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Differential effects of brefeldin A on hormonally regulated Na^+ transport in a model renal epithelial cell line

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Abstract

Na^+ transport in renal epithelia is regulated by a wide variety of endogenous and exogenous cellular factors. Although most natriferic agents have an action on the amiloride-sensitive Na^+ channel, the biochemical pathways which precede activation of the channel remain incompletely defined. One approach to dissecting such intricate pathways is to perturb a specific cellular process and determine its importance in the postulated mechanism. The current studies examine the effect of brefeldin A (BFA), an inhibitor of the central vacuolar system, on basal as well as aldosterone-, insulin-, and forskolin-stimulated Na^+ transport. In the A6 cell line, BFA had a time-dependent effect on basal transport. Aldosterone-induced Na^+ transport was sensitive to BFA while insulin's action was only partially blocked and forskolin-stimulated Na^+ transport was relatively resistant to the action of the inhibitor. These studies highlight differences as well as points of convergence in the natriferic pathways.

Key words: Amiloride sensitivity; Sodium ion channel; Protein trafficking; Insulin; Aldosterone; Forskolin; Protein synthesis

1. Introduction

In Na^+ absorbing epithelia, transcellular Na^+ transport is a multistep process. Under normal conditions, Na^+ enters the cells passively through amiloride-sensitive Na^+ channels in the apical membrane, transverses the cell, and is actively extruded across the basolateral plasma membrane by Na^+/K^+-ATPase [1,2]. While the structure, function and regulation of Na^+/K^+-ATPase have been well characterized [3–5], comparatively little is known about the apical Na^+ channel. Benos et al. have identified this transporter in bovine kidney and the A6 cell line as a heteroligomeric protein of approximately 730 kDa which can be reduced to 5 or 6 non-identical subunits having molecular weights ranging from 40 to 300 kDa [6]. The diuretic amiloride inhibits transport through the holochannel by binding to one of these, a 150-kDa subunit [6].

The apical membrane Na^+ channel appears to be the primary site of action of natriferic hormones such as aldosterone, insulin and vasopressin. Aldosterone's action is dependent on new protein synthesis. While several potential mediators have been postulated [2,5,7–12], the exact nature of the aldosterone-induced proteins remains speculative. Consistent with the requirement for new protein synthesis, aldosterone's stimulation of Na^+ transport is manifested over a period of hours. The peptide hormones, insulin and vasopressin both stimulate Na^+ transport in a more acute manner that is independent of new protein synthesis [13–20]. The action of vasopressin is immediate and is mediated by changes in intracellular cAMP. Insulin's stimulation of Na^+ transport can be detected several minutes after hormone addition and is not associated with changes in intracellular cAMP [16]. Whether the actions of the peptide hormones require membrane protein recycling pathways is an unresolved issue. Therefore, it is known that each of these effectors modulates Na^+ transport in a distinct manner; however, the precise mechanisms remain undefined.

One approach to dissecting pathways involved in the regulation of Na^+ transport is to perturb one or more steps in the chain of events leading to stimulation of the amiloride-sensitive Na^+ channel. One agent that

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has been particularly useful in studies examining intracellular trafficking is brefeldin A (BFA). Physiologically, BFA is known to inhibit secretory pathways; morphologically, this is accompanied by a disintegration of the Golgi apparatus and a redistribution of some Golgi proteins into the ER [21–26]. More recent studies have indicated that, in some cells, BFA also affects the endosomal system and the trans-Golgi network [21,27–30]. We have examined the effects of two concentrations of BFA on basal as well as aldosterone-insulin- and forskolin-stimulated Na^+ transport in a model of the mammalian distal nephron, the A6 cell line.

2. Materials and methods

Materials. Brefeldin A (BFA) was obtained from Epicentre Technologies (Madison, WI). Aldosterone, forskolin and amiloride were purchased from Sigma (St. Louis, MO). Insulin was provided by Eli Lilly (Indianapolis, IN). Culture medium was produced by Gibco (Grand Island, NY) and fetal bovine serum was obtained from ICN/Flow (Costa Mesa, CA). Modified Ussing chambers and automated current-voltage clamps were obtained from World Precision Instruments (New Haven, CT).

