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Study of Enzymes Regulating Vasopressin-Stimulated Cyclic AMP Metabolism in Separated Mitochondria-rich and Granular Epithelial Cells of Toad Urinary Bladder

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Summary. The epithelial cells of the toad urinary bladder are morphologically heterogeneous. In order to relate the effect of vasopressin on cyclic AMP metabolism to cell type, the epithelial cells were separated by the density gradient technique of Scott, Sapirstein and Yoder (*Science* **184:**797, 1974). The separation was verified by electron-microscopy and by observing that the band of cells enriched in mitochondria-rich cells was enriched in carbonic anhydrase activity compared to the band of granular cells. A large portion of the cells collected from the gradient was considered to be nonviable, precluding further study of their function as intact cells. Vasopressin-stimulated adenylate cyclase activity in homogenates of granular cells was similar to that in homogenates of mitochondria-rich cells. Cyclic nucleotide phosphodiesterase activity was also similar in the two types of cell. Thus, the enzymes known to be involved in cyclic AMP metabolism in response to vasopressin appear to be located in both major cell types.

The vasopressin-sensitive epithelial membrane of the toad urinary bladder is composed of cells that are morphologically heterogeneous [2, 16], as are the cells of other anuran and mammalian vasopressin-sensitive epithelial membranes [5, 11, 15, 17]. Electron-micrographs of the toad bladder reveal that about 70 percent of the epithelial cells are "granular cells", 15 percent are "mitochondria-rich cells"; the remainder are "mucuous secreting cells" and "basal cells" [2–4, 16]. The mixed cell population of the toad bladder has been used extensively in studies demonstrating that vasopressin stimulates sodium transport and increases water permeability by stimulating the intracellular production and accumulation of cyclic AMP [1, 9, 10, 14, 20, 23]. If cell function is related to morphology, as is likely, it is apparent that detailed study of the metabolism and function of cyclic AMP in the toad bladder requires separation of the epithelial cells by morphologic type. The report by Scott, Sapirstein and Yoder [21] of such separation using density

gradient centrifugation of cells was of considerable interest. They observed that only the mitochondria-rich cells responded to neurohypophysial hormone with an increase in cell cyclic AMP content. In the present study, the separation of cell types by density gradient centrifugation is confirmed, but the resulting cells are considered to be unsuitable for further study as intact cells. In contrast to the conclusion of the earlier report [21], assays of vasopressin-sensitive adenylate cyclase are interpreted as indicating that both mitochondria-rich and granular cells respond directly to vasopressin.

Materials and Methods

Bufo marinus from the Dominican Republic (National Reagents, Bridgeport, Conn.) or from Colombia (The Pet Farm, Miami, Florida) were maintained on damp san-i-cel. In each experiment, six to twelve toads from one source were used to prepare epithelial cells by the method of Scott, Sapirstein and Yoder [21]. The toads were pithed and each lobe of the urinary bladder excised as a sac. A short length of P.E. 240 tubing (Clay Adams) was tied to the orifice of the sac. The sacs were rinsed with and immersed briefly in amphibian Ringer's solution [21] and then rinsed with and filled with 2 ml of Ringer's solution free of calcium and containing 2 mM EDTA (EDTA Ringer's solution) [21]. Each sac was immersed in the EDTA Ringer's solution in a 30-ml beaker so that the orifice of the tubing protruded above the solution. The beakers were shaken gently at room temperature for 45–60 min. The mucosal solution containing the epithelial cells was collected and pooled at 4 °C. The cells were concentrated by brief centrifugation at 4 °C, resuspended in a small volume of EDTA Ringer's solution, and layered over a discontinuous Ficoll gradient[1] [21]. Cells on the gradient were centrifuged for 45 min at 27,000 rpm in a Beckman SW-27 rotor at 4 °C. Cells in the second and third bands were collected, diluted in EDTA Ringer's solution, and centrifuged briefly at 4 °C. The cell pellet was used for further study.

Cells were prepared for electron-microscopy as described by Scott, Sapirstein and Yoder [21]. Electron-microscopy was performed by Dr. W. Hall of Electro-Nucleonics, Inc., Bethesda, Md.

