Originals

Mechanism of glucose-induced (Na^+ , K^+)-ATPase inhibition in aortic wall of rabbits

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Summary. Hyperglycaemia decreases (Na^+ , K^+)-ATPase activity in specific tissues by a mechanism whose effects are prevented by aldose reductase inhibitors and by raising plasma myo-inositol. This mechanism was activated and studied in vitro in normal rabbit aortic intima-media. Raising medium glucose to 10 mmol/l for 60 min inhibited a major component of (Na^+ , K^+)-ATPase-mediated $^{86}\text{Rb}^+/\text{K}^+$ uptake normally operative in resting aortic intima-media in medium containing normal plasma levels of glucose (5 mmol/l) and myo-inositol (70 $\mu\text{mol/l}$); 20 or 30 mmol/l glucose had no greater effect. This effect occurred under conditions in which the aortic intima-media's normal myo-inositol content is not detectably decreased. The inhibition was prevented by sorbinil (10 $\mu\text{mol/l}$) and by raising medium myo-inositol from 70 to 500 $\mu\text{mol/l}$, which had no effect on (Na^+ , K^+)-ATPase activity when the medium glucose remained at 5 mmol/l. Raising medium glucose selectively inhibited a component of (Na^+ , K^+)-ATPase activity that requires medium myo-inositol, because it is maintained by a regulatory system through rapid basal phosphatidylinositol turnover in a discrete pool, which is replenished by

a fraction of basal de novo phosphatidylinositol synthesis that is selectively dependent on myo-inositol uptake. Medium myo-inositol at a normal plasma level became inadequate to maintain this fraction of basal de novo phosphatidylinositol synthesis ([1,3- ^{14}C]glycerol incorporation) when the medium glucose was raised. When sorbinil was added raising medium glucose did not alter the ability of 70 $\mu\text{mol/l}$ medium myo-inositol to maintain the (Na^+ , K^+)-ATPase activity that requires medium myo-inositol. The inhibition caused by raising medium glucose was reproduced by a competitive inhibitor of active myo-inositol transport, scyllo-inositol (500 $\mu\text{mol/l}$). The effect of medium glucose in an elevated plasma level on (Na^+ , K^+)-ATPase activity in aortic intima-media appears to result from increased polyol pathway activity that makes myo-inositol uptake at a normal plasma level inadequate to maintain the normal operation of a regulatory system.

Key words: Glucose, (Na^+ , K^+)-ATPase, myo-inositol, phosphatidylinositol, aldose reductase.

Hyperglycaemia decreases (Na^+ , K^+)-ATPase activity and induces early functional alterations in components of peripheral nerve, kidney, and retina in diabetic animals by a common mechanism whose identifying characteristic is that its effects are prevented by aldose reductase inhibitors and by raising the otherwise normal plasma myo-inositol (MI) level in diabetic animals some 7-fold [1–9]. It has been assumed that weeks of hyperglycaemia are required to cause the decreased (Na^+ , K^+)-ATPase activities, as well as the functional alterations. Consequently, studies of this mechanism have been restricted to comparisons of metabolite levels and assays of (Na^+ , K^+)-ATPase in tissues from normal animals and from untreated or treated animals with diabetes of some weeks duration. Such studies suggest that hyperglycaemia acts through increased polyol pathway activity to induce some alteration in MI meta-

bolism that impairs (Na^+ , K^+)-ATPase regulation in the affected tissues [1, 2]; the increased polyol pathway activity is postulated to alter MI metabolism by impairing MI uptake. Diabetes of some weeks duration decreases MI in the affected tissues and this is prevented by aldose reductase inhibitors and by raising plasma MI [3, 5–8]; this has led to the hypothesis that “myo-inositol depletion” causes the decreased (Na^+ , K^+)-ATPase activity [2]. However, it is now known that in renal glomeruli the decrease in (Na^+ , K^+)-ATPase activity is present weeks before any detectable decrease in tissue MI [10], and the latter appears to be a delayed secondary effect.

A mechanism by which impaired MI uptake might decrease (Na^+ , K^+)-ATPase activity in specific tissues without detectable decreases in their normal MI contents was suggested by the observation that normal rab-

bit peripheral nerve endoneurium and aortic intima-media (AIM) require their low normal extracellular fluid (ECF) MI levels during incubations with 5 mmol/l glucose to prevent the inhibition of distinct components of their normal resting (Na^+ , K^+)-ATPase activity and phosphatidylinositol (PI) synthesis, although they do not require medium MI to maintain their high normal MI contents [11, 12]. These phenomena were explained by the demonstration that AIM contains a regulatory system that controls and maintains a major component of normal resting (Na^+ , K^+)-ATPase activity through rapid basal PI turnover in a discrete pool, and that this pool is maintained by a distinct fraction of basal PI synthesis that is selectively inhibited when MI uptake is prevented by the absence of medium MI [12]. The polyol pathway is present in rabbit AIM [13] and raising medium glucose from a normal to an elevated plasma level increases AIM sorbitol [14]. We have examined whether raising medium glucose from a normal to an elevated plasma level causes a decrease in normal resting (Na^+ , K^+)-ATPase activity in AIM that can be prevented by adding an aldose reductase inhibitor and by raising the medium MI from a normal plasma level to one 7-fold higher.

