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Two different Na,K-ATPases in the optic nerve: Cells of origin and axonal transport

(sodium transport/retinal ganglion cell/myelinated axon/glia)

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ABSTRACT **Two molecular forms of Na,K-ATPase can be isolated from the central nervous system. The two forms can be distinguished by their sensitivities to cardiac glycosides and by the electrophoretic mobilities of their catalytic subunits, α and $\alpha(+)$. Because Na,K-ATPase is a membrane-bound enzyme, it would be predicted to move in the rapid phase of axonal transport, and this was used as a means to determine which form(s) is made by a defined neuron of the central nervous system. Retinal ganglion cells were labeled *in vivo* by intravitreal injection of [^{35}S]methionine; the Na,K-ATPase that was axonally transported down the optic nerve was purified, and the α and $\alpha(+)$ forms were separated by electrophoresis and detected by fluorography. The two forms were synthesized in the retina in approximately equal amounts. The $\alpha(+)$ form was the predominant form transported from the retinal ganglion cells to the lateral geniculate nucleus and superior colliculus. The oligodendrocytes and other sheath cells of the excised optic nerve, in contrast, synthesized only the α form when incubated *in vitro* with [^{35}S]methionine. The labeled Na,K-ATPase found at the nerve endings always included a small amount of the α form in addition to the $\alpha(+)$ form. The proportions of the two forms did not change with time after transport, and the presence of labeled α was not affected by infusion of cycloheximide to inhibit intracranial protein synthesis. Hence, although $\alpha(+)$ is the predominant form, the evidence suggests that small amounts of the α form are also made and transported by retinal ganglion cells.**

The ouabain-inhibited Na,K-ATPase is the enzyme responsible for active transport of Na^+ and K^+ across the cell membrane (1), and is present in both neurons and glia. When isolated from the kidney, it is comprised of a catalytic subunit of M_r approximately 95,000 (α) and a smaller glycoprotein subunit (β) of unknown function. Preparations of Na,K-ATPase from the brain, however, have two biochemically distinct forms of the catalytic subunit, called α and $\alpha(+)$ (2). These differ in electrophoretic mobility in $NaDodSO_4$-containing gels (2–4) and in sensitivity to cardiac glycosides (2, 5, 6). Purified axolemmal membrane from rat brain white matter contains only $\alpha(+)$ (2, 3), while cultured glia contain only α (2), which suggests that $\alpha(+)$ is characteristic of neurons and α, of nonneuronal cells. Neurons of the sympathetic nervous system contain only the α form, however, and synaptosomes from the cerebral cortex contain both forms (2). Although the presence of the α form in synaptosomes may be due to contamination with membrane of glial origin, the evidence is consistent with the hypothesis that neurons can express either form or both. A selective labeling technique is needed to determine which forms are expressed in different neurons of the central nervous system.

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The Na,K-ATPase of a defined population of neurons was examined by taking advantage of the axonal transport of newly synthesized radioactively labeled protein to sites distant from the cell body (7–9). Although the retina is comprised of many different cell types, only the retinal ganglion cells send axons down the optic nerve and axonally transport protein to nerve endings in the lateral geniculate nucleus and superior colliculus (7). Proteins synthesized by the retina were labeled *in vivo* by intravitreal injection of radioactive precursor amino acid. By the criterion of coelectrophoresis with purified α or $\alpha(+)$, the $\alpha(+)$ form was found to be the predominant form transported to the nerve endings, but a small amount of the α form was also detected. No evidence for interconversion or local synthesis could be found, suggesting that the retinal ganglion cells synthesize and axonally transport both forms of the catalytic subunit of the Na,K-ATPase. The smaller β subunit was not studied, because it is not resolved on 5% acrylamide gels.

MATERIALS AND METHODS

Intravitreal Injection. CD rats (Charles River Breeding Laboratories) were anesthetized with pentobarbital sodium (25 mg/kg), supplemented with ether. Each animal received an intravitreal injection of an aqueous solution of 250 μCi of L-[^{35}S]methionine (1195 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) (7). In some experiments, intraocular radioactivity was diluted after 2 hr by intravitreal injection of unlabeled methionine (25 μl; 0.8 mM).

