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Prolonged Incubation with Elevated Glucose Inhibits the Regulatory Response to Shrinkage of Cultured Human Retinal Pigment Epithelial Cells

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Abstract. Transport defects by retinal pigment epithelial (RPE) and other cells are observed in experimental models of diabetes mellitus. Recent studies have established that glucose concentration, *per se,* is the critical risk factor in the pathogenesis of diabetic complications. This study was designed to test whether transport alterations could be produced in the simplest model of diabetes, sustained exposure of cultured cells to a high-glucose environment. The regulatory transport responses to acute changes in cell volume were measured in order to assess the effects of glucose on a range of transport processes. Continuous lines of nontransformed human retinal pigment epithelial (hRPE) cells were grown for two weeks with either 5.6 low glucose (LG) or 26.0 high glucose (HG) mM in paired experiments. The cell volumes of suspended cells were studied in hypo-, iso- and hypertonic solutions containing the same ionic composition. Hypotonic swelling triggered a regulatory volume decrease (RVD), inhibited by reducing the chemical driving force for K^+ efflux, or blocking K^+ channels (with Ba^{2+}) or Cl^- channels (with NPPB). Thus, the RVD of the hRPE cells likely reflects efflux of K^+ and Cl^- through parallel channels. Shrinkage caused a regulatory volume increase (RVI), which was inhibited by blocking Na^+/H^+ (with dimethylamiloride) or Cl^-/HCO_3^- exchange (with DIDS). Bumetanide inhibited the RVI significantly only when the K^+ concentration was increased above the baseline level. Therefore, the RVI under our baseline conditions likely reflects primarily Na^+/H^+ and Cl^-/HCO_3^- antiport exchange. Growth in high-glucose medium had no substantial effect on the RVD, but reduced the rate constant of the RVI by ≈50%. The RVI was unaffected by growth in high-mannitol medium. Stimulation of protein kinase C (PKC) with DiC_8 increased the RVI of HG-cells, but not of LG-cells. The DiC_8-induced stimulation was bumetanide insensitive and abolished by 1 mM amiloride. Other transport effects of PKC (on the RVD) were unaltered in the HG-cells. We conclude that sustained elevation of extracellular glucose, *per se,* can downregulate the Na^+/H^+ antiport of target cells, an effect noted in streptozotocin-treated rats, and that this downregulation does not reflect interruption of the PKC-signaling pathway.

Key words: Glucose — Protein kinase C — RVD — RVI — Na^+/H^+ antiport — Cl^-/HCO_3^- antiport

Introduction

Defects in membrane transport have been observed in experimental models of diabetes mellitus (MacGregor & Matschinsky, 1986; Greene, Lattimer & Sima, 1987; Winegrad, 1987; Pierce et al., 1990). These data have been interpreted to suggest that the transport defects may play a role in the pathogenesis of the clinical complications of the disease. This hypothesis is not readily addressed in whole-animal, whole-organ and even intact-tissue models, because of the large number of uncontrolled variables. For example, it is unclear whether the transport defects are caused by a lack of circulating insulin or a sustained increase in the extracellular glucose concentration. Insulin clearly has many effects on growth and function, acting both at transcriptional (Oliver et al., 1991) and post-translational (e.g., Siegel &

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Civan, 1976; Häussinger & Lang, 1992) levels. However, several lines of evidence now document that the persistent elevation of the serum glucose concentration, *per se,* is crucial in the pathogenesis of diabetic retinopathy (Engerman, Bloodworth & Nelson, 1977; Klein et al., 1988; The Diabetes Control & Complications Trial Res. Group, 1993; Lasker, 1993) and other complications of the disease.

The aims of the present study have been to address the questions: (i) whether transport defects can be detected in the simplest possible model of diabetes mellitus, namely the sustained exposure to elevated glucose concentrations, and (ii) whether such defects conform to the abnormalities in transport observed with other, more complex models of diabetes mellitus.

The strategy has been to change cell volume and observe the regulatory membrane responses to the perturbation. These regulatory responses are known to reflect the operation of a number of possible transport mechanisms in different cells (Hoffmann, 1987; Hoffmann, Simonsen & Lambert, 1993), and thereby offer the opportunity to assess the effects of elevated glucose concentration on a range of transport processes. We have chosen to use continuous lines of nontransformed human retinal pigment epithelial (RPE) cells because of several considerations: (i) considerable information is already available concerning the volume regulation of RPE cells under different conditions (Adorante & Miller, 1990; Kennedy, 1990, 1992; Miller & Edelman, 1990; Edelman & Miller, 1991; Joseph & Miller, 1991; Lin & Miller, 1991); (ii) the retina, as well as the lens, cornea, peripheral nerve, aortic wall smooth muscle and kidney, is a target organ of diabetes mellitus; (iii) experimental diabetes mellitus has been reported to alter the transport properties of RPE cells (MacGregor & Matschinsky, 1986); and (iv) the use of a continuous line of cultured RPE cells will facilitate focused molecular strategies in examining the effects of high glucose on transport proteins.

Materials and Methods

Cellular Model

Stock cultures of human retinal pigment epithelial cells derived from patients Nos. 17 and 47 (Del Monte & Maumenee, 1980; Dutt et al., 1989, 1990; Del Monte et al., 1991) were received at passage 10. The cells were maintained in T-75 cm^2 flasks with Minimal Essential Medium (MEM) supplemented with 20% calf serum, 0.01–0.05 mg/ml gentamycin and 2 mM L-glutamine.

In preparation for the experiments, cells from passages 13–25 were grown to confluence in MEM supplemented with Earles Balanced Salts, 2 mM L-glutamine, 20% calf serum, 0.01 mg/ml gentamycin and 5.6 mM glucose. After confluence was attained, the concentration of calf serum was reduced to 5% and maintained at this level during the 1–3 days before conducting the experiments. The cells retain at least some of the characteristics of RPE cells in vivo, including microvilli and tight junctions, and the ability to phagocytose rod outer segments (Del Monte et al., 1991).

To assess the effects of growth in high concentrations of glucose, we conducted paired experiments. The RPE cells were first grown to confluence (as described above) and then maintained for two weeks in MEM containing 5% calf serum and (mM) 5.6 glucose (LG), 26.0 glucose (HG), or 5.6 glucose +20.4 mannitol. The cells were harvested by exposure to 0.05% trypsin and 0.2% EDTA for ≈10 min, washed and suspended in one of three solutions. Each solution had the same ionic strength and glucose concentration, and contained (in mM): 55 NaCl, 7.5 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.2 $CaCl_2$, 0.6 $MgCl_2$, 2.4 KCl, 0.6 KH_2PO_4, 15 $NaHCO_3$, and 5.6 glucose. In the absence of added sucrose, the external osmolality was hypotonic (160 mOsm). Isotonic (320 mOsm) and hypertonic (450) solutions were prepared by suitable additions of sucrose to the initially hypotonic solution.