Materials and methods

Male, white New Zealand rabbits (2.0–2.5 kg) were fasted overnight, sedated with diazepam (2 mg/kg i.m.), and 90 min later anaesthetized with sodium pentobarbital (30 mg/kg i.v.) and decapitated. The descending thoracic aorta was rapidly excised and by a previously reported method [15, 16] used to prepare 4 tubular segments of aortic intima-media free of adventitia and retaining an intact endothelium with a normal transmission and scanning electron microscopic appearance. Each AIM segment weighed approximately 30 mg. All media to which the tissue was exposed contained 9% clinical grade dextran, average molecular weight 70000 (Sigma Chemical Co., St. Louis, MO, USA) to provide the oncotic pressure required to maintain this tissue's normal ultrastructure and pattern of energy metabolism [15]. The aorta was dissected in Krebs-Henseleit-bicarbonate (KHB) buffer [17], pH 7.4 at 37°C, bubbled with 5% CO_2 /95% O_2 , that contained glucose (5 mmol/l) and myo-inositol in a normal rabbit plasma level, 70 $\mu\text{mol/l}$ [18]. The dissected aorta was cut into 4 tubular segments, designated 1 to 4 according to their proximal to distal locations in situ. Each anatomically defined segment was systematically rotated into each of the 4 experimental conditions required to compare (Na^+ , K^+)-ATPase activity in paired duplicate samples of AIM from the same aorta in two media that differed only in their levels of glucose or MI, or in the addition of another compound. (Na^+ , K^+)-ATPase activity was assessed by determinations of ouabain-inhibitable intracellular $^{86}\text{Rb}^+$ / K^+ uptake, as described below. Transport activity of (Na^+ , K^+)-ATPase can be monitored with $^{86}\text{Rb}^+$, which substitutes for K^+ in the activation of ATP hydrolysis and the active transport of K^+ [19]. The individual AIM samples were equilibrated at 37°C for 30 min in 3 ml of KHB buffer gassed with 5% CO_2 /air that contained specified levels of glucose and MI in a 10 ml Erlenmeyer flask in a metabolic shaker set at 88 cycles per min. In each experiment duplicate samples were equilibrated in each of the two media to be compared. The samples were then transferred to 3 ml of fresh medium of the same composition to which a tracer level of [^3H]sucrose, 0.64 $\mu\text{Ci/ml}$, 9.5 Ci/mmol, had been added, along with ouabain (0.2 mmol/l) in one of the duplicate samples to be incubated with each medium, and the samples pre-incubated for 10 min. A

tracer quantity of [$^{86}\text{Rb}^+$]RbCl, 0.25 $\mu\text{Ci/ml}$, 0.46 Ci/mmol, was then added to each flask and the samples incubated for 20 min. The tissue was then rapidly removed, drained on filter paper, weighed, and transferred into 2 ml of Protosol (New England Nuclear, Boston, Mass, USA) in a glass scintillation vial and digested for 16 h at room temperature; 0.1 ml of H_2O_2 was then added and the samples incubated for 1 h at 37°C. Econofluor 2 (New England Nuclear) was added and the sample counted for $^{86}\text{Rb}^+$ and ^3H in a liquid scintillation spectrometer with an external standard. The $^{86}\text{Rb}^+$ counts in the sample were corrected for $^{86}\text{Rb}^+$ in extracellular fluid by means of the [^3H]sucrose recovered in the sample and the ratio of $^{86}\text{Rb}^+$ to ^3H counts in the medium. The medium K^+ and the medium $^{86}\text{Rb}^+$ counts/l were determined and these data used to express the corrected recovery of $^{86}\text{Rb}^+$ in the sample as $\mu\text{mol } ^{86}\text{Rb}^+/\text{K}^+ \text{ uptake} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$. The difference between the uptake of the two samples incubated in the absence and presence of ouabain was used as an estimate of the (Na^+ , K^+)-ATPase-mediated $^{86}\text{Rb}^+/\text{K}^+$ uptake. The values obtained by this method were linear in assays conducted over 10 to 20 min in paired samples from the same aorta. KHB buffer contains normal plasma levels of K^+ and Na^+ [17].

To assess basal de novo PI synthesis the 4 AIM samples from each aorta were used to provide two paired samples, which were equilibrated for 30 min in 6 ml of KHB buffer containing specified concentrations of glucose and MI, and then transferred to fresh medium of the same composition to which 0.1 mmol/l [$^{1,3-^{14}\text{C}}$]glycerol, 55 mCi/ml, had been added, and the samples incubated for 15 min at 37°C, while being continuously gassed with 5% CO_2 /air. The tissue was then rapidly removed, frozen in liquid N_2 that had been partially evaporated to its freezing point, weighed, and powdered in liquid N_2 , as previously described [12]. Using methods previously described [12] the phospholipids were extracted, PI isolated by two-dimensional thin layer chromatography, and the PI counted to assess the [$^{1,3-^{14}\text{C}}$]glycerol incorporated into PI.

