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Seasonal Variation in the Na^+, K^+-ATPase Activity in Frog (*Rana dybowskii*) Brain

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Seasonal changes in the activity and characteristics of brain Na^+, K^+-ATPase and Mg^{2+}-ATPase were investigated in frog (*Rana dybowskii*). The brain Na^+, K^+-ATPase activity during hibernation was similar to that in active period in frogs. The Na^+, K^+-ATPase activity increased in December and March, when the frogs enter into and awake from the hibernation. Over 5~35°C temperature range, Na^+, K^+-ATPase showed non-linear Arrhenius kinetics throughout the year. The brain Mg^{2+}-ATPase activity decreased during hibernation, but markedly increased in March. The Arrhenius plots for Mg^{2+}-ATPase activity were linear in frogs both in torpid and active state. The ratio of Na^+, K^+-ATPase activity at 15°C to at 35°C did not change during hibernation. The sensitivity of Na^+, K^+-ATPase to ouabain was also unchanged throughout the year. These results indicate that the activity and characteristics of brain Na^+, K^+-ATPase remain unchanged during hobernation in frog.

KEY WORDS: Frog, *Rana dybowskii*, Na^+, K^+-ATPase, Mg^{2+}-ATPase, Hibernation

The characteristic of animals capable of hibernation is that they are able to maintain their cellular and organ function, such as heart beat, respiration, cellular electrolyte balance and neural activity at such a low body temperature near 5°C, which is quite inhibitory for both the non-hibernators and hibernators in their active state (Strumwasser, 1959; Mendler *et al.*, 1972; Willis, 1979; Aloia and Raison, 1989). This characteristic has been referred to as cold resistance during hibernation.

The brain activity may be essential for animals both in their active and torpid state. It was reported that neural activity and Na^+/K^+ gradients were maintained in the brains of hibernators during torpor (Strumwasser, 1959; Mendeler, 1972; Goldman and Willis, 1973; Beckman and Stanton, 1976; Florant and Heller, 1877). Na^+, K^+-ATPase plays an important role in the maintenance of cation gradients across the cell membrane, which is integral to maintain membrane potential and optimal neuronal activity. Therefore, seasonal changes in the activity and thermal dependence of brain Na^+, K^+-ATPase were studied in a various species, including non-hibernator and hibernator in their active and torpid state, or in induced hypothermic state.

The brain Na^+, K^+-ATPase activity increased in hibernating hamsters and the activity was less reduced at the low experimental temperature in torpid hibernators than in non-hibernators, displaying cold resistance in hibernator during torpor. The brain Na^+, K^+-ATPase of hamsters in torpid state was relatively insensitive to the temperature changes as compared with that of hamsters in active state or that of non-hibernating

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mammals (Goldman and Willis, 1973; Goldman and Albers, 1975). A cold resistance in Na^+, K^+-ATPase from renal cortex of hamster during hibernation was also observed (Willis and Li, 1969; Fang and Willis, 1974).

Charnock and Simonson (1978a) reported that the activity of brain Na^+, K^+-ATPase and Mg^{2+}-ATPase from ground squirrel increased after hibernation. However, the non-linear thermal dependence of these enzymes in Arrhenius plots was not changed during hibernation.

To determine whether there are any seasonal alterations, particularly during hibernation, in the characteristics of Na^+, K^+-ATPase, we examined the seasonal changes in activity, thermal dependence, cold resistance and ouabain sensitivity of brain Na^+, K^+-ATPase of frogs (*Rana dybowskii*) in active state (June, July), in hibernation (December, January) and in October and March, when they enter into and awake from hibernation, respectively.

Materials and Methods

Animal

The frogs (*Rana dybowskii*) were collected all the year round in the vicinity of Kwangju, Korea. These frogs were active from March until the end of October, then they enter into hibernation and stayed in deep hibernation until February. The frogs arouse from hibernation at the end of February. The frogs in their awake season, March, June and July, were captured in the grass near the mountain stream and frogs in their torpid season, October, December and January were collected in the mountain stream. Frogs were killed by decapitation and the brains were quickly removed and then were stored at −70°C until used. In all seasons, the brains of about 15 adult male frogs (20-30 g) were pooled together.

