BBA 75738

(Na^+–K^+)-ACTIVATED ATPase IN ISOLATED MUCOSAL CELLS OF TOAD BLADDER

NADIM CORTAS AND MACKENZIE WALSER

Department of Pharmacology and Experimental Therapeutics and Department of Medicine, The Johns Hopkins School of Medicine, Baltimore, Md., (U.S.A.)

(Received May 24th, 1971)

SUMMARY

1. A method is described for reproducible measurement of ATPase activity in isolated mucosal cells of toad bladder. 47 ± 7 % (S.D.) of the total activity is Na^+- and K^+-dependent, and yields 22 ± 5 (S.D.) μmoles P_i per mg protein per h.

2. The curve depicting this enzymatic activity as a function of Na^+ concentration is sigmoid in shape, suggesting cooperative interaction between binding sites for cations.

3. Graded doses of ouabain were preincubated without ATP with the enzyme for 10 min. In deoxycholate-treated preparations, half-maximal inhibition was seen at 10^{-4} M and complete inhibition at $\geqslant 10^{-3}$ M. In the absence of deoxycholate, 39 % of the activity persisted even at 10^{-2} M ouabain.

INTRODUCTION

Because of its structural simplicity and functional capacity to transport ions as well as respond to hormones, the toad bladder has been widely used for the study of transport phenomena. Following the suggestion of SKOU[1] that the (Na^+–K^+-activated ATPase might be involved in ion transport, many attempts have been made to study this enzyme system in the toad bladder. A reproducible assay would permit correlations to be drawn with observations on the intact bladder. BONTING AND CARAVAGGIO[2] and BONTING AND CANADY[3] demonstrated the presence of (Na^+–K^+)-stimulated ATPase in toad bladder with an activity of 0.3 μmole/mg, dry wt. per h. As they noted, the small fraction which (Na^+–K^+)dependent activity comprised of total ATPase (11–18 %) limited the precision of the assay. HAYS AND BARLAND[4], working with isolated mucosal cells of toad bladder and also with an ultracentrifugal fraction thereof, improved on the activity per mg protein; however, the proportion of the total which was dependent on Na^+ and K^+ remained low (9–15 %). ASANO *et al.*[5] obtained similar results. An improved assay is reported herein.

METHODS

ATPase preparation from the toad bladder

Dominican toads (*Bufo marinus*) were killed by a blow on the head followed by pithing. The bilobed bladder was removed, emptied, and soaked at 0° for 10 min

in a solution containing 1 M NaI, and enough Tris to produce a pH of 7.3. As in frog skin[6], this treatment loosens the attachment of the mucosa to submucosa. Each lobe was then stretched on a petri dish, mucosal side up, and scraped with a glass slide. The scrapings were collected in a chilled centrifuge tube and washed 3 times at 0° with 15 mM Tris, 5 mM EDTA, pH 7.0, and 3 times with 50 mM Tris-HCl buffer,

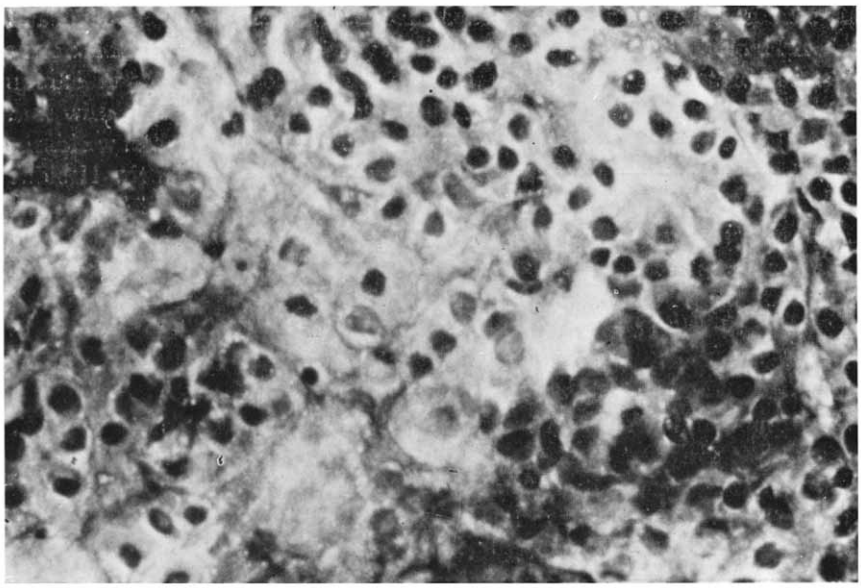

Fig. 1. Mucosal scrapings showing sheets of mucosal cells. Heat fixed, stained with toluidine blue. Approx. × 120.