Measurements of Cell Volume

Cell volume was measured by electronic cell sizing (Yantorno et al., 1989; Civan et al., 1992) with a Coulter counter (initially model ZBI with Channelyzer II, and subsequently model ZM with Channelyzer 256). The technique is based upon the concept that passage of cells through an aperture (100 μm in diameter) will displace equal volumes of conducting medium, producing fluctuations in the resistance across that aperture. The instruments were calibrated with polystyrene microspheres of known dimensions (Epics Div. of Coulter, Hialeah, FL). Measurements of the volume of spherical cells in suspension lead to values in agreement with those estimated from other techniques (Lee et al., 1988).

A 0.5-ml aliquot of each cell suspension in MEM was added to 20 ml of each test solution, and the distribution of cell volumes measured with the Coulter counter. The peak of the distribution function is taken to be the cell volume characteristic of the suspension (Yantorno et al., 1989; Civan et al., 1992).

Chemicals

Chemicals were reagent grade. Strophanthidin, hydrochlorthiazide and sn-1,2-dioctanoyl-glyercol (DiC_8) were obtained from Sigma Chemical (St. Louis, MO) and 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS) from Molecular Probes (Eugene, OR). Staurosporine was purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and okadaic acid from GIBCO BRL Life Technologies (Gaithersburg, MD). NPPB [5-nitro-2-(3-phenylpropyl-amino)-benzoate], dimethylamiloride, amiloride and bumetanide were gracious gifts from Prof. Rainer Gregor (Albert-Ludwigs-Universität, Freiburg, FRG), Dr. Thomas Kleyman (University of Pennsylvania), Dr. George M. Fanelli, Jr. (Merck Institute for Therapeutic Research, West Point, PA), and Hoffmann-La Roche (Nutley, NJ), respectively.

Data Reduction

Values are presented as the means ± 1 SE. The number of experiments is indicated by the symbol *N*. The probability (*P*) of the null hypothesis has been calculated using Student's *t*-test.

Results

Regulatory Responses of Cell Volume

Following suspension in isotonic Tyrode's solution, the initial volumes of the LG- and HG-cells were similar (1,784 ± 64 and 1,735 ± 63 fl, respectively); the mean paired difference in volume (49 ± 90 fl) was statistically insignificant. During the first few minutes following isotonic suspension, the RPE cells shrank by ≈10% and were thereafter stable (open circles, Fig. 1*A*). Suspension in 50% hypotonic solution produced a rapid swelling (filled circles, Fig. 1*A*). Without any further intervention, the cells displayed a regulatory volume decrease (RVD), a spontaneous shrinkage towards the cell volume observed in isotonic suspension.

As in many other cells (Hoffmann, 1987), increasing the external osmolality by 50% to 450 mOsm produced cell shrinkage, but no regulatory response in the test solution used (open triangles, Fig. 1*A*). On the other hand, a regulatory response to shrinking could be elicited by following a "post-RVD RVI" protocol (McCarty & O'Neill, 1992). The cells were first suspended in hypotonic solution. Following this pretreatment, restoring isotonicity by adding nonelectrolyte produced an immediate cell shrinkage, and a subsequent spontaneous return towards the baseline value (filled triangles, Fig. 1*A*). This secondary response to shrinkage is termed the regulatory volume increase (RVI) (Hoffmann, 1987). The RVD and RVI are displayed by many cells but the underlying mechanisms vary from preparation to preparation.

Figure 1*A* and *B* present the results of an initial series of four paired experiments. The glucose concentration in the growth media did not seem to affect the time courses of the volumes of cells suspended simply in isotonic, hypotonic or hypertonic test solutions. However, following the hypotonicity-to-isotonicity protocol, cells grown in high-glucose (HG) media seemed to display a slower, less complete RVI than that of cells grown under baseline low-glucose (LG) conditions (*compare* filled triangles over period 28–60 min in Fig. 1*A* and 1B).

For purposes of comparison, the time course of the RVD is fit to a monoexponential beginning with the time of peak swelling, using nonlinear least-squares analysis (Yantorno et al., 1989; Civan et al., 1992):

$$v_c = (v_c)_\infty + \Delta(v_c)_m e^{-kt} \tag{1}$$

where k is the rate constant and v_c and $(v_c)_\infty$ are the cell volumes at any time t and at the end of the RVD, respectively, and $\Delta(v_c)_m$ is the maximum amount of swelling relative to $(v_c)_\infty$. Changes in the rate constant can be sensitively detected from the time course of the ratio of the magnitudes of the RVD by the control and experimental suspensions:

$$[v_c - (v_c)_\infty]/[v_c - (v_c)_\infty]' = [\Delta(v_c)_m e^{-kt}]/[\Delta(v_c)_m e^{-kt}]' \tag{2}$$

where the experimental and control values are primed and unprimed, respectively. Rearranging,

$$[v_c - (v_c)_\infty]/[v_c - (v_c)_\infty]' = [\Delta(v_c)_m/\Delta(v_c)_m'] e^{-(k-k')t} \tag{3}$$

Fitting the ratio to a single exponential thus permits simple determination of whether $(k - k')$ is significantly different from zero, using Student's t-test. If the experimental volumes decay more rapidly than those of the control, we analyze the time course of the inverse ratio. The transformation of the data will lead to some distortion of the distribution and its variance. However, where differences have been noted, the probabilities are sufficiently different from 0.05 (Table 1) for us to conservatively regard the exponential decays as different.

Data reduction of the RVI can be performed more simply, since the time courses can be fit by straight lines over the periods studied. The rate constant of the RVI is given by the slope of the linear regression analysis. We determine the significance of experimentally induced changes from the difference in slope between the control and experimental regression lines, using Student's t-test. For the convenience of the reader, the experimentally induced changes are presented as percentage changes in Tables 2 and 3, but the significance of those effects is not evaluated from the calculated percentage changes.

The values of the rate constants (k) of both the RVD and RVI varied significantly from week to week, so that comparisons of experimental and control suspensions were always performed with paired samples.

