Basal Phosphatidylinositol Turnover Controls Aortic Na^+/K^+ ATPase Activity

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Abstract

To determine whether basal phosphoinositide turnover plays a role in metabolic regulation in resting rabbit aortic intima-media incubated under steady state conditions, we used deprivation of extracellular *myo*-inositol as a potential means of inhibiting basal phosphatidylinositol (PI) synthesis at restricted sites and of depleting small phosphoinositide pools with a rapid basal turnover. Medium *myo*-inositol in a normal plasma level was required to prevent inhibition of a specific component of basal *de novo* PI synthesis that is necessary to demonstrate a discrete rapidly turning-over [1,3-^{14}C]glycerol-labeled PI pool. Medium *myo*-inositol was also required to label the discrete PI pool with [1-^{14}C]arachidonic acid (AA). The rapid basal turnover of this PI pool, when labeled with glycerol or AA, was not attributable to its utilization for polyphosphoinositide formation, and it seems to reflect basal PI hydrolysis. Depleting endogenous free AA with medium defatted albumin selectively inhibits the component of basal *de novo* PI synthesis that replenishes the rapidly turning-over PI pool. A component of normal resting energy utilization in aortic intima-media also specifically requires medium *myo*-inositol in a normal plasma level and a free AA pool; its magnitude is unaltered by indomethacin, nordihydroguaiaretic acid, or Ca^{2+}-free medium. This energy utilization results primarily from Na^+/K^+ ATPase activity (ouabain-inhibitable O_2 consumption), and in Ca^{2+}-free medium deprivation of medium *myo*-inositol or of free AA inhibits resting Na^+/K^+ ATPase activity to a similar degree (60%, 52%). In aortic intima-media basal PI turnover controls a major fraction of resting Na^+/K^+ ATPase activity.

Introduction

Current understanding of the role of phosphoinositide metabolism in metabolic regulation developed from efforts to relate the acute effects of specific hormones and neurotransmitters on phosphoinositide metabolism in their target tissues to the induction of their biological effects (1). The phosphoinositides are minor phospholipid constituents of cellular membranes in which the parent phosphoinositide, phosphatidylinositol (PI),[1] is found predominantly in the 1-stearoyl-2-arachidonoyl species (2). Phospholipase C hydrolysis of plasma membrane polyphosphoinositides has recently been identified as the transduction mechanism for the receptors of many biological agonists, and its effects related to the release of D-*myo*-inositol 1,4,5-trisphosphate (IP_3) and of diacylglycerol (1, 3–5). IP_3 increases cytosolic Ca^{2+} by mobilization of Ca^{2+} from intracellular stores (3), and diacylglycerol activates protein kinase C (4) and can also serve as substrate for a release of free arachidonic acid (AA), which can contribute to increased eicosanoid production (3, 5). PI hydrolysis is not an initial, receptor-controlled, event in agonist-stimulated phosphoinositide hydrolysis (3), but phospholipase C hydrolysis of PI has recently been shown to be a part of the overall response in some systems (1, 6), although its localization and functions require clarification. Biological agonists selectively stimulate hydrolysis in discrete phosphoinositide pools that must be maintained for the agonist's effectiveness (1, 3). Agonist-stimulated hydrolysis is commonly followed by increased PI synthesis and increased conversion of PI to polyphosphoinositides, which appears to serve to replenish the agonist-sensitive pools (1, 3), and a linkage between agonist-stimulated hydrolysis and the control of PI synthesis for specific agonist-sensitive PI pools has been demonstrated in isolated cells and tissues (1, 7, 8). Both *de novo* PI synthesis and PI resynthesis from diacylglycerol released by phosphoinositide hydrolysis require free *myo*-inositol (2).

Many resting tissues, including aorta, are known to have significant rates of PI synthesis and turnover, as assessed by [^{32}P]Pi labeling studies, that are not decreased by specific antagonists of agonists that stimulate phosphoinositide hydrolysis in the tissue (9–11). There has been no means of determining whether some of this basal PI turnover might reflect basal, non-agonist-dependent, phosphoinositide turnover that has a distinct regulatory function, and no intimation that this might be the case. However, recent observations suggest that basal phosphoinositide turnover may control a major fraction of resting Na^+/K^+ ATPase activity in peripheral nerve, and that a derangement in this regulation resulting from a decrease in nerve *myo*-inositol contributes to the pathogenesis of diabetic polyneuropathy (12, 13). These studies in nerve suggested potential methods for identifying components of normal resting energy utilization in other tissues that might reflect the activities of reactions controlled through basal phosphoinositide turnover. This report presents evidence that in resting aortic intima-media, basal PI turnover, which appears to reflect PI hydrolysis, controls a component of normal resting energy utilization that results primarily from Na^+/K^+ ATPase activity.

Methods

Aortic intima-media preparation. Male, white New Zealand rabbits (2.0–2.5 kg) were fasted overnight, and sedated with diazepam (2 mg/kg i.m.) 90 min before the induction of anesthesia with sodium pentobarbital (30 mg/kg i.v.), and decapitation. The descending thoracic aorta was rapidly excised and used to prepare tubular segments of aortic intima-media that are free of adventitia and retain an intact endothelium with a scanning and transmission electron microscopic appearance similar to

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1. *Abbreviations used in this paper:* AA, arachidonic acid; CoA, coenzyme A; KHB, Krebs-Henseleit-bicarbonate; IP_3, D-*myo*-inositol 1,4,5-triphosphate; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol 4-phosphate; PI-4,5-P_2, phosphatidylinositol 4,5-bis-phosphate.

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that of tissue fixed in situ, by a method previously reported in detail (14, 15). Each aorta yields six tubular segments ~1 cm in length, and alternating anatomical segments were pooled to provide two paired samples, each weighing 50–60 mg. Incubation conditions that preserve the tissue's normal ultrastructure and normal pattern of energy metabolism and maintain a steady state of energy metabolism were defined in previous studies (14, 15). All media to which aortic intima-media is exposed must contain a source of an oncotic pressure roughly equivalent to that in plasma to prevent acute, irreversible endothelial cell injury and the induction of an altered pattern of energy metabolism in the surviving smooth muscle cells (14).

