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Studies on regulation of the ascorbic acid transporter in a cell line derived from rabbit non-pigmented ciliary epithelium

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A cell line was derived from rabbit non-pigmented ciliary epithelium. The non-pigmented ciliary epithelium is one of the two cell layers which secrete aqueous humor into the eye and concentrate ascorbic acid in the newly-formed fluid. The cultured non-pigmented epithelial cells accumulated ascorbic acid at a rate of 3–5 pmol/μg protein per h. As in freshly-isolated native tissue, the ascorbate uptake mechanism was sodium-dependent and could be inhibited by phloretin (apparent $K_i = 2\text{–}10^{-5}$ M). Phorbol 12,13-dibutyrate (PDBu), a protein kinase C activator, reduced the ascorbate uptake rate. The PDBu effect was concentration-dependent; at a concentration of 10^{-6} M, PDBu reduced the ascorbate uptake rate to 65% of the control value. PDBu reduced the maximal rate of ascorbate uptake (determined at 200–500 μM external ascorbate) but caused no detectable change in the K_m for ascorbic acid (approx. 80 μM). The PDBu-induced inhibition of ascorbate uptake persisted in the presence of ouabain and in low sodium (25 mM Na) medium, suggesting that the effect is not secondary to a change in the sodium gradient. Furthermore, no detectable elevation of cell sodium content was seen in cells equilibrated with ^{22}Na prior to PDBu treatment. The PDBu-induced inhibition of ascorbate uptake was apparently mediated by protein kinase C because the effect was not observed in the presence of staurosporine (10^{-6} M), a protein kinase C inhibitor, or in cells in which protein kinase C was downregulated. These observations suggest that activation of protein kinase C causes inhibition of the ascorbate transporter in this cultured cell line.

Introduction

Aqueous humor formation is linked to active sodium transport by the ciliary epithelium bilayer [1]. The electrolyte composition of aqueous humor is only slightly different from that of plasma, but in most mammals ascorbic acid is present at a high concentration [2,3]. In rabbits, aqueous humor contains 1 mM ascorbic acid while the concentration in plasma is less than 50 μM [3]. The high concentration of aqueous humor ascorbic acid has been suggested as a means of guarding against oxidative damage and nocturnal species do not have a high aqueous humor ascorbate content; night-active animals are presumably at a lesser risk for light-catalyzed oxidative chemical reactions in their eye tissues [4,5].

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The rate of aqueous humor formation follows a diurnal thythm [6] and can be altered by hormones and neuropeptides [7]. In the rabbit ciliary processes, noradrenergic nerve terminals have been identified close to the ciliary epithelium [8] and adrenergic and dopaminergic receptors have been demonstrated [9,10]. Taken together, this evidence suggests that there may be a mechanism for altering aqueous humor formation in response to receptor activation. However, there is currently no clearly defined link between receptor activation and actual changes in specific solute transport mechanisms. As in other cells, protein kinases may be involved and earlier studies have demonstrated protein phosphorylation reactions triggered by protein kinase A, protein kinase C and calcium-dependent protein kinase activity in ciliary epithelium [11]. In studies with phorbol esters, Mittag and co-workers [12] suggested that activation of protein kinase C may alter water and electrolyte transport mechanisms in the ciliary processes.

On the basis of earlier work with segments of ciliary body, we have proposed that rabbit ciliary epithelium

has a transporter capable of actively accumulating ascorbic acid from the blood; the ascorbate is probably then be passively released from the ciliary epithelium to the aqueous humor [13,14]. Uptake of labelled ascorbate by cultured pigmented ciliary epithelium has been described by Helbig and his co-workers [15] and active transport of ascorbate across the intact ciliary body/iris has been demonstrated by Chu and Candia [16]. In the present study, we show that activation of protein kinase C alters the ascorbate accumulation rate in a cell line derived from rabbit non-pigmented ciliary epithelium. Because of the small amount of ciliary epithelium in each eye, these studies would be difficult to carry out using ciliary epithelium freshly isolated from laboratory rabbits. However, it needs to be understood that we cannot be certain that this newly-developed cell line responds in the same way as native epithelium.

A portion of this work was presented at a meeting of the Association for Research in Vision and Ophthalmology (ARVO), Sarasota, Florida, May 1992.