Ionic Basis for the Baseline RVD

The RVD of a wide spectrum of biological cells reflects the loss of KCl by means of conductive channels, symports or antiports (Hoffmann, 1987; Hoffmann et al., 1993). In some cells, loss of nonelectrolyte can be important, and the Na,K-exchange pump has also been hypothesized to play a role (Siebens, 1985). Because of the importance of KCl translocation in the RVD of many cells, the speed of the regulatory phase can often be slowed by reducing the concentration gradient fa-

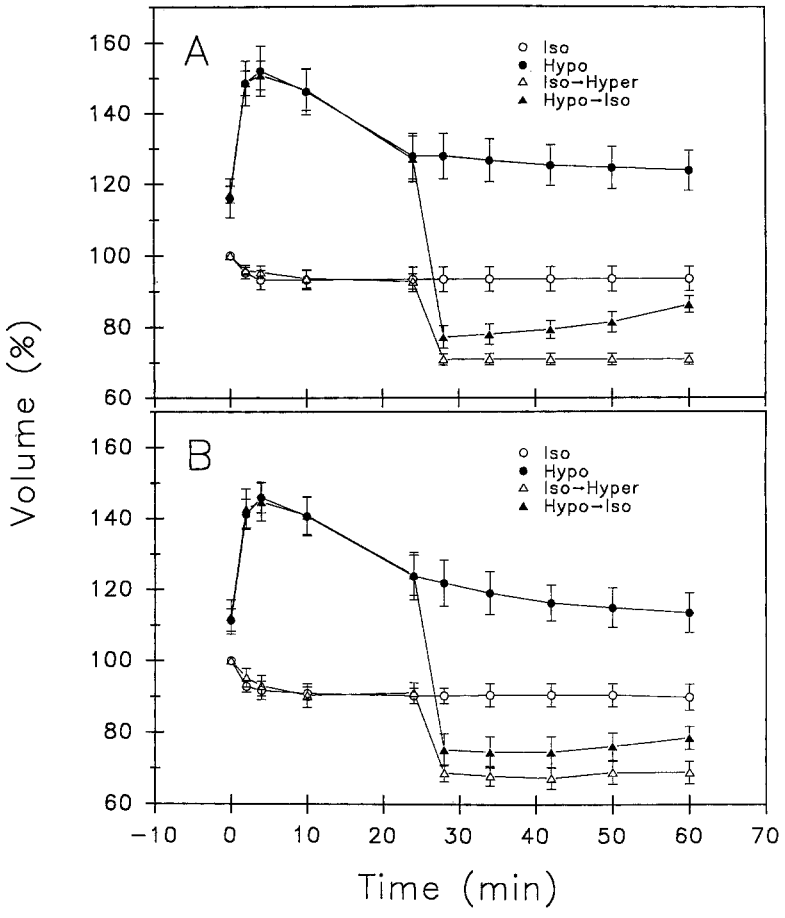

Fig. 1. Paired comparisons of time courses of volumes of cells grown under low-glucose (LG, panel *A*) and high-glucose (HG, panel *B*) conditions ($N = 4$). In this and succeeding figures, cells were suspended in solutions having the same ionic strength, but with or without sucrose to raise the osmolality from 160 to 320 mOsm. Sucrose was added to some of the solutions 25 min after the initial suspension, raising the osmolality of the initially hypotonic solutions to isotonicity (320 mOsm) or imposing hypertonicity (450 mOsm). Unless otherwise stated, the connecting lines are intended to facilitate identification of the data points and have no theoretical significance. The uncertainties are ±1 SE for the mean values presented. The data have been normalized to the initial volumes of the cells suspended in isotonic solution.

voring K^+ exit. In the experiments of Fig. 2, the RVD was studied both with baseline media (as in Fig. 1) and with a reduced K^+ gradient. Here, the gradient was lowered by preincubating the cells with 100 μM strophanthidin, and suspending the cells in strophanthidin-containing medium having an elevated K^+ concentration. The cardiotonic steroid blocks the Na,K-exchange pump, leading to a loss of intracellular K^+. Thus, the experimental perturbation reduced the K^+ gradient by both decreasing the K^+ concentration inside and increasing the concentration outside the cells. Reducing the chemical gradient for K^+ clearly lowered the magnitude and rate constant (Exp. 1a, Table 1) of the RVD.

The efflux of K^+ could proceed by K^+/H^+ antiport or K^+/Cl^- symport activity, as well as through K^+ channels. The observation that 5 mM Ba^{2+} inhibited the RVD (Exp. 7, Table 1) indicates that the anisosmotic swelling triggered K^+ loss at least partially through channels. The principal anion likely to accompany K^+ during the RVD is Cl^-, which could exit through a K^+/Cl^- symport, Cl^-/HCO_3^- antiport or Cl^- channels (Hoffmann, 1987). The Cl^--channel blocker NPPB [5-nitro-2-(3-phenylproplyamino)-benzoate] (Wangemann et al., 1986) inhibited the RVD (Exp. 8, Table 1). In some tissues, NPPB also blocks K^+ channels (Wangemann et al., 1986; Reinach & Schoen, 1990). However, the inhibitory effect of the Cl^--channel inhibitor (NPPB) was far greater than that of the K^+-channel blocker (Ba^{2+}), suggesting that Cl^- channels play a role in the RVD of the human retinal pigment epithelial (hRPE) cells studied.

We conclude that the RVD likely reflects swelling-triggered release of K^+ and Cl^- through parallel ionic channels.

Effect of Prolonged Exposure to High-Glucose Concentration on the RVD

As in the experiments of Fig. 1, anisosmotic swelling in the baseline hypotonic solution produced a similar regulatory volume decrease whether the cells had been grown in low-glucose (*A*) or high-glucose (*B*) media (Fig. 2). Furthermore, the exposure to strophanthidin and elevated K^+ concentrations inhibited the magnitude and speed of the RVD to a similar extent, whether or not the cells had been grown in high-glucose media (Exps. 1a,b, Table 1; Fig. 2).

The results of Figs. 1–2 indicate that sustained ex-

Table 1. Effects of inhibitors and ionic substitutions on rate constant (k) of RVD

Experimental conditions	Baseline k ($10^{-3} \times min^{-1}$)	% change in k	N	P
Part A: *LG-Cells*				
1a *12K$^+$,Stroph* (100 μM)	40.4 ± 3.2	−67 ± 16	4	<0.00005
2a *DiC$_8$* (350 μM)	35.3 ± 1.2	−53 ± 5	6	<0.002
3a *Stauro* (0.3 μM)	35.3 ± 1.2	−15 ± 6	6	>0.1
4a *OkadaicAc* (0.3 μM)	35.3 ± 1.2	−12 ± 3	6	<0.05
5. *Baseline HG*	29.6 ± 1.4	−11 ± 4	10	>0.05
6. *Stauro* (0.3 μM)	64.5 ± 10.8	−19 ± 13	4	>0.2
7. *Ba^{2+}* (5 mM)	55.9 ± 6.2	−25 ± 6	4	<0.02
8. *NPPB* (100 μM)	55.9 ± 6.2	−75 ± 11	4	<0.005
Part B: *HG-Cells*				
1b *12K$^+$,Stroph* (100 μM)	40.8 ± 1.6	−70 ± 6	4	<0.001
2b *DiC$_8$* (350 μM)	27.3 ± 1.1	−60 ± 4	6	<0.0002
3b *Stauro* (0.3 μM)	27.3 ± 1.1	14 ± 1	6	<0.001
4b *OkadaicAc* (0.3 μM)	27.3 ± 1.1	5 ± 5	6	>0.4

Experiments 1–4 were conducted with paired cultures grown in low-glucose (LG) and high-glucose (HG) media. Experiment 5 compares the baseline RVD measurements of the 10 paired experiments of HG- and LG- cells. Experiments 6–8 were conducted with LG-cells alone. In experiments 2–4 and 6–8, three different experimental suspensions were studied in parallel with one control suspension. The symbols have the following meanings: ($12K^+$), the K^+ concentration was raised from 3.0 to 11.8 mM; (Stroph), strophanthidin; (Stauro), staurosporine; (OkadaicAc), okadaic acid; and (Amil), amiloride.

