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Actin depolymerization in the cyclic AMP-stimulated toad bladder epithelial cell, determined by the DNAse method

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Previous studies with the rhodamine phalloidin binding assay have shown that antidiuretic hormone and 8-Br-cAMP rapidly depolymerize F-actin in toad bladder epithelial cells. We have extended these studies with the DNAse inhibition assay and have found that in isolated epithelial cell suspensions, G-actin increases from 37 to 56% of total actin following 8-br-cAMP stimulation. The G-actin concentration in the epithelial cell greatly exceeds its critical concentration, indicating the requirement for a G-actin sequestering protein or proteins in this system.

Actin; Antidiuretic hormone; DNAse inhibition assay

1. INTRODUCTION

Antidiuretic hormone (AHD) increases the osmotic flow of water across its target cells in the renal collecting duct and amphibian bladder by promoting the placement of water channels in the apical cell membrane [1–3]. The channels are normally stored in the membranes of cytoplasmic vesicles (aggrephores); ADH, via cyclic AMP, induces the fusion of these vesicles with the cell membrane. It was recently shown [4] that a rapid 20–30% depolymerization of F-actin is associated with the stimulation of toad bladder epithelial cells by ADH or cylic AMP, (cAMP) suggesting that a breakdown of F-actin may be required for aggrephore movement and fusion. Actin depolymerization occurred prior to the onset of osmotic water flow. These experiments were carried out with the rhodamine-phalloidin labeling technique originally described by Howard and Oresajo [5] and modified by Hall et al. [6]. With this method, only F-actin can be measured.

In the study to be reported, we used the DNAse inhibition assay [7,8] to determine G-actin and total actin content of the toad bladder epithelial cell prior to and following stimulation by 8-bromoadenosine cyclic AMP. By using a suspension of isolated epithelial cells, we were able to estimate the F- and G-actin concentration per cell, and the extent to which the G-actin concentration within the cell exceeds its critical concentration.

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2. EXPERIMENTAL

Toads (*Bufo marinus*) were doubly pithed and their bilobed bladders were tied as paired bags on hollow glass bungs. After washing the inside and outside of the bags with amphibian Ringer's solution (120 mM Na^+, 4 mM K^+, 0.5 mM Ca^{2+}, 116 mM Cl^- and 5 mM phosphate, pH 7.4, osmolality 230 molmol/kg H_2O), the bags were washed inside and out with calcium-free Ringer's solution, filled with 6 ml of calcium-free Ringers, placed in an outside bath of 100 ml and incubated for 45 min in the absence of calcium. Under these conditions, the epithelial cells detach from one another, and from the underlying supporting layer [9]. Following incubation, the two bladder sacs were gently massaged, then cut open and the contents collected in a single test tube. The tube was shaken and rapidly decanted into two test tubes, each finally containing 5 ml of the epithelial cell suspension. To the control suspension, 5 ml of calcium-free Ringer's solution was added: to the second, 5 ml of calcium-free Ringer's containing 3.0 mM 8-Br-cAMP (Sigma) was added to give a final concentration of 1.5 mM. Cell counts were performed on the cell suspensions with a microscope counting chamber prior to decantation and ranged from 1.32 to 2.50 × 10^6 cells per tube. After 12 min of incubation both the control and cAMP suspension were spun briefly in a benchtop centrifuge, the supernatants removed, and the pelleted cells placed in ice. The rest of the assay was carried out with the cells and lysis buffer kept at 4°C, conditions which gave more reproducible values on serial determinations than at room temperature. The cells were lysed by adding 0.7 ml of a lysis buffer identical to the 'MT buffer' described by Blikstad and Carlsson [10] (100 mM PIPES, pH 6.75, 1 mM $MgCl_2$, 1 mM GTP, 1 mM EGTA, 1 M sucrose and 0.5% Triton X-100). 0.2 mM DTT rather than DTE was used in this buffer, and we also added the antiproteolytic agents leupeptin (10 μM) and pepstatin (0.5 mM). We found, as did Blikstad and Carlsson, that this buffer gave consistent values for G- and total actin over a time period of 3–30 min on serial determinations (Fig. 1). Immediately after lysis, the cell suspensions were homogenized in small Dounce homogenizers (Bellco). 0.2 ml of the homogenates were than transferred to an Eppendorff tube and spun at 14 000 × *g*. The G- and total actin content of the supernatant was then determined by the DNAse inhibition method [7,8], in which the inhibition of bovine pancreas DNAse 1 (Boehringer) by G-actin was measured by rapidly mixing 0.025 ml of

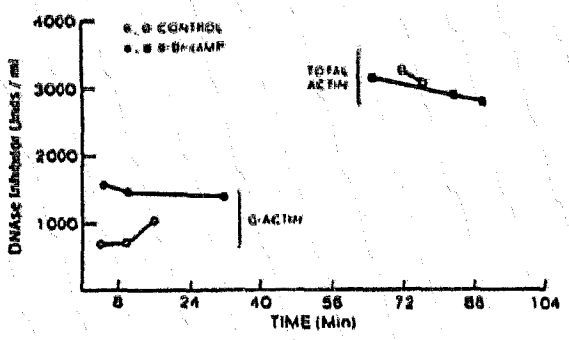

Fig. 1. Experiment showing G- and total actin DNAse inhibitor units in control and 8-Br-cAMP-treated cells. The time is time after lysis.

