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12. Molecular weights as follows: glucagon, 3,500; cytochrome c, 12,400; ribonuclease, 13,700; chromotrypsinogen, 25,000; ovalbumin, 45,000; bovine serum albumin, 67,000.
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Active Transport of Potassium and Chloride in an Identifiable Molluscan Neuron

Abstract. *Direct measurements of intracellular K^+ and Cl^- activities before and after blockage of cellular metabolic processes indicate that K^+ is actively transported inwardly and Cl^- is actively transported outwardly from the giant cell of the abdominal ganglion of* Aplysia. *The rewarming of cells that have been cooled to 1° ± 1°C causes K^+ to be taken up and Cl^- to be extruded against electrochemical gradients.*

The active transport of K^+ and Cl^- has been convincingly demonstrated in only one neural structure, the squid axon (*1*, *2*). In other nervous tissue, the bulk of the evidence for active transport of either ion has been indirect (*3*) or incomplete (*4*). We have directly measured intracellular K^+ activity (a^i_K), intracellular Cl^- activity (a^i_{Cl}), and membrane resting potential (E_M) continuously, and often simultaneously, in the giant neuron R2 (*5*) of the *Aplysia* abdominal ganglion. Control measurements were made over as many as 4 to 6 hours; measurements were also made after exposure to either cooling, ouabain, cyanide, or 2,4-dinitrophenol. From the results, we conclude that (i) K^+ is actively transported into this neuron, (ii) Cl^- is actively transported out of this neuron, and (iii) the transport systems for K^+ and Cl^- are different.

We recorded a^i_K and a^i_{Cl} using liquid ion-exchanger microelectrodes (*6*), calibrated prior to and after the measurement of intracellular activities with KCl solutions varying in activity from $6.0 \times 10^{-1}M$ to $9 \times 10^{-3}M$. We measured membrane potentials using glass micropipettes filled with 3*M* potassium chloride (KCl), 2.0*M* sodium citrate ($Na_3C_6H_5O_7$), or 0.6*M* sodium sulfate (Na_2SO_4) with no difference in results. We found it easier to impale the cell with these electrodes after we removed the connective tissue capsule and then washed the preparation for 30 to 60 minutes with artificial seawater (*7*). After impalement, continuous simultaneous measurements of a^i_K, a^i_{Cl}, and E_M were made in 23 cells until the values became stable (40 to 80 minutes). At this time, the equilibrium potentials for Cl^- and K^+, (E_{Cl}, E_K), and E_M were -55 ± 1.0, -80 ± 0.7, and -50 ± 1.0 mv (mean ± standard error of the mean), respectively. Both E_K and E_{Cl} differ significantly from E_M ($P < .01$). When the control measurements were extended for another 300 minutes (seven cells), E_{Cl}, E_K, and E_M showed little change, having values of -56 ± 2.0, -81 ± 0.9, and -50 ± 2.3 mv, respectively. The giant cells were normally quiescent, maintaining an E_M of about −50 mv and an input membrane resistance (R_T) of about 1.0 megohm and, after stimulation, action potential overshoots of +30 to +50 mv. It seemed reasonable then to assume that the cells were in a steady state within 40 to 80 minutes after impalement (*8*). Since E_K, E_{Cl}, and E_M differ

Table 1. Effects of cooling and treatment with ouabain on K^+ and Cl^- activities in the *Aplysia* giant cell. Values are the mean ± the standard error of the mean; the numbers in parentheses refer to the number of cells; a^i_K and a^i_{Cl} were measured concurrently in five cooled cells and six ouabain-treated cells. All control data refer to steady-state values obtained just before the beginning of treatment. The 70-minute post-treatment time was chosen as the time when $E_{Cl} = E_M$ after cooling to 1°C, the ouabain data being given at the same time for purposes of comparison.

