

Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat

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ABSTRACT Marked increases in intracellular calcium may play a role in mediating cellular dysfunction and death following central nervous system trauma, in part through the activation of the calcium-dependent neutral protease calpain. In this study, we evaluated the effect of the calpain inhibitor AK295 [Z-Leu-aminobutyric acid-CONH(CH₂)₃-morpholine] on cognitive and motor deficits following lateral fluid percussion brain injury in rats. Before injury, male Sprague-Dawley rats (350–425 g) were trained to perform a beam-walking task and to learn a cognitive test using a Morris water maze paradigm. Animals were subjected to fluid percussion injury (2.2–2.4 atm; 1 atm = 101.3 kPa) and, beginning at 15 min postinjury, received a continuous intraarterial infusion of AK295 (120–140 mg/kg, *n* = 15) or vehicle (*n* = 16) for 48 hr. Sham (uninjured) animals received either drug (*n* = 5) or vehicle (*n* = 10). Animals were evaluated for neurobehavioral motor function at 48 hr and 7 days postinjury and were tested in the Morris water maze to evaluate memory retention at 7 days postinjury. At 48 hr, both vehicle- and AK295-treated injured animals showed significant neuromotor deficits (*P* < 0.005). At 7 days, injured animals that received vehicle continued to exhibit significant motor dysfunction (*P* < 0.01). However, brain-injured, AK295-treated animals showed markedly improved motor scores (*P* < 0.02), which were not significantly different from sham (uninjured) animals. Vehicle-treated, injured animals demonstrated a profound cognitive deficit (*P* < 0.001), which was significantly attenuated by AK295 treatment (*P* < 0.05). To our knowledge, this study is the first to use a calpain inhibitor following brain trauma and suggests that calpain plays a role in the posttraumatic events underlying memory and neuromotor dysfunction.

Traumatic brain injury (TBI) results in delayed cell damage and death (1–3) subsequent to acute, widespread neuronal depolarization (4) and massive release of glutamate, as well as other excitatory amino acids (4–7). Activation of glutamate receptors with concomitant depolarization leads to calcium influx through ion channels associated with glutamate receptors, as well as through voltage-sensitive calcium channels. Cellular and tissue calcium levels are significantly elevated following experimental brain injury (8–11), and alterations in calcium flux persist for several days (10). Calcium is thought to mediate a cytotoxic cascade through the activation of proteases, phospholipases, kinases, and phosphatases; impairment of mitochondrial function; and stimulation of excessive neurotransmitter release (12–14). Cellular damage, due in part to calcium-mediated cytotoxicity, may manifest itself as impaired neurobehavioral function following brain injury.

Although recent strategies for reducing posttraumatic cellular damage and neurobehavioral deficits have concentrated on antagonizing presynaptic glutamate release, glutamate receptors, or voltage-sensitive calcium channels (for review,

see refs. 15 and 16), therapeutic strategies focusing on events following the initial calcium entry might offer distinct advantages. Foremost among these is that targeting a downstream event would circumvent the current difficulty in distinguishing which of several receptor and ion channel subclasses are primarily involved in perpetuating the cytotoxic cascade. In addition, therapy directed at more delayed neuropathological events would presumably provide a longer window of opportunity for effective intervention than would treatment aimed at earlier events.

One logical target in such a downstream strategy is the nonlysosomal cysteine protease, calpain. Two isoforms of this protease are found in central nervous system tissues: calpain I, which is inactive until calcium concentrations reach micromolar levels, and calpain II, which is activated by near-millimolar free calcium (17). Activation initiates limited autolysis of calpain subunits and lowers the calcium requirement for subsequent proteolysis (18). Substrates for calpain include cytoskeletal proteins such as spectrin, tubulin, microtubule-associated proteins, and neurofilament proteins (19–23). In addition, calpain is capable of degrading enzymes (e.g., kinases, phosphatases) and membrane-associated proteins (e.g., ion channels and transporters, glutamate receptors, growth factor receptors, adhesion molecules) (24). Consequently, activation of calpain produces irreversible structural and functional alterations that are hypothesized to be cytotoxic to neurons when calpain activation is prolonged and unregulated (25–28). Indeed, evidence accumulated over the past decade suggests that calpain proteolysis contributes to cytotoxicity in many forms of neurodegeneration, including ischemic brain damage, Alzheimer disease, spinal cord injury, and neural muscular degeneration (26, 29). In models of cerebral ischemia, treatment with putative calpain inhibitors has afforded significant neuroprotection, supporting a role for calcium-induced proteolysis by calpain in the pathogenesis of acute ischemic injury (30–35). Recently, ketoamide calpain inhibitors have been shown to be neuroprotective even when administered after initiation of an ischemic event (30, 31).

