THE STIMULATION BY BICARBONATE OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE SEMINIFEROUS TUBULES OF RODENTS AND THE LACK OF EFFECT OF OUABAIN

B. P. SETCHELL, M. W. SMITH AND E. A. MUNN

A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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Summary. Considerable ATPase activity was demonstrated in homogenates of testes and of isolated seminiferous tubules, but ouabain had no consistent effect on the activity suggesting that most of the ATPase is not the sodium and potassium stimulated ATPase usually associated with ion transport. However, the substitution of bicarbonate for chloride ions in assays at pH 8·4 caused a significant stimulation in ATPase activity of homogenates of testes or isolated seminiferous tubules and liver mitochondria. When the homogenates were fractionated on sucrose gradients, most of the ATPase activity was found associated with mitochondria. Because of this large amount of mitochondrial ATPase present, it is unlikely that we could have demonstrated the amounts of transport ATPase which would account for the amount of fluid secreted by the testis.

INTRODUCTION

An adenosine triphosphatase activated by sodium and potassium (Na/K-ATPase) has been found in many secretory tissues and its activity related to the secretion of fluid and ions (Bonting, 1970). Phosphatases which hydrolyse ATP are present in the testis (Tice & Barnett, 1963) and it has been claimed that LH inhibits a Na/K-ATPase in testicular homogenates and by this means increases the flow of fluid from the rete testis (Burgos & Vitale-Calpe, 1969; Vitale-Calpe & Burgos, 1970a, b). These authors do not make it clear how they think that the ATPase would be involved in the secretion of fluid by the testis, but presumably they envisage ultrafiltration of fluid and then reabsorption of sodium using energy derived from the hydrolysis of ATP by a Na/K-ATPase. However, there is evidence from experiments both *in vivo* and with the perfused testis that the flow of fluid from the rete testis is not affected by changes in blood pressure but is depressed by inhibitors of carbonic anhydrase, metabolic alkalosis and inadequate glucose (Linzell & Setchell, 1969; Setchell & Brown, 1971) which is not what one would expect with a process involving ultrafiltration and reabsorption. Nevertheless, if the fluid is formed in this way it ought to be possible to calculate how much Na/K-ATPase would be necessary to account for the reabsorption.

Fluid flowing from the rete testis of various species contains about 15 mequiv. K/l and about 20 μ l fluid/g testis/hr are produced by a rat testis (Setchell, 1970). If this fluid is formed by resorption of Na from a fluid containing 5 mequiv. K/l and 150 mequiv. Na/l, then the testis must resorb about 40 μ l fluid/g testis/hr and about 6 μ equiv. Na/g testis/hr. It has been shown for a number of other tissues that one molecule of ATP is hydrolysed for three sodium ions transported (Bonting, 1970); assuming that the same ratio holds for the testis one would expect to find Na/K-ATPase activity of about 2 μ mol ATP hydrolysed/g testis/hr.

MATERIALS AND METHODS

Homogenates of testes from adult rats (*Rattus norvegicus*, albino CSE strain), hamsters (*Mesocricetus auratus*) and gerbils (*Meriones unguiculatus*) were prepared in 10 mM-histidine HCl using glass homogenizers; ATPase activity was determined in 1 ml medium buffered with 10 mM-histidine at pH 7.4 and containing 6 mM-MgCl₂, 20 mM-KCl, 100 mM-NaCl and 3 mM-ATP with or without 2×10^{-3} M-ouabain. Ouabain was used to measure the Na/K-ATPase activity, since this could not be done directly because of the presence of Na and K in the homogenates. The equivalence of ouabain-inhibition and Na/K-stimulation of ATPase is now generally accepted (Bonting, 1970). The activity of ATPase was also measured in sediments from homogenates of isolated seminiferous tubules and testis homogenates from the same animals. The tubules were isolated using a technique originally described in 1668 by de Graaf and re-introduced by Christensen & Mason (1965). The homogenates were prepared in 0.25 M-sucrose containing 5 mM-EDTA and 0.1% deoxycholic acid buffered with 60 mM-histidine at pH 7.4, 10 ml per testis. Sediments were prepared by centrifuging the homogenates at 100,000 g for 15 min, resuspending the pellets in 10 ml 0.25 M-sucrose buffered at pH 7.4 with 10 mM-histidine; resedimenting and again resuspending, this time in 10 mM-histidine, pH 7.4.