Cell	a^i_K (m*M*)	E_K (mv)	a^i_{Cl} (m*M*)	E_{Cl} (mv)	E_M (mv)
Control	187 ± 7.0 (7)	−83 ± 1.0 (7)	34 ± 1.8 (11)	−59 ± 1.2 (11)	−49 ± 1.6 (11)
70 minutes after cooling	149 ± 4.5 (7)	−78 ± 0.6 (7)	44 ± 2.6 (11)	−48 ± 1.2 (11)	−48 ± 1.4 (11)
Control	165 ± 6.0 (16)	−79 ± 0.9 (16)	36 ± 1.7 (6)	−56 ± 1.7 (6)	−46 ± 1.8 (16)
70 minutes after exposure to ouabain	127 ± 5.4 (16)	−73 ± 1.0 (16)	40 ± 1.1 (6)	−53 ± 1.3 (6)	−37 ± 1.5 (16)

from each other in the steady state, it is clear that neither K^+ nor Cl^- is distributed passively across the cell membrane.

In order to test further if K^+ and Cl^- were actively transported, cell R2 was cooled from room temperature (20° to 22°C) to a temperature (1° to 3°C) that was likely to inhibit metabolic processes (*9*). The liquid ion-exchanger microelectrodes were calibrated at the different temperatures and responded as predicted on the basis of the temperature coefficient of the empirical equation which describes their behavior (*6*). Upon cooling, E_M usually depolarized. Later a steady voltage was attained which could be either more negative or less negative than that of the control; E_M remained at the new voltage for about 50 to 180 minutes and then depolarized further. Occasionally, exposure to low temperatures produced an initial hyperpolarization.

Cooling to 1°C always led to a decrease in a^i_K (Table 1) which could be fitted with a single exponential (Fig. 1A) with a rate constant of $4.7 \pm 0.8 \times 10^{-5}$ sec^{-1} (mean ± standard error of the mean, seven cells). When the cooling was prolonged sufficiently, E_K eventually equaled E_M which was depolarized to −10 mv. Rewarming such a cooled cell to 20° to 22°C caused K^+ to reaccumulate, and E_K once again became more negative than E_M (Fig. 1A).

Cooling to 1°C also led to a rapid influx of Cl^-. In 11 cells, E_{Cl} equaled E_M within 70 minutes of such cooling (Table 1). When a cooled cell was rewarmed to room temperature, a^i_{Cl} decreased and E_{Cl} once again became more negative than E_M (Fig. 1B). An intriguing observation was that in three cells, cooling to 10°C had no effect on a^i_K and a^i_{Cl} although E_M became less negative by 10 to 15 mv.

Effects on a^i_K similar to those produced by cooling to 1°C followed exposure to $2 \times 10^{-4} M$ ouabain (16 cells, Table 1), 10 m*M* cyanide (four cells), and 0.2 m*M* 2,4-dinitrophenol (three cells). The rate constants for K^+ loss (in units of 10^{-5} sec^{-1}) were 6.0 ± 0.6, 4.2 ± 0.3, and 4.3 ± 0.5, respectively, which do not differ significantly from each other nor do they differ significantly from the rate constant during cooling. The effects of exposure to ouabain were not reversible, but we have not determined as yet if the effects of exposure to dinitrophenol or cyanide can be reversed.

Ouabain caused only a small increase in a^i_{Cl} (Table 1), and cyanide had no effect whatsoever even though E_M depolarized an average of 12 mv (four cells). Preliminary experiments indicate that, after exposure to dinitrophenol, E_{Cl} approaches E_M, and this effect appears to be reversible. In squid axon where E_{Cl} was less negative than E_M, only dinitrophenol clearly decreased Cl^- influx, ouabain being ineffective, and the effects of cyanide were uninterpretable (*2*). Thus, there is qualitative agreement between our findings and those of Keynes.

Increases in membrane permeability to K^+ and Cl^- might account in part for the net fluxes of K^+ and Cl^- that occurred in our experiments. We have not measured unidirectional fluxes, but we have indirect evidence that permeability increases to K^+ and Cl^- probably do not occur. Potassium ion left the cell with approximately the same rate constant after exposure to cooling, ouabain, cyanide, and dinitrophenol. Yet ouabain had no effect on input resistance or the transport number for K^+ across the cell membrane as determined by the method described by Brown *et al.* (*7*). Hence K^+ conductance, the product of membrane conductance and the transport number, was unchanged. As for changes in Cl^- permeability, we calculated the Cl^- conductance in six cooled cells using the same method and found that, rather than increasing, it decreased by 25 percent.