Prolonged calpain activation was described after experimental brain injury in regions that correlate with neuronal degeneration and cell death (36), suggesting that calpain inhibitors might exhibit neuroprotective effects in models of TBI. In the present study, a ketoamide calpain inhibitor, AK295 [Z-Leu-aminobutyric acid-CONH(CH₂)₃-morpholine], was administered for 48 hr after fluid percussion (FP) brain injury of moderate severity in the rat. We report that both posttraumatic motor and cognitive deficits were significantly reduced by the administration of this inhibitor. To our knowledge, this study represents the first investigation of a calpain inhibitor in an *in vivo* model of TBI, as well as the first demonstration of

Abbreviations: FP, fluid percussion; MWM, Morris water maze; TBI, traumatic brain injury.

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neurobehavioral efficacy of a calpain inhibitor in an *in vivo* model of neurodegeneration.

METHODS

Adult male Sprague–Dawley rats (394 ± 18 g, $n = 46$) were housed in a Thoren (Hazelton, PA) vivarium on a 12 hr light–dark cycle. Two days before the surgical procedures, each animal was transferred to a plastic bucket lined with wood chips and allowed to acclimate, with food and water provided *ad libitum*. The buckets, needed for housing animals on continuous infusion pumps, had black interiors to minimize environmental stress (37). All procedures described herein were performed in accordance with government guidelines (38).

Experimental Design. To establish preinjury performance criteria, animals were evaluated 2 days before surgery for their ability to perform a series of neurological motor tests. In addition, they received training for a beam-walking task. One day before surgery, animals underwent additional training for the beam-walking task in the morning and initial training for a Morris water maze (MWM) cognitive test in the afternoon. The following morning, animals received final training in the MWM and were then anesthetized for surgical procedures and subjected to FP brain injury. Fifteen minutes following the scheduled injury time, each cannulated animal was returned to its plastic bucket and an infusion was initiated to deliver calpain inhibitor ($n = 15$ injured, $n = 5$ sham) or vehicle ($n = 16$ injured, $n = 10$ sham). After 48 hr, the infusion was discontinued, the cannula was temporarily tied off, and the animal was evaluated with the neurological motor tests. The cannula was then imbedded, and the rat was returned to the vivarium. Seven days after brain injury, each animal was tested for cognitive function in the MWM, and, several hours later, evaluated for neurobehavioral motor function.

Surgical Preparation and FP Brain Injury. Animals received 0.15 ml atropine (i.m.) 10 min before being anesthetized with 60 mg/kg sodium pentobarbital (i.p.). Anesthetized animals were placed in a stereotaxic frame, the scalp and temporal muscle were reflected, and a 5-mm craniectomy was created centered above the left parietal cortex, midway between lambda and bregma. A hollow female Luer-Lok (Becton Dickinson) fitting was rigidly fixed into the craniectomy. The animals were prepared for intraarterial drug administration as described (31). Briefly, the left common carotid artery was dissected free of the vagus nerve and surrounding fascia through a ventral midline cervical incision. The internal and external carotid arteries were exposed, and the external carotid artery was ligated ≈ 4 mm from the carotid bifurcation. An incision was made in the external carotid artery through which PE-10 tubing filled with 50 units/ml heparinized saline was inserted retrogradely ≈ 3 mm and secured with silk thread and tissue adhesive. This allowed for direct drug administration into the internal carotid artery without disturbing internal carotid artery blood flow.

Ninety minutes after anesthesia, one subgroup of animals ($n = 31$) was subjected to lateral FP injury of moderate severity (2.2–2.4 atm; 1 atm = 101.3 kPa) as described (39). Injury was produced by connecting the FP device to an animal via the Luer-Lok fitting. The device rapidly delivered a bolus of saline under pressure into the closed cranial cavity, onto the surface of the intact dura, resulting in a brief rise in intracranial pressure and brain tissue deformation. Sham (uninjured) animals ($n = 15$) received anesthesia and all surgical procedures but were not subjected to FP brain injury. After the scheduled time for injury, the Luer-Lok fitting was removed and the scalp was sutured.