Materials and Methods

L-[*carboxyl*-^{14}C]Ascorbic acid and ^{22}NaCl was purchased from Amersham (Arlington Heights, IL, USA). Phorbol 12,13-dibutyrate (PDBu), staurosporine, phloretin and ouabain were obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA, USA). Water-insoluble substances were dissolved in a minimum volume of either dimethylsulfoxide or ethanol. Equal amounts of dimethyl sulfoxide or ethanol were added to control solutions. In addition, control experiments were performed using reagent blanks to establish that the organic solvent produced no detectable change in [^{14}C]ascorbate uptake.

Cell culture. A cell line derived from Simian-Virus-40-transformed rabbit non-pigmented ciliary epithelial (NPE) cells was used in these studies. A primary culture of rabbit NPE cells was prepared according to a procedure previously described [17], followed by viral transformation with a wild type Simian Virus 40 (SV40). Positive viral transformation was verified by the expression of the large T-antigen (T-Ag) in the nuclei of the SV40-transformed cells. Transformed NPE cells were cultured on glass cover slips to semiconfluency, fixed with methanol-acetone and incubated with antibodies to large T-Ag as described previously [18]. This antibody stained very brightly the nuclei of all SV40-transformed NPE cells, which account for more than 99% of the observed cells, thus indicating that cells were efficiently transformed.

The cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/mg) and gentamicin (0.04 mg/ml) under a humidified atmosphere of 5% CO_2 and 95% air. When a sufficient number of colonies had grown, the cells were trypsinized (0.05%/0.02% trypsin/EDTA solution). Cells were passaged at a split ratio of 1:2–1:4. At this split ratio, the cultures became confluent after a period of 3–5 days. Experiments were carried out using confluent monolayers of cells on 8 mm 24-well multiwell culture plates (Falcon Primaria, Becton Dickinson, Oxnard, CA, USA).

[^{14}C]Ascorbic acid uptake studies. All experiments were performed in Krebs solution containing 110 mM NaCl, 6 mM KCl, 1.1 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose and 25 mM $NaHCO_3$ at pH 7.4. In low sodium solutions, NaCl was replaced with an equimolar amount of choline chloride. Unless otherwise specified, the ascorbic acid concentration was 0.04 mM which is similar to the concentration

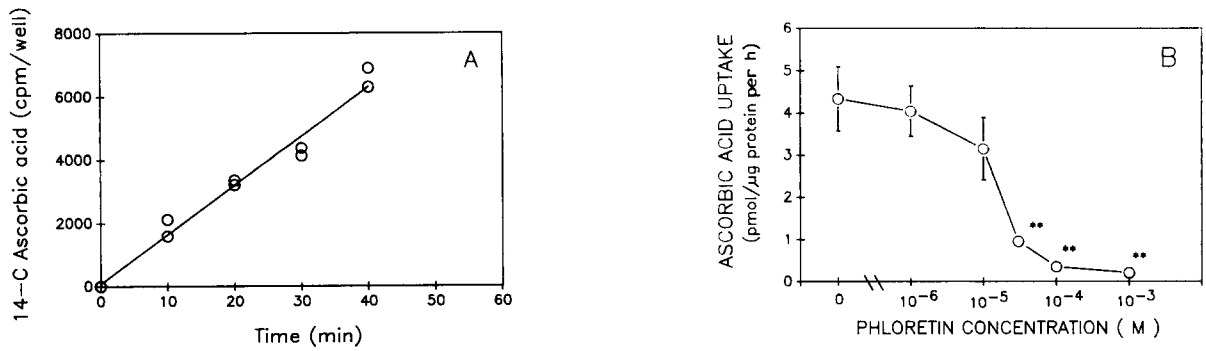

Fig. 1. (a) Time-course of the uptake of [^{14}C]ascorbic acid by cultured rabbit non-pigmented ciliary epithelial cells. The experiment was performed using monolayers of ciliary epithelium grown in 8 mm wells. The results are given as cpm/well. Each point represents the cpm determined in a single well. The ascorbic acid concentration was 0.04 mM. In different batches of cells, the uptake rate varied between 3–5 pmol ascorbate/μg protein per h. (b) The influence of phloretin upon the rate of ascorbic acid uptake. The results are presented as ascorbic acid uptake rates (pmol ascorbic acid accumulated/μg protein per h) and are shown as mean ± S.E. (vertical bar) of data from four experiments (** signficantly different from control, $P < 0.01$).

found in rabbit plasma [3]. Solutions were aerated with 95% O_2/5% CO_2. The cells were washed three times with Krebs solution prior to use.

