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Na^+,K^+-ATPase Activity in Young Chicks After Taste Stimulation

I. HÁJEK,*[1] E. SYKOVÁ,* G. SEDMAN† AND K. T. NG†

*Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Bulovka, Pav. 11, 180 85 Prague 8, Czech Republic
†School of Behavioral Science, La Trobe University, Bundoora, Victoria 3083, Australia

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HÁJEK, I., E. SYKOVÁ, G. SEDMAN AND K. T. NG. *Na^+,K^+-ATPase activity in young chicks after taste stimulation.* BRAIN RES BULL **33**(1) 87–91, 1994.—The activity of specific ouabain-sensitive Na^+,K^+-ATPase was studied in crude membrane fraction of the brain of 1- to 3-day-old chicks after the administration of a chemical aversant methylanthranilate (MeA), shown in previous behavioral studies to induce avoidance of pecking of an otherwise attractive stimulus. Enzyme activity was dramatically decreased (by 40–50%) in the time interval between 10 min–2 h after MeA administration onto the tongue of awake chicks. It was possible to localize these changes in Na^+,K^+-ATPase activity into forebrain structures contained within the dorsal ventricular ridge comprising the hyperstriatum accessorium (HA), hyperstriatum ventrale (HV), hyperstriatum dorsale (HD), and parts of neostriatum (N). In contrast, Na^+,K^+-ATPase activity in the ectostriatum (E), the medial neostriatum (NM), and the paleostriatal complex were unaffected. Results from experiments involving preincubation of membrane fractions and with partial purification using detergents, suggest that some substances with inhibitory effects were produced under the effect of MeA and bound to membrane fractions in their respective areas. A similar decrease of Na^+,K^+-ATPase activity as after MeA administration in vivo was observed when inhibitory mediators (GABA, glycine) were added to membrane fractions in vitro. These findings may have implications for memory processing in chicks following aversive learning using MeA as the aversant.

Na^+,K^+-ATPase Chick brain Aversive stimulus Taste

ONE-DAY-OLD chicks are currently used in a single trial passive avoidance learning task, introduced by Cherkin (4). In this type of experiment, animals are pretrained to peck at a colored glass bead. On a subsequent single training trial, the bead is coated with a chemical taste aversant, methylanthranilate (MeA). Chicks pecking at the bead on the training trial show typical disgust reactions, including the emission of distress vocalization, wiping of the beak on the floor of the cage, and shaking of the head (9). In retention trials that follow, the chicks typically refuse to peck a similar but nonaversive bead, and this refusal is regarded as representing memory for the association of an aversive taste with bead color (9).

It has been demonstrated (6) that a short-time memory stage, lasting for about 10 min following learning, could be abolished by 1, 2, or 154 mM KCl, administered within 2-$^1/_2$ min after learning. It has been suggested (6,7) that hyperpolarization consequent on K^+ conductance changes (15) may be involved in this early stage of memory processing. Recently, it has been found that the direct application of MeA onto the tongue of the chick can cause a long-term increase in neuronal activity (35), manifested as an increase in extracellular potassium concentration $[K^+]_e$ occurring in the forebrain areas that have been shown to be metabolically active during memory formation (18,31,35). Such elevations in potassium concentration can arise from both activated neurones and primary afferent fibres (33,34).

Long-term ionic changes, presumably accompanied by extracellular space shrinkage, may produce long-lasting alterations of neuronal excitability and transmitter release (33,34). For a clearance of K^+ accumulated in extracellular space, several mechanisms are of importance, namely the Na/K pump in neurones and glial cells, K^+ uptake in glial cells, and glial spatial buffering (23,34,38). In addition to the short-term memory stage referred to above, an intermediate stage of memory formation was isolated that could be abolished by inhibitors of the electrogenic Na/K pump, such as ouabain and ethacrynic acid (9,27), when these are applied within 5 min after learning. These drugs had no effect on memory when given after 5 min postlearning. It was proposed (22,27) that the formation of this stage of memory was completed by 10 min following learning and involved a second phase of neuronal hyperpolarization, resulting from the action of the sodium pump (15) shortly after learning.

It was of interest, therefore, to investigate possible changes in the activity of specific, ouabain-sensitive membrane Na^+,K^+-ATPase, the enzyme involved not only in maintenance of the nerve cell potential and ionic gradients across the membrane but also in the release of neurotransmitters from nerve terminals (37),

[1] To whom requests for reprints should be addressed.