Stock solutions of BFA (in ethanol), aldosterone (10^{-3} M in methanol) and forskolin (10^{-2} M in ethanol) were kept at −20°C. Insulin stock solutions (100 μM in 5 mM HCl) were maintained at 4°C.

Cell culture. A6 cells derived from the kidney of *Xenopus laevis* were obtained from the American Type Culture Collection and cultured in an amphibian Dulbecco's modified Eagle's medium as previously described [11]. After amplification in plastic flasks, confluent monolayers were subcultured by mild trypsinization and the cells seeded (10^6 cells/cm^2) on Nucleopore polycarbonate membranes forming the bottom of Transwell chambers (Costar, Cambridge, MA). The Transwells were placed in tissue culture cluster plates forming a two-compartment system and allowing media access to both apical and basolateral faces of the adhered cells. All studies were performed using cells grown on Transwells.

Electrophysiological measurements. For electrophysiological studies, cells which had been maintained in filter culture for 12–20 days were placed in modified Ussing chambers and measurements of potential difference and short circuit current (SCC) were performed as previously described [11]. SCC has been shown to equal net Na^+ flux for basal, aldosterone-and insulin-stimulated tissues [11,14,16,31]. Amiloride-sensitive SCC was used as a measure of the Na^+ flux in forskolin-treated cells.

Electron microscopy studies. Prior to fixation, A6 cells were preincubated with 0.1 or 2.8 μg/ml BFA for 30 min followed by a 30 min incubation in the presence or absence of 10^{-5} M forskolin. Immediately after incubation, the cells were rinsed with PBS and fixed for 1 h in 2% glutaraldehyde, 6 mM eserine in PBS at 4°C. The Transwells were transferred to fresh PBS, washed briefly and postfixed for 1 h at 4°C with 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were washed with deionized water, stained en bloc with 2% aqueous uranyl acetate and dehydrated with a graded ethanol series and propylene oxide. The membrane filters were separated from the plastic inserts and rolled up. The cells were infiltrated with Spurr resin (Electron Microscopy Sciences, Fort Washington, PA). The rolled membranes were cut into several pieces and placed in flat embedding molds. Fresh resin was added and cured at 70°C for 48 h. Thin sections were cut perpendicular to the membrane,

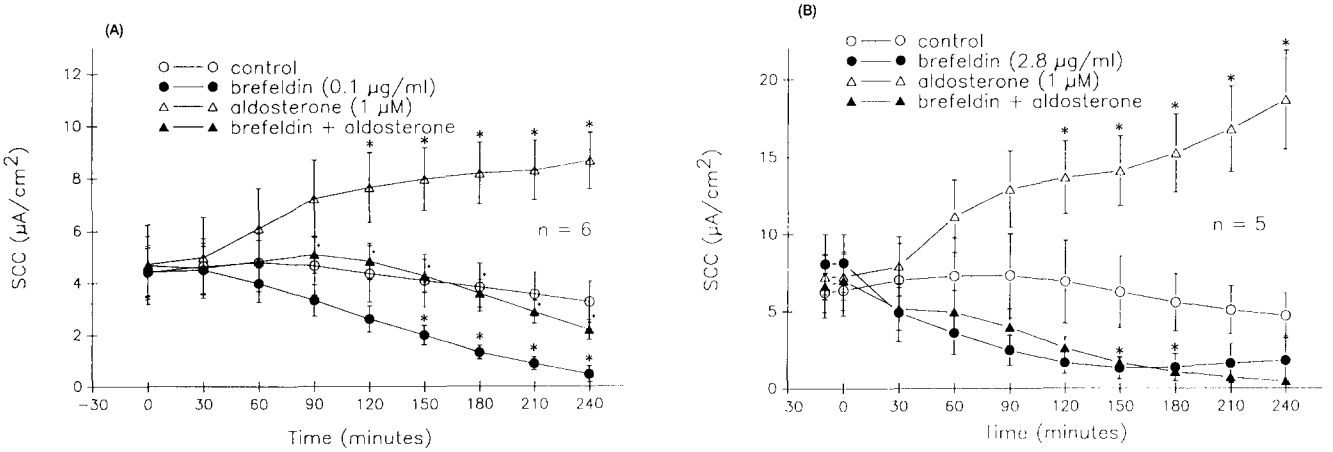

Fig. 1. Effect of brefeldin A on aldosterone-stimulated Na^+ transport. Brefeldin and aldosterone were added to parallel cultures of A6 cells at time 0. The control received diluent only. Bars denote S.E. at each time point. * Denotes values higher (aldosterone-treated) or lower (BFA-treated) than control levels; $P < 0.05$. $^+$ Denotes values higher than BFA-treated; $P < 0.05$.