***In Vitro* Labeling of the Optic Nerve.** The orbit-chiasm segment of optic nerve was dissected, partially desheathed, and placed in a methionine-deficient cell culture medium (Leibowitz L15 lacking methionine, custom formulation from GIBCO, supplemented with bicarbonate and 2.5% rat serum) at 37°C for 30 min in a 5% CO_2/95% air atmosphere. The nerve was then transferred to a similar solution containing 250 μCi of [^{35}S]methionine (9.7 μM), incubated for 6 hr, rinsed twice with medium containing unlabeled methionine, and used for enzyme purification.

Purification of Na,K-ATPase. Animals were killed 24 hr after intravitreal labeling except as noted below. The retinae, orbit-chiasm segment of the optic nerve, and right and left lateral geniculate nucleus and superior colliculus were dissected on ice under a microscope. The lateral geniculate nucleus and superior colliculus from each side were combined in all experiments. Average tissue sample weights (mean ± SEM; mg) were 33 ± 1 for the lateral geniculate nucleus and superior colliculus, 22 ± 2 for the retina, and 5 ± 0.5 for the optic nerve, based on samples from 30 animals. Samples were homogenized in 1.5 ml of 0.315 M sucrose/1 mM EDTA/Tris, pH 7.2. Unlabeled cortex was homogenized in the same solution at 100 mg (wet weight)/ml, and 250-μl aliquots were added to the samples of retina and optic nerve as carrier. The homogenates were centrifuged at 3500 rpm in a Sorvall SS-34 rotor for 10 min, and the pellets were resus-

pended and centrifuged again. The supernatants were pooled and centrifuged at 2200 rpm for 10 min, and the cleared supernatant was centrifuged at 13,000 rpm for 1 hr to pellet a microsomal fraction containing myelin, axon fragments, endoplasmic reticulum, mitochondria, and synaptosomes. The pellet was resuspended in sucrose/EDTA and frozen at −70°C. Na,K-ATPase was purified from the crude microsomal pellet by selective detergent extraction using 0.25 mg of $NaDodSO_4$/mg of protein as described (10). The enzyme was centrifuged on gradients of 7–30% sucrose in a Beckman SW 50.1 rotor at 48,000 rpm for 90 min. The yields were ≈30 μg of microsomal protein and 2 μg of $NaDodSO_4$-extracted enzyme per mg of wet tissue, and the specific activities were 1.6–1.9 μmol/mg of protein per min. Higher specific activities were obtained in previous experiments, when myelin, mitochondria, and other denser particles were removed by prior centrifugation (10) but, because the axonally transported Na,K-ATPase is expected to be in the synaptosomal compartment, a fraction containing synaptosomes was used in this study to obtain a higher and more representative recovery of the labeled enzyme in the lateral geniculate nucleus and superior colliculus. Because this preparation also contains endoplasmic reticulum and axonal membranes, it is appropriate for analysis of newly synthesized retinal enzyme, enzyme in transit in association with endoplasmic reticulum, and enzyme newly inserted into axolemma. Protein concentrations were determined by the method of Lowry *et al.* (11), using bovine serum albumin for standardization.

Gel Electrophoresis and Fluorography. Electrophoresis was carried out at 40 mA in slab gels of 5% acrylamide/0.13% bis(acrylamide) using the Laemmli buffer system (12). The labeled enzyme samples were dissolved at room temperature in Laemmli sample buffer containing 1% phenylmethylsulfonyl fluoride and 5% 2-mercaptoethanol. Approximately 60 μg of protein was loaded per 6.4-mm-wide lane on 1.5-mm-thick gels. Gels were fixed, stained, photographed, impregnated with diphenyloxazole (Biomedical Technologies, Norwood, MA) (13), and dried between cellophane sheets. Fluorography was carried out with Kodak XAR film and required exposures from 3 days to 3 weeks. Figures generally show samples that were electrophoresed on the same gel, but much shorter exposure times were required for enzyme from the retina than for enzyme from the optic nerve or the lateral geniculate nucleus and superior colliculus.

