Quantitative Analysis of Effects of \(\kappa\)-Opioid Agonists on Postischemic Hippocampal CA\(_1\) Neuronal Necrosis in Gerbils

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The ability of the \(\kappa\)-opioid receptor agonists U50488H and U62066E (spiradoline mesylate) compared with the non-\(\kappa\) close structural analogue U54494A to affect postischemic necrosis of the selectively vulnerable hippocampal CA\(_1\) neurons was examined in male Mongolian gerbils. The gerbils were treated with either saline vehicle or 10 mg/kg i.p. of one of the test drugs 30 minutes before and again 2 hours after a 10-minute period of bilateral carotid artery occlusion or sham occlusion under light methoxyflurane anesthesia. Seven days after ischemia and reperfusion the brains were perfusion-fixed, and hippocampal CA\(_1\) cells were counted in a blind fashion. In ischemic gerbils that received only vehicle, there was a 78.9% loss of CA\(_1\) neurons compared with sham-occluded gerbils. In contrast, in U50488H-treated gerbils, mean cell loss was reduced to 33.9% (\(p<0.01\) vs. vehicle-treated group). U62066E was even more effective in reducing postischemic CA\(_1\) degeneration to only 20.7% (\(p<0.0001\) vs. vehicle-treated group). However, treatment with the non-\(\kappa\) analogue U54494A did not cause any apparent protection; the gerbils in this group showed an 80.7% loss of CA\(_1\) neurons. Our results are consistent with the hypothesis that \(\kappa\)-receptor stimulation is associated with improved postischemic neuronal preservation. (Stroke 1988;19:1008-1012)

Following the empirical demonstration of a beneficial effect of the general opiate receptor antagonist naloxone in experimental cerebral ischemia in gerbils,\(^1\) rats,\(^2\) cats,\(^3\) and primates,\(^4,5\) considerable interest has developed concerning the possible role of endogenous opioids in ischemic pathophysiology and neuronal degeneration. Based on the anti-ischemic action of naloxone, an hypothesis has been developed that postischemic neuronal degeneration may in part depend on an excessive activation of endogenous opioid systems, which could be therapeutically antagonized with opiate receptor blocking drugs such as naloxone or naltrexone.

However, some investigators have failed to observe a positive effect of naloxone in their particular ischemia models.\(^6,7\) More recent studies have suggested that pharmacologic stimulation of the \(\kappa\)-opioid receptor subtype can actually ameliorate rather than exacerbate postischemic neuronal degeneration and promote neurologic recovery and survival. The initial study in this regard showed that treatment of cats after permanent unilateral occlusion of a middle cerebral artery with the putative endogenous \(\kappa\)-opioid agonist dynorphin 1-13 resulted in improved long-term survival.\(^8\) In the same study, the non-\(\kappa\) dynorphin 3-13 was ineffective.

Subsequently, Tang\(^7\) and colleagues\(^9\) have demonstrated an anti-ischemic effect of the selective \(\kappa\)-opioid receptor agonist U50488H.\(^10\) Their rationale for testing this compound in various cerebral ischemia paradigms has been based on the \(\kappa\)-related diuretic action\(^11\) that they postulated might serve to reduce postischemic cerebral edema. In an initial study, U50488H was shown to inhibit postischemic motor hyperactivity in gerbils subjected to a 7-minute bilateral occlusion of the common carotid arteries. Ironically, this effect was partially antagonized, rather than promoted, by naloxone. Correlated with the protective effect of U50488H was a semiquantitative finding of a reduction in ischemic cell change in the hippocampal CA\(_1\) subfield.\(^7\) This brain region is selectively vulnerable to brief periods of global ischemia.\(^12\)

In our current study, the ability of the \(\kappa\)-opioid agonist U50488H to retard postischemic CA\(_1\) degeneration in gerbils is examined in more quantitative histologic terms. In addition, the effects of U62066E (spiradoline mesylate), another more potent ana-
logue of U50488H, and U54494A, a non-κ structural analogue, are compared with those of U50488H.

Materials and Methods
Male Mongolian gerbils weighing 50–70 g were anesthetized with methoxyflurane. A 1–2 cm midline throat incision provided access to both carotid arteries while causing minimal tissue damage. After the arteries were located, they were loosely encircled with silk thread to facilitate occlusion with microaneurysm clamps. Thirty minutes before occlusion, intraperitoneal injections of 0.2-ml volumes of either vehicle (0.9% saline) (18 gerbils) or 10 mg/kg U50488H (8 gerbils), U62066E (9 gerbils), or U54494A (7 gerbils) were administered. Following 10 minutes of bilateral carotid occlusion (BCO) the clamps were removed, reperfusion was verified, and the wounds were closed with silk thread. Two hours after reperfusion the gerbils were injected a second time. Seven sham-occluded gerbils underwent the same anesthesia and surgical procedures without BCO.