Preparation of the Na^+, K^+-ATPase fraction

Microsomal Na^+, K^+-ATPase preparations were obtained from the frog brains according to the procedure described previously (Velema and Zaagsma, 1981) with a slight modification. Brains were thawed, minced and homogenized in 4 volumes of cold homogenization buffer solution (20 mM Tris-HCl, pH 7.0) with a glass homogenizer followed by homogenation in a same solution in a motor-driven Teflon pestle-glass homogenizer at medium speed. The resultant homogenate were centrifuged at 8,000 ×g for 30 min and the supernatant was centrifuged at 12,000 ×g for 30 min. The supernatant was centrifuged at 44,000 ×g for 1 hr and the pellet was dissolved in a sample buffer (20mM Tris-HCl, pH 7.4). All procedures were carried out at 4°C

Determination of Na^+, K^+-ATPase activity

The enzyme reaction was started by the addition of 2 mM ATP to a prewarmed (at various temperature) reaction mixture. The final concentrations of the mixture were 120 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 40 mM Tris-HCl, 2 mM ATP and 20 μg protein/ml, pH 7.4 in a total volume of 1 ml. After 1 hr incubation, the reaction was terminated by adding 0.1 ml of 10% SDS. The amount of inorganic phosphate liberated from ATP was determined by the method of Fiske and Subbarow (1925). Ouabain-sensitive Na^+, K^+-ATPase activity was defined as the difference between activities observed in the absence and presence of 1 mM ouabain. Ouabain-insensitive ATPase activity, observed in the presence of 1 mM ouabain, was regarded as Mg^{2+}-ATPase activity. Addition of sodium azide which inhibits ouabain-insensitive mitochondrial ATPase showed no significant effect on the brain microsomal ATPase activity and therefore it was omitted from the reaction solution.

Na^+, K^+-ATPase activity was determined at an interval of 5°C or 10°C and the thermal dependence of Na^+, K^+-ATPase of frog brain was analyzed by Arrhenius plot. To analyze the sensitivity of Na^+, K^+-ATPase to ouabain, Na^+, K^+-ATPase activity was assayed in the presence of various concentrations (10^{-8}~10^{-3} M) of ouabain.

Na^+, K^+-ATPase and Mg^{2+}-ATPase activities were expressed as μmoles of inorganic phosphate liberated from ATP per mg of protein per hour. Data were presented as the means of the triplicates of a group pooled from at least 15 brains or as the means of the mean values from several groups collected in a same season.

The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistical analyses

Statistical analyses were performed with Student's t-test. Criterion for the statistical significance of the difference was a P value of less than 0.05.

Results

Seasonal variation in Na^+, K^+-ATPase activity

Table 1 shows seasonal variations in the activity of Na^+, K^+-ATPase and Mg^{2+}-ATPase of brain membrane preparations from frogs collected in their active period and during hibernation period. The brain Na^+, K^+-ATPase activities of frogs in summer active state were similar to those in their torpid state. There was a marked increase in Na^+, K^+-ATPase activity in March and at the end of October, when the frogs arouse from and enter into hibernation, respectively. On the other hand, Mg^{2+}-ATPase activities decreased significantly during hibernation but increased markedly in March, when the frogs awake from torpor. These results suggest that brain Na^+, K^+-ATPase might play a crucial role for the process of entrance into and arousal from the hibernation, while brain Mg^{2+}-ATPase might be essential for arousal from hibernation.

Temperature dependence of Na^+, K^+-ATPase

The activities of Na^+, K^+-ATPase and Mg^{2+}-ATPase of frog brain were determined at an interval of 5°C or 10°C over a temperature range of 5~35°C and the possible seasonal variation in the temperature dependence of these enzymes was analyzed by Arrhenius plots.

The data was displayed as Arrhenius plots in Fig. 1, where the brain Na^+, K^+-ATPase from frogs in non-hibernating and hibernating periods always yielded non-linear Arrhenius kinetics. Each dependences of Na^+, K^+-ATPase on temperature was quite similar in December and January, while that was also similar in June and July. The thermal dependence of Na^+, K^+-ATPase from frogs in March was similar to that in summer frogs. If Arrhenius plots of the Na^+, K^+-ATPase activity to temperature assumed to be described by a biphasic function, the breaks in the Arrhenius plots appeared at 24°C and 22°C in June and July, respectively, whereas the breaks occurred at the lower temperature between 18~19°C in December, January and March.