Drug Administration. Animals were continuously administered drug or vehicle via the carotid artery according to the methods developed by Bartus *et al.* (31). Briefly, each animal was fitted with a harness jacket to which a spring tether was

attached. A PE-10 cannula, externalized at the back of the animal's neck, was threaded through the tether, looped to ensure strain relief, and connected to a swivel outflow via an intermediate segment of PE-50 tubing. The swivel was clamped into a wire frame across the top of the plastic bucket. Drug or vehicle solution was delivered to the swivel inflow through PE-50 tubing connected to a 20-cc syringe mounted into a continuous-drive syringe pump. This method permitted continuous drug administration in awake, freely moving animals.

For the present study, the infusion rate was varied in three stages over the course of the 48-hr drug-administration period. Animals were randomly assigned to receive infusion of either 2 mM AK295 or saline vehicle solution (pH 5.6), initiated at 15 min after brain injury at a rate of 9 ml/hr. Ten minutes later, the delivery rate was reduced to 1.4 ml/hr. At 24 hr postinjury, the rate was further decreased to 0.7 ml/hr for the final 24 hr. This drug administration paradigm [based upon previously published studies using models of cerebral ischemia (31)] was selected to maximize the amount of calpain inhibitor delivered acutely, while maintaining the infusion well into the pathologic cascade. Sham animals received either calpain inhibitor or vehicle following an identical administration paradigm to that used for injured animals. Thus, drug-treated animals received 52.4 mg (120–140 mg/kg) of AK295 over a period of 48 hr. At 48 hr postinjury, infusion was terminated and the externalized cannula was clamped, cut, and tied off to facilitate behavioral testing. Animals were tested for neurobehavioral motor deficits and then were anesthetized with 2% isoflurane to imbed the cannula beneath the skin.

In a preliminary study, injured animals receiving vehicle solution according to the delivery paradigm described above ($n = 3$) were killed at 48 hr postinjury, and their brains were removed for measurement of tissue edema according to described methods (40). The amount of regional cerebral edema in the animals receiving intraarterial vehicle infusion over 48 hr was comparable with animals injured at a similar level of severity that did not receive an infusion, indicating that the volume infused during the 48-hr administration period did not noticeably exacerbate brain edema (data not shown). Furthermore, AK295 infused intraarterially over several hours does not adversely effect the blood pressure or body temperature of rats (28).

Neurobehavioral Motor Function. Animals were evaluated at 48 hr and 7 days postinjury using a battery of tests designed to measure motor function (for individual test descriptions, see refs. 39 and 41). The evaluator was blinded to both injury status and the compound that the animal received. Two days before injury, each animal's baseline performance in two tasks was assessed. In one task, the maximal angle (up to 45°) at which each animal could stand in each of three directions on an inclined plane was measured. In the second task, each animal's spontaneous locomotor activity was recorded over a 4-min period for vertical and for horizontal movement. Following injury, each animal was retested on the inclined plane, receiving a score (0–4) in each direction based on the difference between the maximal angles achieved pre- and postinjury. Vertical and horizontal activity scores (0–4) were assigned according to the percentage of activity postinjury relative to preinjury. Three motor reflex tests (forelimb flexion, hindlimb flexion, and resistance to lateral pulsion) were also performed postinjury, with the right- and left-sided responses each scored from 0 to 4. Finally, each animal was evaluated in a beam-walking task (adapted from refs. 42 and 43) by grading quality of movement as the animal traversed a narrow (0.75 in), elevated wooden beam. During three training trials on each of 2 days before injury, each animal was placed on the beam at increasing distances from a darkened "escape" or "goal" box. Animals were motivated to traverse the beam into the darkened goal box by turning on a bright light at the opposite end of the beam. During the postinjury testing, each animal was

scored from 0 (worst) to 4 (best) by evaluating its ability to balance and remain on the beam, limb placement during movement, incidence of foot slippage, and body elevation relative to the beam.

A 48-point composite neuroscore was calculated for each animal by summing the scores from the twelve 4-point tests described above. Because lateral FP injury was induced over the left parietal cortex, motor deficits exhibited by injured animals were more profound on the right side. Therefore, a 28-point composite neuroscore was also formulated that more heavily weighted right-sided neurobehavioral motor function but retained contributions from each of the six separate neurobehavioral tasks. The 28-point composite neuroscore was determined by totaling scores from the inclined plane test in the vertical direction, right lateral pulsion, right forelimb flexion, right hindlimb flexion, vertical and horizontal activity, and beam-walking.