Table 2. Effects of inhibitors and ionic substitutions on rate constant (k) of RVI

Experimental conditions	Baseline k (% × min^{-1})	% Change in k[a]	N	P
1. *Gluconate*	0.126 ± 0.009	−183 ± 35	4	<0.01
2. *Bumet* (0.5 mM)[b]	0.314 ± 0.015	−11 ± 12	4	>0.4
3. $2 \times [K^+]_o$	0.189 ± 0.033	11 ± 31	4	>0.7
4. *Bumet* ($20K^+$)[c]	0.152 ± 0.014	−46 ± 13	4	<0.05
5. *HChlor* (100 μM)[d]	0.232 ± 0.009	−15 ± 8	4	>0.1
6. *Amil* (1 mM)[d]	0.178 ± 0.035	−154 ± 25	4	<0.005
7. *Amil* (100 μM)	0.235 ± 0.028	−128 ± 18	4	<0.005
8. *Benz* (10 μM)[d]	0.278 ± 0.023	−12 ± 13	4	>0.4
9. *DMA* (10 μM)[d]	0.191 ± 0.026	−86 ± 7	4	<0.005
10. *Stroph* (100 μM)	0.189 ± 0.033	−100 ± 0	4	<0.005
11. *0 External K$^+$*	0.198 ± 0.016	−350 ± 42	4	<0.001
12. *DIDS* (1 mM)	0.345 ± 0.017	−60 ± 4	4	<0.001

[a] A negative % change in k indicates that the rate of swelling was reduced. Values more negative than −100% indicate that the RVI was reversed in sign, resulting in a further shrinkage with time.
[b] Bumetanide (Bumet).
[c] K^+ (20 mM) was included in all test solutions, and 0.5 mM bumetanide solely in the experimental suspensions.
[d] Hydrochlorthiazide (HChlor), amiloride (Amil), benzamil (Benz), and dimethylamiloride (DMA).

posure to high-glucose concentration has no detectable effect on the RVD of the hRPE cells studied.

Ionic Basis for the Baseline RVI

At least five sets of mechanisms have been considered to play a role in the RVI displayed by different cells (Siebens, 1985; Gilles, 1987; Hoffmann, 1987; Okada & Hazama, 1989; Hoffmann et al., 1993): (i) nonelectrolyte uptake, (ii) a $Na^+/K^+/2Cl^-$ symport, (iii) a Na^+/Cl^- symport, (iv) a conductive Na^+ channel, and (v) paired Na^+/H^+ and Cl^-/HCO_3^- antiports. Our data bear on each of these mechanisms.

Nonelectrolyte Uptake

In the absence of external Cl^-, the RVI is entirely abolished (Exp. 1, Table 2). Given this absolute requirement

Table 3. Effects of high-glucose incubation and PKC activation on rate (k) of RVI

Experimental conditions	Baseline k (% × min^{-1})	% change in k	N	P
1. *HG Baseline*	0.252 ± 0.012	−54 ± 13	12	<0.01
2. *Mannitol* (LG)	0.119 ± 0.032	8 ± 19	4	>0.6
3. DiC_8^c (LG)	0.309 ± 0.026	−11 ± 8	4	>0.2
DiC_8^c (HG)	0.067 ± 0.031	179 ± 130	4	<0.01
4. $Amil,DiC_8^{a,c}$ (HG)	−0.028 ± 0.045	−164 ± 445	4	>0.4
5. $Bumet,DiC_8^{a,b}$ (HG)	0.131 ± 0.027	87 ± 39	4	<0.02
6. *Stauro* (0.3 μM) (LG)	0.200 ± 0.009	−133 ± 7	4	<0.001
7. *OkadaicAc* (0.3 μM) (LG)	0.200 ± 0.009	26 ± 18	4	>0.2

[a] DiC_8 (350 μM).
[b] Bumetanide (0.5 mM) in baseline and experimental suspensions.
[c] Amiloride (100 μM) in baseline and experimental suspensions.

for Cl^-, it is unlikely that nonelectrolyte uptake plays any substantial role in generating the RVI. Furthermore, as noted below, the RVI can be blocked by inhibiting Na^+/H^+ antiport exchange with dimethylamiloride (Exp. 9, Table 2), at a concentration (10 μM) two orders of magnitude lower than the IC_{50} required for blocking Na^+/D-glucose symport activity (Kleyman & Cragoe, 1990).

$Na^+/K^+/2Cl^-$ Symport

This symport has been reported to be of importance under the experimental conditions used for studying a number of preparations of RPE cells (Adorante & Miller, 1990; Kennedy, 1990, 1992; Miller & Edelman, 1990; Edelman & Miller, 1991; Joseph & Miller, 1991; Lin & Miller, 1991). However, under the baseline conditions of the present study, 0.5 mM bumetanide had no significant effect on the RVI (Exp. 2, Table 2). It has long been appreciated that the external K^+ concentration is critical in triggering an RVI (Kregenow, 1971). Increasing the K^+ concentration from 3.0 to 6 mM had no detectable effect on the RVI (Exp. 3, Table 2). On the other hand, increasing the K^+ concentration yet further to 20 mM did elicit a detectable bumetanide-sensitive component of the RVI (Exp. 4, Table 2). We conclude that the RPE cells studied do possess a $Na^+/K^+/2Cl^-$ symport, but that this symport does not substantially contribute to the RVI under our baseline experimental conditions.

Na^+/Cl^- Symport

Two forms of a Na^+/Cl^- symport have been described: one sensitive to furosemide or bumetanide (Hoffmann, 1987) and another sensitive to 100 μM hydrochlorthiazide (Stokes, 1984; Dawson & Frizzell, 1989). Addition of either 0.5 mM bumetanide or 100 μM hydrochlorthiazide had no significant effect under the baseline conditions of the present study (Exps. 2 and 5, Table 2), excluding this form of symport as a major factor in the observed RVI.

