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Low-dose cardiotonic steroids increase sodium–potassium ATPase activity that protects hippocampal slice cultures from experimental ischemia

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ABSTRACT

The sodium–potassium ATPase (Na/K ATPase) is a major ionic transporter in the brain and is responsible for the maintenance of the Na^+ and K^+ gradients across the cell membrane. Cardiotonic steroids such as ouabain, digoxin and marinobufagenin are well-characterized inhibitors of the Na/K ATPase. Recently, cardiotonic steroids have been shown to have additional effects at concentrations below their IC_{50} for pumping. The cardiotonic steroids ouabain, digoxin, and marinobufagenin all show an inverted U-shaped dose–response curve with inhibition of pumping at concentrations near their IC_{50}, while increasing Na/K ATPase activity at doses below their IC_{50}. This stimulatory effect of cardiotonic steroids was observed *in vitro* in hippocampal slice cultures as well as in the hippocampus *in vivo*. Increased Na/K ATPase activity has been shown to protect slice culture neurons from hypoxia–hypoglycemia. Ouabain protected slice culture neurons from experimental ischemia at concentrations that increased Na/K ATPase. This protective effect was observed when ouabain was dosed 30 min before, or 2 h following experimental ischemia. Ouabain no longer protected against experimental ischemia if the increase of Na/K ATPase was blocked. These data suggest that the protective effect of ouabain was due to increased Na/K ATPase activity. The demonstration of a neuroprotective effect of cardiotonic steroids could potentially assist in the treatment of stroke since digoxin, one of the cardiotonic steroids examined in this study, has approval by the Food and Drug Administration and can be safely administered at the concentrations that increase Na/K ATPase activity.

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The Na/K ATPase is a major transporter in the brain that maintains the ionic gradients of Na^+ and K^+ [28]. Disruption of the sodium and potassium gradients depolarizes neurons and disables the Na/H, the Na/Ca and the Na/K/Cl transporters and sodium-dependent glutamate uptake [7]. All of these factors contribute to brain injury following ischemia [7].

Ischemic preconditioning protects the brain from ischemic injury. In preconditioning, a brief period of ischemia protects from a longer, damaging ischemic episode [29]. Cardiac ischemic preconditioning prevented the loss of Na/K ATPase activity that protected hearts from subsequent ischemia [8,18,20,22,27]. Preconditioning also prevented loss of Na/K ATPase in the kidney [1]. In the brain, ischemic preconditioning increased the activity of the Na/K ATPase [6]. In hippocampal slice cultures, increased pump activity protected neurons from an experimental ischemia of hypoxia–hypoglycemia [25]. Even though these studies implicate increased Na/K ATPase activity in protection against ischemia, ischemic preconditioning is unlikely to be used in the clinic [24,29]. This study explores whether cardiotonic steroids are an alternative to ischemic preconditioning to increase basal Na/K ATPase and to test whether the increase in basal pumping protects neurons from ischemia.

Low-dose cardiotonic steroids provide a potential way to increase Na/K ATPase activity. When dosed at concentrations near their IC_{50}, cardiotonic steroids inhibit the Na/K ATPase and are used in the clinic to treat congestive heart disease and arrhythmias [2,10]. There are scattered reports over many decades suggesting that cardiotonic steroids increased pumping at concentrations below their IC_{50} [3,5,11,12,14]. In addition, cardiotonic steroids are synthesized endogenously and are active at nanomolar concentrations [2,28]. This suggests that cardiotonic steroids are potentially regulators of Na/K ATPase. Recent studies have shown that, at high, pharmacological doses, cardiotonic steroids inhibit Na/K ATPase activity. At the lower, physiological concentrations that occur *in vivo*, cardiotonic steroids bind the Na/K ATPase and initiate cascades of intracellular signaling that could increase Na/K ATPase activity [2].

The Na/K ATPase is a heterodimer of α and β subunits [28]. A Na/K ATPase isoform is determined by which α subunit it contains. Rat brain expresses three Na/K ATPase isoforms, α_1, α_2 and