Media and incubation conditions. The invariant components of all media were Krebs-Henseleit-bicarbonate (KHB) buffer (16), pH 7.4 at 37°C, 5 mM glucose, and a source of oncotic pressure. When it was desirable to provide oncotic pressure without risk of depleting endogenous free fatty acids (FFA), 9% (wt/vol) clinical grade dextran, average molecular weight 70,000 (Sigma Chemical Co., St. Louis, MO) was used. The source of oncotic pressure in the standard medium was 9% dialyzed, essentially fatty acid-free bovine serum albumin (BSA) (Sigma Chemical Co.); under these conditions the final calcium content of the medium was adjusted to 3.1 mM to compensate for binding by albumin. Each batch of BSA was dialyzed and lyophylized before use, and the contents of calcium, *myo*-inositol, and FFA in a 9% (wt/vol) solution were determined. Calcium was determined by a calcein fluorometric EGTA titration (17). *myo*-Inositol was determined by gas-liquid chromatographic analysis (18), and only BSA that yielded an undetectable medium *myo*-inositol concentration (<2 μM) was used. FFA was determined by the method of Novak (19), and only BSA that yielded an undetectable medium FFA level (<10 μM) was used. Ca^{2+}-free standard medium differed from standard medium (KHB buffer, 5 mM glucose, 9% BSA) only in the replacement of the buffer calcium by an additional 3.5 mEq of Na^+ and the addition of 0.50 mM EGTA. The gas phase for all media was 5% CO_2/95% O_2 during the dissection of the tissue, and 5% CO_2/air during the equilibration, incubation, and determination of O_2 uptake. In specific experiments arachidonic, linoleic, or γ-linolenic acid was added to the standard medium; the free acid was spread with a stream of N_2 across the bottom of a 250-ml Erlenmeyer flask and standard medium that had been equilibrated with 5% CO_2/95% N_2 was added, the flask was shaken for 10 min in a metabolic shaker with the same gas phase, and aliquots were transferred to incubation flasks and equilibrated with 5% CO_2/air for 5 min before adding the tissue. In specific experiments, albumin-bound palmitic acid solutions prepared by the method of Spector and Hoak (20) were substituted for the 9% BSA in standard medium. Medium albumin concentration was determined by the method of Lowry et al. (21), medium FFA, as described above, and the medium molar ratio of the added free fatty acid/albumin calculated.

The freshly dissected tissue samples were routinely equilibrated for 15 min in 5 ml of a specified medium at 37°C in a 25-ml Erlenmeyer flask shaken at 88 cycles/min in a metabolic shaker while continuously gassed with 5% CO_2/air; the samples were then transferred to 5 ml of a specified incubation medium and incubated under the same conditions. For the determination of O_2 uptake the samples were rapidly transferred to 5 ml of fresh incubation medium maintained at 37°C in a chamber of a Model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) and the O_2 uptake was continuously recorded. The O_2 probe drift rate in the medium used in each experiment was determined, and used as a control.

Extraction and isolation of isotopically labeled phosphoinositides. In specific experiments [1,3-^{14}C]glycerol, [1-^{14}C]arachidonic acid, or [1-^{14}C]lineolic acid was added to the incubation medium. At the end of the incubation or of a subsequent chase, the tissue was rapidly frozen in liquid N_2 that had been partially evaporated to its freezing point (22) and pulverized in liquid N_2 in an all-glass homogenizer. Two methods were employed to extract the frozen tissue powder. For the extraction and isolation of PI and the major phospholipid classes, the homogenizing vessel containing the frozen powder was placed in an ice bath, 3 ml of $CHCl_3/CH_3OH$ (2:1) were added, the sample was homogenized and brought to room temperature. After we added 0.12 ml of 0.25 M KCl, the sample was rehomogenized and allowed to stand for 10 min, centrifuged, and the supernatant was saved. The pellet was reextracted with 3 ml of $CHCl_3/CH_3OH$/0.25 M KCl (64:32:4), the extracts were pooled, and 0.20 volumes of 0.25 M KCl were added. The lower phase was recovered, washed three times with $CHCl_3/CH_3OH$/0.25 M KCl (3:48:47), and dried under a stream of N_2. The lipids were redissolved in $CHCl_3/CH_3OH$ (2:1), and spotted on activated Silica Gel G-60 plates (Analtech, Inc., Newark, DE) and subjected to two-dimensional thin layer chromatography as described by Yavin and Zutra (23). The spots were visualized with I_2 vapor and compared with reference standards; after the I_2 dissipated the spots were scraped into liquid scintillation vials, 15 ml of Aquasol-2 (New England Nuclear, Boston, MA) was added, and the vials were counted in a liquid scintillation spectrometer with an external standard. For the extraction of polyphosphoinositides, the method of Creba et al. (24) was employed. The frozen tissue powder was homogenized in 1 ml of cold 20% TCA in an ice bath, and centrifuged at 4°C. The pellet was washed by homogenization, first in 2 ml of cold 5% TCA, and then in 2 ml of cold water. The washed pellet was extracted twice with 3 ml of $CHCl_3/CH_3OH$/concentrated HCl (100:100:1), then once with 2 ml of $CHCl_3/CH_3OH$/concentrated HCl (200:100:1); the extracts were pooled, and 3 ml of $CHCl_3$ and 2.2 ml of 0.1 HCl containing 0.25 M KCl were added, the lower phase was recovered, washed once with a synthetic upper phase, and dried under a stream of N_2. The lipids were redissolved in $CHCl_3/CH_3OH/H_2O$ (75:25:2) and spotted on activated Silica Gel 60 High Performance Plates (E. Merck, Darmstadt, Federal Republic of Germany) that had been impregnated with potassium oxalate, and subjected to one-dimensional thin layer chromatography as described by Jolles et al. (25). The spots were identified, recovered, and counted as described above. The tissue free *myo*-inositol content was determined as previously described (18).

Materials. myo-Inositol was obtained from Pfanstiehl Laboratories, Inc., Waukegan, IL, and arachidonic acid was obtained from Nu-Chek-Prep, Elysian, MN. Reference standards of PI and of phospholipids other than the polyphosphoinositides were purchased from Serdary Laboratories, New London, Ontario. Reference standards of phosphatidylinositol 4-phosphate and of phosphatidylinositol 4,5-bisphosphate, as well as ouabain, indomethacin, nordihydroguaiaretic acid, linoleic acid, γ-linolenic acid, and palmitic acid were obtained from Sigma Chemical Co. [1,3-^{14}C]Glycerol, [1-^{14}C]AA, and [1-^{14}C]linoleic acid were purchased from New England Nuclear.

Results

The rationale of the initial experiments was as follows. The steady state rate of energy utilization in a resting tissue reflects the combined activities of the energy-requiring reactions normally operative under these conditions, and would include the contributions of any energy-requiring reactions of unknown identity that might be dependent on basal phosphoinositide turnover. The rate of energy utilization in resting aortic intima-media was monitored by the rate of O_2 uptake during incubation in KHB buffer that contained 5 mM glucose and a source of the oncotic pressure required to preserve the tissue's normal pattern of energy metabolism. (Under these conditions the tissue maintains a steady state of energy metabolism, derives 90% of its energy from respiration, and has a glucose uptake that can account for the total O_2 uptake and lactate production (14, 15). Hence its rate of O_2 uptake is a valid measure of the rate of energy utilization, since under steady state conditions the rate of energy utilization determines the rate of substrate oxidation for energy provision (26).) Depriving the tissue of its normal extracellular *myo*-inositol concentration was used as a potential means of inhibiting basal PI synthesis at restricted tissue sites, where it might be dependent on small *myo*-inositol pools susceptible to depletion by this means; this would be expected to deplete any

small phosphoinositide pools with a rapid basal turnover that require the basal PI synthesis at these sites for their maintenance. Inhibiting a fraction of basal PI synthesis per se should not detectably decrease the tissue's total resting energy utilization, because the rates of basal PI synthesis observed in unstimulated aorta (11) have an extremely small energy requirement. However, if deprivation of medium *myo*-inositol depletes a phosphoinositide pool whose rapid basal turnover controls reactions that are responsible for a significant component of normal resting energy utilization, this component should be inhibited. Energy utilization in a resting excitable tissue is restricted largely to essential reactions, and a 20–25% decrease would represent a major change. If the basal turnover in a depletable phosphoinositide pool active in metabolic regulation were normally controlled by some intrinsic regulatory system, the provision of medium *myo*-inositol should restore a component of energy utilization, whose magnitude is independent of the medium *myo*-inositol concentration, once it is adequate to relieve the inhibition of basal PI synthesis.

