J. Membrane Biol. 101, 19–31 (1988)

The Journal of
Membrane Biology

Effects of Internal Na and External K Concentrations on Na/K Coupling of Na,K-Pump in Frog Skeletal Muscle

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Summary. To clarify the dependency of the Na/K coupling of the Na,K-pump on internal Na and external K concentrations in skeletal muscle, the ouabain-induced change in membrane potential, the ouabain-induced change in Na efflux and the membrane resistance were measured at various internal Na and external K concentrations in bullfrog sartorius muscle.

Upon raising the internal Na concentration from 6 mmol/kg muscle water to 20 mmol/kg muscle water, the magnitude of the ouabain-induced change in membrane potential increased about eightfold and the magnitude of the ouabain-induced change in Na efflux increased about fivefold while the membrane resistance was not significantly changed. As the external K concentration increased from 1 to 10 mM, the magnitude of the ouabain-induced change in membrane potential decreased (1/5.5 fold), while the magnitude of the ouabain-induced change in Na efflux increased (about 1.5-fold). The membrane resistance decreased upon raising the external K concentration from 1 to 10 mM (1/2-fold). These observations imply that the values of the Na/K coupling of the Na,K-pump increases upon raising the internal Na concentration and decreases upon raising the external K concentration.

Key Words Na,K-pump · Na/K coupling · membrane potential · internal Na · external K · skeletal muscle

Introduction

Application of insulin hyperpolarizes the membrane of skeletal muscles (Zierler, 1957, 1959; Marunaka & Kitasato, 1985*b*, 1987; Marunaka, 1987*a*, *b*; Marunaka, Murayama & Kitasato, 1987) apparently by stimulating the Na,K-pump (Moore & Rabovsky, 1979; Marunaka & Kitasato, 1985*a*). Insulin has no significant effect on the internal Na and K concentrations and the ratio of Na-permeability to K-permeability at the time when the membrane is hyperpolarized by insulin (30 to 40 min after insulin application; Moore & Rabovsky, 1979; Marunaka, 1986). These reports suggest that the insulin-stimulated Na,K-pump is electrogenic. In addition, the Na/K coupling ratio of the insulin-stimulated Na,K-pump varies when the ionic environment of the pump is changed (Marunaka, 1986, 1987*a*; Marunaka, Murayama & Kitasato, 1986).

Besides the component of the pump which is stimulated by insulin, the Na,K-pump, in general, is electrogenic and the Na/K coupling ratio is 1.5. This was demonstrated by observations in erythrocytes (Gardos, 1964; Sen & Post, 1964; Whittam & Ager, 1965; Post, Albright & Dayani, 1967) and giant axons of squid (Hodgkin & Keynes, 1955). Further, in muscle, it has also been reported that the Na/K coupling is 1.5 (Clausen & Hansen, 1974). However, as mentioned above for the insulin-stimulated Na,K-pump, the Na/K coupling ratio may be different than 1.5 (*see* the review of Sjodin, 1982).

Coupling ratios different than 1.5 have been reported in preparations other than the insulin-stimulated pump. Sjodin and Beaugé (1967) have reported that the Na/K coupling is 2 in squid giant axon. Mullins and Noda (1963) have shown that in frog skeletal muscle the Na/K coupling of the pump is 3. Subsequently in skeletal muscle, Sjodin and Ortiz (1975) determined the ratio to be 1.4 to 1.7. Lederer and Nelson (1984) have reported that in barnacle muscle it is between 1.5 and 2. Further, it has been reported that in squid axon the Na/K coupling is 3 at high internal Na concentration while it is 1.5 at normal internal Na concentration (Mullins & Brinley, 1969).

The purpose of the present study was to investigate whether the Na/K coupling ratio of the basal Na,K-pump varies in response to changes in internal Na and external K concentrations in a manner similar to that observed for the insulin-stimulated Na,K-pump in skeletal muscle.

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Table 1. Ionic composition of the solutions used

Solution	NaCl (mM)	KCl (mM)	$CaCl_2$ (mM)	Tris-Cl (mM)
normal	110	2.5	2	10
5-Na	5	2.5	2	115
0.1-K	110	0.1	2	12.4
1-K	110	1	2	11.5
2.5-K	110	2.5	2	10
5-K	110	5	2	7.5
10-K	110	10	2	2.5

Materials and Methods

Solutions

The composition of the solutions used is summarized in Table 1. The osmolarity and pH were adjusted to 260 mOsm/liter and 7.4 with Tris-HCl, respectively (Kitasato & Marunaka, 1985). The ouabain used to prepare solutions was purchased from Sigma. ^{22}NaCl and ^{14}C-inulin were obtained from New England Nuclear.

Muscles

Sartorius muscles were carefully dissected from bullfrogs (*Rana catesbeiana*) under a microscope, then were stored in normal, 0.1-K or 5-Na solution for 15 hr at 4°C prior to experiments. The purpose of storing muscles in the experimental solutions was to change the internal Na concentration; i.e., muscles enriched with Na (high-Na muscle) were prepared by storing in 0.1-K solution, and muscles with low internal Na concentration (low-Na muscle) were prepared by storing in 5-Na solution. Muscles stored in normal solution are referred to as normal-Na muscle. For studying the effect of the external K concentration, muscles were stored in normal solution for 15 hr at 4°C.

Estimation of the Water Content in the Intracellular Space

The wet weight of the muscle used was 260 to 300 mg. Wet weight and dry weight were estimated by weighing muscles before and after drying at 95°C for 3 hr, respectively. The water content in the extracellular space was estimated by using ^{14}C-inulin. The dry weight was 20% of the wet weight. The water content in the extracellular space was 25% of the wet weight. From these values, the water content in the intracellular space was estimated to be 55% of the wet weight (Marunaka, 1986).

