EFFECT OF OUABAIN ON THE HYPEROSMOLARITY TOLERANT CELLS FROM RAT KIDNEY

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In a previous paper, we reported that the cell line (RHR cells) can survive in hypertonic culture media isolated from the rat kidney (1). Similar hyperosmolarity tolerant cells can be found specifically in the kidneys and such are capable of producing a well-concentrated urine. We have not been able to demonstrate such cells in other organs (unpublished work). When the range of external salts concentration exceeds 3 fold, the concentration of salts in RHR cells is held constant showing that ion regulation is possible. RHR cell membrane is of considerable interest in connection with osmoregulation in the kidney cells. Present detailed knowledge concerning membrane transport has been obtained mainly in red cells, squid axon and muscle cells (2). It is clear that cells in most tissues have some forms of the Na pump. The present experiment was an attempt to clarify the nature of Na pump in RHR cells.

RHR cells were grown in plastic Petri dishes (Falcon, 10 cm diameter) or culture flasks (Ikemoto, TD-15) with Ham's F12 medium supplemented with 30% calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin, in a humidified atmosphere of 3% CO₂-97% air at 37°C for 7 days until a confluent monolayer was established. The medium was then replaced with a hyperosmotic one prepared by addition of NaCl 12.9 g/l to Ham's F12 medium to give a final osmolarity of approx. 600 mOsmole/l. Forty-eight hours later ouabain (Merk) was added to the medium and the effect of this agent on cell proliferation was investigated over the dose range of 5 × 10⁻⁷ M to 10⁻⁸ M (Table 1). At the dose of 10⁻⁶ M, there was stimulation of growth during the 48 hr observation period. On the other hand, with a dose of 10⁻⁸ M, the viable cell number decreased to about 24% of control. These biphasic effects

| Ouabain (M) | Viable cells (×10⁴ cells/flask) | % of control |
|-------------|---------------------------------|--------------|
| control    | 4.6 ± 0.75                       | 100.0        |
| 5 × 10⁻⁷    | 5.4 ± 0.49                       | 117.4        |
| 1 × 10⁻⁶    | 9.1 ± 1.15                       | 197.8        |
| 1 × 10⁻⁸    | 1.1                              | 23.9         |

About 2 × 10⁴ cells in 1.5 ml hyperosmotic culture medium were implanted in a series of replicate culture flasks. Ouabain was administered on the 7th day. The number of viable cell nuclei was counted by hematocytometer 2 days after administration (3). The values represent mean ± standard error obtained 5 replicate cultures.
TABLE 2 Effect of ouabain on intracellular Na and K concentrations of RHR cells

| Ouabain (M) | Na (mEq/1 of cell water) | K (mEq/1 of cell water) |
|-------------|--------------------------|-------------------------|
| 0           | 18.2±0.56                | 120.6±3.69              |
| 10⁻⁶        | 19.1±0.76                | 124.0±4.60              |
| 10⁻⁵        | 47.6±1.95                | 105.2±2.14              |

About $1 \times 10^6$ cells in 10 ml hyperosmotic culture medium were cultured on plastic Petri dishes. Ouabain was administered on the 7th day. After 3 hr incubation in ouabain medium, the intracellular Na and K were measured by washing cells with ice-cold Ca-sucrose solution, extracting in distilled water and measuring on a flame photometer. Water content was measured according to the method of Burrows and Lamb (6). The values represent mean value±standard error obtained 12 replicate cultures.

occur at various concentrations of ouabain. Palmer and Nechay (4) reported that the effect of ouabain on Na⁺—K⁺ ATPase activity and on urinary electrolyte excretion was biphasic. In some in vitro preparations, stimulation of ion transport has occurred at very low concentrations of ouabain (5). Why high concentrations of ouabain exert a stimulatory effect has yet to be elucidated; however, this phenomenon may possibly be related to stimulated Na⁺—K⁺ ATPase activity in RHR cells.

The ouabain treated cells showed signs of cytoplasmic shrinkage after 3 hr in medium containing $10^{-5}$ M ouabain. Based on morphological observations, intracellular concentrations of Na and K in RHR cells after 3 hr in ouabain media were measured (Table 2). At $10^{-6}$ M ouabain, Na and K concentrations were not significantly different from the control value. RHR cells are rather insensitive to ouabain so that with a dose of $10^{-6}$ M, the pump remains unblocked. At the high concentration of $10^{-5}$ M ouabain, RHR cells gained Na 29.4 mEq/1 of cell water and lost K 15.4 mEq/1 of cell water. Thus it is postulated that the loss of K and gain of Na in ouabain treated cells proceeds on a 1:2 basis.

As the Na⁺—K⁺ ATPase inhibitory effect of cardiac glycosides may be reversed by K⁺ (7), the effect of K⁺ on the inhibitory effect of ouabain on cell growth was assessed. In hyperosmotic culture medium with 3 mM K⁺, the percent inhibition of cell growth by $5 \times 10^{-6}$ M ouabain was 31.2%. When K⁺ concentration in the culture medium was increased to 6 and 12 mM, the percent inhibition of cell growth by ouabain was almost 0% and 14%, respectively. Thus it was clarified that the inhibitory effect of ouabain is reversed by K⁺. These results suggest that the Na pump of RHR cells is quite similar to that found in other tissues (8).

For the maintenance of a hypoosmotic state, water must be taken up and salts effluxed against the diffusion gradient. The Na pump may therefore make an important contribution to the efflux of electrolytes.

To determine whether or not the number of Na pump sites per cell is particularly high in RHR cells, measurements on RHR cells and hyperosmolarity non-tolerant cells (RHS cells) from rat kidney were made. The cells were labelled with ouabain from Ham's F12
medium containing $1 \times 10^{-7}$ M $^3$H-ouabain (New England Nuclear Corporation) for 1 hr at 37°C. The specific uptake on the pump site and the number of these sites were calculated using the procedure of Baker and Willis (9). The binding number per cell was $2.7 \times 10^6$ molecules for RHR cells and $2.2 \times 10^6$ for RHS cells. The number of Na pump sites in RHR cells did not differ greatly from that of RHS cells. The Na pump inhibited by ouabain, is apparently not related primarily to the osmoregulatory mechanisms in RHR cells.

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**HYDROLYSIS OF BRADYKININ BY STEM BROMELAIN**

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During the course of studies on the anti-edema and anti-inflammatory actions of stem bromelain, a potent kininase activity was observed (1). The present paper describes the analysis of the bonds of bradykinin which are susceptible to the action of stem bromelain.

Twelve milligrams (12 μmoles) of synthetic bradykinin (Protein Research Foundation, Minoh, Osaka) dissolved in 3 ml of 5 mM phosphate buffer, pH 7.5, containing 2 mM β-mercaptoethanol, was mixed with 3 ml of purified stem bromelain (7.2 mg; 0.218 μmoles) preincubated in the same buffer at 37°C for 20 min for full activation. Twenty microliter aliquots of the reaction mixture incubated at 37°C were withdrawn every 5 min for assay of rat uterus smooth muscle contracting activity. The contracting activity decreased to 0.5% of the original after 15 min incubation. The resultant reaction mixture was stored at −20°C until required. Enzyme solution without added bradykinin was treated in parallel as control. An aliquot of the reaction mixture was heated in a boiling water bath for 10 min to inactivate the enzyme and dinitrophenylated at pH 9.0 at 40°C in the dark for 2 hr (2). The DNP-derivatives were separated into ether-soluble and aqueous fractions by extracting with