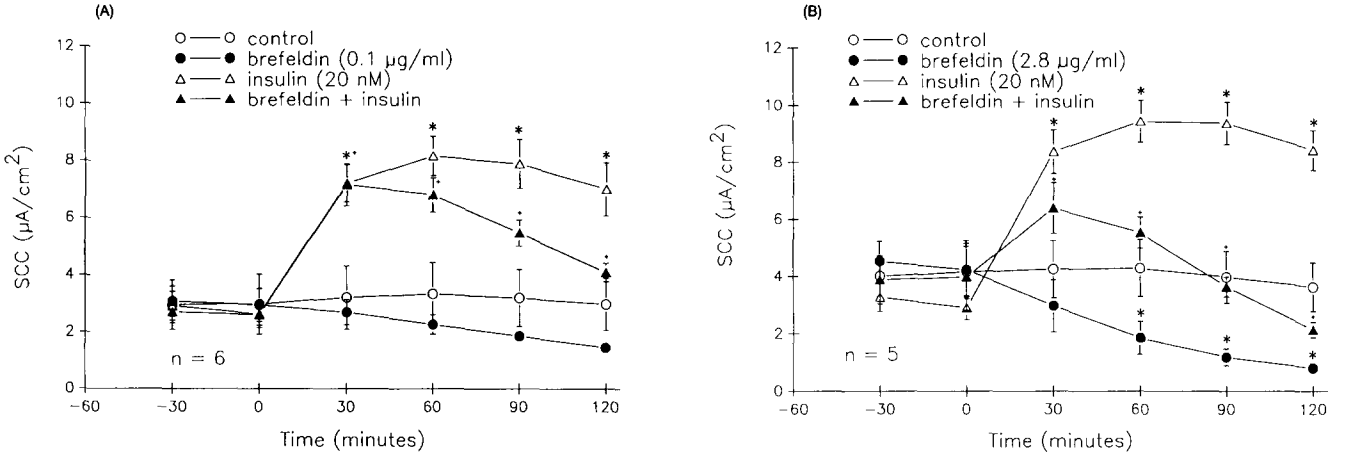

Fig. 2. Effect of brefeldin A on insulin-stimulated Na^+ transport. Brefeldin was added to A6 cell cultures at time −30 min. Insulin was added to the cultures at time 0. The control received diluent only. Bars denote S.E. at each time point. Where no bars are shown, the S.E. is smaller than the symbol used to denote the mean. * Denotes values higher (insulin-treated) or lower (BFA-treated) than control levels; $P < 0.02$ and $P < 0.05$, respectively. $^+$ denotes values higher than BFA-treated; $P < 0.02$.

mounted on copper grids and stained with uranyl acetate and bismuth subnitrate. Cells were observed and photographed in a JEOL 100CX electron microscope.

Statistics. The data are presented as means ± S.E. with n indicating the number of different experiments. Comparisons were performed using Student's t-test for unpaired samples; values of $P < 0.05$ were considered significant.

3. Results

These studies examined the effects of BFA on baseline and hormone-stimulated Na^+ transport in a model of the distal nephron. Aldosterone is a steroid hormone which stimulates Na^+ transport in a protein synthesis dependent manner; insulin is a peptide hormone which stimulates Na^+ transport in a protein synthesis independent manner; forskolin increases the intracellular concentration of cAMP thereby mimicking the effect of vasopressin, another natriferic peptide hormone whose action is independent of new protein synthesis.

