Diabetologia (1991) 34: 157–163
0012186X9100045B

Diabetologia

Elevated extracellular glucose inhibits an adenosine-(Na^+, K^+)-ATPase regulatory system in rabbit aortic wall

D. A. Simmons and A. I. Winegrad

Cox Institute, Department of Medicine, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Summary. The mechanism by which hyperglycaemia causes decreased (Na^+, K^+)-ATPase activity preventable by aldose reductase inhibitors and by raising plasma *myo*-inositol in specific tissues can be activated in vitro in normal rabbit aortic wall; it selectively inhibits a component of resting (Na^+, K^+)-ATPase activity maintained by a novel regulatory system through rapid basal phosphatidylinositol turnover (hydrolysis) in a discrete pool, which is replenished by a fraction of phosphatidylinositol synthesis that selectively requires *myo*-inositol transport. A role for endogenously released adenosine in this regulatory system was examined. Adding adenosine deaminase or 8-phenyltheophylline, an adenosine receptor antagonist, selectively inhibited the component of (Na^+, K^+)-ATPase activity maintained by the regulatory system; when inhibited with adenosine deaminase this component was restored by 2-chloroadenosine, 5′-N-ethylcarboxamidoadenosine, and 1-oleoyl-2-acetylglycerol, but not by forskolin (which also did not inhibit this component). Adenosine deaminase inhibited the rapid basal turnover of the discrete phosphatidylinositol pool, and 2-chloroadenosine then stimulated its turnover. Raising medium glucose from 5 to 10–30 mmol/l inhibits the regulatory system by making *myo*-inositol transport at a normal plasma level inadequate to maintain the replenishment of the discrete phosphatidylinositol pool. 2-Chloroadenosine stimulation of the "adenosine-sensitive" component of (Na^+, K^+)-ATPase activity was inhibited in tissue incubated with 30 mmol/l glucose and *myo*-inositol in a normal plasma level, but this effect was demonstrable when the medium *myo*-inositol was raised seven-fold. Hyperglycaemia-induced decreased (Na^+, K^+)-ATPase activity that is preventable by aldose reductase inhibitors and by raising plasma *myo*-inositol results from the inhibition of a novel adenosine-(Na^+, K^+)-ATPase regulatory system.

Key words: (Na^+, K^+)-ATPase, adenosine, glucose, *myo*-inositol, aorta.

Hyperglycaemia decreases (Na^+, K^+)-ATPase activity and induces functional alterations in peripheral nerve, kidney, and retina by a mechanism whose identifying characteristic is that its effects are preventable by aldose reductase inhibitors and by raising normal plasma *myo*-inositol ~seven-fold [1–4]. This mechanism can be acutely activated and studied in vitro in normal rabbit aortic intima-media (AIM); it was recently found to involve increased polyol pathway activity that impairs *myo*-inositol transport at a normal plasma level and thereby inhibits a novel (Na^+, K^+)-ATPase regulatory system present in the tissue [5]. This system controls and maintains a distinct component of resting (Na^+, K^+)-ATPase activity through rapid basal phosphatidylinositol (PI) turnover in a discrete pool, which reflects PI hydrolysis of some type [6]. The PI pool required for this system's operation is maintained by a distinct fraction of basal de novo PI synthesis that selectively requires *myo*-inositol transport at a normal micromolar plasma level for its maintenance [5, 6]. In AIM provided with medium *myo*-inositol in a normal plasma level raising medium glucose from a normal (5 mmol/l) to a threshold elevated plasma level (10 mmol/l) selectively inhibits the (Na^+, K^+)-ATPase activity maintained by the regulatory system by inhibiting the fraction of PI synthesis that replenishes the PI pool used as its effector arm; this occurs without a measurable change in total tissue *myo*-inositol and is prevented by aldose reductase inhibitors and by raising the medium *myo*-inositol ~seven-fold [5].

Further clarification of the novel (Na^+, K^+)-ATPase regulatory system is required to understand the implications of its inhibition in the tissues in which hyperglycaemia causes decreased (Na^+, K^+)-ATPase activity preventable by aldose reductase inhibitors and by raising plasma *myo*-inositol. Because this system is operative in resting isolated AIM, we examined the possibility that

adenosine, a local hormone, is the stimulus for the component of (Na^+, K^+)-ATPase activity it maintains. Adenosine is released by most tissues in a manner that varies with their activity and energy state and plays an important role in the autoregulation of local metabolism, microcirculatory haemodynamics, and arterial tone [7, 8]. Most adenosine effects are mediated through receptors that inhibit or stimulate adenylate cyclase, but other types of adenosine receptors have been postulated [9–11].