The sources of the material used were: ouabain (Sigma Chemical Co., St. Louis, Mo, USA); myo-inositol (Pfhanstiehl Labs Inc., Waukegan, Ill, USA); scyllo-inositol (Calbiochem, La Jolla, Calif, USA); [$^{86}\text{Rb}^+$]RbCl, [^3H]sucrose, and [$^{1,3-^{14}\text{C}}$]glycerol (New England Nuclear, Boston, Mass, USA); reference standards of PI and other phospholipids (Serdary Labs, New London, Ontario, Canada). Sorbinil was a gift from Pfizer Inc. (Groton, Conn, USA). Tolrestat was a gift from Wyeth-Ayerst Pharmaceuticals (Radnor, Pa, USA).

Statistical analysis

The mean $\Delta \pm \text{SEM}$ between paired samples in each group of paired experiments was analysed for significance by a paired t test analysis. The difference between the mean $\Delta \pm \text{SEM}$ of one group of paired experiments and another group was analysed for significance by an unpaired t test analysis.

Results

Normal resting (Na^+ , K^+)-ATPase activity in AIM is comprised of two distinct components that differ in a requirement for ECF myo-inositol; one component is selectively inhibited when AIM is deprived of medium MI for 30 min leaving another component that remains constant for at least an additional 45 min [12]. In AIM incubated with 5 mmol/l glucose, medium MI in a normal plasma level (70 $\mu\text{mol/l}$) is adequate to maintain the component that requires ECF MI, whose magnitude is constant once the threshold requirement for its maintenance (50 $\mu\text{mol/l}$) is met and is unaltered by fur-

Table 1. Effects of medium myo-inositol on (Na⁺, K⁺)-ATPase activity in normal resting aortic intima-media incubated with 5 mmol/l glucose

		(Na ⁺ , K ⁺)-ATPase-mediated ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
a) Medium MI	0 $\mu\text{mol/l}$	70 $\mu\text{mol/l}$	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	1.31 $\pm$ 0.24	2.65 $\pm$ 0.18	+ 1.34 $\pm$ 0.13	(7)	< 0.001
b) Medium MI	70 $\mu\text{mol/l}$	500 $\mu\text{mol/l}$	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.56 $\pm$ 0.38	2.70 $\pm$ 0.25	+ 0.14 $\pm$ 0.13	(6)	NS
		Non-ouabain-inhibitable ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
c) Medium MI	0 $\mu\text{mol/l}$	70 $\mu\text{mol/l}$	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	3.53 $\pm$ 0.34	3.23 $\pm$ 0.20	- 0.29 $\pm$ 0.34	(7)	NS

Paired duplicate aortic intima-media samples were equilibrated for 30 min and incubated for 30 min in Krebs Henseleit-bicarbonate buffer containing 5 mmol/l glucose and 9% dextran with myo-inositol (MI) added in the concentrations indicated in the table. (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake was assessed during the final 20 min of the incubation. The values are means $\pm$ SEM and the mean $\Delta \pm \text{SEM}$ between paired samples. *p* was determined by a paired *t* test analysis

ther increases in medium MI up to at least 500 $\mu\text{mol/l}$. Under the conditions employed in this study these two components of normal resting (Na⁺, K⁺)-ATPase activity were readily distinguishable. When AIM was equilibrated and incubated in KHB buffer containing 5 mmol/l glucose (and the 9% dextran present in all media) the addition of 70 $\mu\text{mol/l}$ MI to the medium maintained a large component of (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake that was absent in paired samples deprived of medium MI (Table 1, a). Raising the medium MI from 70 to 500 $\mu\text{mol/l}$ had no effect on (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake in AIM incubated with 5 mmol/l glucose (Table 1, b). (The non-ouabain-inhibitable ⁸⁶Rb⁺/K⁺ uptake in AIM incubated with 5 mmol/l glucose was not significantly different in medium lacking or containing 70 $\mu\text{mol/l}$ MI, as shown in Table 1, c).

In AIM deprived of medium MI raising medium glucose from 5 to 30 mmol/l had no effect on (Na⁺, K⁺)-ATPase activity (Table 2, a). However, in AIM provided with 70 $\mu\text{mol/l}$ medium myo-inositol raising medium glucose from 5 to 10 mmol/l caused a marked decrease in (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake (Table 2, b), and raising the medium glucose to 20 or 30 mmol/l had no greater effect (Table 2, c and d). Raising the medium glucose to 10 mmol/l inhibited a component of (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake that was not significantly different from that maintained by medium MI in a normal plasma level in AIM incubated with 5 mmol/l glucose (1.36 $\pm$ 0.25 vs 1.34 $\pm$ 0.13 $\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$, Tables 2, b and 1, a). Thus, in AIM provided with medium MI in a normal plasma level raising medium glucose from 5 to 10 mmol/l for 60 min selectively and completely inhibits the major component of normal resting (Na⁺, K⁺)-ATPase activity that requires ECF MI and is normally maintained by medium MI in a normal plasma level.

