline	293 ± 7.1	7
Fasudil (25 mg kg^{-1})	279 ± 14.2^{NS}	8
Y-27632 (5 mg kg^{-1})	300 ± 0.0^{NS}	6
Y-27632 (10 mg kg^{-1})	300 ± 0.0^{NS}	7

Abbreviations: LSD, least significant difference; NS, nonsignificant; Y-27632, (+)-(*R*)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate.
The Rho-kinase inhibitors, Y-27632 and fasudil, did not change motor coordination in the rota-rod performance test.
Significance differences between means were determined by one-way ANOVA followed by a *post hoc* LSD test.

Discussion

Rho is a member of the Ras family of proteins, which regulate the organization of actin cytoskeleton and mitogenic signalling in response to extracellular signals (Mackay and Hall, 1998). It has been reported that the Rho/Rho-kinase pathway is involved in diverse cellular effects within the CNS, such as axonal outgrowth, dendrogenesis, cell migration, synaptic vesicle recycling, exocytosis and endocytosis (Van Aelst and D'Souza-Schorey, 1997).

In the present study, we investigated the possible effect of two Rho-kinase inhibitors, fasudil and Y-27632, in three experimental models of epilepsy (MES, acute PTZ seizures and the development of PTZ kindling). Furthermore, we measured membrane and cytosolic Rho levels in the whole-brain homogenates obtained from PTZ-kindled mice. Our results showed that both fasudil and Y-27632 significantly reduced the duration of tonic hindlimb extensions and recovery latency for righting reflex in the MES group, and prolonged the onset of PTZ seizures in the acute PTZ seizure test group. Unlike fasudil, repeated administration of Y-27632 prevented the development of PTZ kindling by reducing the mean seizure stage. However, acute single dosing with fasudil or Y-27632 did not change the onset

times of myoclonic jerks and clonic convulsions in PTZ-kindled mice. Moreover, as demonstrated by western blot analysis, chronic administration of sub-convulsive dose of PTZ increased translocation of Rho proteins to the plasma membrane, showing that Rho-induced signalling is activated and it could be involved in the genesis of epileptiform activity.

Maximal electroconvulsive shock-induced seizures may involve several cellular mechanisms mediating neuronal activities. For instance, MES-induced changes in neuronal activity are linked to the regulation of gene expression (for example, c-fos and junB) and intracellular signal-transduction (Pyk2–Ras–Raf–MEK–ERK) pathways (Fochtmann, 1994; Jeon *et al*., 2002). Interestingly, Jeon *et al*. (1997) reported that electroconvulsive shock induced the phosphorylation of a 75-kDa protein in the rat hippocampus, which was later purified and identified as moesin. It has been known that one of the downstream targets of Rho-kinase is the ezrin-radixin-moesin family of proteins. So, electroconvulsive shock may activate Rho-kinase, through the release of glutamate or other neurotransmitters as proposed by Jeon *et al*. (2002), which in turn phosphorylates moesin protein. In this case, we have evidence why Y-27632 and fasudil were effective against MES-induced seizures in our study. Consequently, in addition to the above-mentioned signal-transduction pathways, we also suggest that Rho/Rho-kinase signalling may also be involved in the action of electroconvulsive shock on neurons. However, in this study, we do not have any data about the activation of the Rho kinase enzyme in brain homogenates, although Rho, one of the upstream activators of Rho-kinase, was translocated to the plasma membrane, implying Rho/Rho-kinase pathway activation. Furthermore, we found the Rho-kinase inhibitors effective in the maintenance of the kindling model of epilepsy. Nevertheless, in the kindling phenomenon, Rho kinase activation remains to be demonstrated, using, for instance, a phosphomoesin antibody.

Furthermore, it has been recently demonstrated that electroconvulsive shock activates extracellular signal-regulated kinase (ERK) signalling in the rat frontal cortex (Kang *et al*., 2006). There is a cross-talk between ERK and Rho/Rho-kinase signalling such that Rho-kinase may phosphorylate ERK to activate this protein (Zhao *et al*., 2006). In addition, MES can facilitate Ca^{2+} entry into neuronal cells (Antkiewicz-Michaluk *et al*., 1994), which induces neuronal firing; this Ca^{2+} entry is involved in neuronal death by frequent and prolonged seizures (Raiteri, 2006). Ca^{2+} entry and subsequent activation of the Rho/Rho-kinase signalling has not been studied in neuronal cells. However, based on smooth muscle cell studies (Büyükafşar and Levent, 2003; Büyükafşar *et al*., 2003; Levent and Büyükafşar, 2004), this might also be taking place in neuronal cells and calcium entry-induced seizures might result from the stimulation of Rho signalling. In this case, our results become more reasonable in terms of epileptogenesis, ictogenesis and neuronal death by the accumulation of calcium in neurons after prolonged and frequent seizures. Speculatively, inhibition of this signalling may provide a broad spectrum of antiepileptic effects because the Rho/Rho-kinase pathway seems to be the central downstream pathway that is activated by various upstream stimuli, such as activation of excitatory neurotransmitter receptors, as in the periphery (Büyükafşar and Levent, 2003; Şahan-Fırat *et al*., 2005; Büyükafşar *et al*., 2006b). However, antiepileptic effects of the Rho-kinase inhibitors should be examined in different genetically epilepsy-prone animals, because those may be suitable models for the best evaluation of the characteristics of the effects of Rho-kinase inhibitors.