On the contrary to Na^+, K^+-ATPase, the Arrhenius plots in Fig. 2 illustrate that Mg^{2+}-ATPase activity was described by a linear function to temperature in frogs collected in June and January, in their active and torpid state, respectively. Since the enzyme preparations from July and December also exhibited linear Arrhenius kinetics, the data from those months were excluded from Fig. 2 for clarity of presentation. Clearly, there was not a noticeable difference in the slopes of the plots for active and torpid frogs, indicating that there was no alteration in temperature dependence of Mg^{2+}-ATPase during hibernation. It is noted that Mg^{2+}-ATPase of frogs in March, when the frogs arouse from the torpor, also exhibited a linear relationship but the slope

Table 1. Seasonal variation in specific activity* of Na^+, K^+-ATPase and Mg^{2+}-ATPase of *Rana dybowskii* Brain.

Month	Physiological condition	Na^+, K^+-ATPase	Mg^{2+}-ATPase ATPase
Oct.	entry into hibernation	26.9	6.2
Dec.	hibernation	9.1	4.3
Jan.	hibernation	9.9	3.5
Mar.	arousal	17.5	15.4
Jun.	awake	9.2	5.9
Jul.	awake	15.0	7.5

* Na^+, K^+-ATPase activity and Mg^{2+}-ATPase activity are given as μmol Pi liberated from ATP/mg protein/hr at 35°C

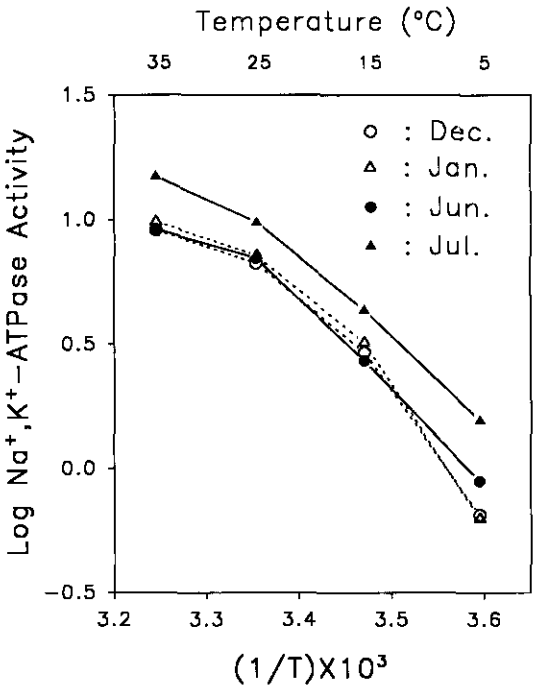

Fig. 1. Arrhenius plots for the Na^+, K^+-ATPase activities in the brain of frogs collected in summer and winter. The logarithm of the Na^+, K^+-ATPase activities are plotted against the reciprocal of the absolute temperature (1/T). The temperature at top abscissa is the experimental temperature in Celsius (°C). The enzyme activity is given as μmol Pi hydrolyzed/mg protein/hr at the various experimental temperatures. Data from summer frogs are plotted as solid line while data from hibernating frogs are plotted as short-dashed line.

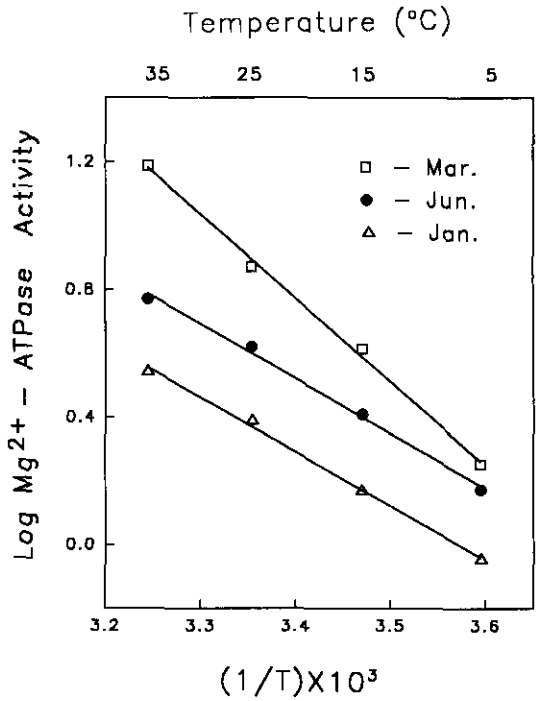

Fig. 2. Arrhenius plots for the Mg^{2+}-ATPase activities in the brain of frogs collected in spring, summer and winter. The logarithm of the Mg^{2+}-ATPase activities are plotted against the reciprocal of the absolute temperature (1/T). The temperature at top abscissa is the experimental temperature in Celsius (°C). The enzyme activity is given as μmol Pi hydrolyzed/mg protein/hr at the various experimental temperatures. The straight lines were determined by least-squares linear regression.

was a little steep compared with those in other seasons.