Cognitive Function. Memory function was assessed following injury using a specialized MWM paradigm that is effective in detecting posttraumatic cognitive deficits (41, 44, 45). A platform was placed at a fixed, eccentric location within a 1-m diameter maze that was filled with water to a level 1 cm above the platform. Styrofoam pieces floating on the water hid the platform so that it was not visible. Each animal was placed in the maze to swim in a series of 10 training trials on each of 2 consecutive days, during which the animal learned the location of the platform using external visual cues. Animals were purposefully overtrained, regularly locating the platform in under 5 sec in the last several trials, compared with latencies approximating 60 sec in the initial trials.

One week following brain injury, the platform was removed from the maze, and the rats were given 60 sec to swim in the maze. The rat's swimming trajectory was recorded by a computerized video system to allow calculation of the times spent in various zones of a standard, computerized grid design used to score the animal's performance in recalling the platform location. The time spent in specific grid zones was weighted according to the proximity of the zone to the location of the platform during training (44). The total distance swum by each animal during the 60-sec test was also recorded.

Data Analyses. During previous studies in this laboratory in which animals were neither cannulated nor placed in harnesses, average body weight loss at 1 week was 5% for sham animals and 10–15% for injured animals. To ensure validity and consistency in behavioral testing, and in consideration of animal welfare, we excluded animals from the present study when their weight loss was greater than 20% above these typical values; that is, sham rats that lost more than 25% ($n = 1$) and injured rats that lost more than 33% ($n = 5$) of their presurgery body weight were killed.

Weight loss and swim distance data are expressed as means \pm SDs. Comparisons between groups were made using a one-way analysis of variance followed by a Bonferroni *t* test. Composite neuroscores and memory scores (nonparametric, ordinal data) are represented by median scores. Comparisons between groups were made using the Mann–Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of AK295 Administration on Sham Animals. No deleterious effects of AK295 were observed in sham animals ($n = 5$) when compared with vehicle-treated sham controls ($n = 9$). Both the mean and the range for percent weight loss were comparable for the two groups (Table 1). Moreover, neither the 28-point composite neuroscore (Fig. 1A) nor the 48-point composite neuroscore (data not shown) showed a significant difference at either 48 hr or 7 days between sham animals that received AK295 and those that received vehicle. The two groups also performed equivalently in the MWM memory

Table 1. Body weight loss at 48 hr and 7 days after surgery with (injured) or without (sham) FP brain injury

	Sham		Injured	
	Vehicle-treated	AK295-treated	Vehicle-treated	AK295-treated
48 hr	12.1 \pm 1.3	13.4 \pm 1.4	14.9 \pm 2.8*	13.4 \pm 2.4
7 days	10.3 \pm 4.8	9.8 \pm 4.9	21.1 \pm 7.2*	23.1 \pm 8.1*

Expressed as percent decrease from body weight before surgery (mean \pm SD).

**P* < 0.05 when compared with vehicle-treated, sham animals at the same time point.

task, receiving median scores that were not significantly different (Fig. 1B). Because AK295 administration had no discernible effect on the behavior of sham animals, subsequent analyses compared the performance of injured animals with vehicle-treated sham animals (henceforth referred to as sham animals).

Body Weight Measurements. Body weight loss, calculated as a percentage of weight before surgery, was monitored for each animal throughout the study as a general means of assessing well-being. Vehicle-treated, brain-injured animals lost significantly more weight than sham animals at 48 hr ($P < 0.05$), whereas AK295-treated, brain-injured animals did not (Table 1). At 1 week, both groups of injured animals showed greater weight loss than at 48 hr. Each of the injured groups' weight loss at 1 week was significantly greater than that for shams ($P < 0.05$). However, weights lost by the vehicle- and AK295-treated injured animals were not significantly different at either 48 hr or 7 days postinjury.