Inhibition of Intracranial Protein Synthesis. Intracranial incorporation of [^{35}S]methionine was prevented in some experiments by infusion of 35 mM cycloheximide (Sigma) together with unlabeled 0.8 mM L-methionine prepared in pyrogen-free sterile saline. One hour after intravitreal injection of [^{35}S]methionine, 10 μl of cycloheximide/methionine solution was injected through a Hamilton syringe placed with a stereotaxic apparatus over the lateral geniculate nucleus and 4.2 mm deep. The cannula of an Alzet osmotic minipump (model 2002, Alza, Palo Alto, CA) was immediately inserted in the same position and fixed to the skull with dental cement. The pump infusion rate was 0.465 μl/hr. Location of the cannula tip was verified at sacrifice.

To measure the extent of inhibition of protein synthesis in the lateral geniculate nucleus and superior colliculus, 2 μCi of L-[4,5-^{3}H]leucine (59.8 Ci/mmol; New England Nuclear) was injected 1 mm from the midline at bregma (4.5 mm deep) either 3 or 23 hr after initiation of treatment with cycloheximide, and the animals were killed 1 hr later. The lateral geniculate nucleus and superior colliculus were fixed in 1 ml of 10% trichloroacetic acid at 4°C overnight, then rinsed, 5- to 10-mg samples were dissected from the superior surfaces, weighed, and dissolved in Nuclear Chicago Solubilizer (Amersham), and radioactivity was determined.

Inhibition of Axonal Transport. Axonal transport was inhibited in nine rats by intravitreal injection of either vinblastine sulfate (0.22 μmol in 25 μl) or colchicine (0.5 μmol in 25 μl) (Sigma) 24 hr prior to intravitreal injection of methionine; a second dose of colchicine or vinblastine (5 μl) was administered at the time of isotope injection or 30 min later.

RESULTS

Regional Distribution of the Two Forms of the Na,K-ATPase. The proportions of the two molecular forms of the Na,K-ATPase are different when isolated from different parts of the central nervous system. There are roughly equal amounts in the retina, but the proportion of α(+) is higher in the optic nerve, still higher in the lateral geniculate nucleus and superior colliculus, and roughly equal again in the visual cortex (Fig. 1). Although it is possible that there may be differences in the recovery of subpopulations of membrane from the different tissue sources, one would expect the recoveries to be comparable in retina, lateral geniculate nucleus and superior colliculus, and visual cortex, all of which contain neuronal cell bodies, synapses, and glia. The cellular or functional basis for the difference is not yet understood, but it may reflect either the distribution of cell types expressing one or the other form or the relative abundance of cell bodies, axons, or synapses.

Axonal Transport of the α and α(+) Forms. In initial studies of the axonal transport of the Na,K-ATPase from the retina, three animals received intravitreal injections of [^{35}S]methionine and were sacrificed 24 hr later. Label in the Na,K-ATPases of the retina was almost equally distributed between α and α(+), while label in the target areas (lateral geniculate nucleus and superior colliculus) was predominantly in α(+), with just a small proportion of α (Fig. 2). On the ipsilateral side of the brain, which receives approximately 5% of the fibers originating in the injected eye, there was almost no detectable label, which argues that the proteins found in the target areas on the contralateral side were conveyed by axonal transport.

The excised optic nerve contains no neuronal cell bodies, and adult mammalian axons are thought to be incapable of significant protein synthesis (14). The excised nerve, however, contains oligodendrocytes and other nonneuronal cells of the nerve sheath. To determine which form of the Na,K-ATPase is synthesized by the nonneuronal cells, the orbitchiasm segment of optic nerve was incubated with [^{35}S]-methionine *in vitro* for 6 hr, and the Na,K-ATPase was purified and analyzed by gel electrophoresis. Although the α(+) form is the predominant form in whole optic nerve (Fig. 1), only the α form was synthesized by nonneuronal cells (Fig. 3).