At the start of each experiment, the cells were equilibrated in control Krebs solution for 60 min. Test agents were then added for 25 min before [^{14}C]ascorbic acid (approx. 0.1 μCi/ml) was added. After a specified uptake period, usually 45 min, the solution was quickly removed and the cells washed for 5 min by immersing the culture plate in one liter of ice-cold, non-radioactive Krebs solution. The cells were then digested in 0.5 M NaOH and the radioactivity in an aliquot of the digest was determined by scintillation counting. Protein in an aliquot of the digest was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Lab, Richmond, CA, USA), using bovine serum albumin as the standard. Uptake was expressed as pmol of ascorbate/μg protein. The data were calculated as mean ± S.E. and statistical significance was examined using a Newman-Keuls test. The number of experiments are given in parentheses.

Results

Characteristics of the ascorbic acid transporter

The cultured rabbit non-pigmented ciliary epithelial cell line accumulated ascorbic acid at a rate of approx. 3–5 pmol/μg protein per h. This resulted in only a small (< 2%) depletion of radiolabelled ascorbate from the bathing medium. The uptake was linear for at least 40 min (Fig. 1a). Ascorbate uptake could be inhibited almost completely by phloretin (Fig. 1b). The apparent K_i for inhibition of ascorbate uptake by phloretin was $2\text{–}10^{-5}$ M.

In earlier studies with fresh tissues, we determined that the ascorbate uptake rate is dependent upon the external sodium concentration and is reduced in the presence of ouabain. The same is true for cultured non-pigmented ciliary epithelium; elevation of internal sodium following a 10-min pretreatment with ouabain for 10 min reduced the ascorbic acid uptake rate from 2.98 ± 0.17 to 1.42 ± 0.07 (mean ± S.E., $n = 6$; $P = 0.01$) pmol/μg protein per h. In addition, the ascorbate uptake rate was diminished when uptake was measured in the presence of a low external sodium concentration (Fig. 2). The data could be fitted to a Hill coefficient of 1.9 ± 0.2 (S.E.).

It should be noted that the ascorbate uptake rates measured in this study may be overestimated because uptake of [^{14}C]dehydroascorbic acid could have contributed to the ^{14}C detected in the cells. Dehydroascorbic acid is accumulated rapidly by ciliary epithelium [15]. We did not measure whether significant oxidation of ascorbic acid occurred during our experimental procedures. However, no significant difference in the measured rate of ascorbate uptake was carried out in the presence of thiourea which was added to prevent ascorbate oxidation as described by Sharma and his co-workers [19]. The rate of ascorbate uptake measured in the presence and absence of 1 mM thiourea was 3.0 ± 0.3 and 3.2 ± 0.1 pmol/μg protein per h, respectively (mean ± S.E., $n = 12$).

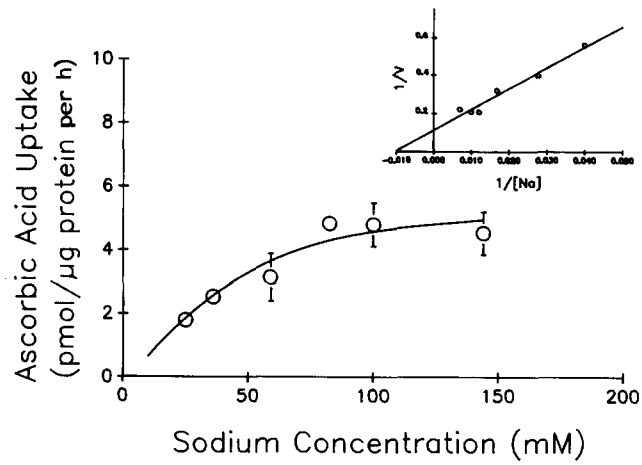

Fig. 2. The influence of sodium upon the rate of ascorbic acid uptake. Each point represents the mean ± S.E. (vertical bar) of results from six experiments. A reciprocal plot of the data (inset) was constructed using a V_{max} of 5.3 pmol/μg protein per h.