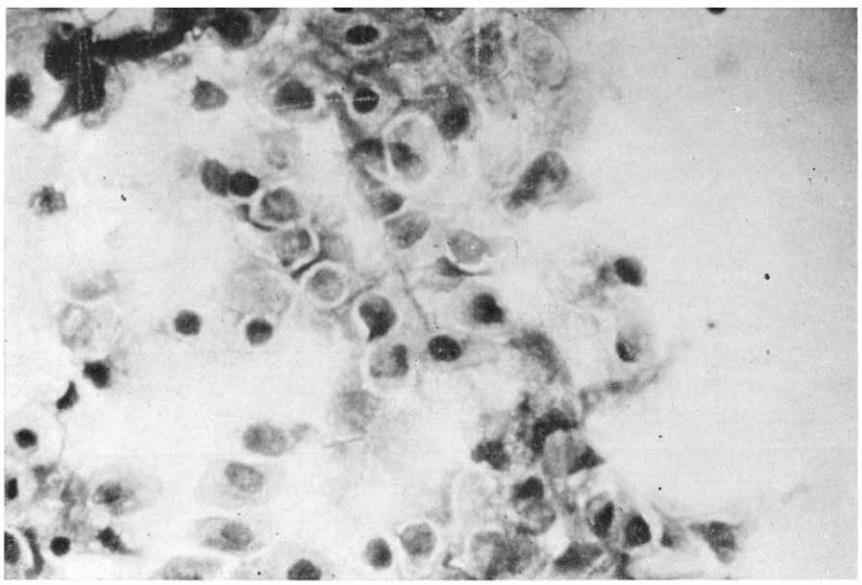

Fig. 2. Washed mucosal scrapings. Heat fixed, stained with toluidine blue. Approx. × 120.

pH 7.0. Prolonged exposure of the scrapings to EDTA resulted in enzyme inactivation. The washed scrapings (Figs. 1, 2 and 3) were immediately transferred to a glass homogenizer (A. H. Thomas No. 4288-A) with 2 ml of the following solution: 5 mM EDTA, 40 mM Tris, 30 mM histidine–HCl, 250 mM sucrose, 0.1 % deoxycholic acid, adjusted with HCl to pH 7.6. Homogenization was carried out in ice with a teflon

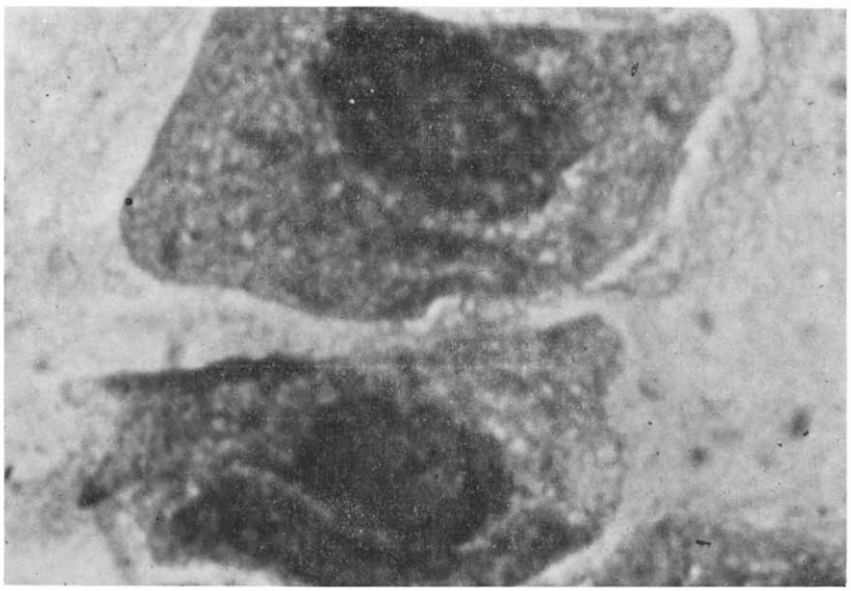

Fig. 3. Mucosal cells. Heat fixed, stained with toluidine blue. Approx. × 1000.

pestle rotated at 650 rev./min, using 20 vertical strokes in 2 min. The homogenate was diluted to 5 ml with the same solution, giving a protein concentration of about 0.2 mg/ml. This was allowed to stand for 1 h at 0°, during which activity was found to be unstable, and was assayed within the ensuing 4 h. Activity was stable for at least 6 h. It fell 15–25 % if left overnight at 5°. Freezing resulted in loss of 70–100 % of activity. Protein was measured by the method of LOWRY *et al.*[7].