In AIM incubated with 30 mmol/l glucose raising

Table 2. Effect of raising medium glucose on (Na⁺, K⁺)-ATPase activity in normal resting aortic intima-media (AIM) in medium lacking or containing a normal plasma myo-inositol (MI) level

		(Na ⁺ , K ⁺)-ATPase-mediated ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
Medium MI	Medium Glucose Concentration				
a) 0 $\mu\text{mol/l}$	5 mmol/l	30 mmol/l	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	1.24 $\pm$ 0.10	1.33 $\pm$ 0.18	+ 0.09 $\pm$ 0.24	(6)	NS
b) 70 $\mu\text{mol/l}$	5 mmol/l	10 mmol/l	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.53 $\pm$ 0.21	1.17 $\pm$ 0.20	- 1.36 $\pm$ 0.25	(5)	< 0.001
c) 70 $\mu\text{mol/l}$	5 mmol/l	20 mmol/l	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.37 $\pm$ 0.16	1.09 $\pm$ 0.17	- 1.28 $\pm$ 0.11 ^a	(5)	< 0.001
d) 70 $\mu\text{mol/l}$	5 mmol/l	30 mmol/l	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.72 $\pm$ 0.28	1.03 $\pm$ 0.25	- 1.69 $\pm$ 0.37 ^a	(6)	< 0.01

Paired duplicate AIM samples were equilibrated for 30 min and incubated for 30 min in Krebs Henseleit-bicarbonate buffer containing 9% dextran and glucose and MI in the concentrations indicated. (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake was assessed during the final 20 min of the incubation. *p* for the mean $\Delta \pm \text{SEM}$ between the paired samples in each group of experiments was determined by a paired *t* test analysis. ^aNot significantly different from the mean $\Delta \pm \text{SEM}$ in b by Student's *t* test analysis

Table 3. Effect of raising medium myo-inositol (MI) from a normal to a pharmacological plasma level on (Na⁺, K⁺)-ATPase activity in aortic intima-media (AIM) exposed to 30 mmol/l glucose

		(Na ⁺ , K ⁺)-ATPase-mediated ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
Medium MI	70 $\mu\text{mol/l}$	500 $\mu\text{mol/l}$	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	1.19 $\pm$ 0.12	2.43 $\pm$ 0.18	+ 1.24 $\pm$ 0.14	(7)	< 0.001

Paired duplicate AIM samples equilibrated for 30 min and incubated for 30 min in Krebs Henseleit-bicarbonate buffer containing 30 mmol/l glucose and 9% dextran with MI in the concentrations indicated. (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake was assessed during the final 20 min of the incubation. *p* for the mean $\Delta \pm \text{SEM}$ between the paired samples was determined by a paired *t* test analysis

Table 4. Effect of raising medium glucose on (Na⁺, K⁺)-ATPase activity in aortic intima-media in medium containing a normal plasma myo-inositol (MI) level when an aldose reductase inhibitor is present

		(Na ⁺ , K ⁺)-ATPase-mediated ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
a) Medium glucose	5 mmol/l	30 mmol/l + sorbinil	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.57 $\pm$ 0.34	2.20 $\pm$ 0.40	-0.37 $\pm$ 0.19	(7)	NS
b) Medium glucose	5 mmol/l	5 mmol/l + sorbinil	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.50 $\pm$ 0.14	2.50 $\pm$ 0.35	0.00 $\pm$ 0.34	(9)	NS
c) Medium glucose	5 mmol/l	30 mmol/l + tolrestat	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
	2.44 $\pm$ 0.17	2.31 $\pm$ 0.20	-0.13 $\pm$ 0.18	(5)	NS

Paired duplicate AIM samples were equilibrated for 30 min and incubated for 30 min in Krebs Henseleit-bicarbonate buffer containing 70 $\mu\text{mol/l}$ MI and 9% dextran with glucose in the concentrations indicated. When added sorbinil (10 $\mu\text{mol/l}$) or tolrestat (1 $\mu\text{mol/l}$) was present during the equilibration and incubation. (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake during the final 20 min of the incubation was compared in the paired samples

Table 5. Effects of scyllo-inositol (500 $\mu\text{mol/l}$) on (Na⁺, K⁺)-ATPase activity in aortic intima-media (AIM) in medium containing 5 mmol/l glucose and 70 or 500 $\mu\text{mol/l}$ myo-inositol (MI)

		(Na ⁺ , K ⁺)-ATPase-mediated ⁸⁶ Rb ⁺ /K ⁺ uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			
Medium MI	Control	+ scyllo-inositol	Mean $\Delta \pm \text{SEM}$	<i>n</i>	<i>p</i>
a) 70 $\mu\text{mol/l}$	2.59 $\pm$ 0.20	1.32 $\pm$ 0.30	-1.27 $\pm$ 0.36	(9)	< 0.01
b) 500 $\mu\text{mol/l}$	2.62 $\pm$ 0.40	2.80 $\pm$ 0.31 + sorbinil	+0.18 $\pm$ 0.33	(7)	NS
c) 70 $\mu\text{mol/l}$	2.43 $\pm$ 0.12	1.38 $\pm$ 0.18	-1.05 $\pm$ 0.19	(9)	< 0.001

