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SODIUM PATHWAY MARKERS IN NORMAL AND KINDLED FROG BRAINS

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The present report evaluates Na,K-ATPase activity as well as Na channel levels in the frog telencephalon after kindling, i.e. the acquisition of an epileptic focus through localized low-voltage electrical stimulation of one hemisphere. K-dependent phosphatase activity and binding of tritiated ouabain were measured, revealing no change in Na,K-ATPase activity 14 h after the last seizure. Na channels were measured by binding assays using a tritiated ethylenediamine tetrodotoxin derivative. Na channels were reduced in kindled brain as compared to controls.

Kindling has been used as a model of experimental epilepsy and also as one of neural plasticity [5]. When certain brain structures are exposed to repeated electrical stimulation, a permanent change in brain function occurs which persists in the absence of further stimulation once seizures have been established. Mechanisms underlying such a change have not yet been clarified. One possible explanation might be related to the role of cationic fluxes and gradients across the cell membrane. Evidence in this sense comes from other epilepsy models [12]. Na–K-pump activity has been studied in epileptic foci provoked by freezing in the rat cortex; these studies suggested an increase of Na and K transport at the synaptic level up to 24 h after the last seizure [17]. In cat with chronic freezing lesions glial and neuronal Na–K-pump activity decreased both in the primary and in the mirror focus [3, 6]. Kalichman reported that ATPase activity was unchanged after amygdaloid kindling in the rat [9]; however, it was demonstrated that in partially kindled rats enzyme activity was increased at various stimulated regions, when measured immediately after a seizure but not when the animals were sacrificed 24 h later [4].

The Na–K-pump is not the only candidate for a membrane-linked mechanism that will alter properties of the cell's excitability. Permanent changes in membrane protein

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molecules may affect other systems as well. We decided to investigate the effect of kindling on the Na,K-ATPase and on the number of tetrodotoxin (TTX) receptors in the brain. These receptors are linked to voltage-dependent sodium channels [16] and are rather plastic since they experience variations during ontogenesis [10]. Changes in these receptors associated to abnormal brain function have not yet been studied.

The frog brain has been shown to be a suitable model for the study of biochemical and electrophysiological mechanisms underlying epilepsy [15]. Temperature effect upon kindling development has already been demonstrated [13] suggesting alterations of membrane properties during kindling. Long-term changes in the number of Na,K-ATPase molecules and voltage-dependent Na channels were explored using ouabain and TTX receptors respectively as markers.

A kindled focus was produced by hourly electrical stimulations on the left telencephalic hemisphere of 10 frogs, *Caudiverbera caudiverbera*, as described before [13, 14]. Briefly, under low temperature anesthesia, the animals were immobilized by spinal transection and the telencephalic hemispheres exposed. The head was fixed and the body immersed in a water bath. Stimulation was given through a bipolar nichrome electrode (insulated except at the tip, 1 mm separation). Electroencephalogram (EEG) recordings were taken using nichrome monopolar electrodes placed on anterior and posterior coordinates of both hippocampi. Stimulus intensity was adjusted to produce just a brief afterdischarge (AD). Such stimuli were given every hour until generalized electrographic seizures occurred (after 6–8 h) and then stimulation was stopped. At this stage of kindling AD duration had grown to 4–5 times the initial value and spikes had become complex and larger in amplitude. Spontaneous interictal activity appeared in interstimulus intervals.

We considered kindling as successful when generalized epileptic activity was recorded on the background EEG after each stimulation and when AD duration was increased. Biochemical analysis was only carried out 14 h later on frogs meeting the above criteria.

Hemispheres were separately dissected and independently homogenized with 20 strokes of a Potter–Elvehjem homogenizer in 1 ml of 0.3 M sucrose. The homogenate was immediately used to determine protein concentration. K-dependent *p*-nitrophenyl phosphatase activity (an activity of Na,K-ATPase [1]) was measured with 0.1 mg of homogenate protein per milliliter and 10^{-6} M tritiated ouabain ([^{3}H]ouabain; 2.6 Ci/mmol) with or without 1 mM unlabelled ouabain using a filtration assay [7]. [^{3}H]ethylenediamine-TTX (spec. act. 26 Ci/mmol) was synthesized and purified according to Chicheportiche et al. [2] and Jaimovich et al. [8]. Binding experiments were performed with brain homogenates by incubation and filtration as described elsewhere [8].

Two independent assays were employed as markers of the Na pump: K-dependent phosphatase activity and binding of the specific inhibitor, [^{3}H]ouabain. The enzymatic activity was the same in both hemispheres of control brains (Table I), and no significant change was observed on brains subjected to kindling. Even when direct Na,K-ATPase activity could not be confidently assayed because of the high level of

TABLE I

K^+-DEPENDENT-*p*-NITROPHENYLPHOSPHATASE ACTIVITIES, [^{3}H]OUABAIN BINDING AND [^{3}H]en-TTX BINDING IN BRAIN HOMOGENATES OF CONTROL AND KINDLED FROGS

Results are expressed as mean ± S.E.M. Numbers in parentheses show the number of assays. Ouabain binding was calculated from the difference between assays containing only saturating (10^{-6}M) [^{3}H]ouabain concentration and those containing an excess (1 mM) of unlabelled ouabain. TTX binding data are B_{max} obtained from experiments as shown in Fig. 1.