The activity of ATPase was also assayed at pH 8.4 with 15 mM-Ampol (2-amino-2-methylpropan-1-ol) buffer as used by Turbeck, Nedergaard & Kruse (1968) for *Hyalophora cecropia* midgut, 6 mM-MgCl₂, 100 mM-KCl or NaCl and 3 mM-ATP. Substitution of 100 mM-KHCO₃ or -NaHCO₃ for the corresponding chloride was then effected.

Homogenates of isolated seminiferous tubules from rat, gerbil and hamster were fractionated by centrifugation (51,500 g average; 9 hr, SW 25 rotor of Spinco centrifuge) on a continuous sucrose density gradient (15 to 60% sucrose in 10 mM-histidine, pH 7.4). The material formed three discrete bands on the gradient and there was a substantial amount in a pellet (Text-fig. 1). The tubes were cut with a tube-cutter (Randolph & Ryan, 1950) in order to obtain the bands separately. The activity of ATPase in fractions from the gradients was measured at pH 7.4 with and without ouabain (2×10^{-3} M) and at pH 8.4 with either chloride or bicarbonate as described above. The cytochrome oxidase activity of the fractions was also measured by the technique of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967). Fractions were also examined in the electron microscope by the negative staining technique.

RESULTS

The total ATPase activity in homogenates of whole testes was 1630 ± 100 S.E.M. (six rats) 2110 ± 64 (six gerbils) and 3370 ± 150 (six hamsters) $\mu\text{mol Pi/g testis/hr}$ at 33°C (protein 10.1 ± 0.37 , $7.70 \pm 0.12\%$ and $10.3 \pm 0.26\%$ of wet weight). The amounts of Na/K-ATPase which would account for fluid transport by the testis would obviously be impossible to detect in the presence of this much greater amount of non-specific ATPase in the whole tissue homogenates, and

TEXT-FIG. 1. The ATPase activity at pH 7.4 and the protein concentration of fractions of homogenized seminiferous tubules from a continuous sucrose density gradient. The fractions are shown diagrammatically and were equivalent to those in Text-fig. 2, except that Fractions 1 and 2 were assayed together., Gerbil; —, rat; ----, hamster.

in fact ouabain had a negligible effect on this activity (+ (i.e. stimulation) 78 ± 81 (six rats), -23 ± 67 (six gerbils) and $+91 \pm 92$ (six hamsters) $\mu\text{mol Pi/g testis/hr}$). An attempt was therefore made to fractionate the ATPase activity in the hope of isolating the transport ATPase and measurements were made with sedimented preparations of homogenates of whole testes and of seminiferous tubules separated from interstitial tissue and blood vessels. The activity, expressed per unit of protein, was of the same order for the two preparations from the rat, gerbil and hamster (24.7 , 30.4 and 46.7 $\mu\text{mol Pi/mg protein/hr}$ for whole testis homogenates and 34.1 , 21.4 and 45.0 $\mu\text{mol Pi/mg protein/hr}$ for homogenates of isolated tubules, respectively) and again 2×10^{-3} M-ouabain

had no consistent effect. However, when the assay was done at pH 8.4, inclusion of 100 mM-K or -Na bicarbonate (pH 8.4) in place of chloride stimulated the ATPase activity. With preparations from gerbil tubules, this stimulation was 94 and 30% with the Na salt and 87 and 51% with the K salt (two experiments).