Diameter of the Muscle Fiber

The diameters of the muscle fibers used for measuring the membrane potential and the input resistance were 45 to 55 μm.

Estimation of the Internal Na Concentration

The internal Na concentration was measured using the method of Keynes and Steinhardt (1968) as previously described by us (Kitasato et al., 1980*a*).

Table 2. Internal Na concentration of low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain[a]

	Internal Na concentration (mmol/kg muscle water)		
Muscle	Low-Na	Normal-Na	High-Na
Ouabain (−)	5.8 ± 1.9	10.3 ± 2.5	19.1 ± 3.1
Ouabain (+)	6.1 ± 2.0	10.8 ± 2.3	19.8 ± 2.8

[a] Each value expresses the mean value of six experiments ± SD of the mean value. Ouabain had no significant effect on the internal Na concentration of low-Na, normal-Na and high-Na muscles. The internal Na concentration of low-Na and high-Na muscles was significantly different from that of normal-Na muscle ($P < 0.005$).

Estimation of the Rate Coefficient of the ^{22}Na Efflux

The rate coefficient of the ^{22}Na efflux is the ratio of the ^{22}Na efflux to the internal ^{22}Na concentration. The rate coefficient of the ^{22}Na efflux was measured by the method of Kitasato et al. (1980*c*).

Calculation of the Na Efflux

The Na efflux is calculated from the product of the internal Na concentration and the rate coefficient of the ^{22}Na efflux at the steady state (Marunaka, 1986).

Measurement of Membrane Electrical Values

The membrane potential of muscles was measured using a glass microelectrode filled with 3 M KCl, whose resistance was about 7 MΩ. The membrane input resistance of the muscles was measured from the potential change in response to a current injection of −10 to 10 nA using the method of Jenerick (1953). The specific membrane resistance was estimated from the value of the input resistance by the following equation (Jenerick, 1953).

$$R_m = \pi^2 d^3 \bar{R}^2 / R_i \quad (1)$$

where R_m is the specific membrane resistance, d is the diameter of the muscle fiber, $\bar{R}$ is the input resistance, and R_i is the specific resistance of the myoplasma.

Calculation of the Na/K Coupling Ratio of the Na,K-Pump

The method of estimating the Na/K coupling ratio of the Na,K-pump is the same as that of Marunaka (1986). At the steady state, the following equation holds:

$$F(J_{Na} + J_K) + I_p = 0 \quad (2)$$

$$\Delta V_m = I_p R_m \quad (3)$$

where F is Faraday's constant, J_{Na} is the Na flux caused by the Na,K-pump, J_K is the K flux caused by the Na,K-pump, I_p is the

Table 3. Membrane potential of low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain[a]

Muscle	Membrane potential (mV)		
	Low-Na	Normal-Na	High-Na
A. Ouabain (−)	−86.2 ± 1.6	−89.2 ± 2.1	−98.3 ± 2.9
B. Ouabain (+)	−85.0 ± 1.8[b]	−86.1 ± 2.4[c]	−89.1 ± 3.1[c]
C. Difference (ΔV_m)	−1.2	−3.1	−9.2

[a] Each value of membrane potential is expressed as the mean value of fifteen experiments ± SD of the mean value.
[b,c] In comparison with ouabain-free, the significant difference is indicated: [b]$P < 0.05$; [c]$P < 0.001$.

Table 4. Rate coefficient of the ^{22}Na efflux from low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain[a]

Muscle	Rate coefficient of ^{22}Na efflux (10^{-6}/sec)		
	Low-Na	Normal-Na	High-Na
Ouabain (−)	76.2 ± 6.9	106.9 ± 10.1	209.5 ± 17.6
Ouabain (+)	56.0 ± 6.5[b]	82.2 ± 9.2[c]	170.8 ± 15.1[c]
Difference	20.2	24.7	38.7

[a] Each value of rate coefficient is expressed as the mean value of six experiments ± SD of the mean value.
[b,c] In comparison with ouabain-free, the significant difference is indicated: [b]$P < 0.025$; [c]$P < 0.005$.

passive current caused by both the J_{Na} and J_K, ΔV_m is the change in the membrane potential caused by I_p; outward currents and efflux of positive ions are defined as positive. From Eqs. (2) and (3), the following equation can be deduced

$$-(J_{Na}/J_K) = 1/(1 + \Delta V_m/(R_m F J_{Na})). \quad (4)$$

Experimental Protocol

To examine the effect of the internal Na concentration on the Na/K coupling ratio, the following protocol was used. The muscles which had been soaked in 5-Na, normal and 0.1-K solutions were, respectively, transferred into 5-Na, normal and normal solutions for 30 min at 20 to 22°C. Just after the preincubation, the muscles in 5-Na, normal and normal solutions were incubated in the same solutions in the presence or absence of 0.1 mM ouabain at 20 to 22°C. Around 30 min after starting to incubate the muscles in the test solutions in the presence or absence of 0.1 mM ouabain, the internal Na concentration, the membrane potential, the rate coefficient of the ^{22}Na efflux and the input resistance of the muscles were measured in the respective solutions in the presence and absence of 0.1 mM ouabain at 20 to 22°C.

To examine the effect of external K concentration on the Na/K coupling ratio, the protocol of experiments was as follows. The muscles stored in normal solution were preincubated in 1-K, 2.5-K, 5-K or 10-K solution for 30 min at 20 to 22°C. After the preincubation, the muscles preincubated in 1-K, 2.5-K, 5-K and 10-K solutions were placed in respective solutions with or without 0.1 mM ouabain at 20 to 22°C. Around 30 min after starting to incubate the muscles in 1-K, 2.5-K, 5-K and 10-K solutions in the presence and absence of 0.1 mM ouabain, the internal Na concentration, membrane potential, the rate coefficient of the ^{22}Na efflux and the input resistance of the muscles were measured in the respective solutions.