Aortic intima-media prepared (i.e., dissected and equilibrated) and incubated for 30 min in a medium designed to prevent any depletion of endogenous *myo*-inositol or FFA maintained a stable O_2 uptake of 210±3 ml/kg per h (n = 7). This medium was KHB buffer that contained 5 mM glucose, 9% dextran, and 0.07 mM *myo*-inositol, a normal rabbit plasma *myo*-inositol level (27). Omitting the *myo*-inositol from this medium during the preparation and incubation of the tissue resulted in a stable, but significantly reduced O_2 uptake of 174±7 ml/kg per h (n = 7), $P < 0.001$; this was restored to a normal resting level when 0.50 mM *myo*-inositol was added to a paired sample during the 30 min incubation (Table I, part A). There was no significant difference (Δ) in the O_2 uptakes of paired samples that were provided with 0.07 mM or 0.50 mM *myo*-inositol during the 30 min incubation; the mean Δ was −7±6 ml/kg per h in eight paired experiments. *myo*-Inositol and *scyllo*-inositol are the only inositols found in plasma and tissues in significant concentrations (27). The reduced O_2 uptake of tissue deprived of medium *myo*-inositol was unaffected by the addition of 0.50 mM *scyllo*-inositol, 100 times the normal rabbit plasma level (27), during a 30-min incubation; the mean Δ was 0±3 ml/kg per h in nine paired experiments. These observations demonstrated that medium *myo*-inositol in a normal plasma concentration is specifically required to maintain a component of normal resting energy utilization in aortic intima-media, and that the magnitude of this component is not a direct function of the medium *myo*-inositol concentration itself once a normal plasma level is available.

To assess the effect of medium *myo*-inositol in a normal plasma concentration on basal *de novo* PI synthesis in aortic intima-media, paired samples were prepared in the medium containing 5 mM glucose, 9% dextran, and 0.07 mM *myo*-inositol, and incubated for 15 min with added 0.10 mM [1,3-^{14}C]glycerol, with one sample deprived of medium *myo*-inositol during its equilibration and incubation. As shown in Table II,

Table I. Effects of Depleting and Repleting Endogenous myo-Inositol (Inositol) or AA on O_2 Uptake of Resting Aortic Intima-Media

		Oxygen uptake				
Experiment	Preparation medium	Incubation medium	Additions	Mean Δ±SEM of paired experiments	n	P
		ml/kg per h	*ml/kg per h*	*ml/kg per h*		
A	Dextran*	Dextran	+0.50 mM Inositol			
		174	210	+36±5	(7)	<0.001
B	Standard‡	Standard	+0.50 mM Inositol			
		182	181	−1±1	(10)	NS
C	Standard	Standard	+AA			
		175	184	+9±7	(7)	NS
D	Standard	Standard	+AA +0.05 mM Inositol			
		184	232	+48±4	(6)	<0.001
E	Standard	Standard	+AA +0.50 mM Inositol			
		172	216	+44±4	(6)	<0.001
F§	Dextran	Dextran +0.50 mM Inositol	Standard +AA +0.50 mM Inositol			
		212	218	+6±4	(6)	NS

Paired tissue samples were dissected and equilibrated for 15 min in the preparation medium indicated, and incubated for 30 min in the incubation medium indicated with additions to one sample. The media consisted of KHB buffer, pH 7.4 at 37°C that contained 5 mM glucose and 9% dextran (*Dextran) or 9% defatted BSA (‡Standard); gas phase 5% CO_2/95% O_2 for preparation medium and 5% CO_2/air for incubation medium. § In Experiment F, one of the paired samples was incubated in Dextran medium and the other in Standard medium. When AA was added to Standard medium the molar ratio of AA/medium albumin was 0.33. The values are the means for the group of paired experiments and the mean Δ±SEM between the paired samples. P was determined by a paired t test analysis.

Table II. A. Effect of Medium myo-Inositol (0.07 mM) on [1,3-^{14}C]Glycerol Incorporation into Aortic Phosphoinositides*

	Nanomoles [1,3-^{14}C]glycerol incorporation per kilogram					
	−*myo*-inositol	+myo-inositol	Mean Δ±SEM	*n*	*P*	Incubation period
						min
PI	65	87	+23±6	10	<0.01	15
PI-4-P	8	8	0±1	6	NS	30
PI-4,5-P_2	0	0	0±0	6	NS	30

B.‡ Decay of [1,3-^{14}C]Glycerol-labeled PI Formed when Medium Lacks or Contains 0.07 mM myo-Inositol

	Nanomoles [1,3-^{14}C]glycerol present in PI per kilogram				
Medium *myo*-inositol	15-min pulse	30-min chase	Mean Δ±SEM	*n*	*P*
mM					
0	55	61	+6±6	6	NS
0.07	83	57	−26±5	7	<0.001

* A. Paired aortic intima-media samples prepared in KHB buffer, pH 7.4 at 37°C, that contained 9% (wt/vol) dextran and 5 mM glucose were incubated for the times indicated with added 0.10 mM [1,3-^{14}C]glycerol, 55 mCi/mmol, with 0.07 mM *myo*-inositol present in one sample. PI, PI-4-P, and PI-4,5-P_2 were isolated by thin layer chromatography and counted as described in Methods. Values are means or the mean Δ±SEM between the paired samples. *P* was determined by a paired *t* test analysis. ‡ B. PI was isolated from paired tissue samples and counted after a 15-min incubation with 0.10 mM [1,3-^{14}C]glycerol, as described under A, or after a subsequent 30-min incubation in medium containing 0.10 mM unlabeled glycerol. In separate series of experiments the medium lacked or contained 0.07 mM *myo*-inositol during the pulse and chase.