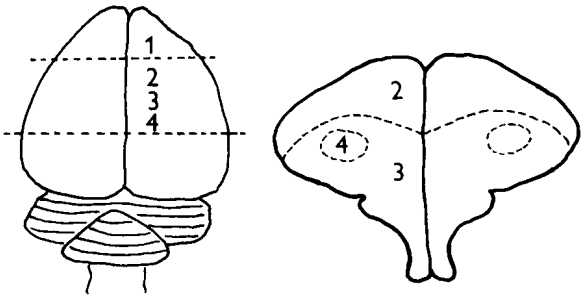

FIG. 1. The scheme of separation of individual forebrain structures. On the left: sectioning of the whole brain, on the right: cross-section of the middle part.

when the chick is stimulated with the aversant used in the learning task. This is an extension of our previous work on ionic changes (35), following MeA stimulation, and glucose utilization (31), following learning.

METHODS

The experiments were performed on 1- to 3-day-old White Leghorn chicks (n = 76), weighing between 35–43 g. Chicks were given 50 μl of either methylanthranilate (MeA) or water onto the tongue. In some chicks (n = 6) MeA was given following pentobarbital anesthesia (50–70 mg/kg), 15 min after IP injection of pentobarbital.

Chicks were killed 5, 10, 30, 60, and 120 min after MeA administration by decapitation and their whole forebrain quickly dissected. The whole isolation procedure from decapitation to immersion of tissue into precooled medium took less than 20 s. Decapitation and subsequent removal of brain was superior to other techniques, as we found on preliminary experiments, that freezing of brains in chicks caused a marked reduction in Na^+,K^+-ATPase activity (by 70%). This finding contrasts with the situation in mammals, where freezing increased enzyme activity (19). In experiments where brain structures were studied separately, forebrains were quickly dissected on a metal plate cooled with crushed ice. Four brain areas (see scheme in Fig. 1) were cut out using a razor blade. Tissues were placed into cool 50 mM Tris-HCl buffer (pH 7.2) and homogenized with a Teflon-glass homogenizer. A crude membrane fraction was then isolated as described previously (11) and used for the estimation of Na^+,K^+-ATPase activity using ouabain (1 mM) as a specific inhibitor. ATPase activity was assayed in 1 ml of the incubation mixture that contained (in mM): NaCl 100, KCl 3, $MgCl_2$ 5, Tris-HCl 100 (pH 7.2). Fifty microliters of membrane suspension containing 50–100 μg of protein as determined by the method of Lowry (21) was added to the incubation mixture. Samples were preincubated for 5 min and the reaction started by the addition of ATP (vanadium free) buffered with Tris, final concentration 2.5 mM. All incubations were carried out for 10 min at 37°C, and the reaction was stopped by the addition of 300 μl of 20% trichloroacetic acid. The resulting inorganic phosphate was determined as previously described (36).

Partial purification of Na^+,K^+-ATPase in membranes was performed according to Jørgensen (17), with the modification of Specht and Sweadner (32), using sodium dodecylsulphate (SDS). Fresh membrane preparations were treated with SDS (0.25 mg/mg protein) for 30 min at 20°C and recentrifuged. This method of membrane preparation is referred to as SDS treated membranes. In a parallel series of experiments, membranes were treated with Triton X-100 (0.06%).

The crude membrane fractions prepared by the above procedure contain bound substances affecting ATPase activity; for example, opioid peptides (11,12). Potential modulators of enzyme activity from membrane preparations such as these were liberated by preincubation in the suspension medium (50 mM Tris-HCl, pH 7.2) for 45 min at 37°C and recentrifugation. This variant of the membrane preparation is referred to as preincubated membranes.

The activity of Na^+,K^+-ATPase was also studied under conditions where the inhibitory neurotransmitters, gamma-amino butyric acid (GABA) and glycine, or the excitatory neurotransmitter glutamic acid, were added directly into the incubation medium (all at concentration 1 mM). In one series of experiments, membrane fraction was preloaded with GABA in order to achieve saturation of GABAergic receptors with the mediator. This was done by incubating the aliquot of membrane suspension in 1 mM GABA for 30 min on ice; the unbound ligand was then separated by centrifugation.

Drugs and all chemicals of analytical grade were obtained from Sigma Chemical Company (St. Louis, MO).

RESULTS

The Effect of Taste Aversant on Na^+,K^+-ATPase Activity in the Chick Forebrain

The effect of oral administration of MeA (taste aversant) on ATPase activity was studied over a range of times from 5–120 min after MeA administration. Control animals received the same amount of tap water. The results are shown in Fig. 2. No change in ATPase activity was observed at the 5-min time interval. However, by 10 min after MeA stimulation, Na^+,K^+-ATPase in the whole forebrain dropped by 40–50%. This decrease lasted until at least 2 h after stimulation. During this period, affected chicks showed no marked behavioral changes, besides the first minutes after MeA application, where aversive signs such as beak wiping and head shaking was seen.

