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The cardiac glycoside ouabain potentiates excitotoxic injury of adult neurons in rat hippocampus

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Abstract

We demonstrate that the enzyme family responsible for the restoration of the transmembrane cation balance, namely the sodium pump (Na^+,K^+-ATPase), plays a critical role in whether glutamate injures adult neurons in vivo. Partial inhibition of the sodium pump by the cardiac glycoside ouabain in young adult rats is not itself damaging. This treatment, however, markedly potentiates ordinarily subtoxic dosages of the glutamate analog kainic acid to produce limbic seizures and widespread neurodegeneration within the hippocampus in a pattern closely resembling that observed for human temporal lobe epilepsy.

Keywords: Sodium pump; Na^+,K^+-ATPase; Epilepsy; Kainic acid; Selective vulnerability; Seizures; Remodeling

Neurodegeneration is a common manifestation of a variety of neurological conditions such as epilepsy, Huntington's chorea, Parkinson's disease and the result of hypoxia or hypoglycemia (reviewed in Refs. [4,13]). Recently, widespread interest has focused on the possibility that injury and death of neurons results from toxic actions of normal neurotransmitters such as glutamate. Considerable evidence derived from the study of fetal and neonatal neurons in vitro suggests that glutamate damages neurons by several mechanisms, including an early phase of osmotic injury (latency of minutes) mediated by intracellular fluxes of Na^+ and Cl^-, and a later phase (hours) arising from increases in intracellular Ca^{2+}. Prevention of these ionic fluxes in model systems can effectively ameliorate neuronal damage [4,13]. A key enzyme involved in moderating these ion fluxes is the Na^+,K^+-ATPase which performs both direct (Na^+/K^+ re-equilibration) and indirect (Na^+-dependent Ca^{2+} exchange and glutamate uptake) roles in neurons and glia (reviewed in Refs. [12,13]). It is therefore likely that the sodium pump is an important factor in neurodegenerative processes.

We studied the role of Na^+,K^+-ATPase in excitotoxic injury using the rat model of kainate-induced seizures [22]. Kainic acid is a potent glutamate analog which elicits characteristic seizures and subsequent neurodegeneration. In rats, the pattern of neuronal damage is widespread throughout the limbic system and has been extensively characterized [22]. Since it is well known that species and developmental variations occur in kainate toxicity [22], we first determined a dose response curve for kainate-induced seizures for young adult rats (~250 g weight; Charles River Breeders, Wilmington, MA). Electroencephalographic electrodes were implanted stereotactically into the hippocampus bilaterally and the superficial frontal cortex using the coordinates of Paxinos and Watson [18], and cemented to a skull connector with acrylic cement. A cannula was also placed into the right lateral ventricle for drug administration (coordinates: AP 0.4 mm; ML +1.3 mm; DV 3.5 mm from bregma). Animals were studied a minimum of one week after surgery. Electrode and cannulae placements were verified by serial sections obtained after animal sacrifice.

We found that the kainate dose-response curve was steep: only 2 of 19 animals exhibited moderate seizures (grade III as per Ref. [23]; 'wet dog' shakes, continuous head nodding, foaming at the mouth, and bilateral forelimb clonus but not falling) for kainate ≤7 mg/kg body weight (given intraperitoneally; i.p.), compared to grade

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IV for 12 of 14 animals given kainate at 8–10 mg/kg. High kainate dosages (≥10 mg/kg i.p.) produced severe, prolonged seizures with a latency of approximately 60–90 min, in the range previously reported [22]. These lasted up to 4–5 h, and included rearing and falling backwards, producing a high animal mortality (>50%). A staining procedure which impregnated dead and dying cells with silver was performed 24 h after treatment, an optimum time to identify somata and dendrites [17]. Animals were narcotized with chloral hydrate and perfused with 4% paraformaldehyde in phosphate-buffered saline for 30 min. The brains were then removed and further fixed in paraformaldehyde for 2 additional days. Subsequently, 40 μm thick sections were cut on a vibratome and stained according to the protocol of Nadler and Evenson [17]. Within the hippocampus, we observed that this stain identified neuronal perikarya and fibers only after overt behavioral seizures. As others have previously reported (reviewed in Ref. [22]), high dose kainate (≥8 mg/kg) primarily damaged neurons within the CA3 subfield of the ventral hippocampus (data not shown), a region which expresses high levels of kainate receptors [16]. In addition, high dosages of kainate were occasionally associated with variable CA1 damage within the dorsal hippocampus of surviving animals. In contrast, low doses of kainate (≤7 mg/kg) produced no hippocampal cell loss. Electroencephalographic (EEG) analysis demonstrated that dosages of kainate 4–7 mg/kg did produce changes in brain EEG activity, such as generalized slowing (Fig. 1B), although behavioral seizures were not observed.