Results

Effect of the Internal Na Concentrations on the Na/K Coupling Ratio

Internal Na Concentration of Low-Na, Normal-Na and High-Na Muscles

Table 2 shows the internal Na concentration of low-Na, normal-Na and high-Na muscles incubated in solutions with and without ouabain. The internal Na concentration of high-Na muscle was about 3 times larger than that of low-Na muscle. Ouabain had no significant effect on the internal Na concentration around 30 min after 0.1 mM ouabain had been applied.

Membrane Potential of Low-Na, Normal-Na and High-Na Muscles

Table 3 indicates the membrane potential of low-Na, normal-Na and high-Na muscles incubated in solutions with and without ouabain. The absolute value of the membrane potential of high-Na muscle was larger than that of low-Na muscle. The difference between the membrane potential in the presence and absence of ouabain was increased when the internal Na concentration was raised. The value of the ouabain-sensitive membrane potential of high-Na muscle was about 8 times larger than that of low-Na muscle.

Rate Coefficient of the ^{22}Na Efflux from Low-Na, Normal-Na and High-Na Muscles

Table 4 shows the rate coefficient of the ^{22}Na efflux from low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain. The rate coefficient of the ^{22}Na efflux increased upon raising the internal Na concentration. The difference between the rate coefficient of the ^{22}Na efflux in the presence and absence of ouabain increased when the internal Na concentration was raised. The value of the ouabain-induced change in the rate coefficient of the ^{22}Na efflux from high-Na muscle was about 2 times larger than that from low-Na muscle.

Na Efflux from Low-Na, Normal-Na and High-Na Muscles

The Na efflux from low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain is

Table 5. Na efflux from low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain

Muscle	Na efflux (nmol/kg muscle water/sec)		
	Low-Na	Normal-Na	High-Na
Ouabain (−)	442	1101	4002
Ouabain (+)	342	888	3382
Difference	100	213	620

Table 6. Input resistance of low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain[a]

Muscle	Input resistance (MΩ)		
	Low-Na	Normal-Na	High-Na
Ouabain (−)	2.5 ± 1.2	2.5 ± 1.0	2.4 ± 1.1
Ouabain (+)	2.6 ± 1.3	2.6 ± 1.4	2.5 ± 1.5

[a] Each value of input resistance is expressed as the mean value of fifteen experiments ± SD of the mean value. Ouabain has no significant effect on the input resistance. No significant difference of the input resistance was observed among low-Na, normal-Na and high-Na muscles.

Table 7. Specific membrane resistance of low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain[a]

Muscle	Relative value of specific membrane resistance		
	Low-Na	Normal-Na	High-Na
Ouabain (−)	1.00	1.00	0.92
Ouabain (+)	1.08	1.08	1.00

[a] Each value expresses the relative value of the specific membrane resistance to that of normal-Na muscle in the absence of ouabain.

Table 8. Na/K coupling of the Na,K-pump of low-Na, normal-Na and high-Na muscles[a]

Muscle	Low-Na	Normal-Na	High-Na
Assumed value:	Calculated value of Na/K coupling:		
A. 1.50	1.38	1.50	1.59
B. 2.00	1.70	2.00	2.24
C. 2.50	1.98	2.50	2.98
D. 3.00	2.22	3.00	3.83

[a] The value of Na/K coupling of normal-Na muscle is assumed to be 1.50 (A), 2.00 (B), 2.50 (C) or 3.00 (D).

shown in Table 5. The Na efflux shown in Table 5 was calculated from the product of the internal Na concentration (Table 2) and the rate coefficient of the ^{22}Na efflux (Table 4). The Na efflux increased upon raising the internal Na concentration. The difference between the Na effluxes in the presence and absence of ouabain also increased as the internal Na concentration increased. The value of the ouabain-induced change in the Na efflux from high-Na muscle was about 5 times larger than that from low-Na muscle.

Membrane Resistance of Low-Na, Normal-Na and High-Na Muscles

Table 6 shows the input resistances of low-Na, normal-Na and high-Na muscles in the presence and absence of ouabain. The input resistance was not significantly affected by changing the internal Na concentration from 6 to 20 mmol/kg muscle water. Ouabain had no significant effect on the input resistance at least around 30 min after 0.1 mM ouabain had been applied. Using the value of the input resistance shown in Table 6, the relative value of the specific membrane resistance to that of normal-Na muscle without ouabain was calculated with Eq. (1) on an assumption that the specific resistance of myoplasma is not affected by changing the internal Na concentration from 6 to 20 mmol/kg muscle water (Table 7).

Calculation of the Na/K Coupling Ratio of the Na,K-Pump of Low-Na, Normal-Na and High-Na Muscles

Using the values of the ouabain-induced change in the membrane potential (Table 3), the ouabain-induced change in the Na efflux (Table 5) and the relative value of the specific membrane resistance (Table 7), the Na/K coupling ratio of the Na,K-pump of low-Na and high-Na muscles was calculated using Eq. (4). Equation (4) allows the calculation of the change in the coupling ratio from a fixed value under control conditions. Therefore, to use Eq. (4) requires an assumption about the value of the Na/K coupling ratio of the pump under control conditions. Since, as mentioned in the Introduction, there is some question about exactly what the coupling ratio of normal muscle is, several different values of coupling ratios were assumed as the value for normal sodium muscle. Specifically, values of 1.5, 2, 2.5 and 3 were assumed as the coupling ratio at normal internal Na concentration (Hodgkin & Keynes, 1955; Mullins & Noda, 1963; Sjodin & Beaugé, 1967; Clausen & Hansen, 1974; Sjodin & Ortiz, 1975; Lederer & Nelson, 1984). Table 8

Table 9. Membrane potential at various external K concentrations in the presence and absence of ouabain[a]