An excitatory neurotransmitter, glutamate, which is held responsible for brain damage and also for epileptogenesis, activates Rho-kinase in neuronal cells (Jeon *et al*., 2002). Furthermore, it has been recently demonstrated that the Rho/Rho-kinase signalling pathway is upregulated and may be involved in NMDA-induced retinal neurotoxicity, because fasudil, a Rho-kinase inhibitor, was found to be neuroprotective against glutamate-related excitotoxicity (Kitaoka *et al*., 2004). NMDA leads to an increase in retinal RhoA protein level and activates Rho-kinase, causing neuronal toxicity. Inhibition of Rho-kinase prevented glutamate-induced neurotoxicity. Moreover, in cultured hippocampal neurons as well as in whole-brain synaptosomal fractions, RhoA is associated with glutamate receptors at the plasma membrane of dendritic spines (Schubert *et al*., 2006). In our study, western blotting demonstrated that PTZ-induced kindling increased the translocation of RhoA proteins to the plasma membrane in the whole-brain homogenates, showing that excitation of the CNS could activate the Rho/Rho-kinase signalling pathway. This may be of both pathophysiological and pharmacological importance. However, to determine the brain areas in which there is more Rho translocation than the others, especially in terms of the areas evoking ictogenic or epileptogenic discharges, requires more detailed studies. On the other hand, the possibility of cross-talk between the Rho/Rho-kinase signalling and other signal-transduction pathways, that is, ERK and p42/p44 remains to be investigated in terms of epilepsy.

One of the most striking results of this study was that Y-27632 was found to be effective against PTZ kindling. Unlike the acute PTZ and MES seizure models, kindled seizures involve a progressive increase in electrographic and behavioural seizure activity resulting from the repeated delivery of short pulses of electrical stimuli to the limbic brain regions. Kindled seizures can also be induced by repeated injections of sub-convulsant doses of PTZ or psychomotor stimulants, and may represent a better method for the evaluation of potential antiepileptic drugs (Loscher, 2002). It was shown by Schroder *et al*. (1993, 1994) that PTZ kindling enhanced glutamate receptor density, mediating its epileptogenic action, in addition to the inhibition of $GABA_A$/benzodiazepine receptor complex. Consequently, as glutamate receptors are associated with Rho/Rho-kinase signalling, kindling may activate this pathway. That is why the Rho-kinase inhibitor Y-27632 was effective against PTZ kindling. As reported with MES (Antkiewicz-Michaluk *et al*., 1994), PTZ-induced neuronal excitation may also involve Ca^{2+} entry into neurons (Onozuka *et al*., 1987), and this Ca^{2+} entry may activate Rho signalling.

The ineffectiveness of fasudil in preventing kindling-induced seizures remains to be investigated especially in terms of its potency, selectivity and pharmacokinetics. The

IC_{50} values of Y-27632 and fasudil in inhibiting Rho-kinase (as the ROCK-2 isoform) are 800 nM and 1.9 μM, respectively. Although fasudil is a potent inhibitor of Rho-kinase, it could also inhibit various other protein kinases (Davies *et al*., 2000), which may possibly counteract each other in the regulation of neuronal excitability. Therefore, we would speculate that inhibition of these other protein kinases could interfere with the inhibitory effects of fasudil on epileptogenesis, through the inhibition of Rho kinase. However, relative to fasudil, Y-27632 is more selective for Rho-kinase and it could substantially diminish kindling-induced seizures. The ineffectiveness of fasudil may also result from its pharmacokinetics. Shibuya *et al*. (2005) have reported that the elimination $t_{1/2}$ of fasudil is approximately 47 min in human subjects, and the authors suggested that successful treatment could be achieved by the administration of fasudil twice daily. However, we administered fasudil once a day in this study. Therefore, more frequent administration of this compound should also be tested. In contrast, based on animal studies (Uehata *et al*., 1997; Kawabuchi *et al*., 2004), Y-27632 is more likely to have a longer $t_{1/2}$ than fasudil. Nonetheless, to make a fuller interpretation of our results, detailed information about the pharmacokinetics of these compounds is needed.

Taken together, this descriptive study suggests that Rho/Rho-kinase signalling may be involved in epileptogenesis and that Rho-kinase inhibitors could be potentially novel anti-epileptic agents.

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Conflict of interest

The authors state no conflict of interest.

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