Na^+ Channel

Epithelial Na^+ channels may contribute to the RVI displayed by cultured human intestinal (Okada & Hazama, 1989) and nonpigmented ciliary epithelial cells (M.M. Civan, M. Coca-Prados and K. Peterson-Yantorno, *in preparation*). Amiloride, in concentrations of 100 μM to 1 mM, did abolish the RVI (Fig. 3; Exps. 6–7, Table 2). However, an amiloride analogue (benzamil) with high specificity for voltage-insensitive Na^+ channels had no detectable effect on the RVI at a concentration of 10 μM (Exp. 8, Table 2). In contrast, an amiloride analogue (dimethylamiloride) with high specificity for the Na^+/H^+ antiport (Kleyman & Cragoe, 1990) did block the RVI (Exp. 9, Table 2). We conclude that it is unlikely that epithelial Na^+ channels significantly contribute to the RVI of the RPE cells studied.

Coupled Na^+/H^+ and Cl^-/HCO_3^- Antiports

The observation that dimethylamiloride, a selective inhibitor of the Na^+/H^+ antiport, blocked the RVI (Exp. 9, Table 2) supported the concept that Na^+/H^+ exchange plays a major role in generating the RVI under the conditions of the present study. Consistent with this interpretation was the observation that the RVI could be inhibited by reducing the chemical driving force for Na^+ entry by blocking the Na,K-exchange pump. The pump was inhibited both by preincubation with 100 μM strophanthidin and (separately) by omitting K^+ from the suspension medium (Exps. 10–11, Table 2).

In parallel experiments, DIDS was applied to block Cl^-/HCO_3^- exchange (Cala, 1986). DIDS was found to significantly slow the RVI (Exp. 12, Table 2).

The foregoing results have led us to conclude that

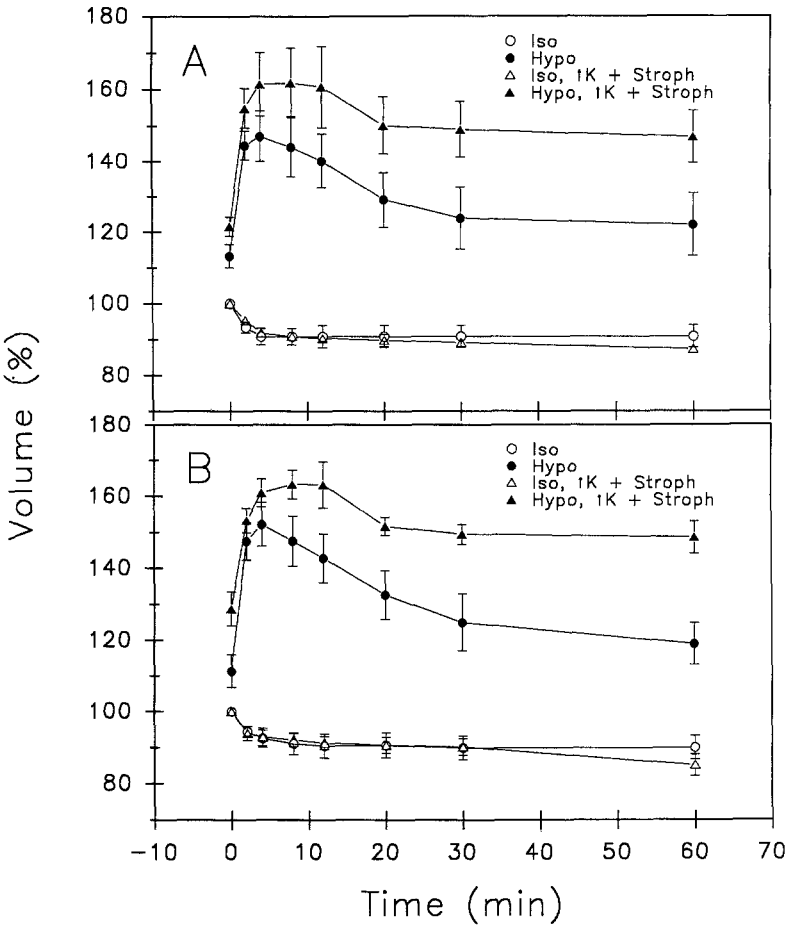

Fig. 2. Effect of reduced K^+ gradient on time courses for volumes of LG- (*A*) and HG-cells (*B*) following suspension in hypo- or isotonic solutions (Exps. 1a and 1b, Table 1). The K^+ gradients of the experimental aliquots were reduced by preincubating the cells in 100 μM strophanthidin, and suspending the cells in solution containing both the cardiotonic steroid and 5.9 mM K^+ (rather than the standard concentration of 3.0 mM).

coupled Na^+/H^+ and Cl^-/HCO_3^- antiports are largely responsible for the regulatory volume increase observed under the current baseline conditions. Elevation of the external K^+ concentration, and possibly other unidentified factors, can stimulate $Na^+/K^+/2Cl^-$ symport activity, as well.

Effect of Prolonged Exposure to High-Glucose Concentration on the RVI

The initial data of Fig. 1 indicated that the RVI was reduced by growing the RPE cells in high glucose. This point was verified by conducting eight additional paired measurements of the RVI of LG- and HG-cells. Figure 4*A* presents the means ± SE for the 12 experimental comparisons. Figure 4*B* presents the data (and linear regression) for the period beginning 28–34 min after first suspending the cells and ending ≈30 min later. In each experiment, isotonicity was restored by adding sucrose 25 min after the initial suspension in 50%-hypotonic solution. Even for LG-cells, the absolute magnitude of the RVI is much smaller than that of the RVD. Nevertheless, growth in high-glucose medium clearly reduced the rate of the regulatory volume increase by more than 50%. This inhibition was highly significant ($P < 0.01$) (Exp. 1, Table 3). This inhibition was specifically caused by the D-glucose. Incubation of cells in similar concentrations of mannitol for the same period produced no detectable change in the RVI (Exp. 2, Table 3).

Signal Transduction of the RVI

Although the signaling pathways involved in regulatory volume responses are incompletely understood, several models have been formulated (e.g., Jennings & Schulz, 1991; Hoffmann et al., 1993). One possibility is that the phosphorylation state of a gating protein may regulate the RVI (Jennings & Schulz, 1991). Of potential relevance to the present study are reports that protein kinase C (PKC) is involved in certain forms of cell volume regulation (MacLeod, Lembessis & Hamilton, 1992) and that PKC-mediated pathways are disturbed in models of diabetes mellitus (Green et al., 1987; Kim, Kyriazi & Greene, 1991; Oliver et al., 1991).