Thus, cooling to 1°C and exposure to ouabain, cyanide, or dinitrophenol, treatments that are known to interfere with the production and utilization of the sources of cellular energy, caused a net efflux of K^+ from the *Aplysia* giant cell, R2. Rewarming reversed the effects of cooling, causing E_K to become more negative faster than E_M. In addition,

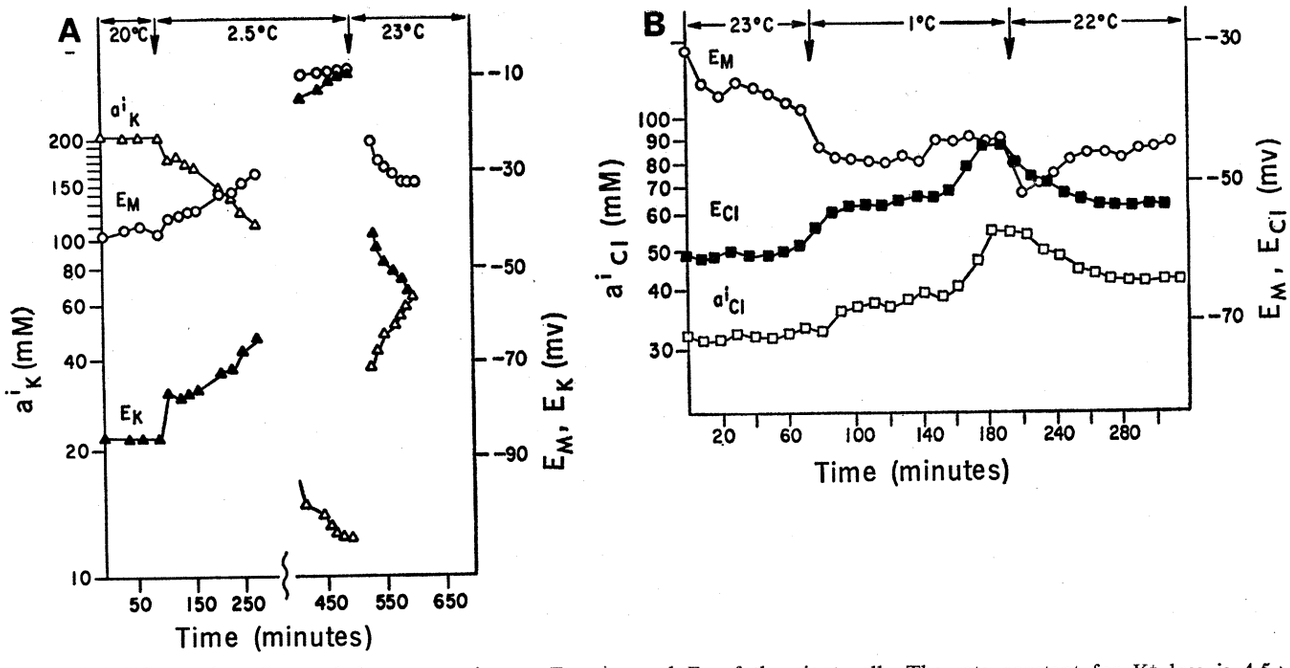

Fig. 1. (A) Effects of cooling and then rewarming on E_M, a^i_K, and E_K of the giant cell. The rate constant for K^+ loss is 4.5×10^{-5} sec^{-1}. (B) Effects of cooling and then rewarming on E_M, a^i_{Cl}, and E_{Cl} of the giant cell.

cooling to 10°C caused significant membrane depolarization, presumably by inhibiting the electrogenic Na^+ pump (*10*), without significant changes in a^i_K. These findings, plus the fact that in the steady state E_K is 20 to 30 mv more negative than E_M, lead to the conclusion that K^+ is actively transported into this cell.

In the steady state, E_{Cl} is more negative than E_M by 2 to 18 mv. The effects of cooling to 1°C and rewarming support the conclusion that Cl^- is actively transported out of cell R2. However, active transport of Cl^- is little affected by ouabain and unaffected by cyanide, an indication that the K^+ and Cl^- transport systems are different.