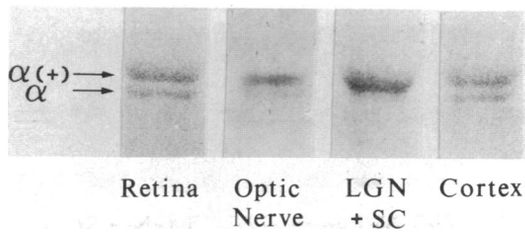

FIG. 1. Regional differences in the proportions of α and α(+) forms. Na,K-ATPase was purified from membrane preparations isolated from the retina, optic nerve, lateral geniculate nucleus/superior colliculus, and visual cortex of the rat; no unlabeled carrier was added. Aliquots of 30–50 μg of the purified enzyme were electrophoresed on a 5% acrylamide gel, using the Laemmli buffer system with $NaDodSO_4$, and were stained with Coomassie brilliant blue. The relative proportions of the two forms of the enzyme are different in these different regions of the visual system. The Na,K-ATPase also contains a smaller glycoprotein subunit (the β subunit), that migrates at the dye front in 5% acrylamide and was not examined in this study.

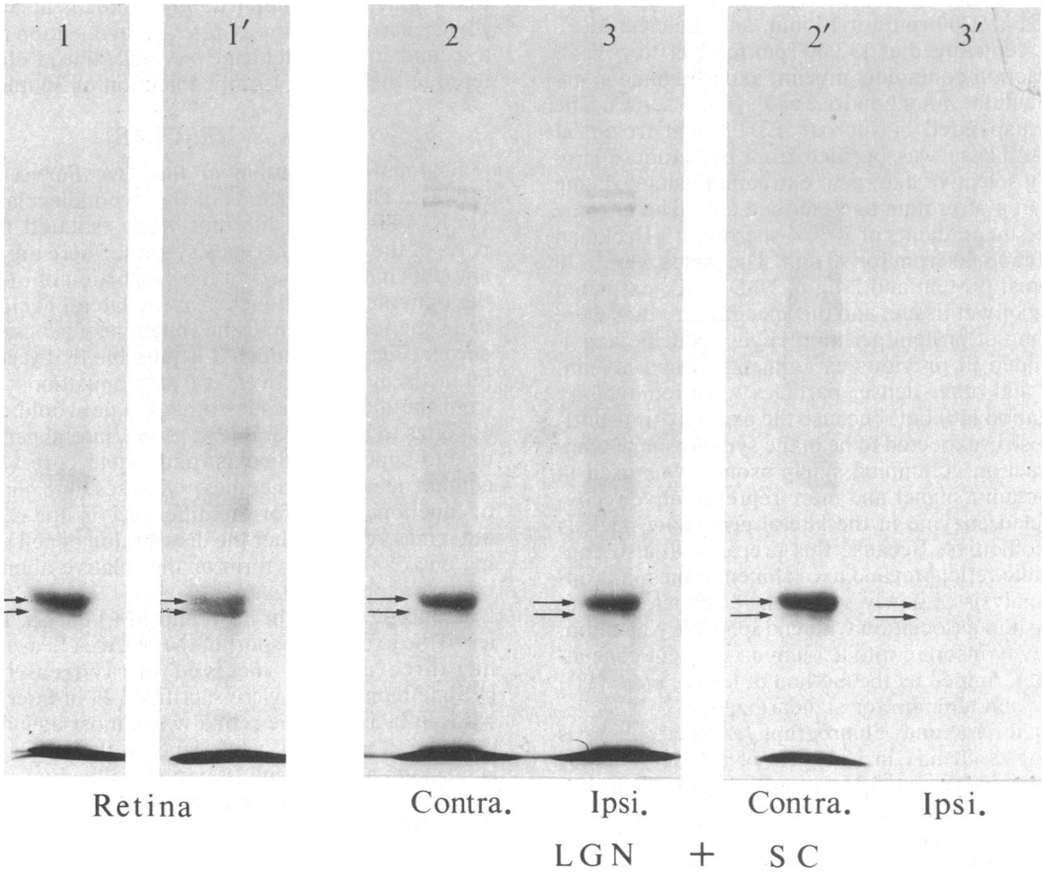

FIG. 2. Axonal transport of the Na,K-ATPase. At 24 hr after injection of [^{35}S]methionine in the right eye, the retina and contralateral (contra.) and ipsilateral (ipsi.) lateral geniculate nucleus and superior colliculus (LGN + SC) were removed and used for purification of the Na,K-ATPase. Lanes 1–3: Coomassie blue staining pattern of the partially purified enzyme electrophoresed on a 5% acrylamide gel. Several minor unrelated proteins copurify with the Na,K-ATPase, as reported earlier (11). Lanes 1′–3′: [^{35}S]Methionine fluorograph of the same gel. $\alpha(+)$ and α are labeled in approximately equal amounts in the retina, while only a small proportion of α is seen in the contralateral target areas and none in the ipsilateral target areas.