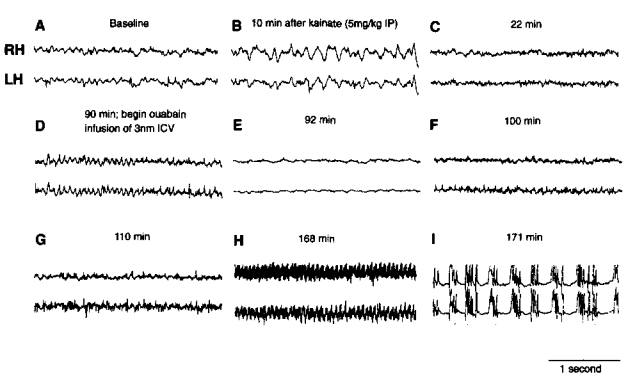

Fig. 1. Representative electroencephalographic recording obtained from a treated animal. (A) shows typical baseline, resting activity prior to drug injection. In (B), 5 mg/kg kainate (below the seizure threshold) was administered i.p. 10 min earlier, with the resulting slowing of EEG activity. Later records (C,D) reverted to baseline, but frequent prolonged periods of staring and 'wet dog' shakes were observed. Ouabain (3 nmol) administered into the right lateral ventricle 90 min after kainate elicited marked motor activity characterized by racing around the cage, generally circling in an ipsilateral direction, but without other signs of agitation or of seizure activity, Within 2 min of this hyperactive phase, EEG activity greatly diminished (E). During this period, the animal was hypotonic with outstretched limbs. Electrical activity soon returned (F,G). Ultimately, Grade IV (as per Sperk et al. [23]) seizures occurred (H,I). RH, right hippocampus; LH, left hippocampus.

The cardiac glycoside ouabain is a member of a class of drugs which inhibits sodium pump function with high specificity, binding near or within the extracellular K^+ site of the phosphorylated catalytic subunit [24]. Since the sodium pump is essential for the maintenance of cellular integrity of all cells, ouabain at high concentrations is highly toxic to both neurons and glia [15]. However, a special and critical dependence of intact sodium pump activity for the integrity of neurons is illustrated by the observation that lower dosages of ouabain directly infused into the hippocampus selectively kills only neurons and indeed, ouabain appears to be one of the most potent neurotoxins [14]. Although most mammalian sodium pump enzyme possesses high (10^{-9}–10^{-8} M) affinity for cardiac glycosides, the rodent $\alpha 1$ isoform exhibits a low affinity (10^{-4}–10^{-3} M) and thus is 'resistant' to glycoside inhibition [25]. Because of this fact, ouabain at appropriate concentrations can differentially inhibit the activity of the $\alpha 2/\alpha 3$ isoforms. We have previously shown using an in vitro model of the cerebral cortex [2] that inhibition of the $\alpha 2/\alpha 3$ isoforms using ouabain (at 1 μM) reduces the total sodium pump activity as assessed by $^{86}Rb^+$ uptake by ~65%. Under basal conditions, this reduction of sodium pump capacity is not neurotoxic. However, if these cultures are subsequently subjected to the additional stress of normally innocuous levels of glutamate (100 μM), neurons are preferentially destroyed. Although this model is convenient and widely used in the study of the pathophysiology of neurodegenerative diseases, the cells are fetal in origin and the findings may not apply to the adult brain. Does a similar amplification of neurotoxicity occur for adult neurons after sodium pump impairment?