Carbonic anhydrase activity was assayed in every experiment. An aliquot from each band was resuspended in 20 mM imidazole, pH 7.5, sonicated, and then centrifuged [21]. The supernatant solution was assayed for carbonic anhydrase activity in an Aminco-Morrow Stop-Flow apparatus using 10^{-4} M *p*-nitrophenol as the indicator [12]. Results are expressed in Maren-type units, i.e. [(time for uncatalyzed change)–(time for catalyzed change)]/(time for catalyzed change).

In studies of the responsiveness of intact cells to vasopressin, the pellet from each band was resuspended in Ringer's solution and divided into two aliquots and the remainder of the experiment was performed in duplicate. The suspensions were shaken gently at room temperature. After equilibration with full Ringer's solution for 60 min, a sample

1 The separation is very sensitive to the composition of the Ficoll solutions. Reproducibility was improved considerably by measuring the refractive index of the gradient solutions before each experiment and adjusting their Ficoll content appropriately.

of each suspension was taken for control cyclic AMP measurement, and vasopressin, 100 mU/ml, was added to the suspensions. After 4 and 8 min, hormone-stimulated samples were taken. Each sample of the cell suspension was rapidly added to chilled TCA (final concentration 8%), vigorously agitated on a vortex mixer, and chilled in ice. Cyclic AMP was extracted, chromatographed, and assayed (7) as described previously [23].

Cells for adenylate cyclase assay were resuspended in 25 mM tris, pH 7.5, 0.5 mM $MgCl_2$, and homogenized with six strokes of an all-glass Tenbroeck homogenizer. In another series, cells were homogenized using six strokes of a tight-fitting Dounce homogenizer. In a third series, cells were disrupted in a Polytron (Brinkman Instrument), using two 5-sec bursts at setting 6. Assays were performed in a volume of 0.12 ml, final composition, 50 mM tris, pH 7.5, 5 mM $MgCl_2$, 1 mM cyclic AMP, 0.2 mM 5′-adenylyl-imidodiphosphate (AMP-PNP), and ~40 μC/ml of α^{32} P-AMP-PNP [18]. After 8 min of incubation at room temperature, assays were stopped by adding 0.1 ml of a solution composed of 0.2% sodium dodecyl sulfate, 1 mM ATP, 1 mM cyclic AMP, and ^{3}H-cyclic AMP, the latter to estimate recovery (50%). The volume in each tube was brought to 1.0 ml with H_2O and the tubes heated at 70 °C for 5 min. Cyclic AMP, separated from other nucleotides by AG 50W X-4 and alumina column chromatography [22], was counted in a Packard Tri-Carb liquid scintillation counter.

Cyclic nucleotide phosphodiesterase activity in the 1,000× *g* supernatant solutions of dounce-prepared homogenates and of polytron-prepared homogenates was assayed at high (70 μM) and at low (0.035 μM) concentrations of cyclic AMP. Assays were performed in a volume of 0.2 ml composed of 50 mM tris, pH 8.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin and approximately 100,000 cpm/ml of recently chromatographed (BioRad AG 50W-X8, 100–200 mesh) ^{3}H-cyclic AMP. Tubes were incubated at 24 °C for 30 min (70 μM cyclic AMP) or for 10 min (0.035 μM cyclic AMP) and reactions stopped and processed as described previously [24]. Enzyme assays were linear with time and homogenate protein.

Protein was measured by the method of Lowry *et al.* [13] using bovine serum albumin as standard.

Cyclic AMP and ATP were purchased from Calbiochem, labeled and unlabeled AMP-PNP from ICN, ^{3}H-cyclic AMP from New England Nuclear, arginine vasopressin from Schwartz/Mann and from Nutritional Biochemical Corp., Ficoll from Pharmacia Fine Chemical, and bovine albumin from the Metrix Division of Armour Pharmaceutical Co.