Phorbol ester inhibition of ascorbic acid uptake

Ascorbic acid uptake was inhibited in cells exposed to phorbol 12,13-dibutyrate (PDBu), a protein kinase C activator (Fig. 3a). The PDBu effect was concentration dependent; at a concentration of 10^{-6} M, PDBu reduced the ascorbic acid uptake rate to 65% of the control value (Fig. 3b). Inhibition of ascorbate uptake by PDBu persisted in the presence of ouabain (Fig. 3c) and in the presence of increased concentrations of ascorbic acid added to the bathing medium (Fig. 4). Under control conditions, the apparent K_m for ascorbic acid was approx. 80 μM. No detectable change in K_m was observed in the presence of PDBu but there was an increase in the apparent maximal rate of ascorbic acid uptake (determined at external ascorbate concentrations of 200–500 μM).

We examined the influence of PDBu on cells which had previously been exposed to 10^{-6} M PDBu for a 24-h period followed by a 90-min period in control (no PDBu) solution. Such a maneuver has been reported to down-regulate protein kinase C [20] and we made the assumption that this was the case in the present study. In the absence of PDBu, control and 'down-regulated' cells had a similar rate of ascorbate uptake (Fig. 5). However, the uptake rate in 'down-regulated' cells was not altered by the addition of 5 μM PDBu. This observation suggests that the PDBu effect may be modulated by protein kinase C. When cells were treated with 10^{-7} M staurosporine, a protein kinase C inhibitor, the ascorbate uptake rate was not significantly

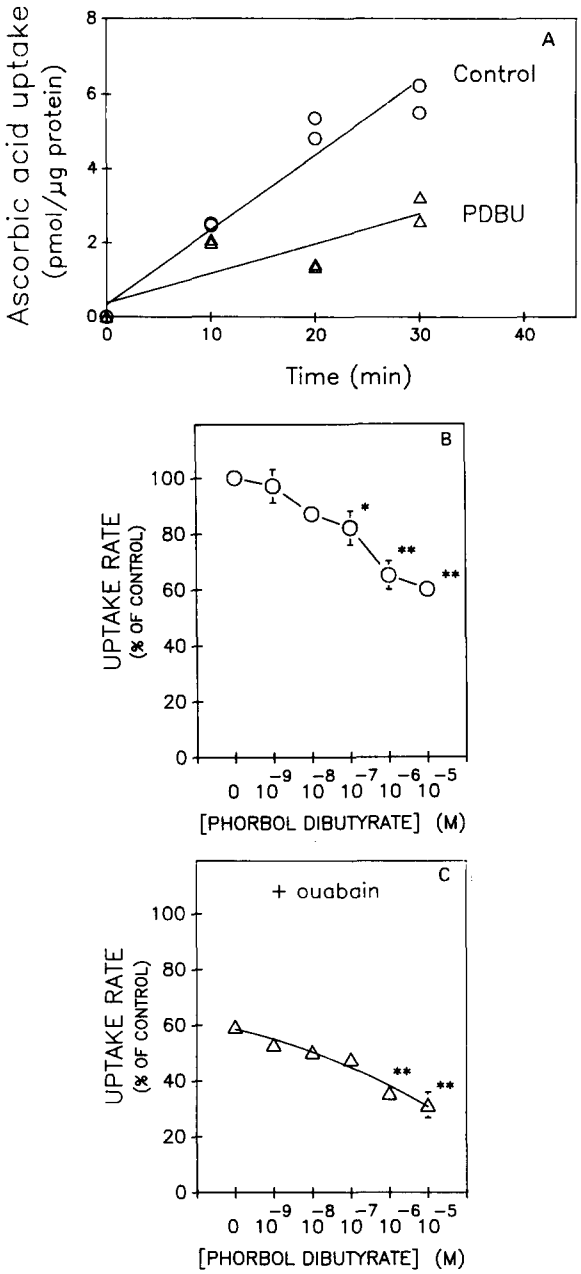

Fig. 3. The influence of phorbol 12,13-dibutyrate (PDBu) upon ascorbic acid uptake. The rate of ascorbic acid uptake in the presence or absence (control) of 10^{-6} M PDBu is shown in (A). The concentration-dependence of PDBu upon the ascorbic acid uptake rate is shown in the absence of ouabain (B) and in the presence of ouabain (C), which alone reduces uptake by approximately 40%. The data are the mean ± S.E. (vertical bar) of results from five experiments except in part A which shows individual data points. (** significantly different from control, $P < 0.01$; * significantly different from control, $P < 0.05$).