Fig. 1 illustrates the effects of two concentrations of BFA on basal and aldosterone-stimulated Na^+ transport. Both concentrations of the inhibitor suppress baseline Na^+ transport after 150 min of incubation.

Compared to control (non-treated) cells, both 0.1 and 2.8 μg/ml BFA completely inhibit aldosterone-stimulated Na^+ transport. However, compared to

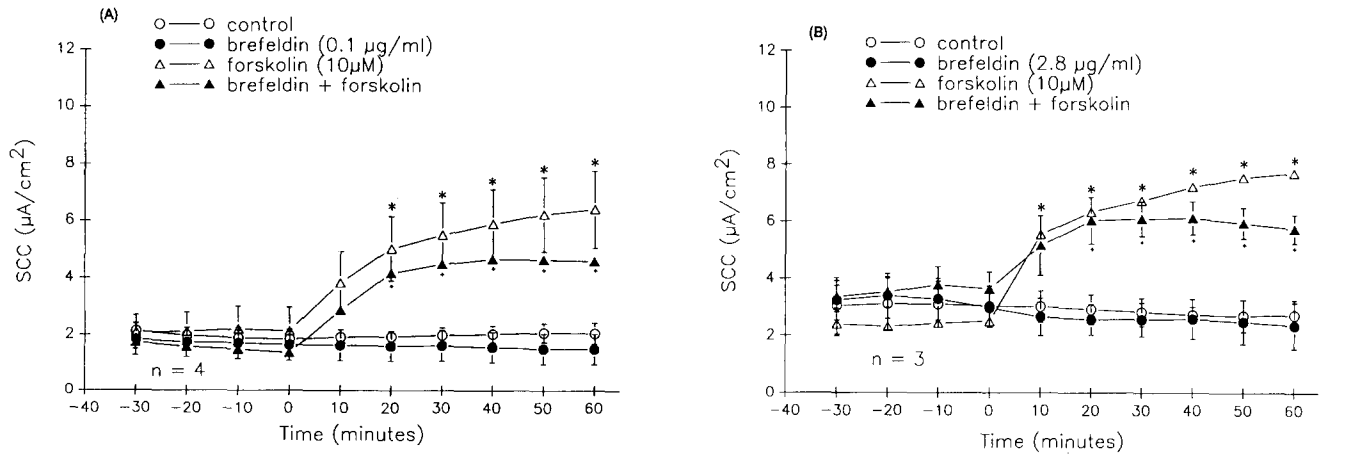

Fig. 3. Effect of brefeldin A on forskolin-stimulated Na^+ transport. Brefeldin was added to A6 cell cultures at time −30 min. Insulin was added to the cells at time 0. The control received diluent only. Bars denote S.E. at each time point. Where no bars are shown the S.E. is smaller than the symbol used to denote the mean. * Denotes values significantly higher than control; $P < 0.05$. $^+$ Denotes values significantly higher than BFA-treated; $P < 0.05$.

BFA-inhibited baseline conditions, aldosterone is able to counteract a portion of the Na^+ transport inhibited by 0.1 μg/ml BFA.

Transepithelial resistances were continuously monitored during the course of these experiments. BFA had no effect on resistances of either basal or hormone-treated monolayers.

Fig. 2 illustrates the effect of BFA on insulin-stimulated Na^+ transport. Since the natriferic action of insulin is acute, inhibitor-treated cells were pre-equilibrated with BFA for 30 min before hormone addition. In matched cell cultures, BFA also inhibits basal Na^+ transport; at 0.1 μg/ml BFA this effect is not statistically significant while at 2.8 μg/ml, the basal inhibition is evident after 1.5 h of BFA treatment.

The addition of insulin to A6 cells stimulates a rapid increase in Na^+ transport which reaches a maximum at approximately 1 h. Thereafter, the level of Na^+ transport reaches a plateau and after another hour begins to decline. 0.1 μg/ml BFA has no effect on the initial insulin-stimulated increase in Na^+ transport. At longer incubation times, the low BFA concentration begins to manifest an inhibitory effect while 2.8 μg/ml BFA partially inhibits insulin's natriferic action at early time points and shows a more substantial inhibition at later time points.

