Activation of the Na^+, K^+ -ATPase in *Narcine brasiliensis*

(electric organ/stimulation/ ^{31}P NMR/ ^{23}Na NMR/shift reagent)

HAYWOOD BLUM^{*†‡}, SHOKO NIOKA^{†§}, AND ROBERT G. JOHNSON, JR.^{*†‡§¶}

^{*}Howard Hughes Medical Institute, and Departments of [†]Biochemistry and Biophysics, [‡]Medicine, and [§]Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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ABSTRACT The *in vivo* activation and turnover rates of the sodium pump (Na^+, K^+ -ATPase) were investigated in the electrocytes of the electric organ of the elasmobranch *Narcine brasiliensis*. The *Narcine* electric organ appears to be an excellent model for the study of sodium pump activation in an excitable tissue. The sodium transmembrane gradient and high-energy phosphagens were concurrently measured by ^{23}Na and ^{31}P NMR spectroscopy. The resting electric organ, which depends primarily on anaerobic metabolism, displays a high concentration of phosphocreatine (PCr). It has an intracellular sodium concentration ($[\text{Na}^+]_i$) of 20 ± 10 milliequivalents/liter as estimated by NMR. Electrical stimulation of the nerves innervating the electric organ results in an increase in $[\text{Na}^+]_i$ in the electrolyte and rapid depletion of PCr. Ouabain causes an 85% decrease in utilization of high-energy phosphagens, indicating that rapid PCr turnover in this tissue is mainly due to Na^+, K^+ -ATPase activity. From these data we can determine that the rate of sodium pump turnover increases by >3 orders of magnitude within several hundred milliseconds. In excised unstimulated electric organ slices, changes in $[\text{Na}^+]_i$ equivalent to those occurring with stimulation, but induced by hyperosmolar conditions, do not result in increased PCr hydrolysis. We conclude that cholinergic stimulation of the electric organ causes a rapid and extremely large increase in sodium pump turnover, which is regulated predominantly by factors other than $[\text{Na}^+]_i$.

The membrane Na^+, K^+ -ATPase, present in virtually every type of animal cell, is vital for Na^+ and K^+ homeostasis (1, 2). *In vivo*, its activity in most cells is thought to be directly regulated physiologically by the intracellular sodium concentration ($[\text{Na}^+]_i$) and/or extracellular $[\text{K}^+]$. The mechanisms by which numerous chemical messengers such as glucose, insulin, glucagon, catecholamines, and circulating cardioglycoside-like factors (3, 4) regulate the Na^+, K^+ -ATPase remain uncertain: it is unclear whether they act indirectly by changing $[\text{Na}^+]_i$. We report on a system in which the sodium pump activity responds to cholinergic stimulation of the cell but not significantly to $[\text{Na}^+]_i$.

The marine elasmobranch *Narcine brasiliensis*, a member of the family Torpedinidae, possesses large bilateral electric organs that are composed of a series of columns of large disc-shaped cells called electrocytes. The electrocytes are stimulated by release of acetylcholine from presynaptic nerve endings whose cell bodies lie in a localized midbrain region termed the electric cortex. Sodium ions enter the electrocyte through the large number of nicotinic channels within the ventral membrane. The combination of stacks of electrocytes in many parallel columns enables the *Narcine* to produce a current sufficient in strength to stun or kill its prey. Sodium ions are extruded from the electrocyte by membrane-bound Na^+, K^+ -ATPase molecules located within the noninnervated

dorsal surface. This membrane is $\approx 10 \mu\text{m}$ away from the ventral surface and contains extensive small tubular infoldings, or canaliculi, which greatly increase the surface area (5), allowing a high density of sodium pumps (6).

Study of the bioenergetic and thermodynamic properties of the *Narcine* electric organ sodium pumps enjoys certain advantages. The organ is large, comprising about one-third of the fish's body weight, and is homogeneous. The concentration of Na^+, K^+ -ATPase is higher than in any other tissue found in nature and its primary amino acid sequence is similar to its mammalian counterpart (7–9). It is poorly vascularized and also poorly supplied with mitochondria; its metabolic pathways are essentially supplied by glycolysis. Each electrocyte is innervated but the relative volume of nerve endings to electrocytes is small. The intracellular spaces are, therefore, effectively a single metabolic compartment.

We have studied the bioenergetics of the organ and its Na^+ membrane pumps *in vivo* by using magnetic resonance spectroscopy to observe intra- and extracellular sodium and intracellular high-energy phosphates, such as phosphocreatine (PCr) and ATP. The *in vivo* studies have been extended and confirmed in isolated organs and in cellular extracts.