altered by PDBu. Staurosporine-treated cells had ascorbate uptake rates of 4.8 ± 0.2 vs 4.5 ± 0.1 pmol/μg protein per h in the presence and absence of 5 μM PDBu, respectively; control (no staurosporine) cells and cells exposed to 5 μM PDBu in the absence of staurosporine had uptake rates of 5.2 ± 0.4 and

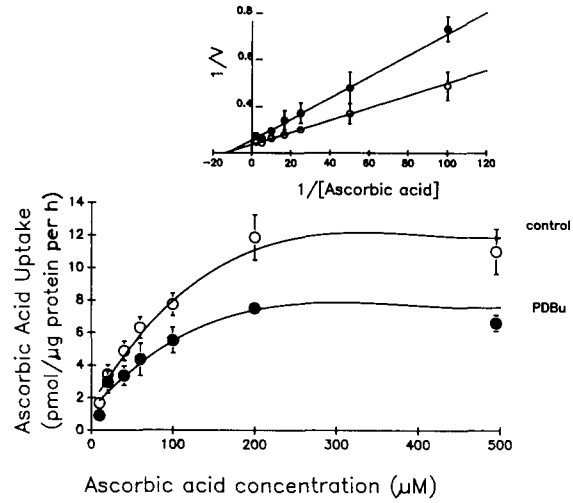

Fig. 4. The influence of ascorbic acid concentration upon the rate of ascorbic acid uptake measured in the presence (●) or absence (○, control) of 10^{-6} M PDBu. A reciprocal plot of the data is shown as an inset. The date are the mean ± S.E. (vertical bar) or results from 4–6 experiments.

* 3.3 ± 0.1 pmol/μg protein per h respectively (mean ± S.E., $n = 8$; * significantly different from control, $P < 0.01$).

The influence of dibutyryl cAMP and A23187 upon ascorbate uptake

There have been reports that cAMP and calcium both participate in signal transduction in ciliary epithelium [9,21]. However, [^{14}C]ascorbate acid uptake was not measurably altered when cells were made calcium-leaky by exposure to calcium ionophore A23187 at concentrations up to 10 μM. In contrast, 1 mM dibutyryl cAMP (dbcAMP), a cell-permeable cAMP analog, caused an approx. 30% reduction in the rate of ascor-

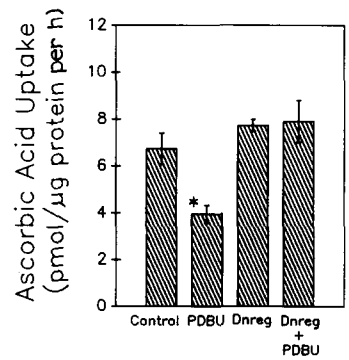

Fig. 5. The influence of protein kinase C downregulation upon the sensitivity of ascorbic acid uptake to PDBu. We assumed that protein kinase C was down-regulated after we exposed the cells to 10^{-6} M PDBu for 24 h; PDBu was removed 90 min prior to the start of the experiment. This maneuver has been reported to downregulate protein kinase C in other cell types [25]. PDBu (5 μM) was added as indicated 25 min prior to the start of the [^{14}C]ascorbic acid uptake period. The data are the mean ± S.E. (vertical bar) of data from eight experiments. (* significantly different from control, $P < 0.05$).

TABLE I

The influence of dibutyryl cAMP and PDBu upon the rate of ascorbate uptake

The data are the mean ± S.E. of results for eight experiments. * Significantly different from control, $P < 0.05$; ** significantly different from control, $P < 0.01$.