Measurement of ATPase activity

The standard assay system for (Na^+–K^+)-ATPase contained 100 mM NaCl, 10 mM KCl, 10 mM KN_3, 2 mM $MgCl_2$, 40 mM Tris–HCl, pH 7.0, and 15–25 μg enzyme protein, in a total volume of 0.25 ml. This was preincubated at 37° for 10 min; the reaction was then started by the addition of 0.05 ml of 6 mM Tris–ATP (Sigma Chemical Co.). After 10 min the reaction was stopped by addition of 0.1 ml 10 % trichloroacetic acid (Eastman Organic Chemicals No. 259). The precipitate was removed by centrifugation for 10 min and P_i was determined promptly on the supernatant by a minor modification of the method of BAGINSKI AND ZAK[8]. Omission of NaCl and KCl yielded the Mg^{2+}-ATPase. There was no difference whether NaN_3, KN_3, or Tris azide was used. Neither Na^+ alone nor K^+ alone stimulated the basal Mg^{2+} activity. Thus KN_3 was used in most preparations. Activity was expressed in μmoles P_i per mg protein per h. The presence of azide was essential to demonstrate Na^+–K^+ acti-

vation. Tris azide was prepared by passing NaN_3 (Eastman Organic Chemical) through a Chelex 100 column (Biorad Laboratories), in the Tris form.

RESULTS

Under the described conditions the (Na^+–K^+)-activated enzyme assay ($n = 14$) yielded 22 ± 5 (S.D.) μmoles P_i per mg protein per h, comprising 47 ± 7 % (S.D.) of the total ATPase. The reaction was linear with time for 45 min and with protein concentrations in the range 20–160 μg/ml.

(Na^+–K^+)-ATPase activity as a function of Na^+ concentration

Na^+ concentration was varied from 1 to 100 mM, keeping other components of the assay medium constant. The results are shown in Fig. 4. Apparent half maximal

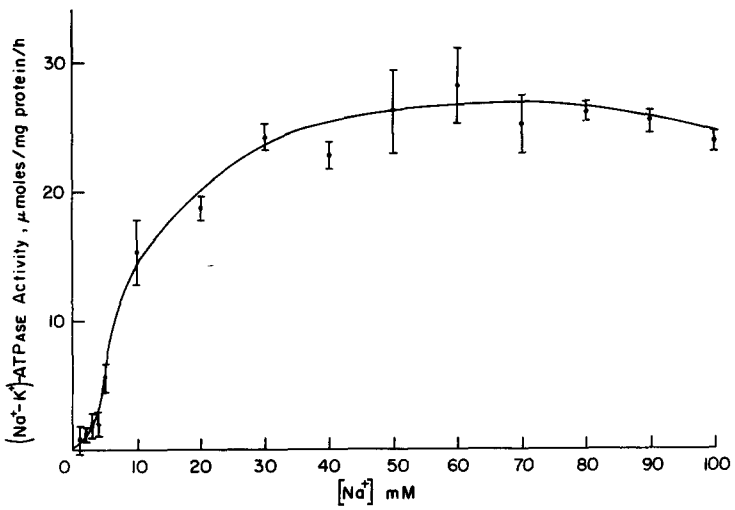

Fig. 4. (Na^+–K^+)-ATPase activity (μmoles P_i per mg protein per h) as a function of sodium concentration. The points represent means ± S.E. (n = 6–8).