	External K concentration (mM)			
	1	2.5	5	10
	Membrane potential (mV)			
A. Ouabain (−)	−102.3 ± 3.8	−88.9 ± 2.9	−79.0 ± 1.0	−56.7 ± 1.2
B. Ouabain (+)	−97.9 ± 4.0[c]	−85.6 ± 3.1[c]	−78.2 ± 1.2[b]	−55.9 ± 1.1[b]
C. Difference (ΔV_m)	−4.4	−3.3	−0.8	−0.8

[a] Each value of membrane potential is expressed as the mean value of fifteen experiments ± SD of the mean value.
[b,c] In comparison with ouabain-free, the significant difference is indicated: [b]$P < 0.05$; [c]$P < 0.005$.

shows the calculated value of the Na/K coupling ratio of low-Na, normal-Na and high-Na muscles. The variation of the Na/K coupling ratio calculated in Table 8 is dependent on the value assumed for the control ratio with the largest change in coupling ratio when the assumed value for normal-Na muscle is 3. But regardless of the value assumed for the coupling ratio of normal sodium muscle, the value of the Na/K coupling ratio is increased in high-sodium muscle and decreased in low-sodium muscle. This result suggests that, like the insulin-stimulated Na,K-pump (Marunaka, 1987*a*), the basal Na,K-pump also has variable Na/K coupling ratio depending on the intracellular Na concentration.

Effect of the External K Concentration on the Na/K Coupling Ratio

Membrane Potential at Various External K Concentrations

The membrane potential was measured at external K concentrations of 1, 2.5, 5 and 10 mM in the presence and absence of ouabain (Table 9). The difference between the membrane potential in the presence and absence of ouabain decreased upon raising the external K concentration from 1 to 10 mM. The ouabain-induced change in membrane potential at the external K concentration of 1 mM was 5.5 times larger than that at the external K concentration of 10 mM.

Internal Na Concentration at Various External K Concentrations

Table 10 shows the internal Na concentrations at external K concentrations of 1, 2.5, 5 and 10 mM in the presence and absence of ouabain. A change in the external K concentration from 1 to 10 mM had no significant effect on the internal Na concentration at the time when the internal Na concentration was measured (at 60 min after the external K concentration had been changed). Application of ouabain also had no significant effect on the internal Na concentration at least at the time when the internal Na concentration was measured (30 min after 0.1 mM ouabain had been applied).

Table 10. Internal Na concentration at various external K concentrations in the presence and absence of ouabain[a]

	External K concentration (mM)			
	1	2.5	5	10
	Internal Na concentration (mmol/kg muscle water)			
Ouabain (−)	11.0 ± 2.8	10.2 ± 2.4	9.7 ± 2.3	9.0 ± 2.7
Ouabain (+)	11.2 ± 3.0	10.5 ± 2.2	10.0 ± 2.5	9.4 ± 2.9

[a] Each value of internal Na concentration is expressed as the mean value of six experiments ± SD of the mean value. The external K concentration had no significant effect on the internal Na concentration. Ouabain had no significant effect on the internal Na concentration.

Rate Coefficient of the ^{22}Na Efflux at Various External K Concentrations

The rate coefficient of the ^{22}Na efflux was increased about 1.5-fold upon raising the external K concentration from 1 to 10 mM regardless of the presence or absence of ouabain (Table 11). However, ouabain does decrease the rate coefficient of the ^{22}Na efflux at all external K concentrations examined (Table 11).

Table 11. Rate coefficient of the ^{22}Na efflux at various external K concentrations in the presence and absence of ouabain[a]

	External K concentration (mM)			
	1	2.5	5	10
	Rate coefficient of ^{22}Na efflux (10^{-6}/sec)			
Ouabain (−)	88.9 ± 7.5	108.5 ± 9.5	128.5 ± 10.1	133.3 ± 11.1
Ouabain (+)	70.2 ± 7.7[b]	82.9 ± 9.9[c]	96.0 ± 11.5[c]	98.0 ± 12.5[c]
Difference	18.7	25.6	32.5	35.3

[a] Each value of rate coefficient of ^{22}Na efflux is expressed as the mean value of six experiments ± SD of the mean value.
[b,c] In comparison with ouabain-free, the significant difference is indicated: [b]$P < 0.005$; [c]$P < 0.001$.

Table 12. Na efflux at various external K concentrations in the presence and absence of ouabain

	External K concentration (mM)			
	1	2.5	5	10
	Na efflux (nmol/kg muscle water/sec)			
Ouabain (−)	978	1107	1246	1200
Oubain (+)	786	870	960	921
Difference	192	237	286	279

Table 13. Input resistance at various external K concentrations in the presence and absence of ouabain[a]

	External K concentration (mM)			
	1	2.5	5	10
	Input resistance (MΩ)			
Ouabain (−)	2.9 ± 1.3	2.4 ± 1.1	2.1 ± 0.9	2.0 ± 0.7
Ouabain (+)	3.0 ± 1.4	2.4 ± 1.5	2.1 ± 1.1	2.1 ± 0.9

[a] Each value of the input resistance is expressed as the mean value of fifteen experiments ± SD of the mean value. Ouabain had no effect on the input resistance.

Na Efflux at Various External K Concentrations

Using the internal Na concentration (Table 10) and the rate coefficient of the ^{22}Na efflux (Table 11), the Na effluxes at external K concentrations of 1, 2.5, 5 and 10 mM were estimated (Table 12). The Na efflux increased when the external K concentration increased from 1 to 10 mM. The difference between the Na effluxes in the presence and absence of ouabain increased as the external K concentration was raised. The ouabain-induced change in Na efflux at an external K concentration of 10 mM was about 1.5 times larger than that at an external K concentration of 1 mM.