METHODS AND MATERIALS

After induction of anesthesia with 3-aminobenzoic acid ethyl ester (Sigma) (0.05 g/liter dissolved in seawater), the fish was placed in an assembly apparatus (Johnson Foundation Biomedical Instrumentation Shop) that pumped aerated seawater into its mouth and over the gills. At the end of an experiment lasting several hours, the fish could usually be revived easily and returned to its regular tank for recovery.

Grass E2 subdermal electrodes (Grass) were inserted into the electric cortex and also placed on the dorsal and ventral surfaces of the fish at the periphery of the electric organ to monitor discharge. Signals were monitored with a Tektronix model 5113 storage oscilloscope with single 4-msec pulses produced with a Grass model S8800 stimulator. A 20-sec delay between pulses prevented down-regulation of the nicotinic receptors, which sometimes occurs at higher stimulation frequencies (data not shown).

Dysprosium chloride (Alfa Products, Danvers, MA) was mixed with a slight excess of triethylenetetraaminehexaacetic acid (TTHA) (Sigma) in water (10) such that the final concentration of $\text{Dy}(\text{TTHA})$ was 300 mM. The pH was titrated to 7.4 with NaOH ; CaCl_2 was then added to a final concentration of 30 mM, and the solution was filtered. After induction of anesthesia, a 20-gauge catheter was placed either in the tail ventral artery for intraarterial perfusion or transcutaneously in the peritoneum for intraperitoneal infusion.

Abbreviations: PCr, phosphocreatine; TTHA, triethylenetetraaminehexaacetic acid; $[\text{Na}^+]_i$, intracellular sodium ion concentration.

[¶]To whom reprint requests should be addressed at: 332 Johnson Pavilion, 36th Street and Hamilton Walk, University of Pennsylvania Medical Center, Philadelphia, PA 19104.

A two-turn NMR surface coil was placed over the electric organ and the entire perfusion apparatus containing the fish was placed in a 1.9-T horizontal bore magnet for NMR spectroscopy utilizing an NMR circuit as described (11). The field was shimmed for maximum uniformity over the electric organ by proton NMR. The Phospho-Energetics (Havertown, PA) model 250-90 spectrometer was then programmed for concurrent ^{31}P and ^{23}Na spectroscopy. The repetition rate for ^{31}P pulsing was 4 sec. The effect of rapid repetition rate was routinely corrected as necessary in all calculations. Gating of the NMR spectrometer with the stimulation pulse was possible but unnecessary since organ stimulation did not degrade the NMR signal.

In vitro NMR experiments were performed on organ slices. After induction of anesthesia as described, the fish were cooled on ice. The electric organ on each side was removed in one piece and placed in a physiological buffer consisting of 250 mM NaCl, 6 mM KCl, 4 mM CaCl_2 , 1 mM MgCl_2 , 0.2 mM NaH_2PO_4 , 12 mM glucose, 360 mM urea, and 20 mM Hepes (pH 7.4). Innervated slices ≈ 3 mm thick were preincubated in appropriate buffers, placed in a 10-mm NMR tube, and spectra were recorded. In some instances, slices were electrically stimulated through the attached nerve trunk while incubating in buffer. Monitor electrodes placed above and below the slice were used to ensure that the stimulation was effective in discharging the organ. Gramicidin, *d*-tubocurarine chloride, choline chloride, and ouabain were all from Sigma.

In vitro membrane ATPase activity was assayed after isolation of the dorsal electrocyte membranes by a modified standard procedure (12). ATPase activity of this membrane fraction was assayed spectrophotometrically by the method of Baginski *et al.* (13). PCr, creatine, ATP, and creatine phosphokinase were extracted and measured by standard spectrophotometric techniques (14, 15).

RESULTS

Measurement of the biochemical properties of the Na^+, K^+ -ATPase in isolated electrocyte dorsal membrane fragments (summarized in Table 1) reveals an apparent $K_{0.5}$ for Na^+ of 10.6 mM in 10 mM K^+ , and a $K_{0.5}$ of 39.6 mM in the presence of 170 mM K^+ . The Na^+, K^+ -ATPase activity was dependent on the substrate concentration in a predictable fashion: at saturating sodium concentration the ATPase was fully active,

Table 1. Biochemical and biophysical properties of cellular extracts of membrane fragments of *N. brasiliensis* electric organ