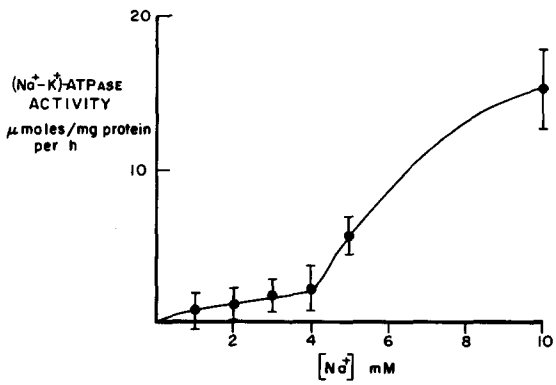

Fig. 5. (Na^+–K^+)-ATPase activity (μmoles P_i per mg protein per h) as a function of sodium concentration, 1–10 mM. The points represent means ± S.E. (n = 8).

activation was 11.8 mM. At the lower concentrations of Na^+ the curve tends to be sigmoid (Fig. 5).

Effect of ouabain on the (Na^+–K^+)-activated ATPase

Ouabain was added at concentrations varying from 10^{-2} to 10^{-10} M. 50% inhibition was obtained at 10^{-4} M (Fig. 6). Complete inhibition occurred at 10^{-3} M. Stimulation, as found in some prior reports[9–11] at low concentrations, was not observed.

If deoxycholate was omitted, 39 ± 9% of the (Na^+–K^+)-stimulated activity was not susceptible to ouabain inhibition, even at 10^{-2} M.

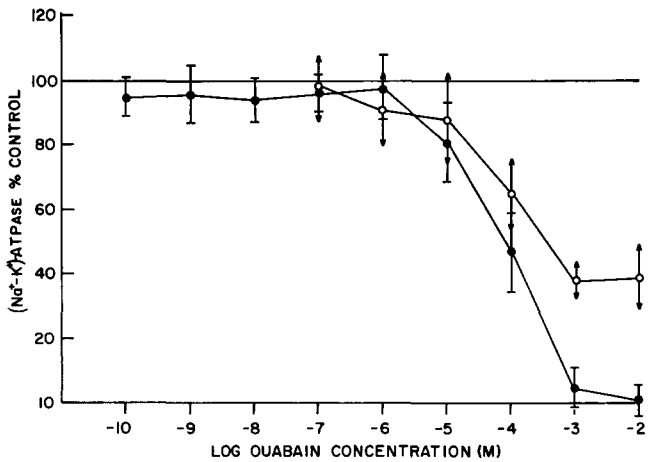

Fig. 6. Ouabain dose response curve. The drug was preincubated with the enzyme for 10 min without ATP which was then added to start the reaction. Incubation time 10 min. ●—●, preparation with deoxycholate; ○—○, preparation without deoxycholate. The points represent means ± S.E.

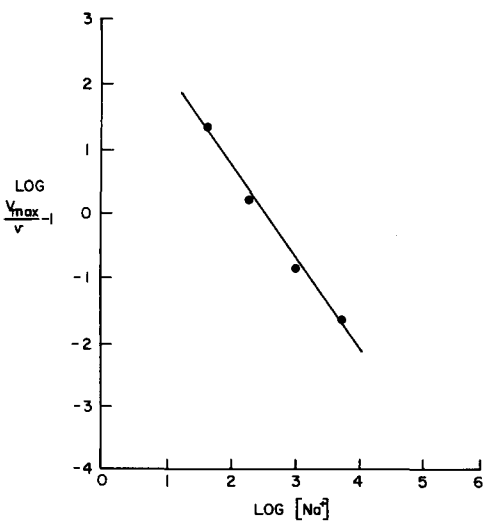

Fig. 7. Hill plot of (Na^+–K^+)-ATPase activity as a function of Na^+ concentration, slope = 1.4 corresponds to n in the expression $\log[(V_{max}/v)-1] = \log 'K' - n \log [S]$. Points in region of apparent K_m are shown.

DISCUSSION

Two features of this assay technique require comment: the technique of homogenization and the use of azide.

Whole bladder is much more difficult to homogenize than isolated epithelial cells and also contains more Mg^{2+}-ATPase derived from muscle. On the other hand, we found that direct scraping of the mucosal epithelium was difficult to perform consistently and prodcued variable degrees of admixture of submucosal and also serosal tissue. NaI treatment yielded sheets of mucosal cells free from other tissue and made possible homogenization in a brief period of time; prolongation of this step inactivated the enzyme. An additional effect of NaI in activating the enzyme may have occurred, similar to that found in other tissues. However, ASANO *et al.*[5] found no increase in toad bladder (Na^+–K^+)-ATPase with this agent.