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 excised and used to prepare four tubular segments of AIM free of adventitia and retaining an intact endothelium with a normal transmission and scanning electron microscopic appearance by a previously described method [12, 13]. Each AIM segment weighed ~30 mg. The aorta was dissected in Krebs-Henseleit bicarbonate buffer [14] bubbled with 5% CO_2/95% O_2, pH 7.4 at 37°C, that contained normal rabbit plasma levels of glucose (5 mmol/l) and *myo*-inositol (70 µmol/l), in addition to 9% clinical grade dextran, average mol. wt. 70,000, to provide the oncotic pressure required to preserve AIM's normal ultrastructure and pattern of energy metabolism [12].

(Na^+, K^+)-ATPase mediated $^{86}Rb^+/K^+$ uptake was compared in paired duplicate AIM samples from the same aorta incubated under conditions that differed in a single parameter using a method detailed in a previous report [5]. Briefly, the individual AIM segments were equilibrated for 30 min in 3 ml of Krebs-Henseleit bicarbonate buffer gassed with 5% CO_2/air that contained 9% dextran, 5 mmol/l glucose, and a specified concentration of *myo*-inositol, in a 10 ml Erlenmeyer flask in a metabolic shaker set at 88 cycles/min. The AIM segment was then transferred to a fresh medium of the same composition containing an added tracer quantity of [^{3}H]sucrose. Ouabain (0.2 mmol/l) was added to one of the two AIM segments in each paired sample. (This ouabain concentration causes maximal inhibition of (Na^+, K^+)-ATPase activity in AIM). The agent whose effect on (Na^+, K^+)-ATPase activity was to be examined was added to one of the paired duplicate AIM samples. The samples were incubated for 30 min, and 20 min prior to the end of the incubation a tracer quantity of [$^{86}Rb^+$]RbCl was added to each flask. At the end of the incubation the AIM segment was removed and rapidly drained on filter paper, weighed, transferred into 2 ml of Protosol (New England Nuclear, Boston, Mass, USA) in a glass liquid scintillation vial, digested for 16 h, and decolourized with H_2O_2. Econofluor-2 (New England Nuclear) was then added and the samples counted in a liquid scintillation spectrometer with an external standard. Samples of incubation medium were counted, and medium K^+ was also determined. The observed tissue $^{86}Rb^+$ counts were corrected for the $^{86}Rb^+$ present in retained extracellular fluid in the tissue by means of the [^{3}H]sucrose counts found in the tissue sample and the ratio of the $^{86}Rb^+$ counts/[^{3}H]sucrose counts in the medium. The resulting estimate of intracellular $^{86}Rb^+$ counts was expressed as µmol of $^{86}Rb^+/K^+$ uptake $\cdot g^{-1} \cdot 20\ min^{-1}$ by means of the ratio of the $^{86}Rb^+$ counts in the incubation medium to the medium K^+ concentration. The difference between the uptakes in the absence and presence of ouabain in the duplicate segments incubated under otherwise identical conditions was used to estimate the (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake. This method yields linear values in 10 or 20 min assays in paired samples from the same aorta incubated under the same conditions.

In specific experiments [1,3-^{14}C]glycerol counts in tissue PI were compared in paired samples from the same aorta after a 15 min pulse and after a 30 min chase. Paired AIM samples (each consisting of two segments) were equilibrated for 30 min in 6 ml of Krebs-Henseleit bicarbonate buffer containing 9% dextran, glucose (5 mmol/l), and a specified *myo*-inositol concentration. The samples were then incubated in fresh medium of the same composition with added 0.1 mmol/l [1,3-^{14}C]glycerol, 55 mCi/mmol, for 15 min; one sample was then immediately processed, as described below, and the other sample transferred to fresh medium of the same composition that contained unlabelled glycerol and incubated for an additional 30 min. Using previously described methods [5] the tissue removed after the pulse or chase were rapidly frozen in liquid N_2 that had been previously partially evaporated to its freezing point, weighed, and pulverized in liquid N_2 in an all glass homogenizer; the phospholipids were extracted from the frozen tissue powder and the PI isolated by two-dimensional thin layer chromatography and counted.

Ouabain, dextran, 2-chloroadenosine, 5′-N-ethylcarboxamidoadenosine, R-N^6-phenylisopropyladenosine, and S-N^6-phenylisopropyladenosine were obtained from Sigma Chemical Co., (St. Louis, Mo., USA). Adenosine deaminase was purchased from Boehringer Mannheim Biochemicals, (Indianapolis, Ind., USA) *myo*-Inositol was obtained from Pfanstiehl Labs Inc., (Waukegan, Ill., USA). Reference standards of PI and other phospholipids were obtained from Serdary Labs, (New London, Ontario, Canada). [$^{86}Rb^+$]RbCl, [1,3-^{14}C]glycerol, and [^{3}H]sucrose were obtained from New England Nuclear.