part A, the sample provided with 0.07 mM *myo*-inositol exhibited a significantly higher (35%) incorporation of [1,3-^{14}C]glycerol into PI. This observation indicates that medium *myo*-inositol in a normal plasma level is required to prevent the inhibition of a component of basal *de novo* PI synthesis. When tissue deprived of medium *myo*-inositol was pulsed with 0.10 mM [1,3-^{14}C]glycerol for 15 min, the labeled PI that was formed exhibited no significant decay during a subsequent 30-min chase (Table II, part B), and appears to be present in PI pools that do not turn over rapidly in resting tissue. In contrast, the additional component of [1,3-^{14}C]glycerol-labeled PI that was formed during a 15-min pulse when 0.07 mM *myo*-inositol was present disappeared completely during a 30-min chase, leaving levels of labeled PI like the stable levels observed in tissue pulsed in medium lacking *myo*-inositol (Table II, part B). The component of basal *de novo* PI synthesis that is inhibited in medium lacking *myo*-inositol thus appears to be specifically required for the maintenance of a discrete PI pool that has a rapid basal turnover. To determine whether the rapid basal turnover of the discrete [1,3-^{14}C]glycerol-labeled PI pool might result from its rapid utilization for polyphosphoinositide formation, the effect of medium *myo*-inositol on [1,3-^{14}C]glycerol incorporation into polyphosphoinositides was examined in incubations extended to 30 min to assure significant turnover of the labeled discrete PI pool in the sample provided with medium *myo*-inositol. In tissue deprived of medium *myo*-inositol there was significant labeling of phosphatidylinositol 4-phosphate (PI-4-P) but not of phosphatidylinositol 4,5-bis-phosphate (PI-4,5-P_2) after 30 min (Table II, part A). However, the presence of 0.07 mM *myo*-inositol in paired samples caused no detectable increase in the labeling of either PI-4-P or of PI-4,5-P_2 (Table II, part A). The rapid basal turnover of the discrete PI pool cannot be attributed to its rapid utilization for polyphosphoinositide formation, and appears to reflect basal PI hydrolysis.

Aortic intima-media has a high content of free *myo*-inositol in pools that are not rapidly depleted in medium lacking *myo*-inositol; tissue incubated in such medium for 30 min contained 5.80±0.43 mmol/kg (n = 5), as compared with the 5.71±0.43 mmol/kg previously found after a 1-h incubation in *myo*-inositol-free medium (28). These large, stable *myo*-inositol pools probably account for the fact that the major fraction of basal *de novo* PI synthesis in this tissue is not susceptible to inhibition by medium lacking *myo*-inositol. Not unexpectedly, a significant increase in total tissue *myo*-inositol content could not be shown when paired samples were incubated for 30 min with 0.07–0.50 mM *myo*-inositol added to one sample; when 0.50 mM *myo*-inositol was added the mean Δ was +0.25±0.21 mmol/kg (n = 5). The requirement for medium *myo*-inositol in a normal plasma concentration to maintain a specific component of basal *de novo* PI synthesis may reflect its requirement at specific tissue sites to prevent the depletion of some very small *myo*-inositol pools that are responsive to the extracellular concentration. Bleasdale et al. (29) proposed a similar explanation for the effect of plasma *myo*-inositol levels on PI synthesis in rat lung, which has a high *myo*-inositol content, and demonstrated that the presence of 0.04 mM *myo*-inositol in the medium (a normal plasma level) critically alters *de novo* PI synthesis in Type II pneumocytes.

Prior observation in nerve (12) suggested that depleting the free AA pool in a resting tissue might provide another probe for energy utilization dependent on basal phosphoinositide turnover, and this was examined in aortic intima-media. The standard medium (KHB buffer containing 5 mM glucose and 9% defatted BSA) was designed to persistently deplete the tissue's endogenous FFA pools and also deprive it of medium *myo*-inositol. Tissue prepared and incubated for 30 min in standard medium had a stable O_2 uptake of 178±3 ml/kg per h (n = 29), which was not significantly different from the reduced O_2 uptake (174±7 ml/kg per h) of tissue that retained its endogenous FFA but was deprived of medium *myo*-inositol. However, the reduced O_2 uptake of tissue prepared and incubated in standard medium was

not increased by the addition of as much as 0.50 mM *myo*-inositol during a 30-min incubation (Table I, part B). These observations suggested that depleting endogenous FFA inhibits the component of normal resting energy utilization that requires medium *myo*-inositol. In tissue prepared and incubated in standard medium the effect of repleting a small free AA pool was examined by adding AA to the standard medium in a molar ratio of AA/albumin of 0.33 during a 30 min incubation; the addition of AA alone to standard medium had no effect on the reduced O_2 uptake of tissue depleted of endogenous FFA and deprived of medium *myo*-inositol (Table I, part C). However, the addition of both AA and of as little as 0.05 mM *myo*-inositol caused a significant increase (Table I, part D), and the magnitude of this effect was not increased when the concentration of the added *myo*-inositol was increased 10-fold (Table I, part E). When paired samples provided with medium *myo*-inositol were incubated under conditions in which one sample retained its endogenous FFA and the other was exposed to 9% defatted BSA with AA added in a molar ratio of 0.33, there was no significant difference in their rates of O_2 consumption (Table I, part F). This indicates that in tissue depleted of endogenous FFA, the replenishment of free AA with medium AA in a molar ratio of AA/albumin of 0.33 restores the normal resting rate of energy utilization. The medium molar ratio of AA/albumin would be expected to be a major determinant of the free AA uptake (30), assuming that no specific, high affinity uptake mechanism for free AA (31) is provided in aortic intima-media. In paired samples prepared and incubated in standard medium, the effects of adding AA in increasing molar ratios and a constant 0.50 mM *myo*-inositol to one sample were examined (Table III). AA in a molar ratio of 0.01 had no effect, but in molar ratios of 0.11 to 1.1 it caused increases in O_2 uptake that were not significantly different in magnitude. Replenishing free AA in tissue depleted of endogenous FFA restores a component of normal resting energy utilization, whose magnitude is independent of the medium molar ratio of AA/albumin, once a threshold level is provided. The molar ratio of AA/albumin in normal rabbit plasma is unknown, but 0.11 might represent a near-physiological molar ratio, since in human plasma estimates of 0.02–0.05 can be derived from the reported percentages of AA in albumin-bound FFA (31, 32) and the normal albumin and FFA concentrations.

When 0.01 mM indomethacin or 0.10 mM nordihydroguaiaretic acid was added to standard medium during the equilibration and incubation of paired tissue samples, the increases in O_2 uptake induced by adding *myo*-inositol and AA in a maximally effective molar ratio (0.33) were not significantly different from that observed when no cyclooxygenase or lipoxygenase inhibitor was present (Table IV). The O_2 uptake of tissue incubated in standard medium was not increased when linoleic, γ-linolenic, or albumin-bound palmitic acid was added along with *myo*-inositol (Table V). (Albumin-bound palmitic acid in a molar ratio of 1.0 caused a small decrease in O_2 uptake, which might reflect some substitution of palmitic acid as substrate for respiration, when it is present in this molar ratio.) The foregoing observations suggest that a tissue free AA pool is a specific requirement for the component of normal resting energy utilization that is dependent on medium *myo*-inositol, and that this cannot be attributed to a requirement for AA for use in eicosanoid production.