Origin of the α Form Found in the Lateral Geniculate Nucleus. The presence of labeled α in the contralateral target areas could be the result of phenomena other than axonal transport from retinal ganglion cells. To test for the interconversion of α and $\alpha(+)$ after synthesis and transport, the time course of appearance of the two forms in both the target areas and the optic nerve was examined after a 2-hr pulse of label in the eye. The distance to the lateral geniculate nucleus from the midretina is ≈20 mm. If a fast axonal transport rate of 240 mm/day is assumed (7, 9), 2 hr after synthesis will be required for the earliest appearance of labeled Na,K-ATPase in the lateral geniculate nucleus. Animals were sacrificed at times ranging from 4 to 24 hr in three complete experiments (data not shown). The $\alpha(+)/\alpha$ ratio in the retina and in the target areas remained constant, with detectable α present in the target areas at the earliest time points and still present after 24 hr. In the optic nerve, the label in α was barely detectable (at all time points) in two experiments, and not detected at all in a third, suggesting that a proportionally smaller fraction of it remains in the optic nerve after a pulse-labeled wave of enzyme passes through. Within the time resolution of the experiment, no evidence was obtained to suggest either that one form of the enzyme arrives sooner than the other or that one form is converted into the other at the nerve endings.

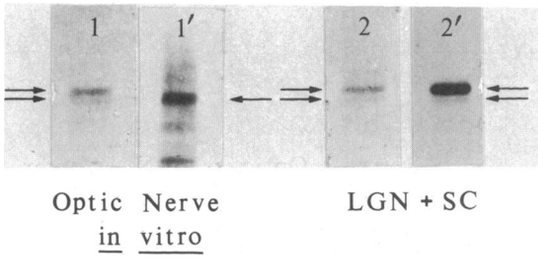

FIG. 3. *In vitro* labeling of the optic nerve. The optic nerve was excised and labeled with [^{35}S]methionine in tissue culture medium at 37°C, then mixed with unlabeled optic nerve pooled from 20 rats, and used for the purification of the Na,K-ATPase. Lanes 1 and 1′: 1, Coomassie blue staining pattern; 1′, fluorograph of the labeled Na,K-ATPase. Only the α form is labeled, which argues that the nonneuronal cells of the optic nerve sheath do not synthesize $\alpha(+)$. Lane 2, staining pattern of the α and $\alpha(+)$ Na,K-ATPase in the target areas (LGN + SC); lane 2′, fluorograph of transported α and $\alpha(+)$ forms after intravitreal labeling.

If a significant quantity of [^{35}S]methionine gained access to the periaxonal space (15–17), it could diffuse to the lateral geniculate nucleus and be incorporated into α subunits by glia. This hypothesis was tested by intravitreal injection of either colchicine or vinblastine to inhibit axonal transport within the optic nerve axons (18) but at the same time leave periaxonal diffusion of unincorporated precursor unimpaired. One would predict that the appearance of $\alpha(+)$ in the target areas would be blocked, but not the appearance of α, if label in α were due to local synthesis. Retinal labeling was ≈50% lower in animals treated with either colchicine or vinblastine, indicating a local effect on precursor uptake or on