Paired duplicate AIM samples were equilibrated for 30 min and incubated for 30 min in Krebs Henseleit-bicarbonate buffer containing 5 mmol/l glucose, 9% dextran and the MI indicated, with scyllo-inositol (500 $\mu\text{mol/l}$) added to one of the paired samples. (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake during the final 20 min of the incubation was compared in the paired samples. In c sorbinil (10 $\mu\text{mol/l}$) was added to sample exposed to scyllo-inositol

the medium MI. From 70 to 500 $\mu\text{mol/l}$ caused an increase in (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake (Table 3) that was not significantly different from the component inhibited by 30 mmol/l glucose in AIM provided with 70 $\mu\text{mol/l}$ medium MI (+1.24 $\pm$ 0.14 vs -1.69 $\pm$ 0.37 $\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$, Tables 3 and 2,d). When sorbinil (10 $\mu\text{mol/l}$) was added raising the medium glucose from 5 to 30 $\mu\text{mol/l}$ did not decrease (Na⁺, K⁺)-ATPase activity in AIM provided with 70 $\mu\text{mol/l}$ medium MI (Table 4,a). The addition of sorbinil (10 $\mu\text{mol/l}$) had no effect on (Na⁺, K⁺)-ATPase activity in AIM incubated with 5 mmol/l glucose and 70 $\mu\text{mol/l}$ MI (Table 4,b), and it does not appear to affect (Na⁺, K⁺)-ATPase activity in AIM unless it is exposed to medium glucose in an elevated plasma level. Tolrestat (1 $\mu\text{mol/l}$), another aldose reductase inhibitor with a significantly different structure [20], also prevented the effect of 30 mmol/l glucose on (Na⁺, K⁺)-ATPase activity in AIM provided with 70 $\mu\text{mol/l}$ medium MI (Table 4,c). These observations suggest that raising medium glucose from a normal to an elevated plasma level causes medium MI in a normal plas-

ma level to become inadequate to maintain a specific component of normal resting (Na⁺, K⁺)-ATPase activity in AIM, and that this effect requires aldose reductase activity.

Scyllo-inositol is a competitive inhibitor of active MI transport [21, 22] that is normally present in rabbit plasma in very low levels, < 5 $\mu\text{mol/l}$ [18]. In AIM incubated with 5 mmol/l glucose in medium lacking MI, adding 500 $\mu\text{mol/l}$ scyllo-inositol has no effect on (Na⁺, K⁺)-ATPase activity [12]. However, as shown in Table 5,a, in AIM incubated with 5 mmol/l glucose and 70 $\mu\text{mol/l}$ MI the addition of 500 $\mu\text{mol/l}$ scyllo-inositol inhibited a component of (Na⁺, K⁺)-ATPase-mediated ⁸⁶Rb⁺/K⁺ uptake, 1.27 $\pm$ 0.36 $\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$, not significantly different from those inhibited by raising the medium glucose to 10–30 mmol/l (see Table 2,b–d). This effect of 500 $\mu\text{mol/l}$ scyllo-inositol on (Na⁺, K⁺)-ATPase activity in AIM incubated with normal plasma levels of glucose and MI was prevented when the medium MI was raised from 70 to 500 $\mu\text{mol/l}$ (Table 5,b), but was not prevented by the addition of 10 $\mu\text{mol/l}$ sorbinil (Table 5,c). Thus, in AIM incubated with normal plasma levels of glucose and MI the effect of an elevated medium glucose on a specific component of normal resting (Na⁺, K⁺)-ATPase activity can be reproduced by the addition of a competitive inhibitor of active MI transport that makes medium MI in a normal plasma level inadequate for its maintenance.

Normal basal de novo PI synthesis in AIM, as assessed by the incorporation of 0.1 mmol/l [1,3-¹⁴C]glycerol into PI in AIM incubated in KHB buffer containing normal plasma levels of glucose and MI, is comprised of two distinct fractions [12]. One fraction does not require medium MI; it forms glycerol-labelled PI during a 15 min pulse that remains stable during chases of at least 30 min. Another distinct fraction requires medium MI and is maintained by 70 $\mu\text{mol/l}$ MI when AIM is incubated with 5 mmol/l glucose; this fraction of basal de novo PI synthesis forms an additional component of glycerol-labelled PI during a 15 min pulse that completely disappears during a 30 min chase [12]. In AIM deprived of medium MI rais-

Table 6. Effects of raising medium glucose on $[1,3\text{-}^{14}\text{C}]$ glycerol incorporation into phosphatidylinositol (PI) in the absence and presence of medium myo-inositol (MI) in a normal plasma level

Medium MI	$[1,3\text{-}^{14}\text{C}]$ glycerol incorporation into PI ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)			<i>n</i>	<i>p</i>
	5 mmol/l glucose	30 mmol/l glucose	Mean $\Delta \pm \text{SEM}$		
a) None	0.046	0.049	$+0.003 \pm 0.009$	(6)	NS
b) 70 $\mu\text{mol/l}$	0.086	0.053	-0.033 ± 0.005	(6)	<0.001