	Ascorbate uptake rate (pmol/μg protein per h)
Control	5.3 ±0.5
PDBu (10^{-6} M)	1.9 ±0.2 **
Dibutyryl cAMP (1 mM)	2.9 ±0.4 *
PDBu (10^{-6} M)+ dibutyryl cAMP (1 mM)	1.76±0.2 **

bate uptake (Table I). Added together, PDBu (10^{-6} M) and dbcAMP (1 mM) elicited no further reduction in the ascorbate uptake rate than PDBu alone.

Sodium content of PDBu-treated cells

We examined whether the PDBu-induced reduction of ascorbate uptake was the result of elevated cell sodium. Cells were allowed to equilibrate with ^{22}Na for 6 h. ^{22}Na-equilibrated cells were then exposed to 5 μM PDBu or 0.1 mM ouabain for 70 min, a period equivalent to that used for ^{14}C uptake experiments. Cell sodium content was calculated from the ^{22}Na remaining in each culture well after washout of radioactivity extracellular space. In ouabain-treated cells, the amount of sodium was increased 3–4-fold. However, no significant change in sodium content was observed in cells treated with PDBu (Table II).

The lack of an increased sodium content of PDBu-treated cells suggests that the PDBu-induced reduction of the ascorbate uptake rate may not be the result of an elevated intracellular sodium concentration. This notion is supported by our observation that the PDBu effect upon ascorbate uptake persists in the presence of ouabain (Fig. 3c). Furthermore, when the sodium gradient across the plasma membrane was decreased by performing experiments in a low sodium solution (25 mM Na), PDBu continued to cause detectable inhibition of ascorbic acid uptake; in 25 mM Na, 10^{-6} PDBu reduced the ascorbate uptake rate from 1.79 ± 0.73 to 1.16 ± 0.16 pmol/μg protein per h (mean ± S.E., $n = 8$; $P < 0.05$).

TABLE II

The influence of PDBu upon cell sodium content

Cells were equilibrated with ^{22}Na for 6 h before being maintained a further 70 min in the presence or absence (control) of either PDBu or ouabain. The cell sodium content was computed from the amount of ^{22}Na remaining following washout of the extracellular space which was accomplished by immersing the culture plate in 1 liter of ice-cold, non-radioactive Krebs solution for 5 min. The data are the mean ± S.E. of results from 12 experiments. * Significantly different from control, $P < 0.05$.

	Sodium content (mol × 10^{-10}/μg protein)
Control	6.6 ± 1.4
PDBu (10^{-6} M)	5.0 ± 1.4
Ouabain (1 mM)	19.8 ± 5.0 *

Discussion

By activating protein kinase C, phorbol esters are known to cause a variety of changes in membrane transport processes. In some cells, challenge with cause phorbol esters activates Na^+/K^+-ATPase activity; other cells respond to phorbol ester exposure with a reduction in sodium pump activity [22–25]. There are also reports that the rate of transport by the Na/K/2Cl cotransporter is altered in cells exposed to phorbol esters [26,27]. In the present study we present evidence that PDBu causes a reduction in the activity of a sodium-dependent ascorbic acid transport mechanism.

In experiments with a cell line derived from rabbit non-pigmented ciliary epithelium, we observed that the rate of ascorbic acid uptake was diminished by PDBu. The PDBu-induced reduction in the ascorbate transport rate was rapid; cells were exposed to PDBu for just 25 min prior to the start of the tracer uptake period. The inhibitory effect of PDBu was observed over a wide range of ascorbic acid concentrations. PDBu appeared to change the maximal rate of ascorbic acid uptake but not the K_m for ascorbic acid.

PDBu-induced changes in the ascorbate uptake rate could not be detected in cells exposed to staurosporine, a protein kinase C inhibitor. The sensitivity to staurosporine suggests that the PDBu effect may indeed be mediated by activation of protein kinase C. The involvement of protein kinase C is further supported by our observation that the PDBu response was almost completely eliminated when cells were pretreated for 24 h with PDBu, a maneuver which we assume down-regulated protein kinase C. Long-term exposure to phorbol esters is understood to cause down-regulation through breakdown of the kinase [25].