Rats given ouabain at very high dosages intraperitoneally [10] or intracerebrally [8] into the lateral ventricle (ICV), exhibit generalized tonic clonic seizures and death. Lower dosages given ICV (<4 nmol [6]) produce a stereotyped motor behavior characterized by vigorous running for ~1–5 min, followed by a temporary hypotonia for ~20–30 min, and then apparent complete recovery. We observed that ICV administration of 3 nmol of ouabain (in 10 μl over 10 min), which, assuming uniform distribution throughout the volume of extracellular brain water [11], provides a concentration ~0.5–1 μM within the brain, reliably elicited running. Although an earlier study found that administration of 1 nmol ouabain directly into the hippocampus produced panneuronal death [14], we observed no limbic behavioral seizures or obvious cell injury (Fig. 2B) after 3 nmol were infused into the ventricle immediately adjacent to the hippocampus. Continuous electroencephalographic analysis during ouabain administration did not reveal spiking activity during or immediately after the running behavior. However, a marked reduction of brain electrical activity occurred during the period of hypotonia, with normalization associated with a resumption of motor activity (Fig. 1E,F).

When subtoxic dosages of kainate (5–7 mg/kg given i.p.) were followed 30 min later by ouabain (3 nmol ICV), seizures (grade III–IV) routinely developed with an average latency of 116 ± 13 min (SEM) after kainate, i.e., at a time when seizures were never observed after kainate alone. These convulsions were typical for those of kainate, were not associated with obvious apneic spells, and continued for 345 ± 9 min (SEM; $n = 8$). In spite of the severity of seizures which routinely occurred after ouabain and kainate, virtually no animal mortality was observed. Delaying ouabain administration by 90 min after kainate injection increased the latency of the onset of seizures (by 70 ± 15 min), although the duration of epileptic discharges under this paradigm shortened notably by ~150 min ($n = 6$). Pairing of ouabain and kainate reliably ($P < 0.001$; Chi-squared analysis) produced seizures characteristic for kainate alone and neuronal degeneration (13 of 14 animals). In contrast, kainate or ouabain alone did not (2 of 16 and 0 of 7 animals respectively). EEG recording showed that the hippocampus adjacent to the ventricle into which ouabain was injected initiated epileptiform activity, which then spread to the contralateral hippocampus, and finally generalized. Analysis of neuronal death by kainate/ouabain treatment revealed a marked difference when compared to the kainate paradigm alone. First, widespread destruction occurred in regions not reliably observed for kainate. For example, within the hippocampus the dorsal region was heavily injured, particularly the CA1 subfield and, to a lesser extent, the CA3 subfield (Fig. 2C,E,F). Regions outside the hippocampus, e.g. frontal cortex, were virtually unaffected, unlike high dose kainate alone. Like kainate, hilar interneurons were heavily damaged by this treatment (Fig. 2D) and the CA2 subfield was relatively spared. These anatomical lesions, particularly in the temporal lobe, recapitulate those observed for human temporal lobe epilepsy [7]. Animals surviving these severe seizures were observed to exhibit spontaneous limbic seizures, especially upon handling, which has been reported [5] after high dose kainate treatment alone.

These results are consistent with an amplification of kainate toxicity by direct, pharmacologic reduction of sodium pump capacity: the dosages of ouabain employed here were well below the epileptogenic threshold and the seizures observed were those of kainate. The mechanism(s) by which ouabain potentiates kainate toxicity remains to be determined. Our hypothesis is that sodium pump impairment will compromise cells with respect to the rectification of ions. This could arise from either a neuronal or glial pool, as both populations actively buffer potentially excitotoxic disturbances by a variety of mechanisms which depend upon the function of the sodium pump [20]. Direct in vitro intracellular recording obtained from hippocampal slices stimulated by glutamate application has supported this concept, as impairment of sodium pump activity markedly reduces the capacity of CA1 pyramidal cells to restore the transmembrane gradient [26]. The more extensive neuronal damage we observed within the hippocampus involving the CA1 area cannot be explained by direct action through kainate receptors, as this region appears to express only low levels of kainate receptors [16], but could occur through activity of aminohydroxymethlyisoxazolepropionic acid (AMPA) receptors [19] which do exhibit a weak affinity for kainate. Alternatively, persistent depolarization produced by kainate increases intrahippocampal glutamate release [9], which could then act indirectly through the high density of *N*-methyl-D-aspartate (NMDA) receptors in this region. The CA1 region of the hippocampus also exhibits extreme sensitivity to hypoxic insults [27]. Although we did not directly measure arterial oxygen saturation during seizures, we did not observe apneic spells and therefore hypoxia is an unlikely explanation for the observed injury. Further, the seizure phenotype was of kainate type alone, which generally produces little CA1 damage. It is unlikely that ouabain acts in other ways in