supernatant with 0.012 ml of DNASE I and 3 ml of salmon testis DNA (Type III, Sigma). The linear segment (generally 5–20 s of the hyperchromicity curve) was read in a Hewlett-Packard diode array spectrophotometer (8452A) at 260 nm, with 2 to 3 serial readings over a period of 3.5–20 min for control and cAMP-treated samples. DNAse I activity was compared to that of a standard solution of DNAse I in lysis buffer, and the difference was expressed as absorbency units (AU) per ml. DNAse I activity in the homogenates was inhibited in the range of 16–35%. Total actin was determined by mixing 0.2 ml of the original homogenate with an equal volume of a solution containing 1.5 M guanidine HCl, 1 mM $CaCl_2$, 1 mM ATP and 20 mM Tris-HCl pH 7.5, and incubating this mixture at 4°C for 5 min. The incubation mixture was then spun at 14 000 × g, and 2 to 3 0.025 ml samples of supernatant were read from both control and cAMP-treated cells. DNAse activity was compared to that of a standard solution of DNAse I in a 1:1 solution of lysis buffer and guanidine HCl solution; we found, that the guanidine HCl buffer slightly inhibited DNAse activity (12.0 ± 3.6 (SEM)%), requiring the use of this separate standard solution for total actin determinations.

To estimate actin concentration in cell water, the mean radius of freshly separated cells in suspension was determined by phase microscopy using a calibrated slide and eyepiece. This proved to be 9.2 ± 0.4 μm, (SEM), with the single cell volume calculated as 3256 μm^3. 80% of the volume was estimated to be cell water. From this and the amount of actin per cell determined by the DNAse inhibition assay, the actin concentration in the cell was calculated [7]. As an independent check of the calculation, we measured the total protein per cell by the Lowry method [11] on known numbers of cells. From densitometry of SDS gels we, (Franki and Hays, unpublished) as well as others [12] have found that actin constitutes 10% of total protein, and using this value, we were able to estimate total actin per cell.

Table I

Effect of 8-Br-cAMP on G- and F-actin (5 paired experiments)

	G-Actin (%)	F-Actin
Control	37 ± 5[a]	63 ± 3
8-Br-cAMP	56 ± 6	44 ± 6

[a]SEM

3. RESULTS AND DISCUSSION

The effect of 1.5 mM 8-Br-cAMP on the G-actin content of the toad bladder epithelial cell is shown in Table I. There was a 19% increase in G-actin, and a reciprocal calculated fall in F-actin from 63 to 44%. Expressed as μg G-actin per ml, the results were 12.2 ± 1.1 and 16.8 ± 1.0 μg per ml (control and 8-Br-cAMP, respectively) $\Delta = 4.6 \pm 1.5$ μg; $p < 0.05$. The 19% decrease in F-actin at 15 min is in the range of the decrease seen in our earlier studies with cell suspensions using the rhodamine phalloidin binding assay [4].

Estimates of total and G-actin concentrations were made from both the DNase inhibition assay and direct determinations of protein per cell (see section 2). These are shown in Table II. Values for total actin by both methods were in the range of 100–150 μM, a relatively low concentration in comparison with platelets [7], but in the range of values reported for lymphocytes [7] and chick skin fibroblasts [13]. G-actin concentration in both control and stimulated cells was considerably greater than its critical concentration of approximately 1 μM [14], indicating that one or more G-actin sequestering proteins are necessary to maintain these high levels of unpolymerized actin, as has been found in a variety of cells. These proposed proteins include profilin [15], and a 5-kDa peptide recently described by Safer et al. [16]. The identity of the sequestering protein or proteins in the toad bladder epithelial cells is not yet known.

Our present and earlier studies place the toad bladder epithelial cell in the category of cells that depolymerize in response to stimulation by specific agonists. Perhaps the most closely related system is the chromaffin cell, where granule exocytosis is accompanied by a rapid increase in G-actin [17] and the transient disappearance of rhodamine phalloidin fluorescence at the outer rim of the cell [17,18]. It has been proposed for this cell [17], as well as the pancreatic β cell, [19] that F-actin filaments at the chromaffin cell periphery act as a bar-

Table II

Concentrations of total and G-actin, determined by the DNAse and Lowry protein methods.

	DNAse Method		Lowry Protein Method	
	G-actin	Total actin (μM)	G-actin	Total actin (μM)
Control	53 ± 5	145 ± 14	34 ± 4	93 ± 10[c]
8-Br-cAMP	77 ± 8[a]	137 ± 14	52 ± 6[b]	

[a]$P < 0.02$
[b]$P < 0.01$
[c]This value used for both control and 8-Br-cAMP stimulated cells

rier to granule fusion; we have suggested the same for the toad bladder epithelial cell, where a subapical layer of F-actin may depolymerize to permit aggrephore fusion [4].

There are differences as well as similarities between the chromaffin cell and the toad bladder epithelial cell. cAMP mediates the depolymerization in the toad bladder, while it inhibits nicotine-induced depolymerization in the chromaffin cell [20]. Vasopressin and cAMP-induced depolymerization is sustained in our system, as in the water flow response, while secretion by the chromaffin cell occurs as a burst, and actin fluorescence at the cell margin is restored by 45 seconds [17].

We do not yet know how cAMP depolymerizes actin. Lamb et al. [21] reported loss of actin microfilament bundles in cultured fibroblasts microinjected with the catalytic unit of cAMP-dependent protein kinase (A kinase), an observation consistent with our findings. Beyond this, the mechanism remains to be determined.

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