As a preliminary step in addressing how sustained glucose elevation might downregulate the RVI, protein

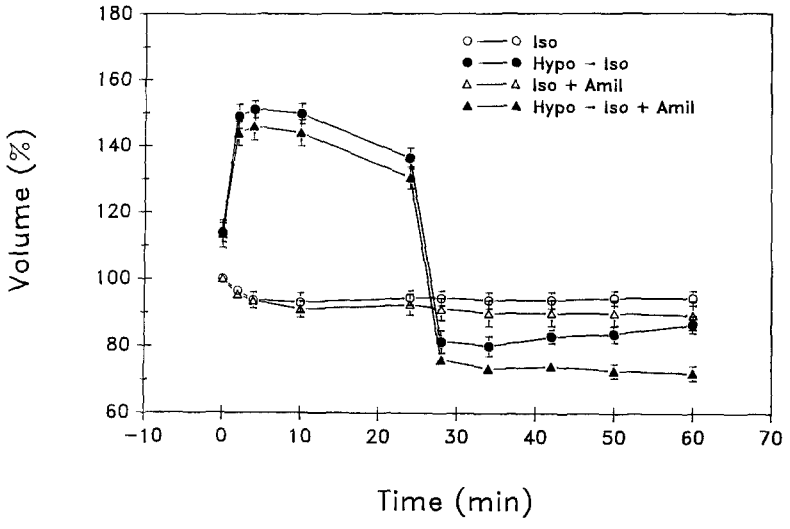

Fig. 3. Effect of 1 mM amiloride on the RVI of LG-cells (Exp. 6, Table 2).

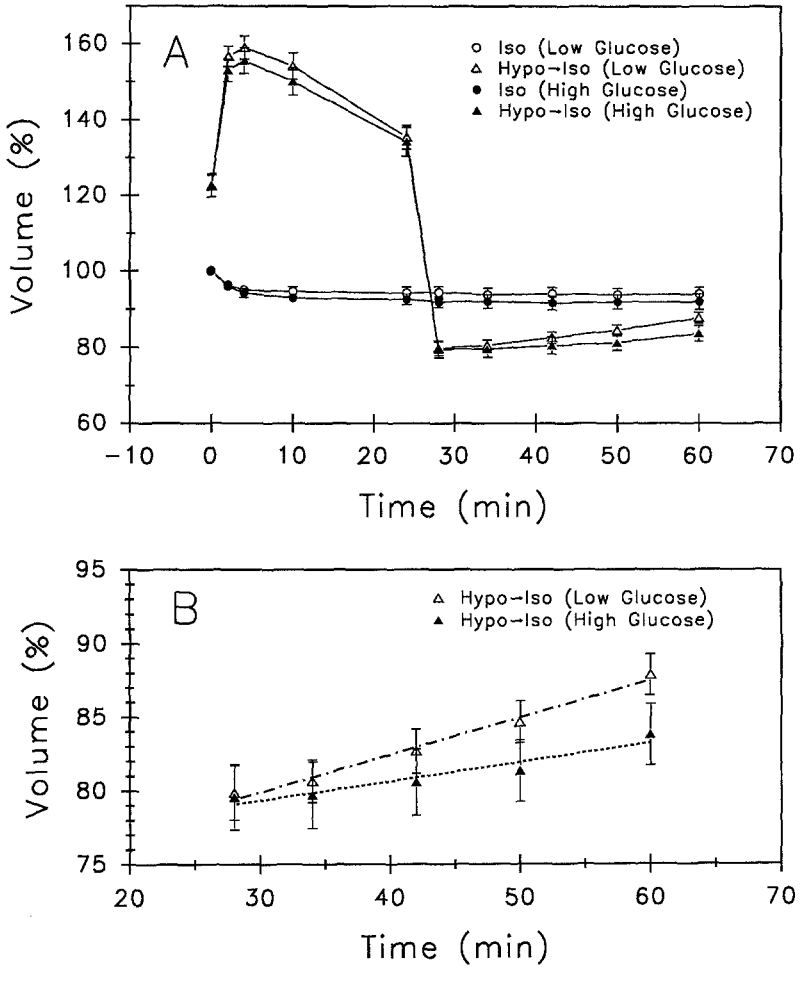

Fig. 4. The effect of growth in high-glucose media on the regulatory volume increase (RVI). The data were reduced from 12 paired experiments (Exp. 1, Table 3). Panel *B* presents the linear regressions for the RVI responses of panel *A*.

kinase C was activated by applying 350 μM DiC_8 to paired preparations of HG- and LG-cells. Activation of PKC had no effect on the RVI of the LG-cells (open squares, Fig. 5; Exp. 3, Table 3), but significantly accelerated the RVI of the HG-cells (filled squares, Fig. 5; Exp. 3, Table 3). That stimulation could have reflected PKC activation either of Na^+/H^+ antiport exchange (Mellas & Hammerman, 1986; Villereal et al.,

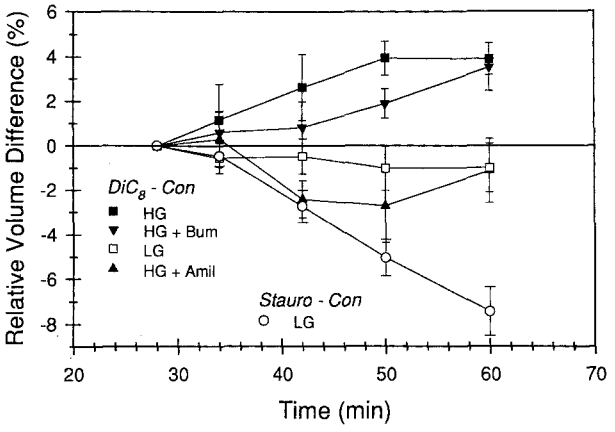

Fig. 5. Changes in RVI induced by DiC_8 or staurosporine. Each trace presents the point-by-point differences between the volumes of experimental suspensions (treated either with DiC_8 or staurosporine) and control suspensions during the RVI. For simplicity, the difference is set at zero at the initial point measured after restoring isotonicity (t = 28 min). LG-cells displayed no significant response to DiC_8 (open squares), but the RVI of the HG-cells was significantly enhanced (filled squares) (Exp. 3, Table 3). The DiC_8-stimulated RVI was abolished by adding 1 mM amiloride (filled upright triangles) (Exp. 4, Table 3), but was not significantly altered by applying bumetanide to the HG-cells (filled inverted triangles) (Exp. 5, Table 3). The time course of the volume differences between staurosporine and control LG-suspensions displayed a negative slope, reflecting a staurosporine-induced shrinkage (open circles) (Exp. 6, Table 3).

1986; Weinman & Shelonikar, 1986; Weinman et al., 1988; Slotki, Schwartz & Alexander, 1990) or of $Na^+/K^+/2Cl^-$ symport activity (Jensen, Jessen & Hoffmann, 1993). This issue was examined by activating PKC when either the $Na^+/K^+/2Cl^-$ symport was blocked with 0.5 mM bumetanide or the Na^+/H^+ antiport was blocked with 100 μM amiloride. The PKC-elicited stimulation was retained in the presence of bumetanide (filled inverted triangles, Fig. 5; Exp. 5, Table 3), but was abolished by amiloride (filled upright triangles, Fig. 5; Exp. 4, Table 3). As illustrated in Fig. 5, in the presence of amiloride, PKC activation produced a transient shrinkage, but the rate of change of cell volume was insignificantly different from zero over the entire period of observation (Exp. 4, Table 3).