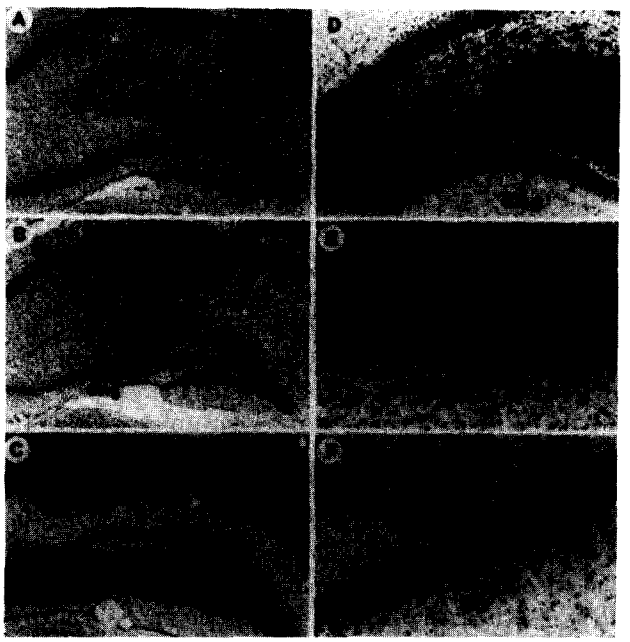

Fig. 2. Neuronal death in the dorsal hippocampus associated with various treatments. Non-seizure producing dosages of kainate (e.g., 7 mg/kg; (A)) do not cause neuronal death as assessed by a silver degeneration stain which shows dead/dying cells and their processes as black. Likewise, ouabain (3 nmol ICV), although producing an extreme pattern of motor behavior did not produce neuronal death (B). In contrast, pairing of these treatments produced widespread death throughout the hilus (Hi) and CA1 and 3 subfields (C). Note fiber staining of apical dendrites of pyramidal cells in stratum radiatum (SR) in (C; small arrows). Similar to human TLE, pyramidal neurons of the CA2 subfield were predominantly spared. A variety of hilar neurons are affected (D) including those of the mossy cell morphology, which are an early target in other animal models of temporal lobe epilepsy [21]. (E,F) correspond to high power views of degenerating CA3 and CA1 pyramidal neurons. Other abbreviations: ML, molecular layer of the dentate; GC, granule cell layer; SO, SP, and SR, stratum oriens, pyramidale, and radiatum respectively; arrow in (B) separates CA2 and CA1 regions.

this model, such as significant modification of cerebrospinal fluid, as the choroid plexus appears to express only the $\alpha 1$ (ouabain resistant) isoform [1].

Although a limited pharmacologic reduction of total sodium pump capacity does not of itself produce neurodegeneration in vivo, even moderate excitation overwhelms neurons with such reduced capacity to accomplish effective homeostasis, and they are injured. These observations have implications for human disease, as cardiac glycosides are widely used to treat congestive heart failure and atrial fibrillation. Neurologic symptoms are a prominent part of glycoside toxicity, which illustrates that these agents do penetrate the blood brain barrier in humans. In contrast to rodent isoforms, however, in humans all three catalytic subunits display high affinity for cardiac glycosides [25]. Thus, it is likely that the neurotoxic threshold for glycosides is much lower for humans than for rodents. Our work suggests that at least under conditions of acute inhibition of sodium pump activity (such as presumably occurs during initiation of therapy) cardiac glycosides may appreciably amplify potential neurologic lesions. Besides pharmacologic impairment, however, functional inhibition could occur whenever the high energy substrates used to operate the pump are limited, such as will occur in hypoglycemia or hypoxemia (reviewed in Refs. [12,13]). In human TLE, for example, study of cytochrome oxidase activity (an enzyme critical for ATP generation with which to power the pump) suggests that its activity may be impaired [3], and therefore sodium pump function as well. Thus, in many ways abnormalities of sodium pump function could reduce its total capacity and contribute to hyperexcitability and an increased potential for neurodegeneration.

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