Omission of azide increased both Mg^{2+}-dependent and (Na^+–K^+)-dependent activity; however, the increase in the latter was much smaller than in the former; furthermore the latter activity became inconsistent. Two factors may explain the effects of azide: (1) inhibition of mitochondrial ATP regeneration[12], which could consume some of the P_i formed by ATPase; (2) inhibition of mitochondrial ATPase[12,13], though this should be relatively small. BOURGOIGNE *et al.*[14] also used azide in a microsomal fraction of turtle bladder cells with similar results.

The shape of the curve relating activity to Na^+ concentration is similar to curves previously obtained in rat kidney[15] and brain[16,17], electric organ of Electrophorus[18], and human erythrocytes[19]. According to SQUIRES[16], the Hill plot[20] of these data indicates a slope greater than 1 and less than 2; the same is true of the present data (Fig. 7). Such a result suggests cooperative interaction of binding sites. In addition to the possibility of two binding sites for Na^+, suggested by these previous investigators, a cooperative interaction with the K^+-binding site might be involved. Thus a conformational change in the phosphorylated enzyme may occur at a critical concentration of Na^+ such that its susceptibility to K^+-activated dephosphorylation is increased.

The ouabain dose-response curve in the deoxycholate-treated preparation is similar to those reported by others[3,5]. It also corresponds closely to the response of short-circuit current to ouabain, at least in the continuously short-circuited tissue. Omission of deoxycholate from the preparation resulted in resistance of part of the (Na^+–K^+)-stimulated ATPase to ouabain. Thus (Na^+–K^+)stimulatable ATPase cannot be equated with ouabain-inhibitable ATPase under all conditions. One explanation may be that deoxycholate permits an interaction between phospholipids and protein, which is essential to the ouabain response. In the absence of deoxycholate it is conceivable that the microenvironment of some, but not all enzyme units is different, and prevents maximal binding of the drug. Further study, involving ouabain inhibition and binding under different conditions of incubation, will be necessary to test these possibilities.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Edward Gfeller for preparing the tissue slides. Supported by U.S. Public Health Service grant AM 02306.

REFERENCES

1 J. C. SKOU, *Biochim. Biophys. Acta*, 23 (1957) 394.
2 S. L. BONTING AND L. L. CARAVAGGIO, *Arch. Biochem. Biophys.*, 101 (1963) 37.
3 S. L. BONTING AND M. R. CANADY, *Am. J. Physiol.*, 207 (1964) 1005.
4 R. M. HAYS AND P. BARLAND, *J. Cell. Biol.*, 31 (1966) 209.
5 Y. ASANO, Y. TASHIMA, H. MATSUI, K. NAGANO AND M. NAKAO, *Biochim. Biophys. Acta*, 219. (1970) 169.
6 J. KAWADA, R. E. TAYLOR, JR. AND S. B. BARKER, *Comp. Biochem. Physiol.*, 30 (1969) 965.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 E. S. BAGINSKI AND B. ZAK, *Clin. Chim. Acta*, 15 (1967) 155.
9 K. REPKE, in W. WILBRANDT, *Proc. 1st Int. Pharmacol. Meeting*, Vol. 3, Pergamon Press, Oxford, 1963, p. 47.
10 K. S. LEE AND D. H. YU, *Biochem. Pharmacol.*, 12 (1963) 1253.
11 R. PALMER AND B. NECHAY, *J. Pharmacol. Exp. Therap.*, 146 (1964) 92.
12 H. E. ROBERTSON AND P. D. BOYER, *J. Biol. Chem.*, 214 (1955) 295.
13 G. A. VIGERS AND F. D. ZIEGLER, *Biochem. Biophys. Res. Commun.*, 30 (1968) 83.
14 J. BOURGOIGNIE, S. KLAHR, J. YATES, L. GUERRA AND N. BRICKER, *Am. J. Physiol.*, 217 (1969) 1496.
15 A. L. GREEN AND C. B. TAYLOR, *Biochem. Biophys. Res. Commun.*, 14 (1964) 118.
16 R. F. SQUIRES, *Biochem. Biophys. Res. Commun.*, 19 (1965) 27.
17 K. AHMED AND J. D. JUDAH, *Biochim. Biophys. Acta*, 120 (1966) 351.
18 G. J. SIEGEL AND R. W. ALBERS, *J. Biol. Chem.*, 242 (1967) 4972.
19 E. K. M. SMITH AND L. G. WELT, *Am. J. Physiol.*, 220 (1971) 119.
20 A. V. HILL, *Biochem. J.*, 7 (1913) 471.