Paired AIM samples were equilibrated for 30 min in Krebs Henseleit-bicarbonate buffer containing 9% dextran and either 5 or 30 mmol/l glucose, and incubated for 15 min in fresh medium of the same composition to which 0.1 mmol/l $[1,3\text{-}^{14}\text{C}]$ glycerol was added. The tissue PI was extracted, isolated by two-dimensional thin layer chromatography and counted. In b 70 $\mu\text{mol/l}$ MI was added to the media. *p* for the mean $\Delta \pm \text{SEM}$ in the incorporations in the paired samples was determined by a paired *t* test analysis

ing the medium glucose from 5 to 30 mmol/l during the equilibration and a 15 min incubation with 0.1 mmol/l $[1,3\text{-}^{14}\text{C}]$ glycerol had no effect on the fraction of basal $[1,3\text{-}^{14}\text{C}]$ glycerol incorporation into PI that does not require ECF MI (Table 6, a). When these experiments were repeated with 70 $\mu\text{mol/l}$ MI added to the media, the samples incubated with 5 mmol/l glucose exhibited the expected increase in $[1,3\text{-}^{14}\text{C}]$ glycerol incorporation into PI, and raising the medium glucose from 5 to 30 mmol/l caused a significant decrease in paired samples (Table 6, b). Thus, in AIM provided with medium MI in a normal plasma level raising the medium glucose from a normal to an elevated plasma level selectively inhibits the fraction of normal basal de novo PI synthesis that requires ECF MI to maintain it. When 0.1 mmol/l $[1,3\text{-}^{14}\text{C}]$ glycerol incorporation into PI during a 15 min incubation was compared in paired AIM samples incubated in medium containing either 5 mmol/l glucose and 70 $\mu\text{mol/l}$ MI or 30 mmol/l glucose and 500 $\mu\text{mol/l}$ MI there was no significant difference; the mean $\Delta \pm \text{SEM}$ between the paired samples was $-0.005 \pm 0.003 \mu\text{mol} [1,3\text{-}^{14}\text{C}]$ glycerol incorporation into PI $\cdot \text{kg}^{-1} \cdot 15 \text{ min}^{-1}$, ($n=7$). Thus, in AIM exposed to medium glucose in an elevated plasma level (30 mmol/l), raising the medium MI from 70 to 500 $\mu\text{mol/l}$ prevents the inhibition of the fraction of basal de novo PI synthesis that requires ECF MI and maintains total basal de novo PI synthesis at normal levels.

Discussion

A major component of the (Na^+ , K^+)-ATPase activity normally operative in resting AIM in medium containing normal plasma levels of glucose, HMI, Na^+ , and K^+ was inhibited when the medium glucose was raised from 5 to 10 mmol/l for 40 min prior to, and during, a 20 min assessment of (Na^+ , K^+)-ATPase-mediated

$^{86}\text{Rb}^+/\text{K}^+$ uptake, and raising medium glucose to 20 or 30 mmol/l had no greater effect. In AIM exposed to 30 mmol/l glucose this inhibition was prevented by two structurally different aldose reductase inhibitors, sorbinil (10 $\mu\text{mol/l}$) and tolrestat (1 $\mu\text{mol/l}$), and also by raising the medium MI from a normal plasma level to one some 7-fold higher. When the medium glucose remained at 5 mmol/l, adding sorbinil or increasing medium MI from 70 to 500 $\mu\text{mol/l}$ had no effect on (Na^+ , K^+)-ATPase activity. These observations suggest that the mechanism by which hyperglycaemia initially decreases (Na^+ , K^+)-ATPase activity in components of peripheral nerve, kidney, and retina in diabetic animals [1, 2] can be acutely activated and studied in vitro in AIM, a model arterial wall.

Raising the medium glucose to a threshold elevated plasma level for 60 min selectively and completely inhibited the component of normal resting (Na^+ , K^+)-ATPase activity that is maintained by a regulatory system recently identified in AIM. This system controls a distinct component of resting (Na^+ , K^+)-ATPase activity through rapid basal PI turnover in a discrete pool, which is not attributable to the use of this pool for polyphosphoinositide formation and appears to reflect rapid basal PI hydrolysis [12]. The stimulus for the rapid basal PI turnover in a discrete pool is postulated to be endogenously released adenosine [1], a "local hormone" released by tissues in a manner that varies with their biological activity and energy state [23]. MI uptake is required to maintain the normal operation of this regulatory system, because the discrete PI pool turns over completely within 30 min in resting AIM and is replenished by a distinct fraction of normal basal de novo PI synthesis that is selectively inhibited when MI uptake is prevented by depriving the tissue of medium MI, which results in the inhibition of the component of resting (Na^+ , K^+)-ATPase activity normally maintained by this system within 30 min [12]. When AIM is incubated with 5 mmol/l glucose, medium MI in a normal plasma level is adequate to maintain the fraction of basal de novo PI synthesis that replenishes the discrete PI pool, and the magnitude of the component of (Na^+ , K^+)-ATPase activity maintained by the regulatory system is constant and independent of the medium MI concentration over the range of 50 to at least 500 $\mu\text{mol/l}$. Our observations suggest that raising medium glucose from 5 to 10–30 mmol/l causes the decrease in normal resting (Na^+ , K^+)-ATPase activity by acting in a manner that requires aldose reductase activity to make medium MI in a normal plasma level inadequate to maintain the normal operation of the regulatory system, presumably by increasing polyol pathway activity that impairs ECF MI uptake at a normal plasma level. This formulation is supported by the following observations. In AIM provided with medium MI in a normal plasma level raising the medium glucose to an elevated plasma level selectively inhibited the fraction of basal de novo PI synthesis and the component of normal resting (Na^+ , K^+)-