Forskolin activates adenylate cyclase and mimics the action of vasopressin in renal epithelia. While vasopressin's action is usually transient [2,14,19], the action of forskolin in these studies resulted in a prolonged activation of Na^+ transport (Fig. 3). We examined the action of forskolin only over the relatively short time-course congruent with the action of vasopressin. Within this short time-frame, BFA has no discernable effect on baseline Na^+ transport. Analogous to the insulin study, the A6 cultures were pretreated with BFA for 30 min. Neither concentration of BFA inhibited the initial natriferic response to the increased intracellular cAMP and only a very modest inhibition was evident 1 h after the addition of forskolin to BFA-pretreated cells.

Other investigators have reported that in A6 cells the increased SCC, which is stimulated in response to cAMP, has an amiloride-insensitive component [20]. Our results substantiate the previous findings (Table 1). Interestingly, it is the amiloride-sensitive portion of the SCC that is sensitive to the high dose of BFA.

Although BFA may modulate several intracellular trafficking patterns, the best described action of the inhibitor is a reversible effect on the structure of the Golgi and a consequent blocking of protein movement through this pathway. It has been reported that in other renal epithelia [25] this inhibition can be reversed by forskolin.

Fig. 4 shows electron micrographs of six A6 cultures which were grown in tandem on nucleopore filters and incubated under various experimental conditions. In each panel a portion of the nucleus is shown on the right-hand side for orientation. Normal Golgi are found in the control cells (Fig. 4 A). No Golgi were identified in cells which had been treated with 0.1 or 2.8 μg/ml BFA for 30 or 60 min (Figs. 4 B–D). In contrast to studies in normal rat kidney (NRK) cells [25], the addition of forskolin was unable to reverse the BFA-induced disruption of the Golgi (Figs. 4 E and F).

Table 1
Effect of amiloride (10^{-5} M) on forskolin-stimulated Na^+ transport

	SCC	SCC after amiloride	ΔSCC
Forskolin ($n = 4$)	6.41 ± 1.36	2.54 ± 0.54	3.86 ± 0.84
Forskolin + 0.1 μg/ml BFA	4.59 ± 0.45	2.25 ± 0.37	2.36 ± 0.28
Forskolin ($n = 3$)	7.70 ± 0.15	2.49 ± 0.26	5.21 ± 0.29
Forskolin + 2.8 μg/ml BFA	5.76 ± 0.50	3.06 ± 0.72	2.70 ± 0.35

Short-circuit current (SCC) in $\mu A/cm^2$ was measured 60 min after the addition of forskolin. Amiloride was added immediately and the SCC after amiloride was determined 10 min later.

4. Discussion

One of the first functional consequences attributed to BFA was an inhibition of protein secretion [24]. Morphologically, this action is associated with a disappearance of the characteristic structures of the Golgi apparatus and retrieval of Golgi proteins into the endoplasmic reticulum [32,33]; biochemically, this appears to be due to an inhibition of the guanine-nucleotide associated binding of a regulatory coat protein of the Golgi [21,23,34,35].

Recently, it has been demonstrated that BFA may have other actions in the central vacuolar system [23,27–29]. In some renal epithelial cells, concentrations of BFA that did not disrupt Golgi structures inhibited targeting of apical (but not basolateral) proteins to the cell surface [28] and also blocked transcytosis of proteins from the basolateral to apical plasma membrane [27].

BFA has been widely used to study protein trafficking [21–23] and is a valuable tool that can be used to dissect the complex pathways involved in hormonally regulated ion transport. We have used two concentrations of BFA to examine hormonally regulated Na^+ transport in A6 cells.