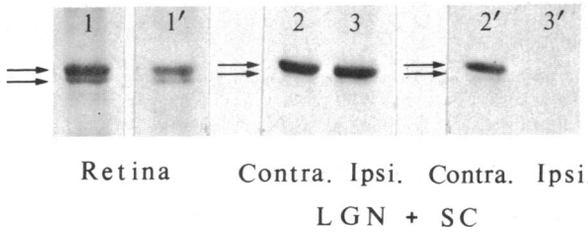

FIG. 4. Axonal transport of the α form during inhibition of local protein synthesis. The experimental protocol was as in Fig. 2, except that protein synthesis in the brain was inhibited by continuous infusion of cycloheximide. Lanes 1–3, Coomassie blue staining pattern of the enzyme purified from retina and the contralateral (contra.) and ipsilateral (ipsi.) lateral geniculate nucleus and superior colliculus (LGN + SC); lanes 1'–3', the corresponding fluorograph, showing that both α and $\alpha(+)$ are labeled and axonally transported, as in control experiments.

protein synthesis. This should not affect periaxonal diffusion of label or the synthesis of protein in the lateral geniculate nucleus, however. The result (not shown) was that the appearance of both $\alpha(+)$ and α in the target areas was inhibited equally when axonal transport was inhibited.

[^{35}S]Methionine from the breakdown of transported protein or from diffusion through the blood, however, may still be available in the target areas for local incorporation into protein. If this is the source of the labeled α subunit found in the target areas, it should be possible to block its appearance by inhibiting protein synthesis. Local inhibition was accomplished by continuous infusion of a mixture of cycloheximide and unlabeled methionine. In control experiments in six animals, incorporation of [^{3}H]leucine injected 4–5 mm from the site of cycloheximide infusion was only 6% of control in the treated region, both 3 hr and 23 hr after beginning infusion. Higher doses proved to be too toxic for the animals. In a total of five animals given intravitreal injections of [^{35}S]methionine, the appearance of both $\alpha(+)$ and α in the target areas was unaffected (Fig. 4). The data argue that both forms of the Na,K-ATPase were transported to the lateral geniculate nucleus and superior colliculus.

DISCUSSION

Fast axonal transport (>240 mm/day) is thought to contribute to membrane renewal at the nerve ending. When analyzed by subcellular fractionation, the rapidly transported proteins are associated with particles with the properties of plasma membrane (19), which are presumably membrane vesicles derived from the Golgi apparatus and destined for insertion at the nerve endings or the axolemma. Since the Na,K-ATPase is known to be an integral membrane protein concentrated in nerve endings, the observation that it is among the rapidly transported proteins fulfills an expectation (8, 9). Protein that electrophoreses in $NaDodSO_4$-containing gels at approximately the right molecular weight to be the catalytic subunit of the Na,K-ATPase can be seen in photographs of one-dimensional gels of axonally transported proteins published by a number of laboratories; its usual absence from two-dimensional gels is a characteristic of the solubility properties of the enzyme in Ampholines. Baitinger and Willard (9) independently found that major protein bands transported to the rabbit superior colliculus coelectrophoresed with Na,K-ATPase standards exhibiting specific K^+-sensitive phosphorylation. In the present study, two different forms of the catalytic subunit of the Na,K-ATPase were identified by the following criteria. (*i*) They copurify with the Na,K-ATPases of the brain. (*ii*) They comigrate on one-dimensional gels with the two forms that have been characterized by their specific Na^+-stimulated and K^+-discharged phosphorylation with [γ-^{32}P]ATP (2) and more recently have been shown to be immunologically cross-reactive (ref. 4; unpublished results). (*iii*) They both display a characteristic insolubility after isoelectric focusing in Ampholines. We cannot rule out the possibility that the putative α and $\alpha(+)$ bands may contain other unrelated but labeled proteins; so far the preparation has been refractory to conventional two-dimensional gel and immunoprecipitation techniques.

The evidence presented here supports the conclusion that retinal ganglion cells transport primarily the $\alpha(+)$ form of the catalytic subunit of the Na,K-ATPase while the nonneuronal cells of the optic nerve sheath express only the α form. This is consistent with the earlier observation that only $\alpha(+)$ is detected in axolemma isolated from myelinated central nervous system axons while cultured central nervous system nonneuronal cells express the α form (2). In the rat, the two forms of the Na,K-ATPase have different affinities for cardiac glycosides (2) and are likely to have other functional differences that are physiologically relevant. It is of particular interest to determine whether any population of central nervous system neurons expresses the α form; hence the small amount of α apparently transported down the optic nerve was examined in more detail. The possibility that the use of the same isolation method for different tissues (optic nerve compared with lateral geniculate nucleus and superior colliculus) might result in differences in the recovery of subpopulations of membrane does not compromise the basic conclusion of this study that both α and $\alpha(+)$ are axonally transported.