The data illustrated in Fig. 1 were further analysed by examining the ratio of Na^+, K^+-ATPase activity at 15°C to that at 35°C, which is referred to as an index of cold resistance. Because the Na^+, K^+-ATPase activity of frog brain falls to such a low value during hibernation (see Fig. 1), even a small error in the measurement of very low level of enzyme activity at 5°C, which is similar to that of ambient water temperature during hibernation, might cause a considerable variation in the ratio. Thus, for the accuracy of this examination, the activity at 15°C was compared with that at 35°C and the data for the ratios of enzyme activity at 15°C to at 35°C in summer and winter frogs was represented in Table 2. At the temperature of 15°C, the Na^+, K^+-ATPase activity decreased to a level of 29% of that at 35°C in frogs in March, June and July, whereas enzyme activity decreased to a level of 31% in December and January. However, there was no statistical significant difference between the summer and winter frogs. The activity ratios at 5° C to at 35°C (data not shown here) also revealed that there was no significant difference between the frogs in summer and winter, indicating no seasonal variation in this parameter.

Sensitivity of Na^+, K^+-ATPase to ouabain

The sensitivity of Na^+, K^+-ATPase to ouabain, a specific Na^+, K^+-ATPase inhibitor, was examined

Table 2. Degree of cold resistance in the brain Na^+, K^+-ATPase from hibernating and non-hibernating frogs.

Condition (Month)	n	Na^+, K^+-ATPase activity 15°C	35°C	Relative % 15°C/35°C
Hibernation (Dec. Jan.)	5	2.75±0.54	8.29±1.78	30.9±4.2
Non-Hibernation (Mar. Jun. Jul.)	3	3.95±0.63	13.89±2.48	28.7±0.7

Data were taken from Fig. 1. Na^+, K^+-ATPase activity is given as mean ± S.E. μmol Pi liberated from ATP/mg protein/hr at indicated temperature.

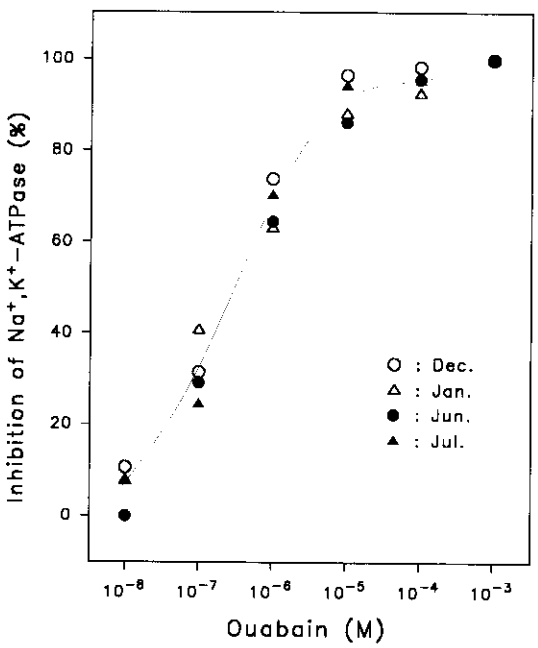

Fig. 3. Dose-response curve for ouabain inhibition of brain Na^+, K^+-ATPase from the frogs collected in summer and in winter. The difference between Na^+, K^+-ATPase activity in the presence and absence of 1 mM ouabain was considered as 100% activity. The concentrations of ouabain giving 50% inhibition (Ki) of brain Na^+, K^+-ATPase activity were 2.5~3.5 × 10^{-7}M in frogs. The solid line was drawed through the mean values at each concentration of ouabain.

in order to study whether there was any seasonal variation in the binding affinity. Na^+, K^+-ATPase activity was determined at different concentrations of ouabain, 10^{-8}~10^{-3} M at 35°C and the inhibition of Na^+, K^+-ATPase by ouabain was analyzed. In the concentration range of ouabain used, ouabain produced typical sigmoidal concentration-effect curves for inhibition of Na^+, K^+-ATPase from the brain of frogs in active state and in torpid state. Because the ouabain concentration-effect curves for Na^+, K^+-ATPase from frogs in October and March were similar to those in summer and winter, the data are excluded from Fig. 3 for simple illustration. There was no considerable shift in the concentration-effect curves upon the season when the frogs were collected. There were also no significant differences in concentrations of ouabain to inhibit Na^+, K^+-ATPase. The concentrations of ouabain that produced half maximal inhibition (Ki) of the Na^+, K^+-ATPase activity were in the range of 0.25~0.35 μM in the frog brains, regardless of the seasons. These results indicate that there was no appreciable seasonal change in the sensitivity of brain Na^+, K^+-ATPase to ouabain.