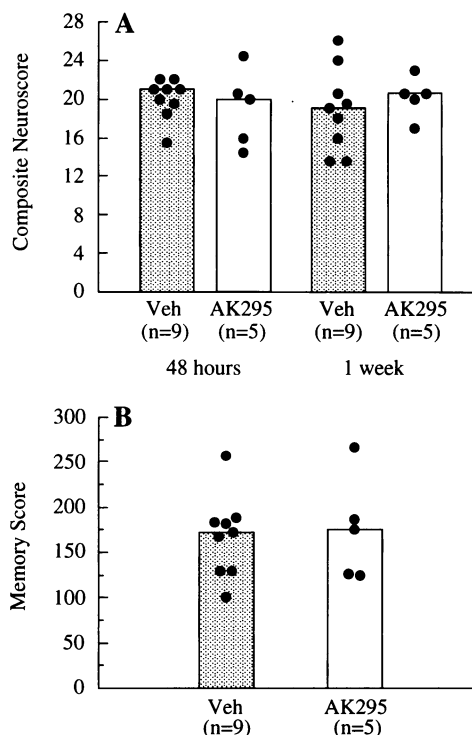


FIG. 1. Effect of AK295 treatment on sham (uninjured) animals. (A) Administration of AK295 to sham animals did not significantly alter neurobehavioral motor function, assessed using a 28-point scale, at either 48 hr or 1 week after surgery. (B) Memory retention of a task learned before surgery was not significantly influenced by AK295 treatment compared with vehicle treatment at 1-week postsurgery. Filled bars and open bars represent median scores for vehicle (Veh)- and AK295-treated animals, respectively. Dots represent scores for individual animals. *n*, Number of animals in a group.

According to the criteria described in *Methods*, six animals with excessive weight loss were removed from the study (one AK295-treated injured, four vehicle-treated injured, and one vehicle-treated sham). If data from these animals were included in the weight loss analysis, the comparisons between groups and the significance levels (P values) reported above were unaffected.

Evaluation of Neurobehavioral Motor Function. At 48 hr after injury, both the injured animals that received vehicle (median score = 23; $P < 0.005$) and those that received calpain inhibitor (median score = 27; $P < 0.01$) showed significant deficits in neuromotor function when compared with sham animals (median score = 37) scored using a 48-point composite scale (Fig. 2A). At the same time, the vehicle- and calpain inhibitor-treated injured groups were not different from each other ($P > 0.05$). The 28-point composite neuroscore evaluation, designed to emphasize right-sided neuromotor function, showed similar trends at 48 hr (Fig. 2B). Both vehicle-treated (median = 10) and AK295-treated (median = 12) animals exhibited significant deficits in motor function ($P < 0.005$) compared with sham animals (median score = 21), whereas no significant difference was observed between AK295- and vehicle-treated injured animals.

Vehicle-treated, injured animals (median = 25.5) continued to show a significant motor deficit relative to sham animals (median score = 33; $P < 0.03$) when evaluated using the 48-point composite neuroscore at 7 days postinjury (Fig. 3A).

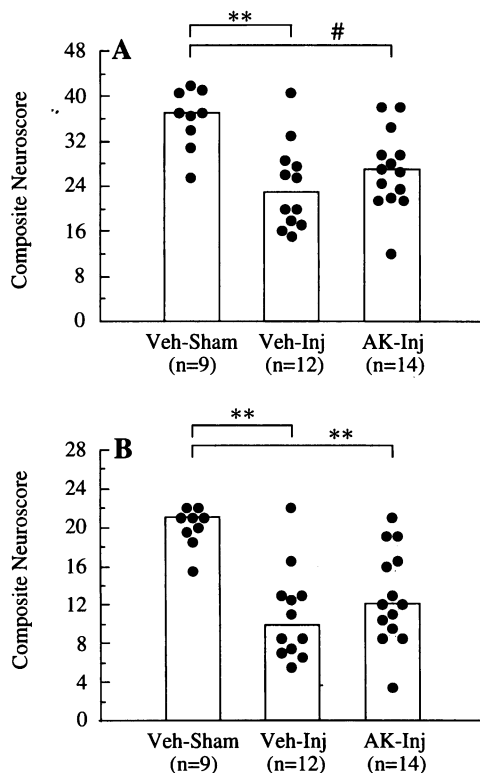


FIG. 2. Effect of AK295 treatment on motor deficits evaluated 48 hr after FP brain injury. (A) Both vehicle- and AK295-treated injured animals showed a significant neurobehavioral motor deficit compared with sham animals evaluated on a 48-point scale. (B) Using a 28-point composite neuroscore intended to increase sensitivity to the anatomic topography of trauma induced by lateral FP, both injured animals receiving vehicle as well as those receiving AK295 showed a significant posttraumatic neurobehavioral motor deficit compared with sham animals. Bars represent median scores for vehicle (Veh)- or AK295 (AK)-treated uninjured (Sham) or injured (Inj) animals; dots represent scores for individual animals. n , Number of animals in a group. Brackets indicate comparisons between two groups, where # is $P < 0.01$ and ** is $P < 0.005$.