Membrane Resistance at Various External K Concentrations

Table 13 shows the input resistances at the external K concentrations of 1, 2.5, 5 and 10 mM in the presence and absence of ouabain. Ouabain had no significant effect on the input resistance at any external K concentrations examined at least around 30 min after 0.1 mM ouabain had been applied. The mean value of the input resistance decreased as the external K concentration increased from 1 to 10 mM regardless of the presence or absence of ouabain. The mean value of the input resistance at the external K concentration of 1 mM was about 1.5 times larger than that at the external K concentration of 10 mM.

Table 14 shows the fractional change in membrane resistance at various different external K concentrations relative to the resistance at the normal external K concentration of 2.5 mM. The specific membrane resistance decreased when the external K concentration was increased from 1 to 10 mM. The specific membrane resistance at the external K concentration of 1 mM was 2.1 times larger than that at the external K concentration of 10 mM.

Calculation of the Na/K Coupling Ratio of the Na,K-Pump at Various External K Concentrations

Using the values of the ouabain-induced change in the membrane potential (Table 9), the ouabain-in-

Table 14. Specific membrane resistance at various external K concentrations in the presence and absence of ouabain[a]

	External K concentration (mM)			
	1	2.5	5	10
	Relative value of specific membrane resistance			
Ouabain (−)	1.46	1.00	0.77	0.69
Ouabain (+)	1.56	1.00	0.77	0.77

[a] Each value expresses the relative value of specific membrane resistance to that at the external K concentration of 2.5 mM in the absence of ouabain.

Table 15. Na/K coupling of the Na,K-pump at various external K concentrations[a]

	External K concentration (mM)			
	1	2.5	5	10
Assumed value:	Calculated value of Na/K coupling:			
A. 1.50	1.60	1.50	1.10	1.11
B. 2.00	2.29	2.00	1.15	1.18
C. 2.50	3.09	2.50	1.19	1.22
D. 3.00	4.02	3.00	1.21	1.25

[a] The value of Na/K coupling at the external K concentration of 2.5 mM is assumed to be 1.50 (A), 2.00 (B), 2.50 (C) or 3.00 (D).

duced change in the Na efflux (Table 12) and the relative value of the specific membrane resistance (Table 14), the Na/K coupling ratio of the Na,K-pump at the external K concentrations of 1, 2.5, 5 and 10 mM was calculated using Eq. (4) with values of 1.5, 2, 2.5 or 3 assumed for the Na/K coupling ratio of the pump in control tissues at an external K concentration of 2.5 mM (Table 15). As was the case for variation of internal Na, the variation of the Na/K coupling ratio calculated in Table 15 is dependent on the value assumed for the control ratio, but regardless of the value assumed for the coupling ratio of normal external potassium, the value of the Na/K coupling ratio is increased in reduced external K and decreased in high external K. This result suggests that, like the insulin-stimulated Na,K-pump (Marunaka, 1986), the basal Na,K-pump also has variable Na/K coupling ratio depending on the extracellular K concentration.

Discussion

The present study suggests that the Na/K coupling ratio of the basal Na,K-pump in frog skeletal muscle is not fixed at 1.5, but varies depending upon the ionic environment. These results support previous observations of variable coupling ratio of Na,K-pump (Mullins & Awad, 1965; Mullins & Brinley, 1969).

Sjodin and Ortiz (1975) have shown that at high values of both internal Na and external K concentrations, the Na/K coupling ratio of the Na,K-pump in frog skeletal muscle is 1.4 to 1.7. Using the data shown in Tables 8 and 15, the value of the Na/K coupling ratio of the pump of high-Na muscle at the external K concentration of 10 mM can be calculated assuming that the effects of internal Na and external K concentrations act independently on the Na/K coupling ratio. The result of the calculation (assuming values for the coupling ratio of control tissues of 1.5 to 3.0) is shown in Table 16. Table 16 shows that the expected value of Na/K coupling ratio is 1.2 to 1.6 at both high internal Na and external K concentrations in the present study. This result indicates a range for the Na/K coupling ratio which suggests that the method used in the present work produces results which are comparable to the method used by Sjodin and Ortiz (1975). Especially, when 2.5 or 3.0 is assumed for the Na/K coupling ratio of normal tissues (normal internal Na and 2.5 mM external K concentration), the result shown in Table 16 (1.45 and 1.60) is within the range (1.4 to 1.7) reported by Sjodin and Ortiz (1975).

Table 16. Na/K coupling of the Na,K-pump at both high internal Na and external K concentrations

Assumed value of Na/K coupling at normal internal Na and external K concentrations	Expected value of Na/K coupling at both high internal Na and external K concentrations
A. 1.50	1.18
B. 2.00	1.32
C. 2.50	1.45
D. 3.00	1.60

When the value of the Na/K coupling ratio at normal internal Na and external K concentrations is assumed to be 1.5, the variation of Na/K coupling ratio (1.1 to 1.6; *see* Tables 8A and 15A) is relatively small for the different internal Na and external K concentrations used in the present study. Nonetheless, all of the present manipulations result in consistent alterations in the apparent Na/K coupling ratio of Na,K-pump. However, because the variation in coupling ratio is relatively small, special attention must be given to potential sources of error which might compromise the conclusions.