Discussion

The activity of brain Na^+, K^+-ATPase of the frog was maintained during hibernation whereas the activity of Mg^{2+}-ATPase decreased during hibernation. This result suggests that maintenance of Na^+, K^+-ATPase activity might be important during hibernation in frog. However, Na^+, K^+-ATPase activity was about twice as much as Mg^{2+}-ATPase activity at 25°C, which is similar to the ambient temperature in summer, whereas those activities were similar at 5°C, which is similar to the ambient water temperature during hibernation. The brain Na^+, K^+-ATPase activity markedly increased in October and March, when they enter into or awake from hibernation, respectively. The brain Mg^{2+}-ATPase activity increased only in March. These results suggest that Na^+, K^+-ATPase is essential for the induction and arousal process while Mg^{2+}-ATPase may be associated with the process of arousal from hibernation in frog

(Beckman and Stanton, 1976; Florant and Heller, 1977).

Charnock and Simonson (1978a) reported that the specific activities of Na^+, K^+-ATPase and Mg^{2+}-ATPase increased in ground squirrel brain after long-term hibernation. The Na^+, K^+-ATPase activity of hamster brain increased during hibernation. The enzyme activity of frog in torpid state was higher at the low reaction temperature than that in active state, indicating cold resistance during hibernation (Goldman and Willis, 1973; Goldman and Albers, 1975). The renal cortical Na^+, K^+-ATPase activity increased after exposure the hamsters to low temperature. Cold resistance in Na^+, K^+-ATPase was also observed (Willis and Li, 1969; Fang and Willis, 1974). By contrast to the results in hamsters and ground squirrels, the Na^+, K^+-ATPase activity from frog brains was maintained and not increased during hibernation, while Mg^{2+}-ATPase significantly decreased during hibernation (Table 1). Furthermore, it appeared that there was no significant difference in the value of cold resistance, the ratio of Na^+, K^+-ATPase activity at low temperature of 5°C or 15°C vs 35 °C between frogs in summer and in winter (Table 2). The absence of cold resistance in the hibernating frogs is also not consistent with the results from the brains of rat and hamster (Willis and Li, 1969).

The activity of renal cortical Na^+, K^+-ATPase of the ground squirrel was markedly reduced during hibernation, whereas Mg^{2+}-ATPase increased clearly. However, there was no evidence for cold resistance both in Na^+, K^+-ATPase and Mg^{2+}-ATPase during hibernation (Charnock and Simonson, 1978b). The activities of these enzymes from the heart of ground squirrel decreased during hibernation (Charnock *et al.*, 1980).

The reason for these differences in these parameters is not clear but it might reveal the tissue-specific or species-specific characteristics among frogs, hamsters and ground squirrels.

The change in thermal dependence of brain Na^+, K^+-ATPase was investigated as an index to a cold adaptation during hibernation. The temperature dependence of Na^+, K^+-ATPase in Arrhenius plot was non-linear in ground squirrel and hamster both in their active state and torpid state (Goldman and Albers, 1975; Charnock and Simonson, 1978a). The transition in temperature dependence occurred at 21°C in ground squirrel and at 15~18°C in hedgehog and hamster, which were not changed during hibernation (Charnock *et al.*, 1980; Charnock and Simonson, 1978a, 1978b). In consistent with those results, brain Na^+, K^+-ATPase of frog also displayed a non-linear temperature dependence (Fig. 1). If the thermal dependence of ATPase activity is assumed to be described a biphasic function with two intersecting line, the breaks appeared at 22~24°C in summer, whereas the breaks appeared at 18~19°C in winter, suggesting that adaptive change in its feature occur in frogs during hibernation. This feature may not be in agreement with those in ground squirrel and hamster. However, it is not clear that the frog Na^+, K^+-ATPase activities would be best described by a biphasic function or by a curvelinear function over the temperature range of 5~35°C, because of the limited number of measurement of Na^+, K^+-ATPase activity.

The thermal dependence of Mg^{2+}-ATPase from the renal cortex, brain and heart was linear or near-linear and unchanged by hibernation in ground squirrels. But, it was biphasic and the transition temperature changed to a low value in hibernating hamster (Charnock *et al.*, 1980; Charnock and Simonson, 1978a, 1978b). In frogs, the linear temperature dependence of brain Mg^{2+}-ATPase was unchanged during hibernation, suggesting that no seasonal alteration in thermal dependence occur.