The free AA pool in a resting tissue would be required to replenish any small rapidly turning-over PI pools that are maintained primarily by *de novo* PI synthesis (rather than PI resynthesis) with PI that contains predominantly arachidonate in position-2. (The incorporation of AA into PI and the polyphosphoinositides results primarily from deacylation and reacylation of PI with arachidonoyl-coenzyme A (CoA) derived from the free AA pool, and not from any preferential incorporation of AA during *de novo* PI synthesis (31, 33).) When paired aortic intima-media samples were incubated in standard medium containing (1-^{14}C)AA, molar ratio 0.33, for 30 min, the addition of 0.50 mM *myo*-inositol caused a significant (45%) increase in [1-^{14}C]AA incorporation into PI, without any associated increase

Table III. Effects of Medium Molar Ratio of AA/Albumin on O_2 Uptake of Tissue Depleted of Endogenous Free Fatty Acid

Molar ratio of added AA/albumin	Mean Δ±SEM in O_2 uptake	*n*	*P*
	ml/kg per h		
0.01	+1±2	6	NS
0.11	+34±6	6	<0.01
0.22	+41±2	6	<0.001
0.33	+44±4	6	<0.001
1.11	+46±9	4	<0.01

Paired tissue samples prepared in standard medium (see legend for Table I) were incubated for 30 min in similar medium with AA in varying molar ratios to medium albumin and 0.50 mM *myo*-inositol added to one sample. The values are the mean difference in the O_2 uptake of paired samples. *P* was determined by a paired *t* test analysis.

Table IV. Effects of Indomethacin and Nordihydroguaiaretic Acid on Increase in O_2 Uptake Induced by Medium myo-Inositol and AA Added to Standard Medium

	Oxygen uptake	Mean Δ±SEM	*n*	*P*
	mg/kg per h			
A. Indomethacin (0.01 mM)				
Standard medium	+*myo*-Inositol +AA			
180	224	+44±5	6	<0.001
B. Nordihydroguaiaretic acid (0.10 mM)				
Standard medium	+*myo*-Inositol +AA			
180	217	+37±6	6	<0.005
C. No inhibitor*				
Standard medium	+*myo*-Inositol +AA			
172	216	+44±4	6	<0.001

Paired tissue samples were dissected and equilibrated in standard medium (see legend for Table I) and then incubated for 30 min in similar medium with 0.50 mM *myo*-inositol and AA in a molar ratio of 0.33 to medium albumin added to one sample. When added, the inhibitor was present in both samples during the 15-min equilibration and the incubation.

* These data are reproduced from Table I, Part E for comparison.

Table V. Effects of Linoleic, γ-Linolenic, and Palmitic Acids on O_2 Uptake of Tissue Depleted of Endogenous FFA

Experiment	Oxygen uptake: Incubation medium	Oxygen uptake: Additions	Mean Δ±SEM of paired experiments	*n*	*P*	Molar ratio of added fatty acid/albumin
	ml/kg per h	*ml/kg per h*	*ml/kg per h*			
A	Standard	+Linoleic A +Inositol*				
	184	178	−6±5	6	NS	0.33
B	Standard	+γ-Linolenic A +Inositol				
	176	180	+4±3	6	NS	0.33
C	Standard	+Palmitic A +Inositol				
	180	183	+3±4	6	NS	0.20
D	Standard	+Palmitic A +Inositol				
	185	179	−7±3	7	<0.05	1.00

Paired tissue samples were prepared in standard medium (KHB buffer, 5 mM glucose, 9% defatted BSA) and incubated for 30 min at 37°C in similar medium, with the addition of 0.50 mM *myo*-inositol (*inositol) and the indicated fatty acid to one sample. The addition of the *myo*-inositol alone has no effect (Table I, part B). *P* determined by a paired *t* test analysis.

in the labeling of PI-4-P or of PI-4,5-P_2 (Table VI, part A). (There was a tendency for increased labeling of some of the major phospholipid classes, but none of these increases was significant.) In resting aortic intima-media, the presence of medium *myo*-inositol is required to maintain a significant component of basal [1-^{14}C]AA incorporation into PI; this probably reflects its requirement to maintain a component of basal *de novo* PI synthesis that provides PI that normally undergoes rapid deacylation and reacylation with arachidonoyl-CoA derived from the free AA pool. The additional component of [1-^{14}C]AA-labeled PI that is formed in tissue provided with medium *myo*-inositol is selectively utilized to replenish the discrete, rapidly turning-over PI pool. When triplicate samples were pulsed with [1-^{14}C]AA for 15 or 30 min in standard medium, the labeled PI that was formed

Table VI. A. Effects of Medium myo-Inositol on [1-^{14}C]AA Incorporation into Phosphoinositides and Other Phospholipids*

Phospholipid	[1-^{14}C]AA incorporation: −*myo*-Inositol	[1-^{14}C]AA incorporation: +*myo*-Inositol	Mean Δ±SEM	*n*	*P*
	nmol/kg per 30 min	*nmol/kg per 30 min*			
Phosphatidylinositol	1,220	1,760	+540±120	6	<0.01
Phosphatidylinositol 4-P	210	220	+10±30	6	NS
Phosphatidylinositol 4,5-P_2	180	190	+20±30	6	NS
Phosphatidic acid	110	140	+40±40	6	NS
Phosphatidylcholine	2,260	3,170	+910±480	6	NS
Phosphatidylethanolamine	350	450	+100±70	6	NS
Phosphatidylserine	280	360	+80±50	6	NS

B.‡ Effect of Medium myo-Inositol on [1-^{14}C]Linoleic Acid Incorporation into Phosphatidylinositol

	[1-^{14}C]Linoleic acid incorporation: −*myo*-Inositol	[1-^{14}C]Linoleic acid incorporation: +*myo*-Inositol	Mean Δ±SEM	*n*	*P*
Phosphatidylinositol	310	280	−30±30	6	NS

* A. Paired samples prepared in standard medium (see legend for Table I) were incubated for 30 min in standard medium containing [1-^{14}C]AA with a molar ratio to medium albumin of 0.33, specific activity 7 mCi/mmol, with 0.50 mM *myo*-inositol added to one sample. Separate experiments were performed to assess the incorporations into the polyphosphoinositides (see Methods). Values are the means for each group of paired experiments, and the mean difference±SEM in the paired samples. *P* was determined by a paired *t* test analysis. ‡ B. The experiments were as described above for part A with the exception of the substitution of [1-^{14}C]linoleic acid, molar ratio to medium albumin of 0.33, specific activity 7 mCi/mmol as the labeled fatty acid.