	Hemisphere	K^+-phosphatase activity (nmol/min/mg)	Specific [^{3}H]ouabain (pmol/mg)	[^{3}H]en-TTX binding (fmol/mg)
Control	left	18.2±3.7 (7)	52.8±10.2 (7)	105±7 (5)
	right	18.8±2.0 (7)	55.1±11.8 (7)	101±6 (5)
Kindled	left	18.5±4.5 (6)	51.3± 8.0 (6)	89±5 (6)
	right	16.7±2.2 (6)	53.4± 6.2 (6)	81±3 (6)

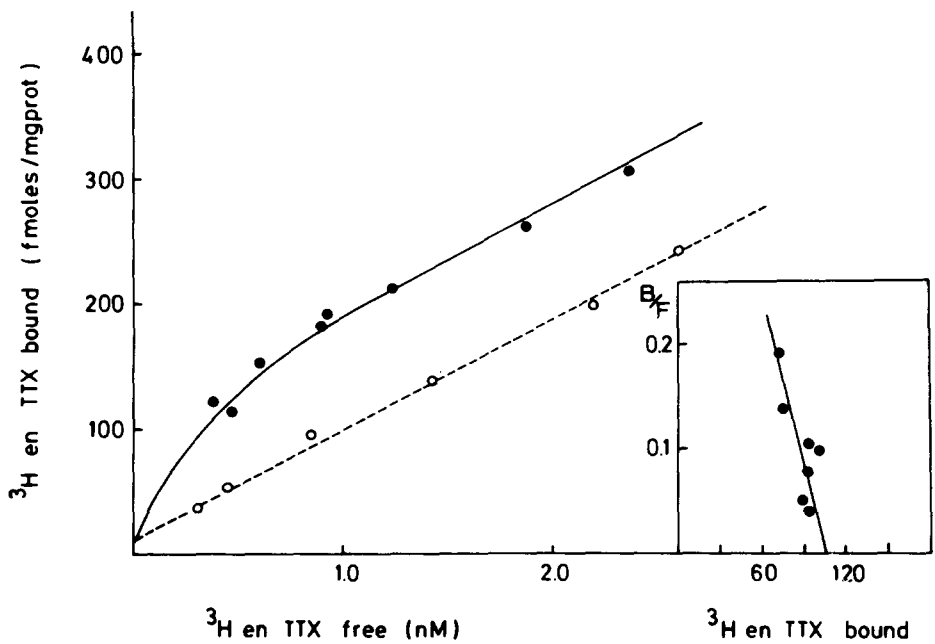

Fig. 1. Equilibrium binding of [^{3}H]en-TTX to frog brain homogenates. Main panel: filled circles represent total binding to right hemisphere homogenates from two control frogs, independently treated. Open circles represent (non-specific) binding in the presence of an excess of unlabelled TTX (10^{-6} M). Inset: Scatchard analysis for the specific (total minus non-specific) binding data. The continuous line (giving B_{max} of 105 fmol/mg prot. and a K_d of 0.44 nM) is the best fit to the experimental points.

Na–K-independent ATPase present, the above results suggest that the Na pump activity and presumably the total number of pump molecules is not affected by kindling in a long-term way. This conclusion is sustained by the experiments measuring [^{3}H]ouabain binding, which is proportional to the number of pumping sites; no difference in specific [^{3}H]ouabain binding was observed upon kindling in the hemisphere homogenate (Table I). Although our results demonstrate no generalized change in membrane structure related to the Na pump 14 h after seizure, previous studies have shown a reduced pump activity in glial cells of acute and chronic epileptogenic lesions in cat cortex [6]. In these studies [3, 6], the possibility of edema is not disregarded; our model is free of edema or brain damage but, because of the minimal focus dimensions, assays cannot be restricted only to the focus area. It is possible that transient changes in ATPase levels might occur during the course of kindling but the enzyme returned to baseline levels when measured 14 h post seizure [4].

As a marker for the number of voltage-dependent Na channels, we measured TTX receptors in brain homogenates. Using a TTX derivative it is possible to see a clear specific binding component in this system (Fig. 1). The Scatchard analysis shows a single class of receptors with a K_d of 0.4×10^{-9} M, and a B_{max} of about 100 fmol/mg prot. In kindled brains a similar K_d was found in 4 experiments, and the maximum number of receptors was smaller than in normal controls as a mean (Table I). Since protein synthesis has been proposed to be required for the development of kindling [15], it might be possible that a voltage-dependent Na channel protein could be mobilized during the process [11], leading to a decrease of TTX binding sites after kindling; furthermore, a reduction in the number of functional channels could imply changes in excitability that might compensate the strong depolarization induced by the seizure. The present study does not provide evidence that changes in Na,K-ATPase activity are involved in the generative mechanism of kindling. However, a reduction in voltage-dependent Na channels was found presumably as a secondary change evoked by kindling.

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