When MeA was introduced onto the tongue of anaesthesized chicks, no change in membrane ATPase activity was observed (Fig. 2, 30 min). Anaesthesia apparently blocked the transmission of taste signals from the beak into the relevant brain structures.

Localization of Na^+,K^+-ATPase Changes in the Brain

Four forebrain parts were isolated as indicated in Fig. 1. The chick brain atlas of Youngren and Phillips (39) was used for the identification of brain areas. The forebrain was first cut transver-

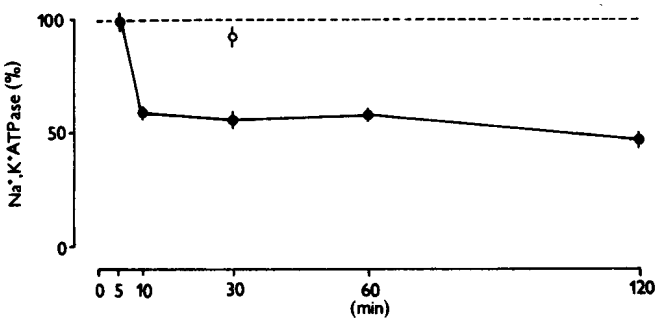

FIG. 2. Time course of Na^+,K^+-ATPase changes in ther chick forebrain after MeA administration in the beak (full circles). The enzyme activity of chick that received water in the same time intervals represent 100%. Open circle—chicks that received MeA under anaesthesia. Each point represents the mean of six to eight experiments ± SE.

TABLE 1

CHANGES OF MEMBRANE Na^+, K^+-ATPASE ACTIVITY IN INDIVIDUAL BRAIN STRUCTURES IN CONTROL CHICKS AND IN THOSE THAT RECEIVED MeA 30 MIN BEFORE SACRIFICE

Structure	Control	MeA
1	9.36 ± 1.19	8.88 ± 1.23
2	11.84 ± 1.74	6.79 ± 1.07*
3	6.50 ± 0.72	5.03 ± 0.60
4	23.71 ± 1.50	21.65 ± 0.72

μmol P_i/mg protein/h; $n = 8$.
Significance of differences against control group (two-tailed t-test): $^*p < 0.02$.

sally into three, approximately equal portions with a razor blade. The rostral part, denoted structure 1, corresponded to the bulbus olfactorius; the middle part was further divided into three parts [2, 3, 4], and the most caudal part of the forebrain section was not used for ATPase studies. Structure denoted 2 contained the hyperstriatum accessorium (HA), the hyperstriatum dorsale (HD), the hyperstriatum ventrale (HV) and the neostriatum (N); part 4 contained the ectostriatum (E), and part 3 contained the remaining basal structures of the middle part of the forebrain, largely the medial neostriatum (see 35), and the paleostriatal complex, comprising the globus parolfactorius, the paleostriatum augmentatum, and the paleostriatum primitivum.

Table 1 shows the ATPase activity in controls and in chicks that received MeA 30 min before the sacrifice. In both control and experimental animals, the highest activity was found in structure 4 (ectostriatum) where the activity exceeded that in other portions of the forebrain by 2 to 4 times. This part of chick brain is the most metabolically active one as previously shown by [^{14}C]-2-deoxyglucose accumulation (35). The application of MeA caused the most marked and significant decrease of Na^+,K^+-ATPase activity in structures comprising the HA, HD, HV, and N. These structures receive afferentation from the beak and tongue (35). Only slight and nonsignificant decrease of enzyme activity was found in the other areas sampled.

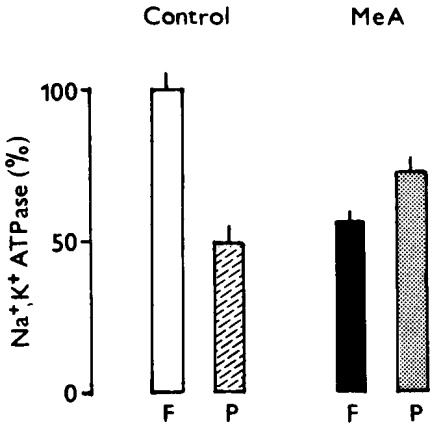

FIG. 3. The effect of preincubation (45 min, 37°C) on Na^+,K^+-ATPase activity in membrane samples isolated from forebrain of control chicks and from those after MeA treatment (30 min). F—fresh samples, P—preincubated samples. Values expressed as percentage of fresh control samples ($n = 6$ in all cases). Note the activity enhancement following preincubation in MeA treated chicks.