Na^+, K^+ -ATPase	1.4 ± 0.3 units per mg of protein*
$K_{0.5}(\text{Na}^+)(\text{K}^+ = 10 \text{ mM})$	10.6 ± 0.5 mM
$K_{0.5}(\text{Na}^+)(\text{K}^+ = 170 \text{ mM})$	39.6 ± 2.0 mM
Na^+ stoichiometry	3.5 ± 0.5
$[\text{Na}^+]_i$	20 ± 10 mM [†]
Creatine + PCr	15 ± 3 μM [‡]
PCr	11.3 ± 0.3 μM [‡] 12.4 ± 2.5 μM [‡]
ATP	3.4 ± 0.2 μM [‡]
CPK	129 ± 35 units per mg of protein

CPK, creatine phosphokinase. Units are μmol per g wet weight of tissue unless otherwise indicated. Results are expressed as means $\pm$ standard error.

*One unit = 1 μmol of ATP hydrolyzed per min.

[†]NMR measurement on electric organ slices calibrated against Na standard using shift reagent and assuming intracellular Na visibility = 0.4 and intracellular volume = 41% of total organ volume (unpublished data).

[‡]Undischarged resting state, biochemical assay after extraction.

[§]Methods of ref. 14.

^{||}Methods of ref. 15.

^{||}NMR measurement on electric organ slices calibrated against P_i standard.

FIG. 1. Typical ^{31}P NMR spectrum of *in vivo* resting *N. brasiliensis* electric organ. Data are averaged over 80 free induction decays (FIDs), taken once every 10 sec. P_i and PCr are not fully relaxed (T_1 , ≈ 4 sec for each). Line broadening of 10 Hz was added to improve signal/noise ratio. PME and PDE, phosphorylated mono- and diesters, respectively.

and the stoichiometry for Na^+ approached 3. The activity of the Na^+, K^+ -ATPase was then investigated in the intact electric organ to examine its cellular regulation.

The ^{31}P NMR spectrum of resting electric organ (Fig. 1) has a low concentration of P_i while PCr is the dominant phosphagen; the ratio of PCr to P_i is >6 . ATP and phosphorylated mono- and diesters are visible. These values are confirmed by biochemical measurements of PCr and ATP (Table 1). From the position of the P_i peak relative to PCr, intracellular pH is calculated to be 7.3 (16).

Stimulation of the electric cortex at low frequency causes a profound depletion of PCr with a commensurate increase in P_i so that total phosphates remain constant (Fig. 2). After cessation of the stimulating pulse, PCr remains stable. With further stimulation, PCr and ATP can be fully hydrolyzed. Recovery of PCr and P_i to almost normal values takes ≈ 24 hr once the fish is removed from the perfusion apparatus and returned to its tank (data not shown).

To evaluate the contribution of the sodium pump to high-energy phosphate turnover, innervated organ slices were preincubated for 20 min in buffer containing 0.1 mM ouabain and then electrically stimulated. The effect of ouabain was to prevent PCr depletion during stimulation by 85% (Fig. 3). Since the cardiac glycoside ouabain is a specific Na^+, K^+ -ATPase inhibitor (17), this result implies that the sodium pump is activated by stimulation of the electrocyte and is the dominant energy utilization reaction.

Transmembrane sodium gradients were measured by using the shift reagent Dy(TTHA) to distinguish intra- from extra-

FIG. 2. Typical time course of depletion of PCr and concomitant increase in P_i with stimulation. Stimulation of the electric organ *in vivo* at 0.1 Hz is confined to the shaded regions.

FIG. 3. Effect of incubation in 0.1 mM ouabain for 20 min prior to electrical stimulation on PCr depletion in *N. brasiliensis* electric organ innervated slices. Difference between preincubation in ouabain and no ouabain is significant ($P < 0.002$). PCr is measured as percent total phosphorus.

cellular sodium. Infusion of Dy(TTHA) causes a downfield shift in the bulk of the sodium signal, leaving a small unshifted intracellular contribution, difficult to resolve at low magnetic fields. Interleaved collection of sodium and phosphorus data with a 5-min time resolution shows no change in the sodium signal with electrical stimulation until full depletion of high-energy phosphates (Fig. 4). If the stimulation is halted with residual high-energy phosphates, there is no decrease in extracellular sodium. Once all the high-energy phosphates

FIG. 4. Typical time course of sodium (extracellular/intracellular ratio) and phosphorus (PCr/ P_i) with stimulation. An intraarterial infusion of 120 mM Dy(TTHA) was started at time zero. Stimulation of the electric cortex was done between 111 and 225 min. Each data point represents 75 free induction decays (FIDs) for ^{31}P and 900 FIDs for ^{23}Na over 6 min. Curves are drawn to help display the data and are not fitted, except Na^+ recovery after cessation of stimulation is least-squares fitted.