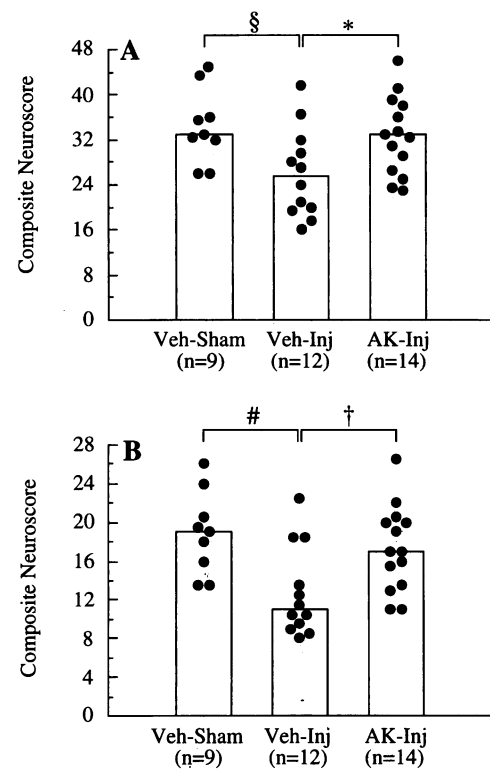


FIG. 3. Effect of AK295 treatment on motor deficits evaluated 1 week after FP brain injury. Motor function of animals treated with calpain inhibitor was significantly increased over the vehicle-treated, injured animals and was not significantly different from that of sham animals. Vehicle-treated, injured animals continued to display a significant motor deficit at 1 week. Results were consistent between (A) the 48-point composite neuroscore and (B) the 28-point composite neuroscore evaluations. Bars represent median scores for vehicle (Veh)- or AK295 (AK)-treated uninjured (Sham) or injured (Inj) animals; dots represent scores for individual animals. n , Number of animals in a group. Brackets indicate comparisons between two groups, where * is $P < 0.05$; § is $P < 0.03$; † is $P < 0.02$; and # is $P < 0.01$.

Treatment with AK295 significantly attenuated motor dysfunction observed in brain-injured animals at 7 days (median score = 33; $P < 0.05$). Remarkably, median neuroscores received by AK295-treated, injured animals and sham animals at 7 days were not significantly different. Using the 28-point composite neuroscore to evaluate neurobehavioral motor function showed a similar improvement in postinjury deficits in injured animals treated with calpain inhibitor (Fig. 3B). At 1 week after injury, no differences were observed between the sham and AK295-treated, injured animals (median = 19 and 17, respectively; $P > 0.05$), while the vehicle-treated, injured animals continued to exhibit a significant motor deficit (median = 11; $P < 0.01$). Importantly, the AK295-treated, injured animals showed significantly improved motor function when compared with vehicle-treated, injured animals ($P < 0.02$).

Evaluation of Cognitive Function. Vehicle-treated, injured animals showed a profound and significant cognitive deficit (median = 52) when compared with sham animals (median = 172; $P < 0.001$) tested 1 week after injury (Fig. 4). Treatment with AK295 significantly attenuated the posttraumatic cognitive dysfunction (median = 103; $P < 0.02$) when compared with vehicle treatment. During the memory test, the total distance swum by each of the sham animals (mean = 2620 \pm 130 cm), the vehicle-treated, injured animals (mean = 2410 \pm 280 cm), and the AK295-treated, injured animals (mean = 2670 \pm 370 cm) were not significantly different.