Statistical analysis

The mean $\Delta \pm$ SEM between the paired samples in each group of paired experiments was analysed for significance by a paired *t*-test analysis.

Results

The (Na^+, K^+)-ATPase activity operative in resting AIM incubated in Krebs-Henseleit bicarbonate buffer containing normal plasma levels of Na^+, K^+, glucose (5 mmol/l), and *myo*-inositol (70 µmol/l), and 9% dextran is comprised of two distinct components; if the medium *myo*-inositol is removed (or if a competitive inhibitor of *myo*-inositol transport is added) the component maintained by a novel regulatory system is selectively inhibited within 30 min (the equilibration period) leaving another stable component of (Na^+, K^+)-ATPase activity that does not require medium *myo*-inositol for its maintenance [5, 6]. (A threshold level of medium *myo*-inositol (50 µmol/l) is required to maintain the component controlled by the regulatory system, whose magnitude is unaffected by further increases in medium *myo*-inositol in tissue incubated with 5 mmol/l glucose [5, 6].) We examined the effects on these two components of resting (Na^+, K^+)-ATPase activity of adding adenosine deaminase (ADA) (1 U/ml) to degrade endogenously released adenosine. ADA inhibited a major fraction of the activity operative in AIM provided with medium *myo*-inositol in a normal plasma level, but had no effect on the component that remains operative in *myo*-inositol-free medium (Table 1). 8-Phenyltheophylline, an adenosine receptor antagonist with a high affinity for both A_1 and A_2 receptors [9, 11], also selectively inhibited the component that requires medium *myo*-inositol and is maintained by the regulatory system (Table 1). When this component was inhibited with ADA it could be restored by adding 2-chloroadenosine (10 µmol/l), a water soluble adenosine analogue that is not a substrate for ADA [9] (Table 1). This 2-chloroadenosine stimulation of

Table 1. Effects of adenosine deaminase (ADA) (1 U/ml), 8-phenyltheophylline (10 μmol/l) and 2-chloroadenosine on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media (AIM) in medium containing or lacking *myo*-inositol

Medium *myo*-inositol	(Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake (μmol·g^{-1}·20 min^{-1})				
	Condition 1	Condition 2	Mean Δ ± SEM	(*n*)	*p*
	No Additions	+ ADA			
(+)	2.65	1.49	− 1.16 ± 0.31	(9)	< 0.01
(−)	1.69	1.64	− 0.05 ± 0.17	(9)	NS
	+ dimethyl sulfoxide	+ 8-phenyltheophylline			
(+)	2.83	1.73	− 1.10 ± 0.20	(9)	< 0.001
(−)	1.57	1.71	+ 0.14 ± 0.27	(6)	NS
	+ ADA	+ ADA + 2-chloroadenosine			
(+)	1.48	1.71 (1 μmol/l)	+ 0.23 ± 0.32	(6)	NS
(+)	1.42	1.56 (5 μmol/l)	+ 0.14 ± 0.22	(6)	NS
(+)	1.46	2.62 (10 μmol/l)	+ 1.14 ± 0.19	(9)	< 0.001
(+)	1.38	2.55 (20 μmol/l)	+ 1.17 ± 0.23	(7)	< 0.01
(+)	1.46	1.75 (10 μmol/l)	+ 0.29 ± 0.38	(6)	NS

Paired duplicate AIM samples were equilibrated (30 min) and incubated (30 min) at 37 °C in Krebs-Henseleit-bicarbonate buffer containing 9% dextran, 5 mmol/l glucose, and 70 μmol/l *myo*-inositol; in specific experiments the *myo*-inositol was omitted (Medium *myo*-inositol (−)). The additions noted were added to the incubation medium. Ouabain (0.2 mmol/l) was added to one of the two AIM segments in each paired sample during the incubation. 8-Phenyltheophylline was added in dimethyl sulfoxide (final medium concentration 0.1%) which was added to the paired controls. (Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake was assessed during the final 20 min of the incubation. Values are means and mean Δ ± SEM between the paired samples. *p* was determined by a paired *t*-test analysis

Table 2. Effects of forskolin on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media

Medium *myo*-inositol	Medium ADA	(Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake (μmol·g^{-1}·20 min^{-1})		Mean Δ ± SEM	(*n*)	*p*
		+ 0.1% Ethanol	+ Forskolin			
(+)	(+)	1.73	1.81	+ 0.08 ± 0.24	(10)	NS
(−)	(−)	1.71	1.99	+ 0.28 ± 0.20	(17)	NS
(+)	(−)	2.49	2.56	+ 0.07 ± 0.39	(7)	NS

The experiments were performed as described in the legend for Table 1. Forskolin (50 μmol/l) was added in an ethanol solution, final medium ethanol concentration 0.1%. The conditions with regard to the presence (+) or absence (−) of medium *myo*-inositol (70 μmol/l) and of medium adenosine deaminase (1 U/ml) were the same for both paired samples. ADA = adenosine deaminase

a distinct component of (Na^+, K^+)-ATPase activity was not demonstrable when the medium *myo*-inositol was omitted (Table 1). These observations demonstrate that endogenously released adenosine is the stimulus for the component of (Na^+, K^+)-ATPase activity maintained by the regulatory system.