are depleted, continued stimulation causes a decrease in extracellular sodium and an increase in intracellular sodium (Fig. 4). After stimulation is halted, the extracellular/intracellular sodium ratio recovers toward its initial value. A best-fit analysis of the recovery of the ratio $\text{Na}_{\text{out}}/\text{Na}_{\text{in}}$ to its equilibrium value yields an exponential time constant of 64 min. Since ATP hydrolysis accompanies sodium extrusion from the intracellular space, this demonstrates the low rate of ATP production from glucose or glycogen through the glycolytic pathway during any dynamic process. The sodium efflux rate under these conditions is >2000 -fold slower than the influx rate during discharge or the subsequent efflux when high-energy phosphates are present (compare Figs. 4 and 5). Once the transmembrane sodium gradient is reestablished, PCr is reaccumulated with a time constant ≈ 3 times longer (3 hr), consistent with the measured stoichiometry of the ATPase.

To determine whether we could observe the rapid shift of sodium into and out of the electric organ in the presence of normal pumping capability, not shown in Fig. 4 because of the 5-min time resolution, we rapidly stimulated the organ three times with a repetition delay of 20 msec. We then observed the ^{23}Na signal recovery in 0.175-sec time blocks for 2.6 sec, as shown in Fig. 5A. Fig. 5B displays representative ^{23}Na spectra taken just after stimulation (FID 3) and upon recovery (FID 10) and their computer deconvolution into two peaks. The data indicate that there is an extremely rapid increase in the Na^+ pump activity after stimulation, which results in normalization of the Na^+ gradient within 2 sec. We estimate sodium efflux rates to be 4 mM/sec per discharge during this time, consistent with the rate of PCr depletion.

If activation of the sodium pumps after electrical stimulation was caused by an increase in $[\text{Na}^+]_i$ (estimated to be $\approx 10\%$ per discharge), incubation of slices in hyperosmolar buffers, identical to the physiological buffer but with the addition of 60 mM choline chloride (13% increase in osmolarity) should also result in the depletion of PCr. Incubation of tissue slices in hyperosmolar medium did result in rapid depletion of PCr (Fig. 6A). However, preincubation of the slices in 0.1 mM *d*-tubocurarine chloride abolished this effect, which is due to nonspecific release of acetylcholine stores from nerve endings (18) (Fig. 6A). Curare, a competitive nicotinic antagonist, blocks the effect of acetylcholine but does not prevent the osmotic water shifts that result in an increase in $[\text{Na}^+]_i$. Similar experiments, with preincubation in curare and then incubation in gramicidin (0.01 mg/ml), did not result in significant PCr depletion (Fig. 6B), although gramicidin will completely equilibrate the intra- and extracellular sodium concentrations (19, 20).

DISCUSSION

Despite the importance of understanding the cellular biology of normal sodium pump regulation and its pathology, little is known about its regulation at a molecular level. One reason for this situation is the difficulty of accurately measuring the ATPase activity, ambient sodium concentration, and sodium fluxes with the cell operating under normal physiologic conditions. We have shown in this investigation that *Narcine* possesses many attributes necessary to optimally investigate the *in vivo* cellular regulation of the Na^+ pump. In *Narcine*, the Na^+ pump has been found to be regulated in a unique fashion not related to $[\text{Na}^+]_i$ and with rapid activation/deactivation kinetics.

The electric organ is well endowed with PCr. Our results show that depletion of PCr in the electrocyte is a result of activation of the Na^+ , K^+ -ATPase. Since, as we have shown, the glycolytic pathway available for reenergizing the organ is so sluggish, PCr, together with cellular ATP stores, repre-

FIG. 5. (A) Recovery of the ^{23}Na NMR in *in vivo* *N. brasiliensis* electric organ (ratio of area of extracellular/intracellular sodium) after three successive stimulations in 40 msec of the electric cortex. Each point is from a single free induction decay (FID) acquired over 0.175 sec. (B) Typical ^{23}Na NMR spectra taken after stimulation (FID 3) and upon recovery (FID 10). These spectra have been deconvoluted to show the underlying intra- and extracellular peaks.

sents the major energy source for driving the membrane pumps after discharge of the organ. PCr replenishes ATP through the creatine phosphokinase reaction, $\text{PCr} + \text{ADP} + \text{H}^+ \rightleftharpoons \text{Cr} + \text{ATP}$, which is generally considered to be in equilibrium (21). Since the flux into PCr is low, we can, therefore, monitor PCr to estimate ATP utilization by the sodium pump—i.e., the rate of PCr decrease is proportional to the ATPase rate.