One interpretation of the foregoing data is that LG-cells responded to swelling by activating PKC to a greater degree than did HG-cells. The activated PKC could then stimulate Na^+/H^+ turnover (Mellas & Hammerman, 1986; Villereal et al., 1986; Weinman & Shelonikar, 1986; Weinman et al., 1988; Slotki et al., 1990), enhancing the RVI. Consistent with this interpretation was the finding that 0.3 μM staurosporine reversed the sign of the RVI, resulting in continued shrinkage of the LG-cells even after restoring isotonicity (open circles, Fig. 5; Exp. 6, Table 3). Addition of the protein-phosphatase inhibitor okadaic acid had no significant effect (Exp. 7, Table 3) at a concentration (0.3 μM) sufficiently high to inhibit protein phosphatase 2A activity (Bialojan & Takai, 1988).

If the defect in the RVI of the HG-cells reflected insufficient activation of PKC, we would expect that other actions of PKC should also be affected in the HG-cells. For this reason, we examined whether inhibition of PKC (with 0.3 μM staurosporine) and activation of PKC (with 350 μM DiC_8) affected the regulatory response to swelling. As illustrated by Fig. 6*A*, adding staurosporine to the LG-cells dramatically increased the magnitude of the RVD without greatly affecting the rate constant (Exp. 6, Table 1). Even in isotonic suspension, staurosporine caused significant cell shrinkage (Fig. 6*A*). As expected, an opposite effect was noted following PKC activation with DiC_8, a slowing of the RVD by the LG-cells (Fig. 6*B;* Exp. 2a, Table 1). Nevertheless, in paired experiments with HG- and LG-cells, comparably large effects were exerted on the rate constant of the RVD by DiC_8, and on the magnitude of the RVD by staurosporine. Only small responses were observed to okadaic acid (Figs. 6*B–C;* Exps. 2a–4a and 2b–4b, Table 1).

Discussion

Baseline Regulatory-Volume Properties

The nontransformed cells (hRPE) used in the present study were derived from human retinal pigment epithelium (Del Monte & Maumenee, 1980; Dutt et al., 1989, 1990; Del Monte et al., 1991), phagocytose rod outer segments (Del Monte et al., 1991), and display at least some structural features of RPE cells in tissue (Del Monte et al., 1991), such as microvilli and tight junctions. In view of these structural and functional characteristics, hRPE cells provide a favorable and convenient model of human RPE cells.

Suspension of the RPE cells in hypotonic medium produced swelling and triggered a secondary regulatory volume decrease (RVD) (Fig. 1*A*). The RVD was inhibited by reducing the concentration gradient for K^+ efflux (Fig. 2*A*, Exp. 1a, Table 1) and by blocking K^+ (Exp. 7, Table 1) and Cl^- channels (Exp. 8, Table 1). It is therefore reasonable to presume that the RVD largely reflects loss of KCl from the hRPE cells through parallel K^+ and Cl^- channels, as has been observed with many other cells (Hoffmann, 1987; Hoffmann et al., 1993).

The hRPE also displayed a regulatory volume increase (RVI), albeit of small magnitude (Fig. 1*A*). The RVI displayed an absolute dependence on external Cl^- (Exp. 1, Table 2), and was inhibited by both DIDS (which blocks Cl^-/HCO_3^- exchange) (Exp. 12, Table 2)

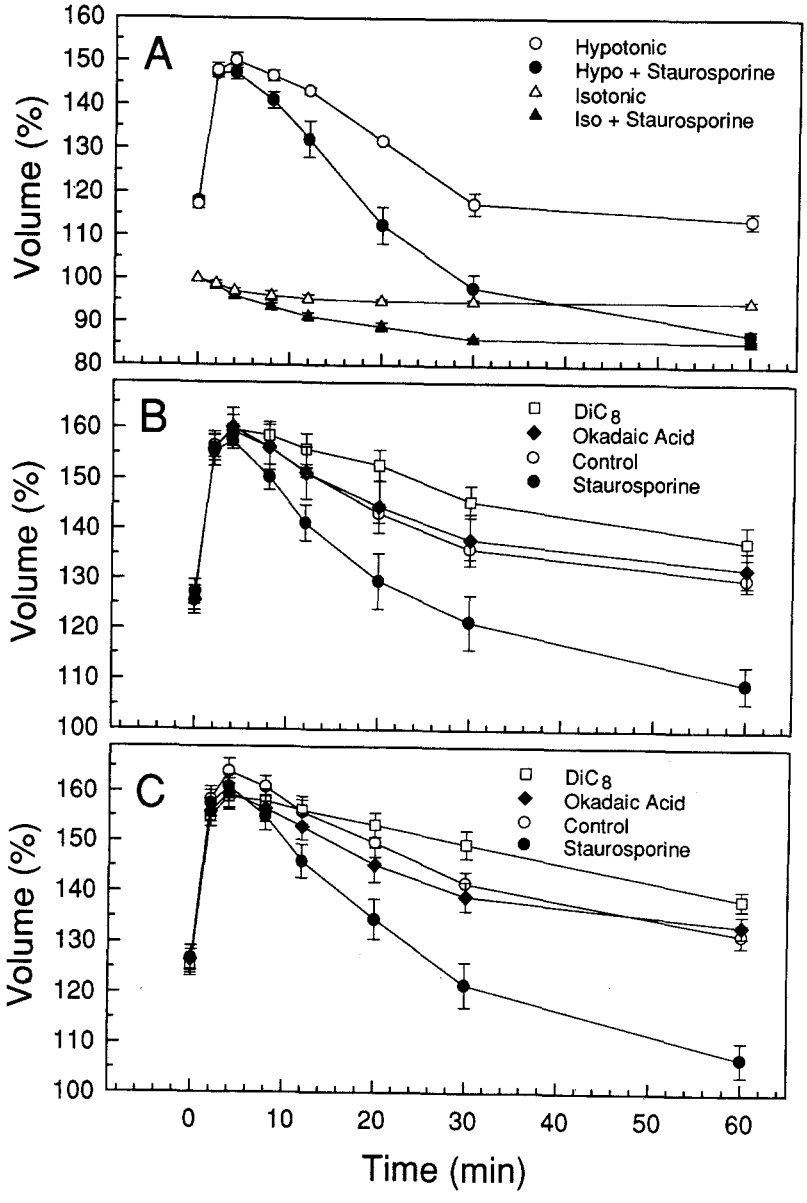

Fig. 6. Effects of DiC_8, staurosporine and okadaic acid on RVD. Panel *A* demonstrates that 0.3 μM staurosporine markedly increased the magnitude of the RVD of LG-cells without greatly affecting the time constant of that response (Exp. 6, Table 1); staurosporine also triggered a significant shrinkage of LG-cells suspended in isotonic solution. Panels *B* and *C* present the RVD responses of LG- and HG-cells, respectively (Exps. 2–4, Table 1). The major effects were a DiC_8-triggered slowing of the RVD, and a staurosporine-elicited increase in the magnitude of the RVD (with little change in the time constant). DiC_8 exerted similar effects on the RVD of the LG- and HG-cells, in contrast to the different actions noted on the RVI (Fig. 5).