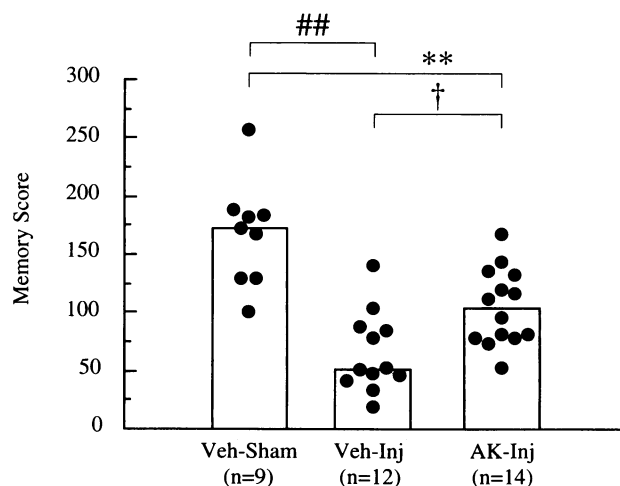


FIG. 4. Effect of AK295 treatment on cognitive dysfunction in a MWM task assessed 1 week after FP brain injury. AK295 treatment significantly improved brain-injured animals' retention of a previously learned task when compared with vehicle treatment. However, both groups continued to show impairments relative to sham controls. Bars represent median scores for vehicle (Veh)- or AK295 (AK)-treated uninjured (Sham) or injured (Inj) animals; dots represent scores for individual animals. *n*, Number of animals in a group. Brackets indicate comparisons between two groups, where † is $P < 0.02$; * is $P < 0.005$; and ## is $P < 0.001$.

DISCUSSION

Postinjury administration of the calpain inhibitor AK295 resulted in significant attenuation of cognitive deficits evaluated 1 week following traumatic brain injury. At this same time, AK295-treated, brain-injured animals showed significantly improved performance in a battery of neurobehavioral motor tests, reaching levels equivalent to uninjured (sham) animals. These findings support the hypothesis that trauma-induced activation of the calcium-dependent protease, calpain, contributes to posttraumatic morbidity. Furthermore, our results suggest that novel calpain inhibitors may be useful in treating the posttraumatic pathogenic cascade, as demonstrated by the significant improvement in behavioral outcome measures following administration of AK295.

An alternative therapeutic approach to the pharmacological prevention of posttraumatic calcium entry into cells is the inhibition of deleterious secondary events triggered by elevated intracellular calcium. Inhibition of neuronal calpain, for example, might attenuate consequences of trauma-induced calcium overload, particularly in the soma and dendrites. Moreover, inhibition of glial calpain might interfere with cytoskeletal changes likely to occur during posttraumatic glial hypertrophy (2, 46). Calpain inhibition is a powerful strategy because calpain is located in many neuronal and glial populations (46, 47). Targeting calpain, then, provides protection across many brain regions while maximizing beneficial effects in the most vulnerable cellular structures. In addition, because calpain degrades a wide range of cellular proteins (24), calpain inhibition has the potential for attenuating or preventing multiple pathophysiologic events triggered by a common activator. From a therapeutic perspective, calpain inhibitors may have several advantages over other more conventional receptor antagonists: (i) targeting calpain may provide a significantly enhanced window of opportunity, because calpain activation occurs after the influx of calcium, a particularly important consideration in cases where immediate posttraumatic treatment is not feasible; (ii) antagonizing calpain may have few deleterious effects on normal cell function because the physiologic role of calpain, compared with that of calcium channels and glutamate receptors, may be limited under

normal conditions; and (iii) beneficial or protective events triggered by elevated ionic calcium (48) can proceed because calpain inhibition does not prevent intracellular calcium increases.

The calpain inhibitor AK295 has several characteristics that make it well suited for therapeutic as well as mechanistic studies. This ketoamide inhibitor has a greater potency ($K_i < 50$ nM for both calpain I and II) than do other commonly used inhibitors such as leupeptin or calpain inhibitor I (28). Relative to these compounds, AK295 is also more selective for calpain ($K_i = 0.027\text{--}0.042$ μM) over other cysteine proteases (for example, K_i for cathepsin B = 24 μM) (28). The primary effect of AK295 administration, therefore, is presumed to be the selective inhibition of calpain. However, inhibition of other cysteine proteases may also contribute to the neurobehavioral efficacy of AK295 following FP injury. The increased membrane permeability of AK295 with respect to the aforementioned aldehyde inhibitors (28) obviates the need for administration over extended periods prior to experimental brain injury. A transient, regional breakdown of the blood-brain barrier following lateral FP injury (2) may also facilitate access of the inhibitor to injured neural tissue. Although AK295 has no known vasoactive properties (28), it is conceivable that the initial hypervolemic infusion of drug increased local tissue perfusion, consequently improving blood flow and drug delivery to the injured tissue.