Direct measurement of the forward reaction rate in the creatine kinase reaction by saturation transfer (unpublished results) confirms the finding that there is zero flux in the

forward direction, implying that [ADP] and ATPase activity are extremely low. Direct electrical stimulation produces events that activate the sodium pump; with stimulation, pump activity (measured by PCr depletion or by sodium flux) increases by >3 orders of magnitude. This occurs with an $[\text{Na}^+]_i$ increase of 10%. We have shown that the $K_{0.5}$ for sodium of the Na^+, K^+ -ATPase of isolated membrane fragments is 39.6 mM in the presence of normal intracellular K^+ and that the maximum *in vitro* pump velocity is only 4- to 5-fold greater than the rate at normal $[\text{Na}^+]_i$ (20 mM) (Table 1). Since the sodium pump is electrogenic, its rate will change

FIG. 6. Effect on depletion of PCr in *N. brasiliensis* electric organ slices with or without preincubation with 0.1 mM *d*-tubocurarine chloride for 20 min. (A) Increasing osmolarity of incubating buffer from normal by 120 mosM with choline chloride. (B) Incubating in gramicidin (0.1 mg/ml). * $P < 0.001$; ** $P < 0.0001$. PCr is measured as percent total phosphorus.

as the membrane is depolarized by stimulation (22). However, this effect, measured in reconstituted vesicles, amounts at most to a 2- to 3-fold increase in pump activity (23). Thus, the observed bursts in ATPase rate cannot be explained by changes in $[\text{Na}^+]_i$ alone acting on a membrane pump but must also depend on recruitment of additional pumps. The rapid kinetics of the Na^+ efflux across the membrane of the electrocyte after stimulation points toward a tightly regulated mechanism.

In the rat adipocyte, Lytton *et al.* (24, 25) have described two kinetically different forms of the ATPase in intact cells: namely, α , a high-affinity form ($K_{0.5} = 10$ mM) and $\alpha(+)$, a low-affinity form ($K_{0.5} = 80$ mM). In the presence of insulin, the $\alpha(+)$ form is modified to the high-affinity α form. The disappearance of this effect in isolated scattered membranes and adipocyte ghosts (26)—i.e., all of the ATPase is in the α form—has been interpreted as consistent with an allosteric or labile covalent effector that modulates the affinity of the $\alpha(+)$ form. Since the above report in 1985, molecular cloning of the ATPase has demonstrated at least three α unit subtypes (27). Recent preliminary reports of partially reconstituted adipocytes and brain synaptosomes (28) have suggested that hormonal additions may affect not only the $K_{0.5}$ of the ATPase but also the $V_{\max}$. Additional reports citing the effect of cAMP on the Na^+, K^+ -ATPase within the shark rectal gland (29) and of phorbol esters (30) on the Na^+ pump in the liver provide pharmacologic evidence for this view. Moreover, a body of literature exists on the effects of hyperglycemia, myo-inositol depletion, and sorbitol on Na^+, K^+ -ATPase activity within rabbit aorta and peripheral nerve (31–33). While the central theme is that the Na^+ pump activity can be regulated by factors other than intracellular sodium, these studies can all be criticized by their inability to totally negate the possibility that the experimental manipulation results in perturbations in the intracellular sodium and consequential increase or decrease in the Na^+, K^+ -ATPase activity. Furthermore, the changes in ATPase rate found in these studies are small. The ability to accurately and quantitatively monitor ATPase activity and transmembrane Na^+ gradients in a rapid temporal fashion in *Narcine* has permitted a more defined setting for the study of the regulation of this Na^+, K^+ -ATPase.

In summary, we have described the following observations: (i) an extremely low basal turnover of the ATPase, (ii) a >2000-fold increase in the ATPase activity after stimulation, (iii) activation of Na^+ efflux in the millisecond time domain, (iv) minimal average increase in the intracellular sodium concentration with stimulation, and (v) the absence of an increase in the ATPase activity when the intracellular Na^+ concentration is increased by mechanisms other than stimulation. These findings are consistent with a mechanism of Na^+, K^+ -ATPase operation by which most of the Na^+ pumps are not functionally operative during resting conditions, but with stimulation the majority of the ATPases are rapidly activated and then deactivated. Further investigation of the regulation of the *Narcine* sodium pump may provide useful insights into the cellular biology of Na^+, K^+ -ATPases.

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