Table 17. Estimated value of the internal Cl concentration of low-Na, normal-Na and high-Na muscles

	Internal Cl concentration (mmol/kg muscle water)		
Muscle	Low-Na	Normal-Na	High-Na
A. Ouabain (−)	4.25	3.78	2.64
B. Ouabain (+)	4.46	4.27	3.79
C. Difference	0.21	0.49	1.15

Relation Between the Steady and Nonsteady State

In the present study, the value of the Na/K coupling ratio of the Na,K-pump is calculated assuming that an ionic steady state has been reached in the muscles examined. If the muscles are not in ionic steady state, then the calculation of coupling ratio will be changed. When there is no net transmembrane current, the following equation holds even if the muscle cell is not in steady state.

$$F(J_{Na} + J_K) + I_p + C_m(dE_m/dt) = 0 \quad (5)$$

where C_m is the membrane capacitance and E_m is the membrane potential. In the present study, Eq. (2) was used to calculate the value of the Na/K coupling ratio given in Tables 8 and 15. The difference between Eqs. (2) and (5) is $C_m(dE_m/dt)$. In frog skeletal muscle, C_m is approximately 6 μF/cm^2 (Katz, 1966). Moore and Rabovsky (1979) showed that the change in membrane potential is 5 mV/hr in the presence of 0.1 mM ouabain. In experiments on muscles under the same conditions as that in the present study the change in membrane potential was less than 1 mV/10 min in the presence of 0.1 mM ouabain. Based on a maximum rate of change of voltage of no more than 6 mV/hr, the maximum value of $C_m(dE_m/dt)$ is 10 pA/cm^2. On the other hand, the pump current I_p can be calculated using Eq. (3). Using the smallest value for ΔV_m (0.8 mV) and a membrane resistance of 4×10^3 Ωcm^2 (Fatt & Katz, 1951; Hodgkin & Horowicz, 1959; Katz, 1966), the minimum value of I_p would be 0.2 μA/cm^2. Therefore, the maximum value of $C_m(dE_m/dt)$ (10 pA/cm^2) is negligibly small compared to the minimum value of I_p (0.2 μA/cm^2) under the conditions of the present study. Thus, the muscles used in the present study are so close to a steady-state condition that the steady-state assumption of Eq. (2) is not significantly violated in the present study.

Changes in Membrane Potential due to Ouabain-Induced Changes in Internal Ion Concentrations and its Effect on the Na/K Coupling Ratio

An additional potential problem involves the attribution of the entire potential change only to the electrogenicity of the pump. The difficulty is that the ouabain-induced change in the membrane potential measured in the present study could be due to directly blocking the electrogenic Na,K-pump; alternatively, some component of the potential change could be due to a ouabain-induced alteration in intracellular ion activities. Therefore, it is necessary to estimate if treatment with ouabain for 30 min can change the internal ion concentrations to an extent which would alter the membrane potential and, thus, cause an overestimate of the potential due to pump current (Tables 3 and 9). Although no significant change in the internal Na concentration was detectable 30 min after the application of 0.1 mM ouabain (Tables 2 and 10), over longer periods of time the mean value of the internal Na concentration is likely to increase in the presence of ouabain. Further, the internal K and Cl concentrations which were not directly measured might also change in the presence of ouabain. Internal chloride ion, in particular, may change in response to the ouabain-induced change in membrane potential. Based on the present results and known properties of frog skeletal muscle, it is possible to place an upper limit on the magnitude of the error in the determination of coupling ratio which might be introduced by ouabain-induced changes in internal ion concentrations which lead to changes in the membrane potentials shown in Tables 3 and 9. Determination of the upper limit of the error due to changes in intracellular ion concentrations depends upon two assumptions. First, the determination of error assumes that chloride ion is likely to be at or very close to electrochemical equilibrium at the time when the measurements in the current study were made. Second, the determination assumes that sodium, chloride, and potassium are the only major permeable ions, and, therefore, changes in cation concentrations (sodium and potassium) must be equal to the changes in anion (chloride) concentration.

The first assumption is supported by the work of Hodgkin and Horowicz (1959) who reported that, although transmembrane movements of Cl^- are quite rapid, in frog skeletal muscle Cl^- is passively distributed across the plasma membrane. The redistribution of Cl^- to equilibrium in response to a change in the electrochemical gradient for chloride takes place within 15 min (Hodgkin & Horowicz,

Table 18. Estimated value of the internal Cl concentration at various external K concentrations

	External K concentration (mM)			
	1	2.5	5	10
	Internal Cl concentration (mmol/kg muscle water)			
A. Ouabain (−)	2.26	3.82	5.64	13.56
B. Ouabain (+)	2.68	4.35	5.82	14.01
C. Difference	0.42	0.53	0.18	0.45

1959). In this study, for measurements in the absence of ouabain, Cl^- would have reached equilibrium prior to the time when measurements were made (60 min). In fact, for all the ionic conditions examined in this study in the absence of ouabain, there was no measurable change in membrane potential occurring at the time when experimental values were determined. This supports the idea that Cl^- is at equilibrium. If Cl^- reaches an electrochemical equilibrium, the internal Cl concentration can be calculated from the following equation:

$$\text{Membrane potential} = (RT/F) \ln ([Cl]_i/[Cl]_o). \quad (6)$$

Tables 17A and 18A show the calculated equilibrium value of the internal Cl concentration in the absence of ouabain based on Eq.(6) and the data of Tables 3 and 9 ($[Cl]_o$ = 126.5 mM).

If Cl^- reaches electrochemical equilibrium at the time when measurements are made, potassium, sodium and chloride are the only major permeable ions, and there is no significant change in intracellular sodium or potassium; then the membrane potential (E_m) after blocking the electrogenic component of the Na,K-pump with ouabain is:

$$E_m = (RT/F) \ln ((([K]_o + \alpha[Na]_o)/([K]_i + \alpha[Na]_i)) \quad (7)$$

where α is the ratio of Na permeability-to-K permeability. The difference between this potential and the potential in the absence of ouabain can be thought of as being due to a current I_p, the "pump" current. Actually, since intracellular sodium and potassium concentration may change slightly, the measured "pump" current will consist of two components. One component is attributable to a "true" current associated with the charge translocation of the electrogenic Na/K pump; while the other component can be attributed to a membrane potential

Table 19. Estimated value of the ouabain-sensitive membrane potential caused by the change in the internal ion concentrations of low-Na, normal-Na and high-Na muscles[a]

Muscle	Low-Na	Normal-Na	High-Na
ΔE_m (mV)	0.053	0.124	0.290

[a] $\Delta[Cl]_i$ is assumed to be the value shown in Table 17C.