These studies were carried out with a newly-developed cell line and we cannot judge how closely the cells resemble the native rabbit non-pigmented ciliary epithelium. In the single previous study of these cells, Chu and co-workers [28] have presented evidence which suggests that the cells respond to furosemide similarly to freshly isolated rabbit ciliary epithelium. The characteristics of sodium-dependence and phloretin inhibition which we observed in this cell line are similar to the characteristics of ascorbate uptake determined in isolated segments of rabbit iris/ciliary body [14] and in cultured bovine pigmented ciliary epithelium [15]. The ascorbic acid uptake mechanism in the cultured non-

pigmented epithelial cells was sodium-dependent; lowering the external sodium concentration lowered the rate of uptake. This observation is consistent with an uptake mechanism which co-transports sodium into the cell. As reported in both freshly isolated tissues [13,14] and cultured ciliary epithelium [15], cells exposed to ouabain had a diminished rate of ascorbate uptake. We interpret this finding to signify that increasing cytoplasmic sodium slows ascorbate uptake. Because the ascorbate transporter may be electrogenic [15], the ascorbate uptake rate may increase as the result of cell depolarization when cytoplasmic sodium rises in the presence of ouabain. Alternately, increasing cytoplasmic sodium could perhaps inhibit the release of transported molecules on the inside of the cell membrane. It is also possible that the transporter is bidirectional and outward transport becomes more significant at high cytoplasmic sodium concentrations.

Helbig and his co-workers reported that in pigmented ciliary epithelium the Hill coefficient for sodium-ascorbate cotransport was 1.94 and suggested that the transport stoichiometry is at least 2 Na^+ : 1 ascorbic acid [15]. We also calculated a Hill coefficient of 1.9, suggesting that both non-pigmented and pigmented ciliary epithelium could have a sodium-dependent ascorbate transporter with similar stoichiometry. However, the presence of a sodium-dependent ascorbate transporter in non-pigmented ciliary epithelium is not fully consistent with the transepithelial flux studies of Chu and Candia [16] who suggested that the pigmented epithelial cell layer alone might account for transepithelial ascorbate transport in a divided-chamber in-vitro preparation. Sodium-dependent ascorbic acid accumulation mechanisms have been identified in a number of cell types, including retinal pigment epithelium [29], the kidney [30], mammalian intestine [29] and fish intestine [32]. However, a sodium-independent ascorbate uptake mechanism is responsible for ascorbate entry into retinal capillary pericytes [33].

We examined whether the PDBu-induced reduction in the rate of ascorbate uptake was related to a change in the cytoplasmic sodium concentration but found no evidence to support such a scheme. First, the PDBu effect persisted in the presence of ouabain. In fact, no change in the ouabain-sensitive component of ascorbic acid uptake was detected in cells exposed to PDBu; the net effect of PDBu upon ascorbate uptake could be largely accounted for by a change in the ouabain-insensitive component of ascorbate uptake. Second, in experiments where ^{22}Na equilibration was used to obtain an index of cell sodium content, there was no sign of a sodium increase in cells exposed to PDBu for 45 min, whereas ouabain caused a three-fold increase in cell sodium. In addition, the PDBu-induced inhibition of ascorbate uptake did not appear to require high external sodium; PDBu-induced inhibition could still be observed when the sodium concentration in the bathing medium was reduced to 25 mM.

By activating protein kinase C with PDBu we have examined the response of the ascorbate transporter to one of the key components of signal transduction. To examine other signal transduction pathways we challenged the cells with dbcAMP (to activate cAMP-dependent pathways) and A23187 (to activate calcium-dependent pathways). A23187 did not cause a detectable change in the rate of ascorbate uptake. In contrast, dbcAMP (1 mM) decreased the rate of ascorbate uptake by 35%. Inhibition of ascorbate uptake by cAMP has been reported previously in guinea pig kidney cortex slices [19]. When added together, PDBu and dbcAMP caused no greater degree of transport inhibition than that seen with PDBu alone.

In summary, we have determined that the ascorbic acid transporter of cultured non-pigmented ciliary epithelium can be slowed following the activation of protein kinase C. cAMP-dependent mechanisms may also be able to alter ascorbate transport. These experiments with cultured cells suggest a possible linkage of an ascorbate transport mechanism to signal transduction pathways.

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