Arrhenius plot for glutamate oxidase was biphasic while that for succinate oxidase was linear in frog skeletal muscle. Arrhenius plot for mitochondrial succinate oxidase was biphasic, and the transition temperature was changed from 23 °C in summer animals to 13°C in winter animals (Augee *et al.*, 1984). Linear Arrhenius kinetics has been reported for succinate oxidase from liver of toad, trout and catfish. The feature of adaptation to low temperature during hibernation seems quite diverse depending upon the enzyme, tissue and animal species.

It was thought that the Arrhenius discontinuity

of enzyme activity at certain temperature might be associated with a phase transition of the membrane lipids (Aloia and Raison, 1989; Charnock *et al.*, 1980). This concept is supported by the fact that transitions in thermal dependence of succinate oxidase and membrane structure occurred at similar temperature in sheep and rat (Raison and McMurchie, 1974). Augee *et al.* (1984) reported that the Arrhenius plots for the succinate oxidase activity and spin label motion exhibited a discontinuity at similar temperature in ground squirrel liver. Thus, it seems that enzyme activity may be associated with lipid phase transition of membrane. There were seasonal alterations in the composition of membrane phospholipids and fatty acids in hibernators, such as ground squirrel and hedgehog (Goldman, 1975; Blaker and Moscatelli, 1978; Aloia, 1980; Charnock *et al.*, 1980; Demediuk and Moscatelli, 1983). Such changes in membrane lipid composition might maintain the fluidity of membrane that, in turn, maintain enzyme activity during hibernation. Therefore, changes in transition temperature for brain Na^+, K^+-ATPase in hibernating animals may be associated with the changes in lipid composition of the membrane.

The Na^+/K^+ balance was maintained in the brain during hibernation while the Na^+, K^+-ATPase activity was reduced (Charnock and Simonson 1978a). The result suggests that the brain Na^+, K^+-ATPase in hibernating animal may have higher affinity for Na^+ or K^+. There was no considerable seasonal change in the inhibitory effect of ouabain on the Na^+, K^+-ATPase activity in frog brain (Fig. 3). Half maximal inhibition of Na^+, K^+-ATPase activity occurred at ouabain concentrations in the range $2.5\sim3.5 \times 10^{-7}$ M in frogs, exhibiting no appreciable change in ouabain sensitivity. The sensitivity of brain Na^+, K^+-ATPase to ouabain was significantly reduced after hibernation in ground squirrel. But, that of renal cortical Na^+, K^+-ATPase from the same species was not unchanged during hibernation (Charnock and Simonson, 1978a, 1978b). The values of Ki of ouabain for brain Na^+, K^+-ATPase were quite similar between frog and ground squirrel, despite of the difference in species.

Taken together, these results suggest that the characteristics of Na^+, K^+-ATPase is maintained during hibernation in frog.

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개구리 뇌에서 Na^+, K^+-ATPase 특성의 계절적 변화

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북방산개구리(*Rana dybowskii*) 뇌 조직의 Na^+, K^+-ATPase와 Mg^{2+}-ATPase 활성도와 특성의 계절에 따른 변화를 연구하였다. 북방산개구리 뇌에서 Na^+, K^+-ATPase 활성도는 활동기와 동면기에 비슷한 활성도를 나타내고, 동면에서 깨어나는 각성기와 동면으로 들어가는 시기에 높은 활성도를 나타내었다. 5~35°C의 온도에서 각 계절 전반에 걸쳐서 Na^+, K^+-ATPase는 non-linear Arrhenius kinetics를 보였다. Mg^{2+}-ATPase는 동면기에 분명하게 감소하였으며 각성기에는 뚜렷하게 증가하였다. Mg^{2+}-ATPase는 모든 계절에 걸쳐서 linear Arrhenius kinetics를 나타내었다. Na^+, K^+-ATPase의 15°C/35°C에서 활성도의 비에는 동면기와 활동기에 유의성 있는 차이가 나타나지 않았다. ouabain에 의한 Na^+, K^+-ATPase의 활성 저해 양상도 계절에 따른 변화가 없었다. 본 결과는 동면기에도 활동기와 마찬가지로 개구리 뇌의 Na^+, K^+-ATPase의 생화학적 특성과 활성도가 유지됨을 시사한다.