Results

Assay of carbonic anhydrase activity and anatomic typing of the cells of band 2 and band 3 confirmed the separation by density gradient centrifugation described by Scott, Sapirstein, and Yoder [21]. The cells of band 2 had more than twice the carbonic anhydrase activity of band 3, $\frac{\text{Activity band 2}}{\text{Activity band 3}} = 2.24$, $p < 0.001$, $n = 15$, presumably reflecting the enrichment of band 2 in mitochondria-rich cells. Electron-microscopy confirmed the enrichment. In two experiments, 60 to 100 cells in each band were typed on a morphologic basis. The ratio of identified mitochondria-rich cells to identified granular cells in band 2 divided by the ratio of

these cells in band 3 was 9.2 and 14, indicating enrichment of band 2 in mitochondria-rich cells and of band 3 in granular cells. It was observed, however, that 25 to 40 percent of the cells examined in each band had lost their normal appearance and could not be identified as mitochondria-rich, granular, mucous secreting, or basal cells. Many cells were vacuolated and broken.

Scott, Sapirstein and Yoder [21] reported a stimulatory effect of oxytocin on the cyclic AMP content of the mitochondria-rich cells of band 2 and no effect on the granular cells of band 3. In similar experiments, however, we found that vasopressin had a small and inconsistent effect on the cyclic AMP content of the separated cells of band 2 and band 3. The lack of effect of vasopressin in the present study and the damaged appearance of the cells on electron-microscopy support the concept that a large portion of the cells are not viable after incubation in calcium-free Ringer's solution containing 2 mM EDTA for the three hours required for the separation procedure.

Although the cells were judged to be insufficiently viable for testing responses of the intact cell to vasopressin, the two bands were clearly enriched in the major cell types and were suitable for determining enrichment in the activity of enzymes involved in the response to vasopressin. Basal and vasopressin-stimulated adenylate cyclase activity were assayed (Table 1) as well as cyclic nucleotide phosphodiesterase activity (Table 2). Basal adenylate cyclase activity was similar in the two bands. In contrast to the report of Scott, Sapirstein and Yoder [21], in which only the cells of band 2 (mitochondria-rich cells) responded to neurohypophysial hormone with elevated levels of cyclic AMP, the adenylate cyclase activity of both bands was stimulated by hormone. Preparations enriched in granular cells (band 3) clearly responded to vasopressin as well as those

Table 1. Adenylate cyclase activity

Band 2	Band 3	Band 2-Band 3
Basal activity		
15.3	15.6	-0.3 ± 1.4
Vasopressin-stimulated increment in activity		
44.7	50.5	-5.8 ± 5.6

Activity is expressed as pmoles cyclic AMP formed $\times$ mg protein $^{-1} \times 8$ min^{-1}. Differences are mean $\pm$ SE. $n = 15$.

Table 2. Cyclic AMP phosphodiesterase activity

Band 2	Band 3	Band 2-Band 3
Concentration = 70 μM		
15.3	14.9	0.4 ± 2.7
Concentration = 0.035 μM		
53.1	68.7	−15.6 ± 4.3[a]

Results are expressed as nmoles × mg protein^{-1} × 30 min^{-1} for substrate = 70 μM cyclic AMP, and pmoles × mg prot^{-1} × 10 min^{-1} for substrate = 0.035 μM cyclic AMP. Differences are expressed as mean ± SE. The series, $n=8$ includes homogenates prepared with a Dounce homogenizer ($n=2$), and a polytron ($n=6$).
[a] $p<0.01$.

enriched in mitochondria-rich cells (band 2); there was no difference between the two bands in hormone-stimulated activity (Table 1). Cyclic AMP phosphodiesterase activity assayed with a high concentration of substrate was similar in the two bands but at a low substrate concentration, activity in band 2 was less than that in band 3 (Table 2).

Using histochemical techniques [19] and enzymatic assay of the entire epithelial cell population [25], it has been found that carbonic anhydrase activity is greater in epithelial cells of urinary bladders of Colombian toads than of Dominican toads. Carbonic anhydrase activity was greater in each band of separated cells prepared from Colombian toads than in cells in the corresponding band prepared from Dominican toads (Table 3), in contrast to the previous report of similar activity in separated cells from toads of the two origins [21]. It has been suggested that the difference in carbonic anhydrase activity is related to the ability to acidify urine [18, 25].