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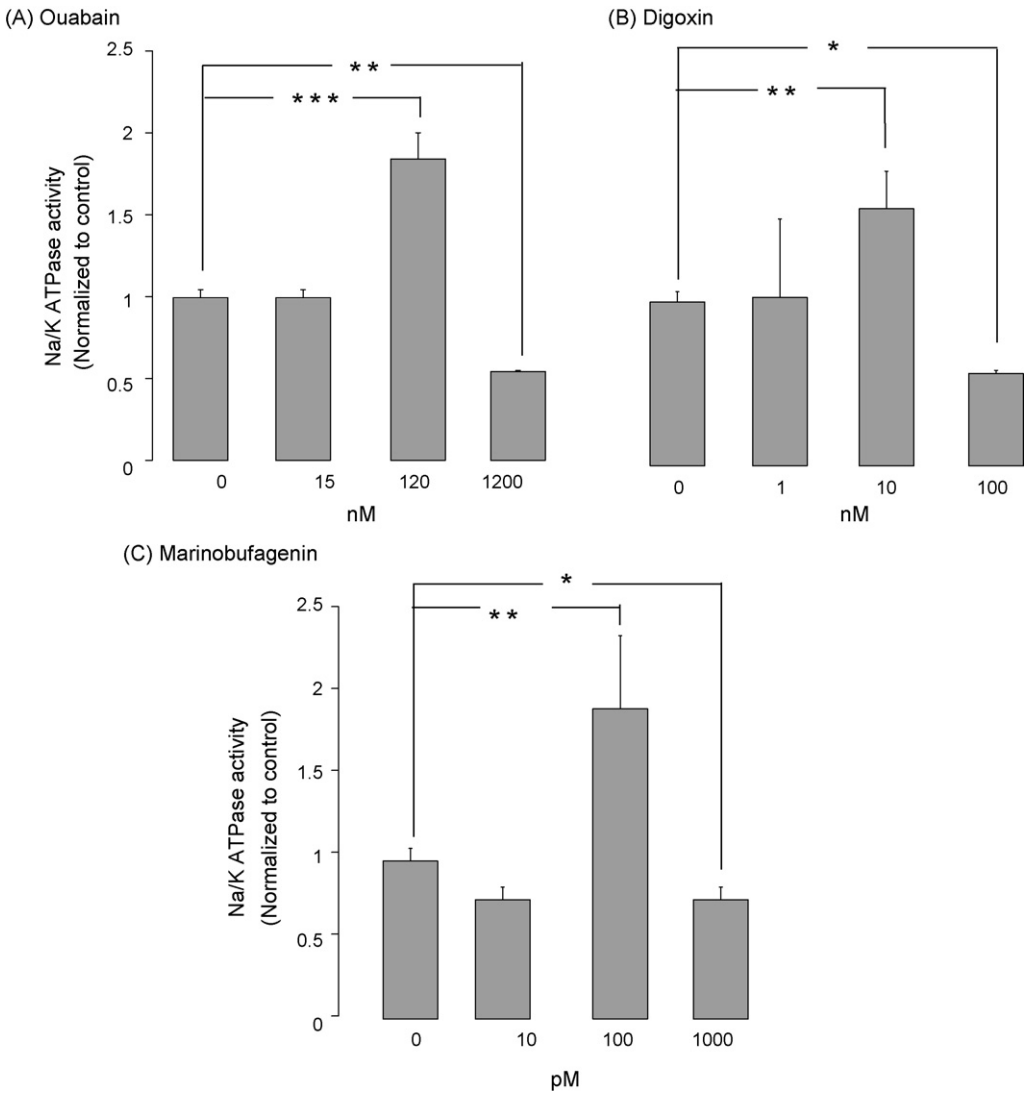

Fig. 1. Ouabain, digoxin and marinobufagenin show an inverted U-shaped dose–response curve for Na/K ATPase. Na/K ATPase activity was assayed by ^{86}Rb uptake after drug or control treatment and normalized to control. Panel A: ouabain. Ouabain (120 nM) significantly increased pumping while ouabain (1200 nM) significantly decreased pumping (ANOVA, $F_{3,23} = 38.37$, $p < 0.0001$; post hoc test, $^{***}p < 0.0001$, $^{**}p < 0.01$). Panel B: digoxin. Digoxin (10 nM) significantly increased pumping while digoxin (100 nM) significantly decreased pumping (ANOVA, $F_{3,49} = 12.06$, $p < 0.0001$; post hoc test, $^{**}p < 0.01$, $^{*}p < 0.05$). Panel C: marinobufagenin. MBG (100 pM) significantly increased pumping while MBG (1 nM) significantly decreased pumping (ANOVA, $F_{3,33} = 12.24$, $p < 0.0001$; post hoc test, $p < 0.01$, $^{*}p < 0.05$). All values are average ± SEM.

α_3 [16,17]. Na/K ATPase isoforms bind cardiotonic steroids with differing affinities [2,9,21]. Digoxin, ouabain and marinobufagenin (MBG) are the cardiotonic steroids examined in this study. Digoxin and ouabain are members of the cardenolide family of cardiotonic steroids [2]. The α_2 and α_3 isoforms of Na/K ATPase bind ouabain and digoxin with higher affinity than the α_1 isoform [9]. MBG is a member of the bufadienolide family [2]. MBG binds the α_1 isoform of the Na/K ATPase with much higher affinity than the α_2 and α_3 [9].

This study examined whether ouabain, digoxin, or MBG increase Na/K ATPase activity at concentrations below their IC_{50} for pumping. In addition, hippocampal slice cultures were used to test whether cardiotonic steroids can protect against *in vitro* experimental ischemia.

Hippocampal slice cultures were prepared as described by Hassen et al. [15]. Cultures were maintained for 2 weeks before experiments at 32 °C in a 5% CO_2 incubator. ^{86}Rb uptake (PerkinElmer, Waltham, MA) was done as described by Tian et al. [25]. Briefly, ^{86}RbCl (1 μCi, >1 μCi/μg, PerkinElmer, Boston, MA) was added to the slice cultures in Earles Balanced Salt Solution containing 2.3 mM KCl (2.3 mM K EBSS) for 30 min at 37 °C. The reaction was stopped with 3 washes of ice-chilled 2.3 mM K EBSS and slices homogenized with 1 ml 0.1N NaOH. An aliquot of the homogenized sample was counted on a Beckman LS6000IC scintillation counter (Beckman Instruments, Fullerton, CA). Protein content was measured using a BCA assay (Pierce Chemical, Rockford, IL). Experimental ischemia was induced as described by Hassen et al. [15]. One day before an experiment, the cultures were shifted to a 37 °C incubator in a 5% CO_2 atmosphere. Experimental ischemia was induced by submerging slice cultures for 10 min in Earles Balanced Salt solution (BSS) without glucose that was bubbled vigorously with 95% N_2, 5% CO_2. A mock ischemia group was submerged in BSS with 5 mM glucose for 10 min and bubbled vigorously with 20% O_2, 75% N_2, 5% CO_2. Cell loss assays using propidium iodide (PI) were performed as described by Hassen et al. [15]. PI is a standard method to assay cell loss in slice cultures [19]. Briefly, slice cultures were incubated for 30 min with 0.5% PI and PI epifluorescence images were obtained with a CCD camera on a Zeiss Axiovert 100 microscope using rhodamine optics. Fluorescence images were analyzed using NIH Image J. Experiments were analyzed by one-way ANOVA.

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