Table VII. Comparison of the Decay of the [1-^{14}C]AA-labeled Phosphatidylinositol Formed in Absence or Presence of Medium myo-Inositol

	Nanomoles of [1-^{14}C]AA in PtdIns/kg tissue weight			
Experiment A	15-min pulse	30-min chase	60-min chase	*n*
−*myo*-Inositol	550±38	600±51	650±74	4
+*myo*-Inositol	880±42*	610±35‡	600±75‡	3
Experiment B	30-min pulse	15-min chase	30-min chase	*n*
−*myo*-Inositol	1,140±190	1,280±210	1,230±170	6
+*myo*-Inositol	1,820±160§	1,760±250	1,420±210‡	7

Triplicate samples from each aorta were prepared in standard medium (see legend to Table I), and pulsed with added [1-^{14}C]AA, molar ratio of AA/albumin 0.33, specific activity 10.5 mCi/mmol to label PtdIns; two samples were then subjected to a chase in standard medium containing unlabeled AA in a similar molar ratio. In separate experiments 0.50 mM *myo*-inositol was present during the pulse and chase. Values are means±SEM. The differences in the presence and absence of medium *myo*-inositol were analyzed by the *t* test; * $P < 0.01$; ‡ No significant difference. § $P < 0.05$. (In Experiments B, the mean Δ±SEM in paired samples in the initial level of [1-^{14}C]AA in PtdIns and the level after a 30-min chase was +90±70 nmol/kg in *myo*-inositol-free medium, not significantly different, and −400±70 nmol/kg in *myo*-inositol containing medium, $P < 0.001$. *P* was determined by a paired *t* test analysis.)

exhibited no significant decay during chases as long as 1 h (Table VII). (In tissue pulsed for 30 min the nonsignificant increase in labeled PI during the first 15 min of the chase probably reflects some delay in effectively diluting the labeled arachidonoyl-CoA pool.) When medium *myo*-inositol was present during a 15- or 30-min pulse with [1-^{14}C]AA, a large additional component of [1-^{14}C]AA-labeled PI was formed, which disappeared during a 30 min chase leaving levels of labeled PI similar to the stable levels in tissue pulsed in medium lacking *myo*-inositol (Table VII). These observations provided additional support for the conclusion that medium *myo*-inositol is necessary to maintain a discrete PI pool that has a rapid basal turnover, and support the inference that the free AA pool is required to replenish the discrete PI pool with arachidonate-containing PI. They also provide additional evidence that the rapid basal turnover of the discrete PI pool does not result from its utilization for polyphosphoinositide formation, for although medium *myo*-inositol is required to label the discrete PI pool with [1-^{14}C]AA, it does not increase [1-^{14}C] labeling of the polyphosphoinositides (Table VI).

In tissue depleted of endogenous FFA it was impossible to label the discrete PI pool with substrates that are incorporated into PI solely by *de novo* synthesis, because the component of basal *de novo* PI synthesis that requires medium *myo*-inositol is selectively inhibited. When paired samples prepared in standard medium were incubated for 30 min with added [1-^{14}C]linoleic acid, there was significant labeling of PI, but the addition of *myo*-inositol failed to restore an additional component of basal *de novo* PI synthesis (Table VI, part B). Similarly, when paired samples were incubated for 30 min in standard medium with added 0.10 mM [1,3-^{14}C]glycerol, there was significant incorporation of labeled glycerol into PI, but the addition of 0.50 mM *myo*-inositol caused no significant increase: the mean Δ was +2±2 nmol [1,3-^{14}C]glycerol incorporated/kg ($n = 8$). However, when these experiments were repeated and both AA, molar ratio 0.33, and 0.50 mM *myo*-inositol were added to the standard medium an additional component of basal glycerol incorporation into PI was restored; the mean Δ in six paired experiments was +37±10 nmol [1,3-^{14}C]glycerol incorporated/kg, $P < 0.05$. Depleting the free AA pool selectively inhibits the component of basal *de novo* PI synthesis that is required for the maintenance of the discrete PI pool. The conclusion that rapid basal PI turnover in this pool controls the component of normal resting energy utilization that is inhibited when the tissue is depleted of free AA or deprived of medium *myo*-inositol is supported by the fact that in each instance there is a selective inhibition of the basal *de novo* PI synthesis required to maintain the discrete PI pool.

Aortic intima-media prepared and incubated for 30 min in Ca^{2+}-free standard medium that contains 0.50 mM EGTA (see Methods) had an O_2 uptake higher than that observed in Ca^{2+}-containing standard medium, but the addition of 0.50 mM *myo*-inositol and AA in a molar ratio of 0.33 caused an increase in O_2 uptake similar to that observed in Ca^{2+}-containing medium (Table VIII). The component of resting energy utilization that is controlled through basal PI turnover does not require Ca^{2+}-influx, and is identifiable and unaltered in magnitude in Ca^{2+}-free medium.

The ouabain-inhibitable O_2 consumption was used to assess the rates of Na^+/K^+-ATPase activity in aortic intima-media un-

Table VIII. Effect of Ca^{2+}-free Medium on the Component of Energy Utilization That Requires Medium myo-Inositol and AA

	O_2 uptake				
Medium	Control	+AA +*myo*-Inositol	Mean Δ±SEM	*n*	*P*
	ml/kg per h	*ml/kg per h*			
Standard	172	216	+44±4	6	<0.001
Ca^{2+}-free standard	215	261	+46±6	6	<0.001

Paired samples were prepared in standard medium (KHB buffer, pH 7.4 at 37°C containing 5 mM glucose and 9% defatted BSA) or in similar medium containing Ca^{2+}-free KHB and 0.50 mM EGTA, and incubated for 30 min with 0.50 mM *myo*-inositol and AA with a molar ratio to medium albumin of 0.33 added to one sample. *P* determined by paired *t* test analysis.

der conditions in which the component of energy utilization that is controlled through basal PI turnover was maintained or inhibited. This was examined in Ca^{2+}-free medium to prevent any distortion of the decrease in energy utilization resulting from ouabain inhibition of Na^+/K^+ ATPase activity by the effects of a potential secondary increase in Ca^{2+}-influx when total resting Na^+/K^+ ATPase activity is inhibited in aortic smooth muscle. (Electrogenic Na^+/K^+ ATPase activity contributes to the resting membrane potential in vascular smooth muscle, and, in contrast to nerve, its voltage-dependent Ca^{2+}-channels appear to open in a graded manner in response to graded depolarizations of sufficient magnitude (34–37).) In aortic intima-media incubated in Ca^{2+}-free standard medium that contained 0.50 mM *myo*-inositol and AA in a molar ratio of 0.33, the rate of O_2 uptake resulting from energy utilization for Na^+/K^+-ATPase activity was 61±6 ml/kg per h (Table IX). This was decreased by 60% when the tissue was deprived only of medium *myo*-inositol, and by a similar percentage (52%) when it was deprived only of free AA (Table IX). Na^+/K^+ ATPase activity accounted for roughly 76% of the total energy utilization that is dependent on the provision of the medium *myo*-inositol and AA necessary to maintain the discrete PI pool that has a rapid basal turnover.