In MDCK cells, 10 μM BFA selectively inhibits transcytosis of proteins from the basolateral to apical plasma membrane with no effect on the Golgi apparatus [27]. In contrast to those findings, we were unable to observe intact Golgi after incubation in the presence of either 2.8 μg/ml (10 μM) or 0.1 μg/ml BFA (Figs. 4 B–D). In this regard, the sensitivity of the A6 cell line is more analogous to normal rat kidney cells [25],

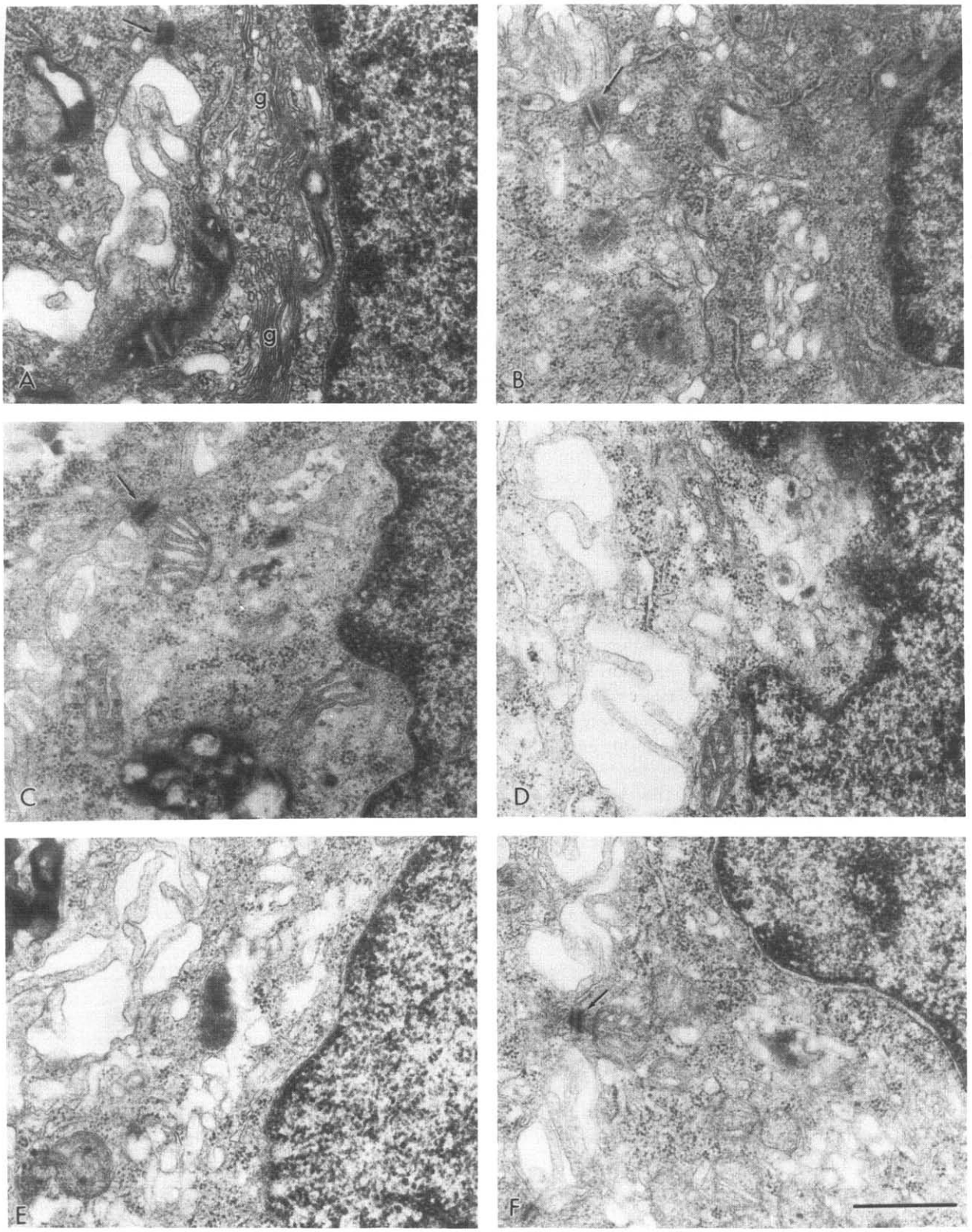

Fig. 4. Effect of brefeldin A on Golgi apparatus in A6 cells. A6 cells were prepared for electron microscopy after incubation under the following conditions: (A) Control, cells were incubated in media for 1 h; (B) low dose brefeldin, cells were incubated with 0.1 μg/ml brefeldin A for 60 min; (C) high dose brefeldin, cells were incubated with 2.8 μg/ml brefeldin A for 30 min; (D) high dose brefeldin, cells were incubated with 2.8 μg/ml brefeldin A for 60 min; (E) low dose brefeldin + forskolin, cells were incubated with 0.1 μg/ml brefeldin A for 60 min, 10 μM forskolin was added to the media for the final 30 min of this incubation; (F) high dose brefeldin + forskolin, cells were incubated with 2.8 μg/ml brefeldin A for 60 min, 10 μM forskolin was added to the media for the final 30 min of this incubation. g = Golgi apparatus; arrows indicate junctional complexes. Magnification: 20000 ×; scale bar = 1 μm.