Table 3. Carbonic anhydrase activity

	Dominican Toads	Colombian Toads
Band 2	7.2 ± 1.0	18.4 ± 1.6
Band 3	3.7 ± 0.6 ($n=11$)	7.2 ± 0.4 ($n=4$)

Results (mean ± SE) are expressed as "Maren type" enzyme units × mg protein^{-1}. The activity in each band is significantly less in Dominican toads than in Colombian toads, Band 2 $p<0.001$, Band 3 $p<0.01$.

Discussion

Although the transport functions of the toad urinary bladder reside in a single layer of cells, the cells are morphologically heterogeneous. Since there may well be a corresponding heterogeneity of cell function, further understanding of the biochemical events in the response to vasopressin requires analysis of the response of each type of cell. The separation of cell types by density gradient centrifugation described by Scott, Sapirstein and Yoder [21], and confirmed in this study, may offer considerable utility for studying the function of the different cell types. It is concluded, however, that the preparation is unsuitable for study of intact cells. The conclusion is based on the vacuolated appearance of the cells collected after density gradient centrifugation and their small and variable increment in cyclic AMP content in response to vasopressin. Similar conclusions may be drawn from earlier studies [20, 21]. Although 5×10^{-8} M oxytocin elicited a significant increment in the cyclic AMP content of cells of band 2 [21], the mitochondria-rich cells, the increment was only 2.4 times basal levels. There was no effect of oxytocin on the cells of band 3 [21], the granular cells. Generally, the mitochondria-rich cells are described as less than 20 percent of the epithelial cell population [2–4, 16]. In an earlier report [20] of studies of intact bladders, 4×10^{-8} M oxytocin caused an increment in cyclic AMP content 4 times basal levels. The fourfold increment in the cyclic AMP content of the entire cell population cannot be explained by the 2.4-fold increment found in the mitochondria-rich cells of band 2 [21]. Deterioration of the cells collected from the Ficoll gradients is not surprising since at that time, the cells have been incubating in a calcium-free, 2 mM EDTA Ringer's solution for about three hours.

Despite reservations regarding the viability of the cells, their separation by major cell types is confirmed, and within limits should prove useful in studying properties of the two types of cell. The results of the present study indicate that cells from the band rich in mitochondria-rich cells, and cells from the band rich in granular cells contain vasopressin-sensitive adenylate cyclase of similar specific activity. Both types of cell have cyclic nucleotide phosphodiesterase activity. At low concentrations of substrate the enzyme in granular cells is more active than the enzyme in mitochondria-rich cells[2].

2 In preliminary experiments, homogenates from both bands were found to contain cyclic AMP-dependent protein kinase activity (Ausiello, Preston, & Handler, *unpublished observations*).

The granular cells form the bulk of the epithelial cells of the bladder. Previous reports have ascribed to the granular cells [3, 4, 6] or to both granular and mitochondria-rich cells [11] the cyclic AMP-mediated water permeability response to vasopressin. If only the mitochondria-rich cells make cyclic AMP in response to vasopressin, as suggested [21], it becomes necessary to postulate that cylic AMP or another signal generated in mitochondria-rich cells alters permeability in granular cells. A recent study utilized immunofluorescent staining for cyclic AMP in the intact bladder in order to explore that postulate. The results were interpreted as demonstrating increased levels of cyclic AMP in all epithelial cells within 2 min (the earliest sample reported) of exposure to vasopressin [8]. The site of origin of the cyclic AMP could not be determined with these techniques. The description of vasopressin-sensitive adenylate cyclase in the granular and the mitochondria-rich cells (Table 1) rather than in the mitochondria-rich cells exclusively [21] eliminates the need for postulating that cyclic AMP generated in mitochondria-rich cells alters permeability in granular cells. The granular cells as well as the mitochondria-rich cells contain the principal enzymes known to be involved in cyclic AMP metabolism.

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