and by 10 μM dimethylamiloride (a selective blocker of Na^+/H^+ exchange) (Exp. 9, Table 2). Benzamil, which is a selective blocker of epithelial Na^+ channels, had no effect at the same 10 μM concentration (Exp. 8, Table 2). We conclude that parallel Na^+/H^+ and Cl^-/HCO_3^- antiports were primarily responsible for the RVI observed under our baseline conditions. This conclusion conforms to evidence indicating an apical Na^+/H^+ antiport and a basolateral Cl^-/HCO_3^- antiport in RPE cells studied in different preparations (Lin & Miller, 1991). A detectable bumetanide-sensitive component of the RVI could be elicited by increasing the K^+ concentration of the bath (Exp. 4, Table 2). This likely corresponds to the $Na^+/K^+/2Cl^-$ symport reported to be of importance under the experimental conditions used for studying a number of other preparations of RPE cells (Adorante & Miller, 1990; Kennedy, 1990, 1992; Miller & Edelman, 1990; Edelman & Miller, 1991; Joseph & Miller, 1991; Lin & Miller, 1991). It has long been known that the external K^+ concentration is critical in triggering an RVI (Kregenow, 1971). Thus, the hRPE cells likely possess a $Na^+/K^+/2Cl^-$ symport, which did not significantly contribute to the RVI under our baseline experimental conditions.

Effects of Sustained Exposure to High-Glucose Concentrations

Determination of the baseline RVD and RVI properties of the hRPE permitted us to examine possible defects in membrane transport triggered by sustained elevation in extracellular glucose concentration. This issue is timely for two reasons. First, transport defects have been found in target tissues of diabetes mellitus in experimental models of that disease (MacGregor & Matschinsky, 1986; Greene et al., 1987; Winegrad, 1987; Pierce et al., 1990). Second, after decades of uncertainty, it is now clear that the elevation in glucose concentration, *per se,* is the major risk factor in the pathogenesis of the complications of diabetes (Engerman et al., 1977; Klein et al., 1988; Lasker, 1993; The Diabetes Control & Complications Trial Res. Group, 1993). Despite this information, several issues require clarification: (i) What transport processes are reproducibly affected by elevated glucose levels? (ii) How does the glucose elevation trigger these changes? (iii) Do the transport defects play a significant role in the pathogenesis of the complications of diabetes? In the present work, we have begun to address the first two of these issues.

The strategy of the current work was to study a cell (the RPE) reported to develop an abnormality in transport [of the Na,K-exchange pump (MacGregor & Matschinsky, 1986)], using the simplest possible experimental model of diabetes mellitus. The RVD of most vertebrate cells reflects release of cell K^+ and Cl^- (Hoffmann, 1987). In turn, the accumulation of the cell K^+ poised for release is critically dependent on the integrity of the Na,K-exchange pump. We have found that sustained exposure to elevated glucose levels has very little effect on the baseline RVD (Fig. 1; Exp. 5, Table 1*A*) or on the responses to inhibitors (Figs. 2, 6*B–C;* Exp. 2, Table 1) or to an enhancer of the RVD (Figs. 6*B–C*). These data suggest that sustained glucose elevation does not substantially inhibit the Na,K-activated ATPase of the hRPE cells. This conclusion, based on indirect evidence in the present study, is consistent with measurements of ouabain-inhibitable $^{86}Rb^+$ uptake in the same lines incubated under similar conditions (Marano et al., 1990). Thus, growth of cultured cells in high-glucose concentration may be an incomplete model for generating the defect in Na,K-activated ATPase observed in certain other experimental models of diabetes mellitus (MacGregor & Matschinsky, 1986; Greene et al., 1987; Winegrad, 1987). Alternatively, depression of the pump may not be an exorable sequel to diabetes in all target cells.

In contrast, sustained exposure to an elevated glucose concentration did inhibit another transport phenomenon, the regulatory volume increase (RVI) (Fig. 4; Exp. 1, Table 3), suggesting that either the underlying transporters (the Na^+/H^+ and Cl^-/HCO_3^- antiports) or the control of those transporters had been blocked.

Possible Basis for Glucose-Triggered Inhibition of the RVI

The possibility that glucose elevation may lead to defective intracellular signaling has received considerable attention (Kinoshita, 1974; Greene et al., 1987; Winegrad, 1987; Matschinsky & MacGregor, 1988), but a unified and compelling general hypothesis is not yet available. Of interest has been the polyol accumulation in multiple tissues resulting from aldose reductase action on an increased load of glucose (Matschinsky & MacGregor, 1988). A small, possibly nonmeasurable, pool of myoinositol has been hypothesized to be of pathogenic importance (Winegrad, 1987). The hRPE cells studied here also respond to growth in high-glucose medium with increased sorbitol and reduced myoinositol concentrations; the sorbitol accumulation can be blocked by inhibiting aldose reductase activity with sorbinil (Marano et al., 1990).

Of particular interest in the present context is the observation that models of diabetes mellitus have been associated with disturbances of PKC-mediated pathways (Greene et al., 1987; Kim, Kyriazi & Greene, 1991; Oliver et al., 1991). Furthermore, PKC is involved in certain forms of cell volume regulation (MacLeod, Lembessis & Hamilton, 1992). In the present study, activation of PKC stimulated the RVI of HG-cells by a bumetanide-insensitive, amiloride- inhibitable mechanism (Fig. 5; Exp. 3, Table 3). This is likely to reflect stimulation of baseline Na^+/H^+ antiport activity, as reported for Na^+/H^+ antiports in several other preparations (Mellas & Hammerman, 1986; Villereal et al., 1986; Weinman & Shelonikar, 1986; Weinman et al., 1988; Slotki et al., 1990). These observations have led us to conclude that high glucose levels lead to inhibition of the response of the Na^+/H^+ antiport to anisosmotic swelling. This conclusion is consistent with the report that Na^+/H^+ antiport exchange is downregulated in a more complex model of diabetes mellitus (Pierce et al., 1990).

In principle, the downregulation of the Na^+/H^+ antiport could reflect either inhibition of the transporter itself or interruption of the PKC-signaling pathway. To distinguish between these possibilities, we examined the effects of stimulating PKC (with DiC_8) or inhibiting PKC (with staurosporine) on other transport pathways of the same cells. Growth in high-glucose medium had no substantial effect on the responses of the RVD to either DiC_8 or staurosporine (Figs. 6*B–C*). We conclude that sustained exposure to high-glucose con-

centration downregulates the Na^+/H^+ antiport without disrupting the PKC-signaling pathway of hRPE cells.

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