hepatocytes [32], and mouse pituitary cells [33] than to the MDCK cell line. Therefore, it is likely that any process requiring protein trafficking through the Golgi will be blocked at the concentration used in these studies.

Perhaps the most intriguing and unexpected finding from these studies was the effect of BFA on basal Na^+ transport. Although the magnitude of the effect was directly proportional to the initial transport rate (measured as SCC), BFA clearly inhibited basal Na^+ transport within 2–3 h of inhibitor addition (Figs. 1 and 2). Our results are in agreement with a preliminary report from Johnson and Grillo [36] and suggest that some component of the basal Na^+ transport machinery is undergoing active synthesis and/or recycling in a manner that is BFA-sensitive. Whether the effect is manifested on the holochannel, a subunit of the channel or another component of the transport pathway is unknown. However, it is interesting that this dynamic flux in the transport pathway exhibits a relatively short time-course.

The precise mechanisms by which natriferic hormones modulate the activity of the amiloride-sensitive Na^+ channel remain undefined. It is, therefore, difficult to assess the correct formulation of data presentation. Empirically, we have noted that aldosterone and vasopressin stimulate a geometric increase in Na^+ transport while insulin-stimulated transport is best expressed as an arithmetic increase (Blazer-Yost, B. and Cox, M., unpublished observations). Therefore, we have chosen to present the transport data in an unmodified form (Figs. 1–3).

Numerous studies have shown that stimulation of transcellular Na^+ transport by aldosterone requires new protein synthesis [2,5,7–9,11,12]. However, the exact nature of the newly synthesized proteins is unclear. Immunolocalization and biochemical labelling studies have suggested that aldosterone does not induce the holochannel form of the Na^+ transporter [10,37,38]. However, a 2D-PAGE comparison of complexes from control and aldosterone-treated toad urinary bladder and A6 cells has demonstrated that a single heterogeneous subunit ($M_r \sim 65$–70 kDa) is induced by aldosterone [10–12]. This appears to be the only channel subunit which is actively synthesized in response to aldosterone [10]. As expected from the protein-synthesis dependent nature of the aldosterone response, the action of this steroid hormone is sensitive to both concentrations of BFA.

In contrast to aldosterone, the stimulatory effect of insulin on Na^+ transport is independent of new protein synthesis. Insulin's action is initiated by peptide binding to a basolateral receptor and within minutes culminates in a stimulation of amiloride-sensitive Na^+ transport [13,14,16,39]. Although the intracellular pathways linking receptor binding to channel activation are unknown, the level of cAMP does not change in response to insulin [16]. Recently, Marunaka et al. have used patch clamp techniques in A6 cells to demonstrate that insulin increases the open probability of the amiloride-sensitive Na^+ channel with no effect on single channel conductance [39]. This study has elegantly validated earlier, less direct, investigations suggesting that the insulin-stimulated natriferic pathway has a primary effect on the amiloride-sensitive Na^+ channel [40,41].

0.1 μg/ml BFA has no effect on the initial increase in insulin-stimulated Na^+ transport (Fig. 2 A). At the low inhibitor concentration, the moderate inhibition of insulin-stimulated Na^+ transport at later time points ($t > 30$ min) parallels a BFA-induced decrease in basal transport and the degree of hormone stimulation does not appear to be affected by the inhibitor. In contrast, 2.8 μg/ml BFA partially inhibits insulin's action in A6 cells and the magnitude of the inhibition appears to be greater than the suppression of basal transport.