The requirement for medium *myo*-inositol for the adenosine effect on (Na^+, K^+)-ATPase activity in AIM distinguishes this effect from all previously reported adenosine effects in isolated tissues and cultured cells, most of which are attributable to receptors that inhibit (A_1) or stimulate (A_2) adenylate cyclase [9–11]. When the adenosine-stimulated component of (Na^+, K^+)-ATPase activity was inhibited with ADA or by using *myo*-inositol-free medium it was not restored by forskolin, a direct stimulator of adenylate cyclase [15], in concentrations as high as 50 μmol/l (Table 2). Forskolin also had no effect on (Na^+, K^+)-ATPase activity when added to AIM in which the adenosine-stimulated component was operative (Table 2), but would have been expected to cause a decrease if the adenosine effect were mediated through a receptor that inhibits adenylate cyclase [9]. These observations suggest that the adenosine effect is not mediated through a receptor whose transduction mechanism is an inhibition or stimulation of adenylate cyclase. When the adenosine-stimulated component of (Na^+, K^+)-ATPase activity was inhibited with ADA it was restored by 10 μmol/l 2-chloroadenosine (Table 1) and by 0.2 μmol/l 5'-N-ethylcarboxamidoadenosine (Table 3), but in concentrations as high as 100 μmol/l neither R-N^6-phenylisopropyladenosine or S-N^6-phenylisopropyladenosine had an effect (Table 3). The apparent relative potencies of these adenosine analogues suggest that the adenosine effect is probably mediated through a variant type of A_2 receptor [9, 11].

The relationship between endogenously released adenosine and the rapid basal turnover of the discrete PI pool was examined in studies that had the following basis. In AIM incubated with normal plasma levels of glucose and *myo*-inositol basal de novo PI synthesis is comprised of two distinct fractions, one of which is selectively inhibited if the medium *myo*-inositol is removed to inhibit *myo*-inositol transport [6]. The fraction that remains operative in *myo*-inositol-free medium forms labelled PI during a 15 min pulse with [1,3-^{14}C]glycerol that remains stable during a 30 min chase [6], as illustrated by the pulse-chase study in *myo*-inositol-free medium in Table 4. The fraction of basal de novo PI synthesis that requires medium *myo*-inositol in a normal plasma level replenishes the discrete PI pool with a rapid basal turnover; this frac-

Table 3. Effects of adenosine deaminase and adenosine analogues on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media in medium containing 0.1% dimethyl sulfoxide

(Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake ($\mu mol \cdot g^{-1} \cdot 20\ min^{-1}$)				
Condition 1	Condition 2	Mean Δ ± SEM	(*n*)	*p*
− ADA	+ ADA (1 U/ml)			
2.59	1.67	− 0.92 ± 0.23	(7)	< 0.01
+ ADA	+ ADA + 5′-N-ethylcarboxamido-adenosine (0.2 μmol/l)			
1.59	2.48	+ 0.89 ± 0.24	(9)	< 0.01
+ ADA	+ ADA + R-N^6-phenylisopropyl-adenosine (100 μmol/l)			
1.64	1.75	+ 0.11 ± 0.26	(10)	NS
+ ADA	+ ADA + S-N^6-phenylisopropyl-adenosine (100 μmol/l)			
1.75	1.71	− 0.04 ± 0.17	(6)	NS

The experiments were performed as described in legend for Table 1. All incubation media contained *myo*-inositol (70 μmol/l) and 0.1% dimethyl sulfoxide, the solvent for the adenosine analogues tested. ADA = adenosine deaminase

Table 4. Comparison of cpm in phosphatidylinositol in paired rabbit aortic intima-media (AIM) samples after a 15 min pulse with [1,3-^{14}C]glycerol and after a 30 min chase

Medium *myo*-inositol	cpm in phosphatidylinositol/g tissue				
	Pulse	Chase	Mean Δ ± SEM	(*n*)	*p*
(−)	5527	5931	+ 404 ± 381	(6)	NS
(+)	7989	6069	− 1920 ± 197	(6)	< 0.001
		(+ ADA)			
(+)	8231	8617	+ 386 ± 295	(7)	NS
		(+ ADA + 2-chloroadenosine)			
(+)	7908	6631	− 1277 ± 200	(7)	< 0.001