TABLE 2

CHANGES OF MEMBRANE Na^+, K^+-ATPASE ACTIVITY IN CONTROL CHICKS AND AFTER MeA ADMINISTRATION, THE EFFECT OF SODIUM DODECYLSULPHATE OR TRITON X-100 PURIFICATION

Fresh	Detergent Treated	Soluble Fraction
	Controls	
9.47 ± 0.71	+ SDS 22.60 ± 1.04	6.94 ± 0.52
	+ Triton 19.00 ± 2.07	5.40 ± 2.07
	Methylanthranilate	
6.01 ± 0.70*	+ SDS 24.46 ± 0.78	4.59 ± 0.44*
	+ Triton 14.27 ± 1.00*	4.62 ± 0.85

μmol P_i/mg protein/h; $n = 7$.
Significance of differences against respective controls (two-tailed t-test): $^*p < 0.005$.

The Mechanism of Na^+,K^+-ATPase Decrease

As indicated in Fig. 3, preincubation of membranes from the brain of control chicks resulted in depression of enzyme activity by about 50 ± 5%. In contrast, when membranes from MeA-treated chicks, which have lower initial ATPase activity, were similarly preincubated, a significant increase (by 15 ± 4%) was observed. On the basis of these results, fresh membrane fractions were subjected to purification using sodium dodecylsulphate or Triton X-100. The changes in specific activity in control and MeA-treated chicks (30 min) under those conditions are shown in Table 2. It can be seen that freshly prepared membranes from chicks after MeA treatment were less active than those from controls, but this difference disappeared after purification with detergents. Lower Na^+,K^+-ATPase activity of MeA treated chicks, however, could still be found in that part of ATPase, which was solubilized, presumably because some inhibitory substances were extracted during the purification procedure. Treatment with either detergent solubilized about 40–50% of the protein from the membrane fraction into the supernatant. The solubilized protein had lower specific ATPase activity and participated on the total activity by only 15–20% in individual preparations. The decreased specific enzyme activity in the supernatant suggest the possibility, that proteins without Na^+,K^+-ATPase activity were also liberated by detergent treatment.

Na^+,K^+-ATPase activity following MeA administration was assessed in the presence of the inhibitory transmitters GABA and glycine, and the excitatory transmitter, glutamic acid, added to the membrane fractions in vitro. As can be seen from the Table 3, both GABA and glycine decreased significantly the enzyme activity by more than 50%, whereas glutamic acid at the same

TABLE 3

Na^+, K^+-ATPASE ACTIVITY IN CONTROL FRESH SAMPLES IN COMPARISON WITH THAT AFTER ADDITION OF 1 mM GABA, GLYCINE, OR GLUTAMATE INTO THE INCUBATION MIXTURE

Control	GABA	Glycine	Glutamate
7.50 ± 0.47	3.47 ± 0.87*	4.25 ± 0.68*	6.52 ± 0.58

μmol P_i/mg protein/h; $n = 11$ in all groups.
Significance of differences against control group (two-tailed t-test): $^*p < 0.001$.

concentration (1 mM) had no significant effect. Beside these experiments, in a second series of experiments, fresh membranes were preloaded with GABA in order to saturate GABAergic receptors with the specific ligand. After preincubation with GABA and separation of the unbound ligand, ATPase activity was compared with that in fresh membrane aliquots. It was found that even here, GABA was able to depress Na^+,K^+-ATPase significantly ($p < 0.001$), and to the same extent as when it was present in high concentrations during the whole incubation period (from 7.50 ± 0.47 μmol P_i/mg protein/h ($n = 11$) in controls to 4.00 ± 0.75 ($n = 4$) in GABA-preloaded samples).

DISCUSSION

Our results show that after the application of chemical taste aversant, MeA, a long-lasting depression in the activity of Na^+,K^+-ATPase takes place in several brain structures. This phenomenon occurred especially in brain areas that were shown in a previous study (35) to respond to MeA or electrical stimulation by an elevation of extracellular potassium; namely, the hyperstriatum, but also possibly the region of the neostriatum (35).

These changes in potassium accumulation have previously been described in nervous tissue and in receptor organs [for review see (33,34)]. Although the Na/K pump, both in neurones (20) and in glia (14), has been suggested to be the effective mechanism of K^+ clearance, several other mechanisms are known to participate in K^+ clearance, including glial spatial buffering and K^+ uptake [review (34,35,38)]. In our previous experiments, MeA was shown to evoke increases in $[K^+]_e$ that lasted for several minutes (40 min) before returning to the original resting $[K^+]_e$ baseline. It is possible that this long-term K^+ elevation caused by long-lasting neural activity persists because the Na/K pump is not yet fully efficient in the neonate chick. Glial cells in neonate chicks may be only partly developed and, therefore, they do not efficiently control the extracellular K^+ homeostasis (16).