Arginine vasopressin also acutely stimulates transcellular Na^+ transport in a protein synthesis independent manner. Like insulin, vasopressin binds to a basolateral receptor; however, in contrast to insulin, receptor binding stimulates adenylate cyclase resulting in an increase in cAMP and a stimulation of protein kinase A. The action of vasopressin is associated with a protein kinase A-dependent phosphorylation of the 300-kDa subunit of the Na^+ channel [42].

To determine the physical mechanism by which vasopressin stimulates the Na^+ channel, Garty and Edelman [43] studied Na^+ channel functional expression under conditions of limited proteolysis. In the toad urinary bladder, incubation of the mucosal surface with trypsin irreversibly inhibits 50% of basal Na^+ transport. In contrast, trypsin incubation did not inhibit a subsequent response to vasopressin suggesting that one of the actions of this peptide hormone is to facilitate the insertion of functional Na^+ channels from a trypsin-insensitive pool.

More recently, labeling studies in the A6 cell line have indicated that vasopressin did not induce the apical insertion of new Na^+ channels. In addition, protein kinase A phosphorylation of the 300-kDa subunit of the channel was accompanied by an increase in the open probability of reconstituted channels. The authors interpret these results to indicate that vasopressin activates channels already present in the apical membrane [42].

The A6 cell line is moderately sensitive to arginine vasopressin while the analogous amphibian peptide hormone, arginine vasotocin, exhibits a greater potency [18]. However, the natriferic action of both peptides is accompanied by an increase in intracellular cAMP. The diterpene, forskolin, stimulates the catalytic subunit of adenylate cyclase [44] and induces a higher level

of intracellular cAMP than arginine vasopressin [45] and a more stable transport response in the A6 cell line [20]. In the current studies, forskolin was used to mimic the action of vasopressin.

In the normal rat kidney cell line (NRK), forskolin reverses the morphological effect of BFA by a cAMP-independent mechanism [25]. In our studies, the concentration of forskolin (10 μM) necessary to stimulate cAMP and, consequently, Na^+ transport, was unable to reverse the morphological effect of BFA (Figs. 4 E–F). This may represent a difference between the two cell lines or a concentration-dependent phenomena. Concentrations greater than 50 μM were necessary to reverse BFA effects in NRK cells [25]. We have assumed, therefore, that the major action of forskolin in the natriferic pathway is a cAMP-mediated stimulation of ion transport.

In amphibian model systems, basal as well as aldosterone and insulin-stimulated Na^+ transport is completely amiloride-sensitive [14,31]. However, as noted by previous investigators [20,45], the forskolin-stimulated increase in transcellular transport was not completely blocked by amiloride (Table 1). The amiloride-insensitive component of this response has been shown by others to be due to an increase in transcellular Cl^- flux [20].

Over the time course where vasopressin would be expected to manifest its actions, the forskolin-induced increase in transport is not significantly affected by BFA (Fig. 3). After 1 h, 2.8 μg/ml of the inhibitor can suppress a minor portion of the forskolin-stimulated transport and the inhibited transport appears to be amiloride-sensitive Na^+ flux (Table 1). In view of the time-course of action of this peptide hormone, inhibition after 30 min is unlikely to be physiologically relevant. If the major mechanism for forskolin action is the insertion of new channels from a trypsin-insensitive pool (presumably an intracellular pool) then this shuttling/insertion is not sensitive to BFA.

These studies have provided additional insight into the complex question of how the biochemical pathways involved in transcellular Na^+ transport are regulated. The inhibitory effect of BFA on normal, basal Na^+ transport suggests a dynamic process occurring in the absence of hormonal stimulation. This normal synthesis and/or cycling of components of the Na^+ transport machinery should be considered as a factor when designing controlled studies of the mechanisms of hormone-stimulated transport phenomena.

The differential effects of BFA on basal and hormonal-regulated Na^+ transport may represent diverse sensitivity of signal transduction pathways, diverse actions on the amiloride-sensitive Na^+ channel and/or the presence of multiple forms of the channel which are regulated independently. Future studies examining the effect of BFA on known steps in the Na^+ transport pathway or biochemical modification of the Na^+ channel will provide answers to some of the issues raised by these studies.

5. Acknowledgements

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