When the homogenates were fractionated on a sucrose gradient, most of the activity remained on the upper part of the gradient while most of the protein was sedimented (Text-fig. 1), but the activity per unit weight of protein was

TEXT-FIG. 2. The ATPase activity at pH 8.4, the increment in ATPase activity resulting from the substitution of 100 mM-KHCO₃ for the KCl, the cytochrome oxidase activity, and the protein concentration of fractions of homogenized seminiferous tubules of two gerbils from a continuous sucrose density gradient.

no higher than that of unfractionated preparations. Ouabain (2×10^{-3} M) had no effect on the ATPase activity of any of the fractions at pH 7.4, but bicarbonate ions stimulated the activity of some fractions at pH 8.4. The fractions which had both the highest increment in ATPase activity in the presence of HCO₃⁻ and the highest unstimulated ATPase activity were also the fractions with the highest cytochrome oxidase activity (Text-fig. 2). When the fractions were examined in the electron microscope, Fractions 1 (the least dense, see Text-fig. 1), 2 and 6 which were clear contained no particulate material. Fractions 3

and 4 contained some mitochondria but most of the material consisted of fragments of membranes of unknown origin. Fraction 5 was richest in mitochondria, many of which appeared to be derived from spermatids; there were also some fragments of other membranes and occasional axonemal filaments. Fraction 7 (the pellet) contained large amounts of fibrous sheaths and dense outer fibres, axonemal filaments and heads of spermatozoa; a few mitochondria were also present.

We have also found that an Ampol-buffered medium containing 100 mM-KHCO₃ stimulated rat liver mitochondrial ATPase some sixfold when compared with a similar medium containing 100 mM-KCl and that ATPase activity was principally associated with the inner membrane separated as described by McMurray & Dawson (1969) (31.4 and 30.8 (two experiments) μ mol Pi/mg protein/hr at 37° C with 100 mM-Cl⁻ and 63.1 and 42.4 μ mol Pi/mg protein/hr with 100 mM-HCO₃⁻ for inner membranes compared with 2.6 μ mol Pi/mg protein/hr for outer membranes with 100 mM-Cl⁻ and 0.8 with 100 mM-HCO₃⁻). Furthermore, the ATPase activity in mitochondria broken by freezing and thawing was about six times greater than with intact mitochondria (52.4 and 36.8 μ mol Pi/mg protein/hr compared with 7.9 and 8.1 (two experiments) with 100 mM-HCO₃⁻).

DISCUSSION

It seems likely from these results that most of the ATPase activity in testicular homogenates is of mitochondrial origin, since a large proportion of the ATPase is found in fractions from sucrose gradients which contain identifiable mitochondria and high concentrations of cytochrome oxidase, an enzyme found exclusively in mitochondria. Because of the large amount of mitochondrial ATPase present in the testis, it is unlikely that one could detect the small amounts of transport ATPase necessary to account for the rate of fluid secretion by the testis. Our observation that bicarbonate ions stimulate mitochondrial ATPase activity at pH 8.4 confirms the findings of Fanestil, Hastings & Mahowald (1963), who also described ATPase activity in rat liver mitochondria that was stimulated by HCO₃⁻ at pH 8.5. However, the effect of bicarbonate in stimulating the rat liver mitochondrial ATPase under these conditions does not appear to be due to the uncoupling of oxidative phosphorylation, as suggested by Fanestil *et al.* (1963) (E. A. Munn, unpublished observations). We suppose the same to be true of the testicular mitochondrial ATPase and the rôle of the bicarbonate-activated ATPase still remains obscure. The mitochondrial origin of the enzyme found in the liver and testis would appear to differentiate it from the microsomal bicarbonate-stimulated ATPase which has been found in the oxyntic cells of gastric mucosa, where it is thought to have a rôle in ion transport (Durbin & Kasbekar, 1965; Wiebelhaus, Sung, Helander, Shah, Blum & Sachs, 1971).

Our finding that no ouabain-inhibited Na/K-ATPase could be detected in any of the testicular preparations we examined, because of the high amount of non-specific ATPase present, is directly contrary to the report of Burgos & Vitale-Calpe (1969), who reported that about 30% of testicular ATPase was

Na/K dependent. Our total ATPase activities were some seven times higher than theirs but we cannot explain the difference between the two results. Some clarification is necessary before the mode of action for LH which they suggested can be accepted.

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