Paired AIM samples were equilibrated (30 min), incubated for 15 min with 0.1 mmol/l [1,3^{14}C]glycerol, 55 mCi/mmol, and one sample subjected to a 30 min chase. The medium was Krebs-Henseleit-bicarbonate buffer containing 9% dextran, 5 mmol/l glucose and 70 μmol/l *myo*-inositol; in specific experiments the *myo*-inositol was omitted (Medium *myo*-inositol (−)). In specific experiments additions to the medium were made during the chase, adenosine deaminase (ADA) (1 U/ml) alone or in combination with 2-chloroadenosine (10 μmol/l). Values are means and mean Δ ± SEM between the paired samples. *p* was determined by a paired *t*-test analysis

tion forms an additional component of glycerol-labelled PI during a 15 min chase that disappears during a 30 min chase [6]. Thus, as illustrated in Table 4, when paired AIM samples are provided with 70 μmol/l medium *myo*-inositol during a 15 min pulse with 0.1 mmol/l [1,3-^{14}C]glycerol and one sample is then subjected to a 30 min chase, there is a significant decrease in the counts in PI during the chase that reflects the rapid basal turnover of the labelled PI in the discrete PI pool. This decrease in labelled PI was inhibited when ADA (1 U/ml) was added during the chase (Table 4), and under these conditions adding 2-chloroadenosine (10 μmol/l) stimulated a loss of PI (Table 4). These observations support the conclusion that endogenously released adenosine is the stimulus for the rapid basal PI turnover in a discrete pool that is the effector arm of the (Na^+, K^+)-ATPase regulatory system.

The basal turnover of the discrete PI pool is not attributable to its use for phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-biphosphate formation and reflects PI hydrolysis of some type [6]. When the adenosine-stimulated component of (Na^+, K^+)-ATPase activity was selectively inhibited with ADA or by omitting medium *myo*-inositol it was restored by 1-oleoyl-2-acetylglycerol (100 μmol/l), a synthetic sn-1,2-diacylglycerol that can activate some protein kinase(s) C when added to intact cells [16] (Table 5). In contrast, 1-oleoyl-2-acetylglycerol did not stimulate (Na^+, K^+)-ATPase activity in AIM in which the adenosine-stimulated component was operative, but rather caused a small decrease (Table 5). These observations suggest that the adenosine stimulation of a distinct component of (Na^+, K^+)-ATPase activity is mediated through diacylglycerol release and that adenosine probably stimulates a phospholipase C hydrolysis of PI in the discrete PI pool.

In AIM provided with medium *myo*-inositol in a normal plasma level raising the medium glucose to 30 mmol/l selectively inhibits the component of (Na^+, K^+)-ATPase activity controlled by the regulatory system by inhibiting the fraction of PI synthesis that replenishes the discrete PI pool, and these effects are prevented if the medium *myo*-inositol is raised from 70 to 500 μmol/l [5]. We examined the ability of 2-chloroadenosine (10 μmol/l) to stimulate the "adenosine-sensitive" component of (Na^+, K^+)-ATPase activity under these two conditions, with ADA added to degrade endogenously released adenosine. 2-Chloroadenosine did not stimulate (Na^+, K^+)-ATPase activity in AIM incubated with 30 mmol/l glucose and *myo*-inositol in a normal plasma level, but did cause a stimulation in tissue incubated with 30 mmol/l glucose when the medium *myo*-inositol was raised ~seven-fold (Table 6). This supports the inference that exposing AIM to medium glucose in an elevated plasma level decreases

Table 5. Effects of 1-oleoyl-2-acetylglycerol on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media

Medium *myo*-inositol	Medium ADA	(Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake ($\mu mol \cdot g^{-1} \cdot 20\ min^{-1}$)				
		+ dimethyl sulfoxide	+ 1-oleoyl-2-acetylglycerol	Mean $\Delta \pm$ SEM	(n)	p
(+)	(+)	1.85	2.86	$+1.01 \pm 0.21$	(7)	< 0.01
(−)	(−)	1.48	2.37	$+0.89 \pm 0.17$	(10)	< 0.001
(+)	(−)	2.39	1.98	-0.41 ± 0.12	(6)	< 0.05

The experiments were performed as described in legend for Table 1. The conditions with regard to the presence (+) or absence (−) of medium *myo*-inositol (70 μmol/l) and of medium adenosine deaminase (ADA) (1 U/ml) were the same for both paired samples. The 1-oleoyl-2-acetylglycerol (100 μmol/l) was added in a dimethyl sulfoxide suspension sonicated to prepare micelles [17]; the same final medium dimethyl sulfoxide concentration (0.3%) was added to the paired controls

Table 6. 2-Chloroadenosine effects on (Na^+, K^+)-ATPase activity in rabbit aortic intima-media incubated with 30 mmol/l glucose and *myo*-inositol in a normal or elevated plasma level