Treatment with the calpain inhibitor AK295 significantly improved motor function of brain-injured animals at 1 week, but not at 48 hr, postinjury. This finding is not inconsistent with our expectations. Much of the cell loss that results from TBI follows a "delayed cell death" pattern (2, 49), and is accompanied by a chronic neurobehavioral motor deficit beginning as early as 1 day and persisting beyond 1 month postinjury (39). However, a number of transient pathogenic phenomena have been characterized that may impair normal brain function during the acute posttraumatic period. For example, significant brain edema occurs within hours after injury and resolves in most regions by 3 to 5 days (40). In addition, the excessive extracellular glutamate measured immediately following experimental brain injury seems to decrease with a time constant on the order of minutes to hours (4-7). It is expected that calpain inhibition would not provide significant attenuation of the transient neuronal dysfunction that results from these phenomena, because the inhibitor is intended to protect neurons and glia against an intracellular, calcium-mediated cytotoxic cascade. However, once these phenomena resolve, the effects of continuing calpain inhibition (presumed to be the reduction of intracellular proteolysis and its ensuing long-term structural and functional damage) may be detected. The data presented here indicate that timely administration of a selective, membrane permeant calpain inhibitor can significantly reduce the more lasting neurobehavioral consequences of trauma.

Lateral FP brain injury results in neuronal cell death in an extensive region of the ipsilateral parietotemporal cortex (1, 2). Within hours of the traumatic insult, neurons with abnormal morphology are detectable. As the pathogenic cascade progresses, necrosis and cell loss are evident in the cortex, and other brain regions are recruited into the total neurodegenerative pattern. Activation of calpain seems to be a part of this posttraumatic neurodegenerative cascade, as indicated by the early onset and the prolonged nature of calpain-mediated spectrin breakdown in the cortex and hippocampus following lateral FP injury (36). Acutely diminished levels of microtubule-associated protein 2 (MAP2) and neurofilament proteins in the hippocampus and/or cortex following experimental brain injury (49-51) suggest that calpain may be acting on other structural proteins as well. MAP2 degradation accompanying experimental cerebral ischemia (35) and loss of neurofilament protein resulting from experimental spinal cord injury (52) can be attenuated by inhibition of cysteine proteases. Inhibition of calpain I, in particular, may be beneficial

in reducing trauma-induced degradation of neuronal cytoarchitecture, as calpain I is distributed throughout neuronal perikarya and dendrites (46).

The present study showed that AK295 administration significantly improved memory retention following TBI, indicating that calpain inhibition attenuated posttraumatic retrograde amnesia. Because each group of animals was able to swim equivalent distances during the MWM test, the difference in memory scores between the groups could not be attributed to accompanying motor deficits. It is reasonable to postulate that impaired hippocampal function is at least partially responsible for the posttraumatic memory deficit, in light of the important role the hippocampus plays in the formation and short-term retention of memory (53, 54). The degree of retrograde amnesia following lateral FP brain injury was correlated with the extent of bilateral neuronal loss in the dentate hilus (55) that, in addition to the ipsilateral CA3 region, is a primary site of selective, hippocampal neuronal damage (2, 45, 56, 57). Inhibitors of cysteine proteases, and thus of calpain, have been reported to affect hippocampal cell function, improving the recovery of synaptic potentials in hippocampal slices subjected to hypoxia (58, 59) and promoting retention of the capability for long-term potentiation following cerebral ischemia (34). In addition, such inhibitors reduced hippocampal cell loss in the CA1 region in experimental models of cerebral ischemia (28, 33, 34). Studies are needed to determine if calpain inhibitors, in addition to attenuating posttraumatic cognitive dysfunction, promote cell survival in hippocampal regions that typically exhibit delayed cell death following brain trauma.

In conclusion, postinjury administration of the calpain inhibitor AK295 produced significant attenuation of both motor and cognitive deficits associated with TBI in rats. These findings suggest that calpain plays an important role in the posttraumatic sequelae that lead to persistent neurobehavioral dysfunction, and indicate that the inhibition of calpain may be a beneficial therapeutic approach toward reducing posttraumatic morbidity.

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