change due to an alteration in intracellular ion concentrations. Since there is little if any change in the intracellular sodium concentration 30 min after the application of ouabain, any significant change in cellular potential must be primarily due to changes in the concentration of intracellular potassium. If potassium, sodium, and chloride are the only major permeable ions and there is no significant change in intracellular sodium concentration, then the change in intracellular potassium can be no larger than the change in intracellular chloride. Under these conditions with a small change in intracellular potassium, the measured change in the membrane potential (E'_m) will be:

$$E'_m = (RT/F) \ln ((([K]_o + \alpha[Na]_o)/([K]_i + \Delta[K]_i + \alpha([Na]_i))) \quad (8)$$

where $\Delta[K]_i$ is the change in the internal K concentration. Therefore, the component of the ouabain-induced change in membrane potential caused by changes in intracellular ion concentrations (ΔE_m) is:

$$\Delta E_m = E_m - E'_m = (RT/F) \ln ((([K]_i + \Delta[K]_i + \alpha[Na]_i)/([K]_i + \alpha[Na]_i)). \quad (9)$$

As mentioned above, the maximum change in intracellular potassium would be equal to the change in chloride associated with passive electrochemical redistribution (Tables 17 and 18). Using Eq. (9) with $\Delta[K]_i$ equated with the maximum chloride change of Tables 17C and 18C, the maximum value ΔE_m can be calculated assuming that $[K]_i$ is 100 mmol/kg muscle water, α is 0.01, and $[Na]_i$ is taken from Tables 2 and 10. The calculated value of ΔE_m is given in Tables 19 and 20. The values of ΔE_m are much smaller than the magnitude of the total ouabain-induced potential change given in Tables 3C and 9C. Although ΔE_m is small, the Na/K coupling ratio was recalculated by subtracting the value (ΔE_m) shown in Tables 19 and 20 from the ouabain-induced potential change given in Tables 3C and 9C. In fact, the result of the calculation of the Na/K coupling ratio (Tables 21 and 22) using the corrected

Table 20. Estimated value of the ouabain-sensitive membrane potential caused by the change in the internal ion concentrations at various external K concentrations[a]

	External K concentration (mM)			
	1	2.5	5	10
ΔE_m	0.106	0.134	0.046	0.114

[a] $\Delta[Cl]_i$ is assumed to be the value shown in Table 18C.

Table 21. Na/K coupling of the Na,K-pump of low-Na, normal-Na and high-Na muscles[a]

Muscle	Low-Na	Normal-Na	High-Na
Assumed value:	Estimated value of Na/K coupling:		
A. 1.50	1.40	1.53	1.62
B. 2.00	1.76	2.08	2.33
C. 2.50	2.07	2.66	3.18
D. 3.00	2.35	3.26	4.20

[a] The values of Na/K coupling are calculated on assumption that the ouabain-sensitive membrane potential of normal-Na muscle (−3.1 mV) shown in Table 3 is caused by the Na,K-pump whose Na/K coupling is 1.50 (A), 2.00 (B), 2.50 (C) or 3.00 (D).

value of the pump electrogenic potential indicates that the result is very similar to the original coupling ratios calculated using the total ouabain-induced potential (Tables 8 and 15). The results shown in Tables 8 and 21 and Tables 15 and 22 are summarized in Tables 23 and 24, respectively.

Thus, even when the maximal error which could be introduced by changes in intracellular ion concentrations is considered, the qualitative conclusion that the Na/K coupling ratios of the pump vary in response to change in extracellular K and intracellular Na remains unchanged.

Effect of Changes in Internal Na Concentration on Na Efflux and Estimation of the Effect of the External K Concentration on the Na/K Coupling Ratio

When the effect of the external K concentration on the Na/K coupling ratio was estimated, the mean value of the internal Na concentration decreases slightly with increase of the external K concentration (Table 10). A decrease in the internal Na concentration of the magnitude measured in this study is known to reduce Na efflux (Keynes & Swan, 1959; Mullins & Frumento, 1963; Kitasato et al., 1980*b*, c; Eisner, Lederer & Vaughan-Jones, 1981; Marunaka & Kitasato, 1985*a*). However, if the calculated value of Na efflux at high external K concentration is underestimated, then the change in Na/K coupling ratio at high external K concentration is larger (*see* Eq. (4); ΔV_m is a minus value). Therefore, the conclusion that the coupling ratio varies with external potassium concentration is not qualitatively changed.

Table 22. Na/K coupling of the Na,K-pump at various external K concentrations[a]

	External K concentration (mM)			
	1	2.5	5	10
Assumed value:	Estimated value of Na/K coupling:			
A. 1.50	1.63	1.53	1.10	1.13
B. 2.00	2.37	2.08	1.16	1.21
C. 2.50	3.25	2.66	1.20	1.26
D. 3.00	4.47	3.33	1.23	1.30

[a] The values of Na/K coupling is calculated on the assumption that the ouabain-sensitive membrane potential at external K concentration of 2.5 mM (−3.3 mV) shown in Table 9 is caused by the Na,K-pump whose Na/K coupling is 1.50 (A), 2.00 (B), 2.50 (C) or 3.00 (D).

Direct Effect of the Change in the Internal Na Concentration on the Na/K Coupling Ratio at Various External K Concentrations

The present results show that an increase in the internal Na concentration increases the Na/K coupling ratio of the pump and that the coupling ratio is also larger at low external K concentration than at high external K concentration. However, a reduction in external K concentration produces an increase in the mean value of the internal Na concentration (Table 10). Thus the variation in coupling ratio associated with changing external potassium may be due to an indirect increase in internal Na concentration rather than a direct effect of external potassium.