Medium *myo*-inositol	(Na^+, K^+)-ATPase-mediated $^{86}Rb^+/K^+$ uptake ($\mu mol \cdot g^{-1} \cdot 20\ min^{-1}$)				
	+ ADA	+ ADA + 2-chloroadenosine	Mean $\Delta \pm$ SEM	(n)	p
70 μmol/l	1.25	1.46	$+0.21 \pm 0.22$	(7)	NS
500 μmol/l	1.32	2.18	$+0.89 \pm 0.06$	(5)	< 0.001

Experiments performed as described in legend for Table 1, but with the medium glucose raised to 30 mmol/l during the equilibration (30 min) and incubation (30 min). The adenosine deaminase (ADA) (1 U/ml) and 2-chloroadenosine (10 μmol/l) were added to the incubation medium

(Na^+, K^+)-ATPase activity by inhibiting a specific adenosine effect.

Discussion

When the mechanism by which hyperglycaemia causes decreased (Na^+, K^+)-ATPase activity in specific tissues that is preventable by aldose reductase inhibitors and by raising plasma *myo*-inositol is activated in vitro in AIM it inhibits a novel (Na^+, K^+)-ATPase regulatory system, whose effector arm is rapid basal PI turnover in a discrete pool [5, 6]. The studies reported above demonstrate that this is a previously unrecognized adenosine (Na^+, K^+)-ATPase regulatory system, by which endogenously released adenosine regulates (stimulates) and normally maintains a distinct component of (Na^+, K^+)-ATPase activity in resting AIM. This adenosine effect is mediated through a novel type of adenosine receptor, apparently a variant type of A_2 receptor, that stimulates rapid basal PI turnover in a discrete pool. The rapid basal turnover of this PI pool is not attributable to its use for polyphosphoinositide formation and reflects PI hydrolysis of some type [6]. When the adenosine-stimulated component is selectively inhibited by adding adenosine deaminase or by using *myo*-inositol-free medium to inhibit the replenishment of the discrete PI pool, it can be restored by adding a synthetic sn-1,2-diacylglycerol, 1-oleoyl-2-acetylglycerol, which can activate some protein kinase(s) C in intact cells [16]; whereas in tissue in which the adenosine-stimulated component is operative 1-oleoyl-2-acetylglycerol does not stimulate (Na^+, K^+)-ATPase activity. These observations suggest that adenosine probably stimulates a phospholipase C hydrolysis of PI in the discrete PI pool that acts by a release of diacylglycerol at specific membrane sites to stimulate the distinct component of (Na^+, K^+)-ATPase activity. Whether adenosine selectively stimulates the activity of a specific (Na^+, K^+)-ATPase isoform in one or both major cell types in AIM remains to be clarified.

A striking feature of this regulatory system is its requirement for *myo*-inositol transport at a normal micromolar plasma level to maintain its operation [5, 6]. The PI pool used by this system is replenished by a distinct fraction of de novo PI synthesis that behaves as though it were localized in a specific cell type or subcellular site (PI synthesis occurs in both plasma membrane and endoplasmic reticulum [17]), where it requires a small *myo*-inositol pool that is peculiarly dependent on *myo*-inositol transport for its maintenance [5, 6]. *Myo*-inositol transport is not one of the major mechanisms by which most tissues maintain high *myo*-inositol contents comprised of heterogeneous pools [18]. AIM does not require medium *myo*-inositol during incubations with glucose in a normal or elevated plasma level to prevent any measurable decrease in its high normal *myo*-inositol content (5.80 ± 0.43 mmol/kg), to maintain another major fraction of de novo PI synthesis, or to maintain its normal low basal incorporations of labelled glycerol and arachidonic acid into polyphosphoinositides [5, 6, 19]. The biological rationale for having the adenosine effect on (Na^+, K^+)-ATPase activity mediated through a PI pool that is critically dependent on *myo*-inositol transport for its maintenance remains to be clarified. However, this explains why the effect of endogenously released adenosine (and of 2-chloroadenosine) on (Na^+, K^+)-ATPase activity is subject to quite rapid inhibition when an elevated extracellular glucose level induces increased polpol pathway activity that makes *myo*-inositol transport at a normal extracellular level inadequate to maintain the replenishment of the discrete PI pool [5]. It is of note that in normal resting periph-

eral nerve endoneurium inhibiting *myo*-inositol transport by omitting it from the medium also causes rapid and selective inhibitions of distinct fractions of normal PI synthesis and (Na^+, K^+)-ATPase activity [20], and that the decreased (Na^+, K^+)-ATPase activity in endoneurium from diabetic rabbits is acutely corrected by a synthetic diacylglycerol that can activate some protein kinase(s) C [21].