Discussion

The observations presented in this report support the hypothesis derived from previous studies in resting nerve (12) that some basal phosphoinositide turnover functions in metabolic regulation, which in resting excitable tissues can include the control of a major fraction of Na^+/K^+ ATPase activity. Identifying metabolic activity in a resting tissue that is dependent on basal phosphoinositide turnover presents a novel methodological problem. Some approaches suggested by our previous observations in nerve were useful in resting rabbit aortic intima-media, and they may have broad applicability.

The initial aim was to identify a component of normal resting energy utilization that might reflect the activities of reactions that are dependent on basal phosphoinositide turnover in pools that are depleted when any basal PI synthesis that requires the presence of extracellular *myo*-inositol is inhibited. Steady state energy utilization was used to monitor the combined activities of the energy-requiring reactions operative in the resting tissue; this could be assessed by the steady state rates of O_2 uptake under incubation conditions in which respiration is the major mechanism for energy provision and glucose is the principal respiratory substrate (14, 15). In tissue that retained its endogenous FFA, or that was depleted of endogenous FFA but repleted with free AA, a component of normal resting energy utilization was identified that requires medium *myo*-inositol in a normal plasma concentration (0.05–0.07 mM), but whose magnitude is not dependent on the medium *myo*-inositol concentration over the range of 0.05 to 0.50 mM. This requirement appears to be specific, since *scyllo*-inositol at 100 times its normal plasma level is not an effective substitute for *myo*-inositol. The presence of medium *myo*-inositol in a normal plasma concentration was found to be necessary to prevent the inhibition of a specific component of basal *de novo* PI synthesis that is required to demonstrate a discrete [1,3-^{14}C]glycerol-labeled PI pool that exhibits a rapid basal turnover in pulse-chase experiments. Medium *myo*-inositol is also required to maintain a specific component of basal [1-^{14}C]AA incorporation, which supports the expectation that the PI derived from the basal *de novo* synthesis that requires medium *myo*-inositol normally undergoes deacylation and reacylation with arachidonoyl-CoA derived from the free AA pool (31, 33). The component of basal [1-^{14}C]AA-labeled PI formation that requires medium *myo*-inositol is selectively utilized to replenish the discrete PI pool, which exhibits a similar rapid basal turnover when it is labeled with [1-^{14}C]AA. The requirement for medium *myo*-inositol to maintain a specific component of normal resting energy utilization in resting aortic intima-media appears to result from its requirement to maintain a discrete PI pool in which both the glycerol and arachidonate moieties of PI turnover rapidly. This rapid basal PI turnover appears to reflect basal PI hydrolysis, since it cannot be attributed to the rapid utilization of PI in the discrete PI pool for polyphosphoinositide formation. Medium *myo*-inositol is required to label the discrete PI pool with [1,3-^{14}C]glycerol or [1-^{14}C]AA but does not increase their incorporations into PI-4-P and PI-4,5-P_2 during incubations that should permit significant turnover of labeled PI in the discrete rapidly turning-over pool.

The mechanism of this basal PI hydrolysis remains to be clarified, but phospholipase C hydrolysis is one logical candidate, particularly since a markedly elevated cytosolic Ca^{2+} would not be expected in a resting tissue, and this seems to be required for the activation of phospholipase A_2 hydrolysis of PI in agonist-stimulated tissues (3, 38). There is a theoretical basis for speculation that basal phospholipase C hydrolysis of PI at specific sites in cellular membranes might be adapted to serve regulatory functions distinct from those served by receptor-controlled polyphosphoinositide hydrolysis. For although the phosphorylated *myo*-inositols released by phospholipase C hydrolysis of PI do not mobilize Ca^{2+} from intracellular stores (3), the other product, diacylglycerol, activates protein kinase C and its local accumulation in cellular membranes perturbs the bilayer structure and has the potential of altering the microenvironment and activity of membrane-bound enzymes (4).

The requirement for medium *myo*-inositol in a low, normal plasma concentration to maintain a specific component of basal *de novo* PI synthesis in aortic intima-media, despite a total tissue free *myo*-inositol content (~5.80 nmol/kg) that appears high relative to the reported K_m's of 1.5–2.5 mM of cytidinephosphodiacylglycerol:inositol phosphatidyltransferase (39, 40) is not a

Table IX. Alterations in the Rate of O_2 Uptake Related to Na^+/K^+ ATPase Activity Induced by Depriving the Tissue of Medium myo-Inositol or AA

Additions to medium	O_2 uptake				
	Control	+ Ouabain	Mean Δ±SEM	*n*	*P* vs. A*
A. *myo*-Inositol and AA	268	207	−61±6	6	
B. AA	221	197	−24±5	6	<0.001
C. *myo*-Inositol	213	184	−29±8	11	<0.01

Paired tissue samples prepared in Ca^{2+}-free standard medium (Ca^{2+}-free KHB buffer, pH 7.4 at 37°C, containing 0.50 mM EGTA, 5 mM glucose, and 9% defatted BSA), were equilibrated and incubated in similar medium with the additions noted, and 0.20 mM ouabain was added to one sample during the incubation. When present, the *myo*-inositol concentration was 0.50 mM, and the AA was in a molar ratio of 0.33 to medium albumin. The mean Δ in the O_2 uptake of the paired samples represents the O_2 uptake attributable to Na^+/K^+ ATPase activity under conditions A, B, or C.

* *P* was determined by a *t* test analysis.

unique situation; there are counterparts in rat lung (29) and peripheral nerve (12). This could reflect a requirement for extracellular *myo*-inositol in a specific constituent cell type, such as that demonstrated in Type II pneumocytes in lung (29). However, also note that the subcellular distribution of *myo*-inositol in mammalian cells is presently unknown, and consequently the existence of specific very small *myo*-inositol pools in aortic smooth muscle and/or endothelial cells that are selectively susceptible to depletion in medium lacking *myo*-inositol cannot be excluded.