To clarify whether the large value of the Na/K coupling ratio at low external K concentration was induced by the direct action of lowering external K concentration or by its indirect action through increasing the internal Na concentration, I measured the ouabain-sensitive membrane potential when the internal Na concentration was lower than that produced by high external K concentration. At low external K concentration (1 mM), the membrane potential of low-Na muscle (internal Na concentration of about 6 mmol/kg muscle water) was depolarized about 1.5 mV after 30 min in 0.1 mM ouabain. This value of the ouabain-induced depolarization (1.5 mV) is larger than the ouabain-induced depolariza-

Table 23. Na/K coupling of the Na,K-pump of low-Na, normal-Na and high-Na muscles[a]

Muscle	Low-Na	Normal-Na	High-Na
Assumed value:	Estimated value of Na/K coupling:		
A. 1.50	1.38–1.40	1.50–1.53	1.59–1.62
B. 2.00	1.70–1.76	2.00–2.08	2.24–2.33
C. 2.50	1.98–2.07	2.50–2.66	2.98–3.18
D. 3.00	2.22–2.35	3.00–3.26	3.83–4.20

[a] All values shown in Tables are the same as those in Tables 8 and 21.

Table 24. Na/K coupling of the Na,K-pump at various external K concentrations[a]

	External K concentration (mM)			
	1	2.5	5	10
Assumed value:	Estimated value of Na/K coupling:			
A. 1.50	1.60–1.63	1.50–1.53	1.10–1.10	1.11–1.13
B. 2.00	2.29–2.37	2.00–2.08	1.15–1.16	1.18–1.21
C. 2.50	3.09–3.25	2.50–2.66	1.19–1.20	1.22–1.26
D. 3.00	4.02–4.47	3.00–3.33	1.21–1.23	1.25-1.30

[a] All values shown in Tables are the same as those in Tables 15 and 22.

tion (0.8 mV) at high external K concentration (5 and 10 mM) when the internal Na concentration is 9 to 10 mmol/kg muscle water. On the other hand, 0.1 mM ouabain decreased the Na efflux from low-Na muscle with an internal Na concentration of about 6 mmol/kg muscle water at an external K concentration of 1 mM after 30 min in 0.1 mM ouabain. This ouabain-induced change in the Na efflux was two- or threefold smaller than the value of the ouabain-induced change in the Na efflux at high external K concentration (5 and 10 mM) and normal Na concentration (9 to 10 mmol/kg muscle water) (my preliminary data). At the external K concentration of 1 mM, the input resistance was not significantly affected by lowering the internal Na concentration from 9 to 10 mmol/kg muscle water (normal-Na muscle) to 6 mmol/kg muscle water (low-Na muscle), regardless of the presence of 0.1 mM ouabain.

These observations indicate that, even when the internal Na concentration is low, the value of the Na/K coupling ratio at low external K concentration (1 mM) is larger than that at high external K (5 and 10 mM) and normal Na concentrations. This suggests that lowering of the external K concentration increases the value of the Na/K coupling ratio through a direct effect of potassium rather than by raising the internal Na concentration, although a part of the increase in the Na/K coupling ratio seen in reduced external K concentrations may be due to raising the internal Na concentration. Therefore, the conclusion the coupling ratio varies with external potassium concentration is not qualitatively changed.

Effect of the Membrane Potential on the Na,K-Pump Activity

In the present study, internal Na and external K concentrations were shown to affect the Na/K coupling ratio of the pump. However, as the internal Na concentration or the external K concentration was changed, the membrane potential also changed. This change in the membrane potential might have an effect on the Na/K coupling ratio. Apparently, the Na,K-pump activity in squid axons is independent of the membrane potential (Brinley & Mullins, 1974). In addition, Beaugé, Sjodin and Ortiz (1975) and Beaugé and Sjodin (1976) have shown that the activation of the Na,K-pump in frog skeletal muscles by increasing external K concentration is independent of the change in the membrane potential induced by the rise in the external K concentration. However, recently it has been reported that the Na,K-pump activity is dependent on membrane potential (Gadsby, Kimura & Noma, 1985; Eisner, Valdeolmillos & Wray, 1986; DeWeer, Rakowski & Gadsby, 1987; Gadsby & Nakao, 1987); specifically these investigators suggest that the net pump current increases when the membrane is depolarized. However, for all the experimental conditions described in this paper, sodium pump current *decreases* when the membrane is depolarized and *increases* when the membrane is hyperpolarized (Tables 3 and 9). If there is a voltage effect on the pump in the present study, it would tend to reduce rather than increase the variation in coupling ratio and correction for such an effect would only make the variation in the coupling ratio larger.

Conclusions of the Present Study

I have discussed the problems of investigating the effect of varying the internal Na and external K concentrations on the Na/K coupling ratio of the pump. Despite several potential problems, the fundamental qualitative result of this study remains unchanged; that is, that an increase in intracellular Na concentration increases the Na/K coupling ratio while an increase in extracellular K concentration decreases the Na/K coupling ratio of the Na,K-pump.

I express my gratitude to Drs. Hiroshi Kitasato and Koichi Murayama (Department of Physiology, Shiga University of Medical Science, Ohtsu, 520-21 Japan) for giving me useful suggestions, useful discussion and constant encouragement. I also thank Dr. Douglas C. Eaton (Department of Physiology, Emory University School of Medicine, Atlanta, Georgia 30322), for giving me useful discussion and suggestions, giving every convenience in preparing the manuscript, critical reading of the manuscript, and editorial help. This work was supported by grants from the Ministry of Education, Science and Culture of Japan to Dr. Y. Marunaka (57770067, 58770068, 59770063, 60770077) and Dr. H. Kitasato (57570029, 58570036, 60570037) and from National Institutes of Health of the USA to Dr. D. C. Eaton (DHHS DK-38830). Dr. Y. Marunaka was supported by a Research Fellowship Abroad from the Ministry of Education, Science and Culture of Japan.

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Received 17 August 1987