The general pattern of adenosine's diverse effects is believed to be designed to modulate local tissue metabolism and circulation and the effects of external stimuli (e.g. specific hormones and neurotransmitters) in accordance with information about local tissue activity and energy state provided by adenosine release, and to thereby help to maintain normal energy balance [7]. The adenosine-(Na^+, K^+)-ATPase regulatory system identified in AIM has an obvious potential for use in mediating such acute autoregulatory effects. An acute increase in electrogenic (Na^+, K^+)-ATPase activity that raises the membrane potential is one of the mechanisms by which factors released by blood vessels normally modulate vascular smooth muscle tone and its contractile response to neurotransmitters and hormones [22], and adenosine's previously unknown effect on aortic (Na^+, K^+)-ATPase activity may well reflect one of the mechanisms by which it plays its role in the autoregulation of arterial tone and of microcirculatory haemodynamics [8]. Thus a hyperglycaemia-induced inhibition of the adenosine-(Na^+, K^+)-ATPase regulatory system in a tissue in which it normally operates has the potential of causing effects that are not restricted to the diverse effects that can be evoked by a persistent abnormally low level of (Na^+, K^+)-ATPase activity; for it might also cause effects that result from inhibiting one of the adenosine effects normally utilized in acute vascular autoregulation. This is a potential explanation for the relationship between the mechanism by which hyperglycaemia causes decreased (Na^+, K^+)-ATPase activity preventable by aldose reductase inhibitors and by raising plasma *myo*-inositol and the development in some of the affected tissues of functional alterations that reflect a derangement in vascular regulation (e.g. glomerular hyperfiltration, increased retinal blood flow) [3, 4]. This speculation is supported by the recent observation that adenosine's normal effect on glomerular filtration rate (a decrease) is inhibited in diabetic rats and is restored when they are treated with an aldose reductase inhibitor [23].

The distinction between the basal PI turnover involved in the adenosine-(Na^+, K^+)-ATPase regulatory system and classic agonist stimulated phosphoinositide turnover requires emphasis. Stimulation of a resting tissue by a hormone or neurotransmitter whose receptor's transduction mechanism is activation of phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphase and a release of D-*myo*-inositol 1,4,5-P_3 (which raises cytosolic Ca^{2+}) and of diacylglycerol characteristically evokes acute sequential increases in the turnovers of discrete pools of phosphatidylinositol 4,5-bisphosphate and of PI; the increased PI turnover reflects, in part, a secondary, non-receptor controlled increase in PI hydrolysis in specific PI pools [25, 26]. Phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate has been thought to be the only receptor transduction mechanism that involes a phosphoinositide [25, 26]. It has long been known that in a number of resting tissues a small PI pool exhibits persistent rapid basal turnover [27, 28], but its significance has been unknown. Our observations demonstrate that in resting AIM rapid basal PI turnover in a discrete pool reflects the transduction mechanism of a novel adenosine receptor. It is of note that in contrast to the requirement for medium *myo*-inositol for the adenosine effect on (Na^+, K^+)-ATPase activity, the effects of agonists that stimulate phosphatidylinositol 4,5-bisphosphate hydrolysis and a secondary increase in PI turnover in specific PI pools in aorta and cultured aortic cell types (e.g. angiotensin II, norepinephrine) have been routinely demonstrated in *myo*-inositol-free medium [29–31], and the maintenance of the phosphatidylinositol 4,5-bisphosphate and PI pools required for their effects does not appear to be critically dependent on *myo*-inositol transport. Aortae from diabetic rats exhibit an increased contractile response to angiotensin II and norepinephrine [32, 33]; whether this is a secondary consequence of inhibition of the adenosine-(Na^+, K^+)-ATPase regulatory system remains to be clarified, but this is a possible explanation.

Acknowledgements. The authors thank K. Henschel and C. Young for expert technical assistance. Dr. D. A. Simmons was the recipient of a Research and Development Award from the Juvenile Diabetes Foundation. This study was also supported, in part, by NIH grant DK 32308, a grant from Pfizer, Inc., and gifts from the Ware Foundation.