One week after BCO, the gerbils were anesthetized again with methoxyflurane and the brains were fixed via cardiac perfusion with a combination of 10% formaldehyde, 10% acetic acid, and 80% methanol (FAM) following a saline flush. Immediately following perfusion, the brains were removed, placed in FAM overnight, and then embedded in paraffin. Five-micrometer-thick cross sections were taken between 1.4 and 3.0 mm posterior to the bregma, stained with cresyl violet, and examined by light microscopy at x 320 magnification. Slide labels were covered with tape to enable blind evaluation. All normal-appearing pyramidal cells in a 315-fim line from postischemic neuronal degeneration secondarily to a brief nonlethal episode of global brain ischemia. The probability that this anti-ischemic effect is related to U50488H's κ-opioid agonist properties is supported by the slightly greater protective effect of the more potent U50488H analogue U62066E. Furthermore, if the structure of U50488H is altered slightly but to the point of destroying the κ-opioid agonism, then the anti-ischemic action disappears. This is clear in the lack of effect of the non-κ compound U54494A. In a similar fashion, Tang showed that the more κ-active levo-enantiomer of the racemic U50488 is much more effective than the less κ-active dextro-enantiomer in protecting gerbils from postischemic hyperactivity.

The precise mechanism of the apparently κ-opioid receptor-mediated cerebroprotective action of U50488H and U62066E is still uncertain although three distinct possibilities can be gleaned from related cerebral ischemia and trauma studies. First, the original rationale for testing U50488H was the expectation that the diuretic action of this compound U50488H analogue U62066E. Furthermore, if the structure of U50488H is altered slightly but to the point of destroying the κ-opioid agonism, then the anti-ischemic action disappears. This is clear in the lack of effect of the non-κ compound U54494A. In a similar fashion, Tang showed that the more κ-active levo-enantiomer of the racemic U50488 is much more effective than the less κ-active dextro-enantiomer in protecting gerbils from postischemic hyperactivity.

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administration of antidiuretic hormone prevents the plasma hyperosmolar action of U50488H as well as the reduction of ischemic cerebral edema. Thus, it is conceivable that the protection of the gerbil CA1 region from degeneration could be in part the result of a k-opioid-mediated water diuresis and a possible reduction of cellular edema in the peculiarly susceptible CA1 pyramidal neurons. Tang in fact noted greater swelling of the CA1 cells in untreated than in U50488H-treated gerbils 24 hours after ischemia (i.e., prior to actual degeneration), which is consistent with this possibility.

A second candidate mechanism concerns the purported role of an excitatory amino acid-induced calcium influx in the susceptibility of the hippocampal CA1 region to brief periods of ischemia. A recent study strongly supports the involvement of disturbed calcium homeostasis in postischemic damage to the gerbil CA1 region. In this regard, U50488H has been shown to antagonize excitatory amino acid (e.g., kainate, N-methyl-D-aspartate, and quisqualate)-triggered seizures in mice and to decrease potassium-induced calcium uptake by rat brain synaptosomes. However, the likelihood that these effects are related to the anti-ischemic action of U50488H (or U62066E) seems remote since they are also shared by the non-k analogue U54494A, which was completely ineffective in retarding postischemic CA1 degeneration.

A third potential contributing factor in the antiischemic action of k-opioid agonists has to do with the demonstrated effect of U50488H to significantly attenuate posttraumatic hypoperfusion as shown in injured mouse brain and cat spinal cord. A similar improvement in blood flow maintenance by the k-opioid agonists after reperfusion of the ischemic gerbil brain could serve to promote postischemic neuronal survival. However, this possible scenario seems of little importance to our current protection of the CA1 region since brain hypoperfusion does not occur in the gerbil brain after only brief periods of ischemia.

**TABLE 1. Incidence of Gerbils With >50% Hippocampal CA1 Neuronal Preservation 1 Week After 10-Minute Bilateral Carotid Artery Occlusion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>No.</th>
<th>%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>18</td>
<td>3</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>U50488H</td>
<td>8</td>
<td>6</td>
<td>75.0</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>U62066E</td>
<td>9</td>
<td>8</td>
<td>88.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U54494A</td>
<td>7</td>
<td>1</td>
<td>14.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Probability assessed using \( \chi^2 \) compared with vehicle.
Thus, of the three mechanisms discussed, the most tenable explanation for the antagonism of delayed postischemic CA$_1$ degeneration by U50488H and U62066E appears to be a reduction in cellular edema secondary to a $\kappa$-opioid receptor-mediated diuretic action as proposed by Tang and coworkers. This apparent inhibition of cellular edema is probably complex in that other antiedema agents such as the glucocorticoid steroids can actually exacerbate postischemic CA$_1$ necrosis. Such an effect has been reported for corticosterone pretreatment of rats subjected to 20 minutes of severe forebrain ischemia. Furthermore, we have observed that high-dose treatment of gerbils with methylprednisolone significantly increases CA$_1$ neuronal loss in the currently employed model (E.D. Hall and K.E. Pazara, unpublished observations).

In addition to a complex antiedema effect, a direct protective action of the $\kappa$-opioid agonists on the hippocampal CA$_1$ region should not be dismissed. A recent report showed that concentrations of the endogenous $\kappa$-opioid agonist peptide dynorphin in the gerbil hippocampus, which are higher than in other brain regions except the hypothalamus, decrease by approximately 50% and remain decreased for at least a week after a 5-minute episode of BCO. The decrease in dynorphin precedes the onset of ischemic cell change in the CA$_1$ subfield. It is tempting to speculate from this that the partial loss of dynorphin in some way contributes to the degenerative pathophysiology and that administration of an exogenous $\kappa$-opioid receptor agonist serves to restore protection in a replacement therapy fashion. Confirmation of this possible
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KEY WORDS • cerebral ischemia • hippocampus • endorphins • gerbils
Quantitative analysis of effects of kappa-opioid agonists on postischemic hippocampal CA1 neuronal necrosis in gerbils.
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Stroke. 1988;19:1008-1012
doi: 10.1161/01.STR.19.8.1008

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