ATPase activity that require ECF MI to maintain them, but when the medium MI was raised to 500 $\mu\text{mol/l}$ the effects of 30 mmol/l glucose on normal basal de novo PI synthesis and (Na^+ , K^+)-ATPase activity were prevented. When sorbinil or tolrestat was added during incubations with 30 mmol/l glucose, medium MI in a normal plasma level remained adequate to maintain the component of (Na^+ , K^+)-ATPase activity that requires ECF MI. Raising medium glucose from a normal to an elevated plasma level during a 60 min incubation was previously shown to cause increases in sorbitol in AIM [14], and increased polyol pathway activity is known to impair active MI transport in a number of tissues [24, 25], although the basis of this effect is still obscure. Finally, in AIM incubated with normal plasma levels of glucose and MI the addition of scyllo-inositol (500 $\mu\text{mol/l}$), a competitive inhibitor of active MI transport [21, 22], reproduced the effect of raising the medium glucose level and inhibited the component of (Na^+ , K^+)-ATPase activity that is normally maintained by the regulatory system by making medium MI in a normal plasma level inadequate for its maintenance.

Medium glucose in an elevated plasma level inhibited a major component of normal resting (Na^+ , K^+)-ATPase activity under conditions in which there is no detectable decrease in tissue MI. The high normal MI content of AIM, 5.80 ± 0.43 mmol/kg, does not require medium MI to prevent any significant decrease during a 60 min incubation with glucose in a normal or in an elevated plasma level [12, 14], and it is not significantly increased when 70 or 500 $\mu\text{mol/l}$ MI is added during incubations with 5 mmol/l glucose [12]. The major mechanisms by which most tissues maintain high MI contents that are not dependent on dietary MI are endogenous MI synthesis and the recovery of MI from phosphoinositide turnover [26], and the quantitative importance of MI uptake as a source of MI is unclear in most tissues. Although MI uptake does not appear to be essential for the maintenance of the major MI pools in AIM, the fraction of basal PI synthesis that replenishes the discrete PI pool behaves as though it is localized in a specific cell type or subcellular site where it requires a very small MI pool that is peculiarly dependent on ECF MI uptake for its maintenance. (It is of note that PI synthesis occurs in plasma membrane, as well as in endoplasmic reticulum, and that a rat plasma membrane PI synthetase with a remarkably low K_m for MI, 67 $\mu\text{mol/l}$, has recently been identified [27]). The effect of an elevated medium glucose on basal de novo PI synthesis in AIM was restricted to a specific fraction, it did not inhibit the major fraction of the tissue's normal basal de novo PI synthesis that does not require ECF MI, which is the fraction that accounts for all of the [$1,3\text{-}^{14}\text{C}$]glycerol incorporation into polyphosphoinositides in resting AIM [12]. This is in accordance with the evidence that the effect of diabetes on (Na^+ , K^+)-ATPase activity in peripheral nerve is not associated with an inhibition of polyphosphoinositide synthesis or turnover [28].

Our observations would suggest that in those tissues in which hyperglycaemia causes a decrease in (Na^+ , K^+)-ATPase activity that is prevented by aldose reductase inhibitors and by raising plasma MI it initially acts by increasing polyol pathway activity and impairing MI uptake at a normal ECF MI level, which inhibits a specific component of normal (Na^+ , K^+)-ATPase activity, because some tissue components contain a (Na^+ , K^+)-ATPase regulatory system like that identified in AIM, which requires ECF MI uptake to maintain its normal operation. (Our observations would also suggest that this initial effect of hyperglycaemia probably occurs without a detectable decrease in tissue MI and with only selective alterations in PI synthesis and phosphoinositide metabolism, which agrees with observations in renal glomeruli and peripheral nerve from diabetic rats [10, 28]). There is evidence suggesting that this formulation is probably valid in peripheral nerve endoneurium, in which raising medium glucose from 5 to 30 mmol/l causes an acute inhibition of active MI transport that is largely prevented by 10 $\mu\text{mol/l}$ sorbinil [24]. For depriving endoneurium of medium MI inhibits a specific fraction of normal basal PI synthesis and a distinct component of normal resting (Na^+ , K^+)-ATPase activity that appears to be controlled by a regulatory system like that in AIM (11).

The ability of a rise in medium glucose to 10 mmol/l for 60 min to activate this mechanism for (Na^+ , K^+)-ATPase inhibition in AIM is of note. This may explain why insulin treatment that does not persistently prevent any detectable hyperglycaemia is ineffective in preventing the effects attributed to this mechanism in peripheral nerve [6]. This observation also raises a serious question about the untested assumption that some weeks of hyperglycaemia are required to cause decreased (Na^+ , K^+)-ATPase activity in components of nerve, kidney, and retina, which has been the premise on which the design of all previous studies of this mechanism have been based. Until this question is resolved caution is required in interpreting alterations observed in these tissues after some weeks of diabetes, since there is the possibility that some of these alterations reflect secondary effects of, and complex adaptations to, the initial effects of hyperglycaemia in these tissues.