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. N Engl J Med 316: 599–606
3. 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
4. Pugliese G, Tilton RG, Speedy A et al. (1990) Modulation of hemodynamic and vascular filtration changes in diabetic rats by dietary *myo*-inositol. Diabetes 39: 312–322
5. Simmons DA, Winegrad AI (1989) Mechanism of glucose-induced (Na^+, K^+)-ATPase inhibition in aortic wall of rabbits. Diabetologia 32: 402–408
6. Simmons DA, Kern EFO, Winegrad AI, Martin DB (1986) Basal phosphatidylinositol turnover controls aortic (Na^+, K^+)-ATPase activity. J Clin Invest 77: 503–513
7. Newby AC, Adenosine and the concept of retaliatory metabolites (1984) Trends Biol Sci 9: 42–44
8. Berne RM, Knabb RM, Ely RW, Rubio R (1983) Adenosine in the local regulation of blood flow: a brief overview. Fed Proc 42: 3136–3142
9. Daly JW (1982) Adenosine receptors: targets for future drugs. J Med Chem 25: 197–207
10. Stiles GL (1986) Adenosine receptors: structure function and regulation. Trends Physiol Sci 7: 486–490
11. Daly JW, Ukena D, Jacobson KA (1987) Analogues of adenosine, theophylline, and caffeine: selective interactions with A_1 and A_2 adenosine receptors. In: Gerlach E, Becker BF (eds) Topics and perspectives in adenosine research. Springer, Berlin Heidelberg New York, pp 23–26
12. 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
13. Morrison AD, Orci L, Berwick L, 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
14. Krebs HA, Henseleit K (1932) Untersuchungen für die Harnstoffbildung im Tierkörper. Hoppe-Seylers Z Physiol Chem 210: 33–36
15. Daly JW (1984) Forskolin, adenylate cyclase and cell physiology: an overview. In: Greengard P (ed) Advances in cyclic nucleotide and protein phosphorylation research, vol 17. Raven Press, New York, pp 81–89
16. Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J Biol Chem 258: 6701–6704
17. Imai A, Gershengorn MC (1987) Independent PI synthesis in pituitary plasma membrane and endoplasmic reticulum. Nature 325: 726–728
18. Sherman WR, Munsell LY, Gish BG, Honchar MO (1985) Effects of systemically administered lithium on phosphoinositide metabolism in rat brain, kidney, and testes. J Neurochem 44: 798–807
19. 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 aortic intima-media preparation. Diabetes 28: 720–723
20. Simmons DA, Winegrad AI, Martin DB (1982) Significance of tissue myo-inositol concentration in metabolic regulation in nerve. Science 217: 848–851
21. Greene DA, Lattimer SA (1986) Protein kinase C agonists acutely normalize decreased ouabain-inhibitable respiration in diabetic rabbit nerve: implications for Na^+/K^+ ATPase regulation and diabetic complications. Diabetes 35: 242–245
22. Vanhoutte PM (1988) The endothelium-modulator of vascular smooth muscle tone. N Engl J Med 319: 512–513
23. Perlmutter J, Jacobs J, Ziyadeh F, Senesky D, Simmons DA, Kern EFO, Goldfarb S (1989) Reduced renal vasoconstrictive response to adenosine and reversal by aldose reductase inhibition in acute experimental diabetes. Clin Res 37: 583 (Abstract)
24. Hokin LE (1985) Receptors and phosphoinositide generated second messengers. Ann Rev Biochem 54: 205–235
25. Dixon JF, Hokin LE (1989) Kinetic analysis of the formation of inositol 1:2 cyclic phosphate in carbachol-stimulated pancreatic minilobules. J Biol Chem 264: 11721–11724
26. Berridge MJ (1987) Inositol triphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 56: 159–193
27. Agranoff B, Bleasdale JE (1978) The acetylcholine phospholipid effect: what has it told us? In: Wells WW, Eisenberg Jr F (eds) Cyclitols and phosphoinositides. Academic Press, New York, pp 105–120
28. Takhar APS, Kirk CJ (1981) Stimulation of inorganic phosphate incorporation into phosphatidylinositol in rat thoracic aorta mediated through V_1-vasopressin receptors. Biochem J 194: 167–172
29. Campbell MD, Deth RC, Payne RA, Honeyman TW (1985) Phosphoinositide hydrolysis is correlated with agonist-induced calcium flux and contraction in the rabbit aorta. Eur J Pharmacol 116: 129–136
30. Griendling KK, Rittenhouse SE, Brock TA, Ekstein LS, Gimbrone Jr MA, Alexander RW (1986) Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. J Biol Chem 261: 5901–5906
31. Griendling KK, Berk BC, Ganz P, Gimbrone Jr MA, Alexander RW (1987) Angiotensin II stimulation of vascular smooth muscle phosphoinositide metabolism. State of the art lecture. Hypertension 9 [Suppl 3]: 181–185
32. Brody MJ, Dixon RL (1964) Vascular reactivity in experimental diabetes. Circ Res 14: 494–501
33. Scarborough NL, Carrier GO (1983) Increased alpha-2 adrenoreceptor mediated vascular contraction in diabetic rats. J Auton Pharmacol 3: 177–183

Received: 16 July 1990
and in revised form: 6 November 1990

Dr. D. A. Simmons
Cox Institute
School of Medicine
University of Pennsylvania
Philadelphia PA 19104–6056
USA