In anesthetized chicks, the decrease in ATPase activity was small (7.2 ± 5.4%) and not statistically significant. It is possible to assume that under anesthesia the transmission of impulses from the taste receptors into specific structures in the brain is markedly reduced. However, Syková et al. (35) found long-lasting elevation of $[K^+]_e$ in several brain structures after MeA administration in anesthetized chicks. In our present experiments the anesthesia was probably deeper than in the electrophysiological investigations. It is also possible that in awake chicks the application of MeA may cause even higher accumulation and elevation in potassium concentration, a possibility that cannot be investigated in day-old chicks with available procedures.

Our findings suggest that substances with inhibitory effects may be released after MeA administration and bind to specific receptors in the membrane fraction. Their effect disappeared after preincubation of the membrane fraction, due, perhaps, to the action of intrinsic degrading enzymes. From our experiments with partially purified membranes using detergents (SDS and Triton X-100), it would appear that some inhibitory substance(s) may be released in several brain structures due to massive stimulation of neurones by a strong taste stimulus. It appears that the inhibitor of Na^+,K^+-ATPase is bound to membrane fractions and may be liberated by detergent treatment or be metabolized in the membrane itself. While endogenous opioids bound to membrane receptors may increase Na/K pump activity (11,12), an inhibitor that is massively released in certain brain areas may bind to its respective receptors and depress ATPase activity. Some inhibitors of ATPase activity have been described by several authors; the list contains both endogenous substances with ouabain-like effects (13,27) and mediators, such as dopamine (2,3).

The production of an inhibitor in our experiments is apparently localized to certain brain structures, which receive strong afferent input after MeA application. These structures correspond to primary taste areas: the hyperstriatum accessorium (HA), the hyperstriatum ventrale (HV), the hyperstriatum dorsale (HD), and parts of the neostriatum (N). However, when the forebrain is homogenized as a whole, inhibitory substances may interact with other structures as well, bringing their ATPase activity down. Similar decreases as in vivo were obtained in vitro using the inhibitory mediators GABA and glycine. It has been demonstrated that avian brain contains a high density of GABAergic receptors, especially in optic areas and in the hyperstriatum ventrale (5).

The observed changes in Na^+,K^+-ATPase activity in brain areas involved in processing the taste aversive signals may play an important physiological role in modulating neuronal excitability in specific areas of the chick brain by affecting the level of $[K^+]_e$. The early changes in ionic environment and enzyme activity observed after adequate stimulation may be associated with later-occurring changes in brain metabolism, such as amino acid transport (27), protein synthesis (30), and, finally, even morphological rearrangements in areas active during memory formation, including the enhanced formation of dendritic spines (24).

It is premature to speculate on the precise implications of the present findings for the role of the sodium pump in the early stages of memory processing (22). The fact that sodium pump inhibitors can induce amnesia (22,25,26) poses an interpretative problem for the depressed Na^+,K^+-ATPase activity reported here. However, it is noteworthy that this depression did not occur until 10 min after stimulation, while ouabain and ethacrynic acid had an amnesic effect only when administered by 5 min following learning (22). Loss of memory following application of these drugs occurred after 10 min postlearning (22). Furthermore, there is evidence to suggest that the uptake of at least some amino acids necessary for long-term memory-related protein synthesis is dependent on the sodium-potassium transport system and occurs before 10 min after training, with the amnesic effect of interference with this uptake apparent only after 50 min postlearning (26). It is possible that sodium pump activity within the first 5 min or so after learning is necessary for initiating later cellular processes associated with memory formation, with the subsequent depression of enzyme activity reported here playing a different role, perhaps one of memory maintenance.

Any attempt to relate the present findings to previous theorizing about cellular processes underlying memory formation (22,25) needs to take into account the following: a) the Na^+,K^+-ATPase activity monitored in the present experiments did not occur in the presence of normal learning-induced neuronal and glial responses; b) the amount of MeA used considerably exceeded that employed in the learning experience; and c) the possibility that responses to taste stimulation may be lateralized. Andrew (1) cites evidence to suggest that memory for the taste component of the aversive learning paradigm used with neonate chicks may be preferentially represented in one hemisphere. What appears to be unequivocal from the present findings is that a strong taste stimulation evoked by MeA administration can exert a considerable effect on the activity of Na^+,K^+-ATPase activity, linked directly with ion homeostasis and transmitter release in the chick CNS.

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