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Vesicular Stomatitis Virus (Indiana Serotype): Transovarial Transmission by Phlebotomine Sandflies

Abstract. *Transovarial transmission of vesicular stomatitis virus (Indiana serotype) by experimentally infected* Lutzomyia trapidoi *and* Lutzomyia ylephiletrix *to their progeny was demonstrated. Virus was recovered from all developmental stages; mean virus titers from egg to first generation adult showed a four-log increase, indicating that virus multiplication occurred during development of the sandflies. Virus titers in first generation adult females were comparable to those found in their parents. These infected female sandflies transmitted vesicular stomatitus virus Indiana by bite to susceptible animals and transmitted the virus transovarially to their offspring (second generation). Results demonstrate a possible mechanism for transmission and maintenance of this virus in nature without a vertebrate (heat) host reservoir.*

Because of its clinical similarity to foot-and-mouth disease, vesicular stomatitis is of economic and veterinary importance. The disease occurs in both epizootic and enzootic forms. Immunologic surveys indicate that vesicular stomatitis virus (VSV) naturally infects many species, including man (*1, 2*). Antibodies are frequently found among humans living in areas where VSV is endemic, an indication that the virus may be of public health importance (*1, 2*).

The recovery of VSV Indiana from wild phlebotomine sandflies (*3, 4*) along with the evidence of virus multiplication in, and bite transmission by, experimentally infected sandflies (*4*), suggests that these blood-sucking insects may be vectors of the virus. However, a susceptible vertebrate species that develops a viremia sufficient to infect a biting arthropod (*5*) has not been found. Although VSV is classified as an arbovirus (*6*), available data suggest that it does not follow the conventional insect-vertebrate cycle of mosquito-borne viruses; the natural source of VSV has also remained a mystery (*5*). In view of earlier reports suggesting "transovarial transmission" of sandfly (*papatasi*) fever virus (*7, 8*) together with recent isolation of several other viral agents from male sandflies (*9–12*), we investigated the possibility of insect-to-insect transmission of VSV Indiana. We report here generation-to-generation transmission of VSV Indiana by experimentally infected phlebotomine sandflies, and we thus demonstrate a possible mechanism for transmission and maintenance of the virus without a vertebrate host reservoir.

Infant hamsters were inoculated subcutaneously with 10^4 plaque-forming units of a third-passage strain of VSV Indiana. Approximately 24 hours later, one of the hamsters was exposed for 3 hours in a cage with wild sandflies. The hamster was then bled, and the extent of viremia was determined. Blood-engorged flies were removed from the cage, were placed individually in numbered clay pots (*13–15*), and were maintained at 26° to 28°C until oviposition occurred. After oviposition, females were removed from the pots and were frozen at −60°C for subsequent virus titration. Decomposing leaf material was added to the pots as a food source for emerging larvae. At various intervals during development, eggs, larvae (first, second, third, and fourth instar), pupae and F_1 adults were removed from the pots and tested for the presence of virus.

All sandfly specimens except eggs and first instar larvae were triturated individually in sterile 2-ml tissue grinders (Ten Broeck) containing 1.0 ml of medium 199 with 10 percent fetal bovine serum inactivated by heat, penicillin (10,000 unit/ml), streptomycin (16 μg/ml), and amphotericin B (2.5 μg/ml). Because of their tiny size, eggs and first instar larvae were processed under a dissecting microscope. These specimens were placed in separate wells of a microplate containing 0.1 or 0.2 ml of the aforementioned diluent and were triturated individually with a metal probe. Suspensions of parent female sandflies and specimens of hamster blood were prepared in serial, tenfold dilutions and titrated in Vero cell (a continuous line of African green monkey kidney) microplate monolayer cultures; four wells were used for each dilution (*16*). Microplates were incubated at 37°C and were examined after 48 hours for VSV cytopathic effect under an inverted microscope (*16*); results were recorded as the tissue culture infectious dose, 50 percent effective ($TCID_{50}$) per insect specimen or per milliliter of hamster blood (*17*).