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References

1. Winegrad AI (1987) Does a common mechanism induce the diverse complications of diabetes? *Diabetes* 36: 396-406
2. Greene DA, Lattimer SA, Sima AAF (1987) Sorbitol, phosphoinositides, and sodium-potassium ATPase in the pathogenesis of diabetic complications. *New Engl J Med* 316: 599-606

3. Cohen MP (1986) Aldose reductase, glomerular metabolism, and diabetic nephropathy. *Metabolism* 35 [Suppl 1]: 55-59
4. Goldfarb S, Simmons DA, Kern EFO (1986) Amelioration of glomerular hyperfiltration in acute experimental diabetes by dietary myo-inositol supplementation and aldose reductase inhibition. *Trans Assoc Am Phys* 99: 67-72
5. MacGregor LC, Matschinsky FM (1986) Experimental diabetes impairs function of the retinal pigmented epithelium. *Metabolism* 35 [Suppl 1]: 28-34
6. Greene DA, DeJesus PV, Winegrad AI (1975) Effects of insulin and dietary myo-inositol on impaired peripheral motor nerve conduction velocity in acute streptozotocin diabetes. *J Clin Invest* 55: 1326-1336
7. Greene DA, Lattimer SA (1983) Impaired rat sciatic nerve sodium-potassium adenosine triphosphate in acute streptozotocin diabetes and its correction by dietary myo-inositol supplementation. *J Clin Invest* 72: 1058-1063
8. Gillon KRW, Hawthorne JN, Tomlinson DR (1983) myo-Inositol and sorbitol metabolism in relation to peripheral nerve function in experimental diabetes in the rat: the effect of aldose reductase inhibition. *Diabetologia* 25: 365-371
9. MacGregor LC, Matschinsky FM (1985) Treatment with aldose reductase inhibitors or with myo-inositol arrests deterioration of the electroretinogram of diabetic rats. *J Clin Invest* 76: 887-889
10. Cohen MP (in press) Effect of experimental diabetes and aldose reductase inhibitors on glomerular phosphoinositide metabolism. *Proceedings of 3rd International Symposium on Diabetes Mellitus*. Elsevier, Amsterdam
11. Simmons DA, Winegrad AI, Martin DB (1982) Significance of tissue myo-inositol concentrations in metabolic regulation in nerve. *Science* 217: 848-851
12. Simmons DA, Kern EFO, Winegrad AI, Martin DB (1986) Basal phosphatidylinositol turnover controls aortic (Na^+ , K^+)-ATPase activity. *J Clin Invest* 77: 503-513
13. Morrison AD, Clements RS Jr, Winegrad AI (1972) Effects of elevated glucose concentrations on the metabolism of the aortic wall. *J Clin Invest* 51: 3114-3123
14. Morrison AD, Orci L, Perrelet A, Winegrad AI (1979) Studies of the effects of an elevated glucose concentration on the ultrastructure and composite metabolism of the intact rabbit aortic intima-media preparation. *Diabetes* 28: 720-723
15. Morrison AD, Berwick L, Orci L, Winegrad AI (1976) Morphology and metabolism of an aortic intima-media preparation in which an intact endothelium is preserved. *J Clin Invest* 57: 650-660
16. Morrison AD, Orci L, Berwick L, Perrelet A, Winegrad AI (1977) The effects of anoxia on the morphology and composite metabolism of the intact aortic intima-media preparation. *J Clin Invest* 59: 1027-1037
17. Krebs HA, Henseleit K (1932) Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z Physiol Chem* 210: 33-36
18. Sherman WR, Stewart MA, Kurien MM, Goodwin SL (1986) The measurement of myo-inositol, myo-inosose-2, and scyllo-inositol in mammalian tissues. *Biochim Biophys Acta* 158: 197-205
19. Resh MD, Nemenoff RA, Guidotti G (1980) Insulin stimulation of (Na^+ , K^+)-adenosine triphosphate-dependent $^{86}\text{Rb}^+$ uptake in rat adipocytes. *J Biol Chem* 255: 10938-10945
20. Dvornik D (1987) Aldose reductase inhibitors. In: Dvornik D, Porte D (eds) *Aldose Reductase Inhibition*. McGraw-Hill, New York, pp 221-323
21. Takenawa T, Tsumita T (1974) myo-Inositol transport in plasma membrane of rat kidney. *Biochim Biophys Acta* 373: 106-114
22. Spector R (1978) Cyclohexitol Transport in the Central Nervous System. In: Wells WW, Eisenburg F Jr (eds) *Cyclitols and phosphoinositides*. Academic Press, New York, pp 499-506
23. Daly J (1982) Adenosine receptors: targets for future drugs. *J Med Chem* 25: 197-207
24. Gillon KRW, Hawthorne JN (1983) Transport of MI into endoneurial preparations of sciatic nerve from normal and STZ-diabetic rats. *Biochem J* 210: 775-781
25. Broekhuysen RM (1968) Changes in myo-inositol permeability in the lens due to cataractous conditions. *Biochim Biophys Acta* 163: 269-272
26. Sherman WR, Munsell LY, Gish BG, Honchar MP (1985) Effects of systemically administered lithium on phosphoinositide metabolism in rat brain, kidney, and testes. *J Neurochem* 44: 798-807
27. Imai A, Gershengorn MC (1987) Independent PI synthesis in pituitary plasma membrane and endoplasmic reticulum. *Nature* 325: 726-728
28. Eichberg J, Berti-Mattera L, Bell ME, Peterson RG (1985) Changes in peripheral nerve polyphosphoinositide metabolism in experimental diabetes: nature and significance. In: Bleasdale JE, Eichberg J, Hauser G (eds) *Inositol and phosphoinositides: metabolism and regulation*. Humana Press, Clifton, NJ, pp 583-599

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