The possibility that depleting the free AA pool in a resting tissue might provide another probe for energy utilization that is dependent on basal phosphoinositide turnover was suggested by previous observations in nerve (12), and proved to be valid in aortic intima-media. In tissue provided with medium *myo*-inositol, depleting endogenous FFA with medium defatted albumin inhibits the component of normal resting energy utilization that is dependent on medium *myo*-inositol in tissue that retains its endogenous FFA. This component is restored by medium AA in a molar ratio of AA/albumin of 0.11 (roughly two to five times the estimated molar ratios in human plasma that can be derived from the available data [31, 32]), and its magnitude is independent of further increases in the molar ratio of AA/albumin over a 10-fold range. In tissue depleted of endogenous FFA the provision of medium AA in a maximally effective molar ratio restores the resting rate of energy utilization observed in paired samples that retain their endogenous FFA. The requirement for free AA appears to be specific, since linoleic, γ-linolenic, and palmitic acid are not effective substitutes. Depleting or repleting free AA has no effect on the reduced energy utilization of tissue deprived of medium *myo*-inositol, and the presence or absence of medium *myo*-inositol does not affect the similarly reduced O_2 uptake of tissue depleted of endogenous free AA. The presence of a free AA pool is an independent requirement for the component of energy utilization that requires medium *myo*-inositol. This cannot be attributed to a requirement for free AA for eicosanoid production, since the magnitude of this component of energy utilization is not decreased by 0.01 mM indomethacin or by 0.10 mM nordihydroguaiaretic acid. However, the presence of a free AA pool was found to be required to prevent a selective inhibition of the component of basal *de novo* PI synthesis that is dependent on medium *myo*-inositol and is specifically utilized for the replenishment of the rapidly turning-over PI pool. In tissue depleted of endogenous FFA, [1,3-^{14}C]glycerol and [1-^{14}C]linoleic acid are incorporated into PI by the component of basal *de novo* PI synthesis that does not require medium *myo*-inositol, but the basal *de novo* PI synthesis that requires medium *myo*-inositol is inhibited, and the discrete PI pool cannot be demonstrated with these labeled substrates. In tissue depleted of endogenous FFA, medium AA restores the basal *de novo* PI synthesis that is dependent on medium *myo*-inositol; this effect appears to be specific, since linoleic acid and palmitic acid are not effective substitutes. Thus the presence of a free AA pool appears to be specifically required both to maintain the component of normal resting energy utilization that requires medium *myo*-inositol and to maintain the basal *de novo* PI synthesis that replenishes the discrete PI pool. This provides additional support for the conclusion that the rapid basal PI turnover in the discrete PI pool controls a specific component of normal resting energy utilization. The mechanism by which depleting free AA selectively inhibits the basal *de novo* PI synthesis that normally replenishes the discrete PI pool requires comment. The levels of free AA in mammalian tissues are low relative to the free levels of the other major fatty acids, and disproportionately so when the free and esterified levels are compared (41). The endogenous free AA pool in a resting isolated tissue reflects the balance between free AA release from lipids and its conversion to arachidonoyl-CoA for reincorporation, and the conservation of the free AA released from lipids is required to provide the arachidonoyl-CoA necessary for phospholipid remodeling, such as the conversion of PI derived from *de novo* PI synthesis to PI with a predominance of arachidonate in position 2 (31, 33). Consequently depleting the free AA pool in resting aortic intima-media would be expected to deplete arachidonyl-CoA and induce a deficiency of arachidonate-containing PI in the rapidly turning-over PI pool. The significance of the normal predominance of arachidonate in position 2 of membrane PI is not completely understood, but phosphoinositides that contain arachidonate are preferentially hydrolyzed in response to receptor stimulation (3). The selective inhibition of the component of basal *de novo* PI synthesis that replenishes the discrete PI pool observed in tissue depleted of free AA cannot be attributed to any obligatory requirement for arachidonoyl-CoA for *de novo* PI synthesis per se (33), and probably reflects the loss of the normal stimulus for this specific component of basal *de novo* synthesis. This could be an expected secondary consequence of a primary inhibition of basal PI hydrolysis in the discrete PI pool (1, 7) induced by a deficiency of arachidonate-containing PI.

The component of energy utilization in resting aortic intima-media that requires medium *myo*-inositol and a free AA pool is identifiable and unaltered in magnitude in Ca^{2+}-free medium, and the identity of the reactions responsible for this energy utilization could be validly examined under these conditions. In Ca^{2+}-free medium ouabain can be used to assess the rate of energy utilization resulting from Na^+/K^+ ATPase activity in aortic intima-media without risk of distortion by the metabolic effects of a potential secondary increase in Ca^{2+} influx in aortic smooth muscle (34–37). Depriving the tissue of either medium *myo*-inositol or of free AA inhibits a similar major fraction (60%, 52%) of the total Na^+/K^+ ATPase activity, which accounts for most (76%) of the component of energy utilization that is controlled through basal PI turnover in the discrete PI pool. (Since Ca^{2+}-free medium, which contains 0.50 mM EGTA, increases the component of energy utilization that does not require medium *myo*-inositol and free AA, the potential error in extrapolating these results to the conditions that exist when medium Ca^{2+} is present is restricted to a potential underestimation of the percentage of the total tissue Na^+/K^+ ATPase activity that is controlled through basal PI turnover.)

The origin and nature of the stimulus for basal PI hydrolysis, the mechanism of this hydrolysis, and the manner in which this affects the activity of a component of Na^+/K^+ ATPase remain to be clarified. However our observations raise the possibility that some intrinsic regulatory system utilizes PI hydrolysis in specific PI pools to control the activity of a component of Na^+/K^+ ATPase in order to regulate some biological parameter that is affected by the activity of this enzyme. A similar potential for regulation of this type appears to exist in nerve (12), but whether this is peculiar to excitable tissues, in which special requirements for the regulation of Na^+/K^+ ATPase activity may exist, remains to be determined. The demonstrated capacity of insulin to stimulate Na^+/K^+ ATPase activity in intact adipocytes without any increase in enzyme copy number, recruitment of enzyme from

cytoplasmic membranes, or increase in "substrate" (42) supports the postulated existence of mechanisms for the regulation of this enzyme in addition to those already identified (43). Two molecular forms of the catalytic subunit of this enzyme have been demonstrated in adipocytes, muscle, and brain, and recent evidence suggests that the two forms in adipocytes have radically different affinities for intracellular Na^+, and that in intact adipocytes insulin selectively alters the activity and affinity for intracellular Na^+ of one of these forms (44). Friend erythroleukemia cells have been found to contain a plasma membrane-bound cAMP-independent protein kinase that phosphorylates the catalytic subunit of plasma membrane Na^+/K^+ ATPase both in intact cells and purified plasma membranes at a site between the active site aspartate residue and the ATP-binding site, and it is postulated that phosphorylation in this region may serve to regulate the activity of this enzyme (43, 45). In an earlier era Hokin-Neaverson (46) speculated that acetylcholine stimulation of Na^+/K^+ ATPase activity in avian salt gland might be mediated by PI hydrolysis in the plasma membrane in proximity to the enzyme; the mechanism of this effect is still unknown, since, as yet, receptor-stimulated polyphosphoinositide hydrolysis has not been directly linked to the control of Na^+/K^+ ATPase activity (3).

In peripheral nerve hyperglycemia induces a moderate decrease in *myo*-inositol content that is causally related to the development of decreased conduction velocity, which initially results from an elevated Na^+ attributed to decreased axonal Na^+/K^+-ATPase activity (18, 47). Our previous studies in nerve led us to propose that the effect of the reduced nerve *myo*-inositol content results from the inhibition of basal PI synthesis that is required to maintain small phosphoinositide pools with a rapid basal turnover that controls a major fraction of resting Na^+/K^+ ATPase activity in nerve (12, 13). Our observations in aortic intima-media widen the range of possible mechanisms for derangements in vascular Na^+/K^+ ATPase activity in disease states.

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