Because it is not yet known whether the two forms of the Na,K-ATPase are derived from separate genes or whether one arises from post-translational modification of the other, we examined the time course of appearance of the two forms at the target areas for evidence of interconversion. Other proteins are thought to be modified during axonal transport: there is proteolysis of neurophysin, accompanying peptide processing (20); axonal glycosylation of proteins in a giant neuron of *Aplysia* (21); and proteolysis or regional sorting of cytoskeletal components of slowly transported proteins (22, 23). Here the proportion of α, however, neither increased nor decreased significantly with time after its appearance in the optic nerve and the target areas. If interconversion of the two forms of the Na,K-ATPase occurred in the ganglion cell body prior to their axonal transport, it would not have been detected.

If [^{35}S]methionine gained access to the lateral geniculate nucleus by diffusion through the periaxonal space, it would be expected to make equal contributions to protein labeling on the ipsilateral and contralateral sides of the brain, yet the α form of the Na,K-ATPase was seen only on the contralateral side, suggesting that its presence is the direct or indirect result of axonal transport. In addition, when axonal transport was inhibited with colchicine or vinblastine injected into the vitreal chamber, the appearance of both α and $\alpha(+)$ in the target areas was inhibited equally well. The possibility that the α form in the transported proteins is due to local synthesis by glia or other neurons, however, is a critical hypothesis to be tested. Transfer of axonally transported label to myelin-associated proteins has been shown to occur in garfish olfactory nerve (24). Transfer of labeled proteins in the opposite direction, from glia to axons, has also been seen in the squid giant axon (25). Within the mammalian visual system, the phenomenon of "transneuronal transfer" of radioactive substances from optic nerve endings to neurons projecting to the visual cortex (26) may be due at least in part to the capture and reutilization of substances released from the neurons, such as transmitters, nerve terminal proteins shed from the surface, and the breakdown products of normal turnover (27). Transneuronal transfer in at least one sys-

tem (the chicken ciliary ganglion) is reduced by inhibition of local protein synthesis (28).

Studies of transneuronal transfer in the visual system indicate that material is transferred not only from optic nerve endings to synaptically linked cortical afferents, but also, albeit to a lesser degree, to nonsynaptically linked cells in the vincinity of the nerve endings and preterminal axons (29–32). It is such a "transcellular" transfer radioactivity that might account for the small amount of the α form of the Na,K-ATPase found to be labeled in the target areas. In the present experiments, we were concerned that even a small amount of local synthesis of α would be detected because the sensitivity of the assay has been increased by purifying the Na,K-ATPase and eliminating much of the background of unrelated proteins.

Cycloheximide has been used previously to inhibit intracranial protein synthesis by administering a single intracisternal injection, supplemented at 3-hr intervals with subcutaneous injections (29). Protein synthesis was inhibited by only 50–70%, however. In the present experiments, significantly better inhibition was obtained by infusing cycloheximide continuously through an osmotic minipump implanted under the skin and connected to a cannula over the lateral geniculate nucleus. A 94% inhibition of local protein synthesis was achieved in this way, with no significant reduction in the appearance of the labeled form of the Na,K-ATPase in the target areas. Within the technical limitations of the experiment, then, the conclusion is that the labeled α form is derived from the retinal ganglion cells.

Localization of the two forms of the Na,K-ATPase in the lateral geniculate nucleus and superior colliculus will require the use of specific antibodies in immunohistological experiments. Assuming that antibodies could be produced that would react with only α or $\alpha(+)$ and only on the inside surface of the cell, one could determine whether the $\alpha(+)$ form is restricted to myelinated regions of axon and whether the α form is present in the ganglion cell terminals or only in surrounding glia or other cells. Until such antibodies are available, however, the weight of the evidence suggests that a small amount of the α form is a normal constituent of the ganglion cell endings.

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